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RESEARCH ARTICLE

CONTAMINATION LEVEL AND BIOMARKERS RESPONSES IN *Aphanius fasciatus* FISHES FROM TUNISIAN COAST

***Annabi, A., Kessabi, K., Said, K. and Messaoudi, I.**

Laboratoire de Recherche (LR11-ES41): Génétique, Biodiversité et Valorisation des Bio-ressources, Institut Supérieur de Biotechnologie de Monastir, Université de Monastir, Tunisie

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ABSTRACT

The environmental impact in natural populations of female Mediterranean killifish *Aphanius fasciatus* was monitored, in four sites of the Tunisian coast, by measuring metal contents (Cd, Cu, Zn) as well as levels of three oxidative stress biomarkers: Metallothioneins (MTs), Protein Sulfhydryl (PSH) and Thiobarbituric Acid-Reactive Substances (TBARS) in liver and gonad tissues. Site 2 appeared as the most polluted site, since it contains the highest concentrations of analyzed pollutants (Cu, Cd, Zn and PAHs) and presents the most important levels of metals in the liver and gonad tissues ($P < 0.05$). The significantly-highest values of both Hepato-, Gonado-somatic indexes (HSI, GSI respectively) were noticed in Site 4 and Site 3. In liver tissues, the analysis of MTs and PSH levels revealed a significant difference between the studied sites ($P < 0.05$) while TBARS levels were similar. In gonad tissues, we noticed a significant difference for PSH contents between studied sites and the highest value was observed in female captured from the Site 4. Interestingly, these results suggest a possible sensitivity of liver and gonad tissue on the studied biomarkers content in *A. fasciatus* and will be helpful in pollution monitoring.

Key words: *Aphanius fasciatus*, Biomarkers, Brackish water, Coastal Pollution

INTRODUCTION

Pollution of the aquatic environment with metals has become a serious health concern in recent years. Although environmental concentrations of metals are rarely directly dangerous for fish survival, are known to accumulate in fish tissues reaching concentrations of up to thousands of times higher than in the surrounding water environment and becoming extremely harmful (Henry *et al.*, 2004; Dietrich *et al.*, 2010). Oxidative damages to living beings affect nucleic acids, proteins, lipids and carbohydrates (Sies, 1986). The majority of organisms have their own cellular antioxidative defence system, composed of both enzymatic (e.g: glutathione peroxidase (GSH-Px), superoxide dismutase SOD) as well as non-enzymatic components (e.g: lipid peroxydation (LPO), Metallothioneins (MTs)). Formation of oxidized DNA or protein adducts has been proposed as biomarkers of oxidative stress (Sies, 1986; Lopez-Barea, 1994). Lipids, especially the polyunsaturated so abundant in fish, are highly prone to oxidative attack generating aldehydes e.g Malondialdehyde (Esterbauer and Zollner, 1989). Lipid peroxidation is essentially a toxic response to oxidative damage to cellular and tissue components (Storey, 1996; Kelly *et al.*, 1998). Metallothioneins are cysteine-rich low molecular weight proteins which bind transition metals, regulate endogenous copper (Cu) and Zinc (Zn), detoxicate excess of these and other pollutant metals, act as free radical scavengers and are induced as a part of a general stress response (Kagi and Kojima, 1987). Increased transcription of metallothionein genes by several metals is the basis for its use as a biomarker (Kille *et al.*, 1992). Reactions between protein molecules and reactive oxygen species (ROS) often lead to the modification of certain amino acid residues (e.g. histidine, lysine, arginine, proline and threonine) forming carbonyl derivatives. Alterations in protein function caused by protein thiol oxidation can profoundly affect cell function. Reversible protein thiol (PSH) oxidation has been demonstrated in proteins with a range of functions including signal transduction, ion transport, contractility, metabolism, protein synthesis and catabolism (Lui *et al.*, 2010).

The physiological and biochemical changes in fish exposed to contaminants are complex, and although liver, gill and brain are generally the targets in aquatic toxicological studies (Li *et al.*, 2010*a*, 2010*b*). For this purpose, it is important to identify responses in other organs. The Mediterranean killifish, *Aphanius fasciatus* (Valenciennes, 1821) is a small cyprinodont fish inhabiting brackish waters in coastal brackish habitats of the central and eastern coastal ponds or lagoons of the Mediterranean area. It is able to tolerate a wide range of physico-chemical parameters, such as temperature (5–39 °C) and salinity (0–180 ppt). The Eastern coast of Tunisia hosts a mosaic of *A. fasciatus* populations and presents a unique blend of industrial, residential and seafaring carried out at relatively proximal areas. Gulf of Gabès, is chronically polluted by heavy metal (especially cadmium: Cd) and accumulation and biological effects in local population of fish have been

Corresponding author: medannabi@yahoo.fr

evaluated only recently (Annabi *et al.,* 2009; Messaoudi *et al.,* 2009*b*). From a conservation perspective, gonad is an important organ for species perenity. Although, it is not mainly responsible for detoxification and reproductive success is one of the key factors in determining species survival. Pollution by metals could impair reproductive success of adult organisms through decreasing the quality of gametes, which in turn may affect fertilization success, hatching of embryos and subsequent survival of offspring (Au *et al.,* 2001; Li *et al.,* 2010*c*). Nonetheless, information on the effects of metal on gonad tissue is largely lacking.

According to available data, most studies of the adverse effects of pollutants on fish have focused on lethal or sublethal effects of single metal at higher concentrations. Despite the fact that fishes are normally affected by a combination of metals in their natural environment, few studies have investigated the effects of metal mixtures. Moreover, no attempts have been made to evaluate environmental concentrations of metals mixtures on fish gonadic tissues. To our knowledge, no study has investigated the effect of pollutants on the biomarker responses in the Mediterranean killifish *A. fasciatus*. Hence, as its use as a sentinel species for aquatic pollution biomonitoring has been suggested (Kessabi *et al.*, 2010), there is still a need to characterize biomarker responses to widespread environmental contaminants in this fish species. The aim of the present study was to investigate the influence of contamination levels, on the antioxidant response of three stress biomarkers (MTs, PSH and TBARS levels) in gonad and liver tissues of *A. fasciatus* captured from Tunisian coast. In addition, we followed up the progression of the Hepato- , gonado-somatic indexes and the condition factor (K-factor) to provide a general quantitative estimation of the physiological state between *A. fasciatus* populations.

MATERIALS AND METHODS

Study sites

A. fasciatus samples were collected from different sites located in the south eastern coast of Tunisia (Fig. 1). Sampling sites were selected according to their pollution level. We showed, previously, that analytical data presented a different pattern of distribution for pollutant contents (metals and PAHs) in these studied sites (Annabi *et al.,* 2012). The first site (S1, Luza) was considered a reference site since the absence of industrial and human activities. The second site (S2) is located near the industrialized coast of "Sfax" in the gulf of "Gabès". This area is especially characterized by shallow waters, high salinity, important tides and temperature. This ecosystem is chronically polluted by Cd. We evaluated, recently, the biological effects of metallic pollution in local populations of fishes (Annabi *et al.*, 2009; Messaoudi *et al*., 2009*b*). The third site (S3, Oued Hamdoun) is located in the southern coastal zone of "Sousse". Sousse, bordered in the south by "Oued Hamdoun", is considered as the most important town with approximately 173.000 inhabitants and represents, the unique freshwater source feeding this coast. Additionally, it contains a power station (located near "Hamdoun Oued") that pumps the seawater to cool its engines and expels the heated water directly into the site. The first signs of pollution in the "Oued Hamdoun" site were claimed by Afli and Ben Mustapha (2004). Many industrialized dumps (especially textile industry) as well as urban discharges characterized the last site (S4, Khniss zone) which is located in the south of Monastir town. Therefore, this site constitutes a significant source of pollution (Fig. 1).

Field sampling

Adult *A. fasciatus* female specimens (> 4 cm) were captured with held net (April 2010). Before dissection, fishes were anesthetized on ice and weighed. Liver and gonad excised from fishes in each sex and each site were weighed. These samples were kept on dry ice while being prepared and then stored at – 80 °C until they were analyzed.

Metal analysis (Cd, Cu, Zn)

Liver and gonad tissues were mineralized in the presence of 0.5 ml nitric acid and then incubated on a hot plate for 24 hours at 120 °C. The product was adjusted to 5 ml with deionized water (Bervoets and Blust, 2003). All samples were analyzed using the "Flame Atomic Absorption spectrophotometer" (AAS) technique. Measurements were implemented using a ZEEnit 700-Analytik-Jena, Germany (Flame and Graphite-Furnace AAS), equipped with Deuterium and Zeeman background corrections, respectively, as recommended by the manufacturer. The accuracy and the precision of our analyses for tissues metals contents were based on the analysis of Cd, Cu and Zn in a standard reference bovine liver. Our methodology shows that analytical results of the present work are of satisfactory quality. Furthermore, samples were analyzed in triplicate and variation coefficient was less than 10%. Concentrations of metals were expressed as $\mu g\ g^{-1}$ dry weight.

Physiological parameters

In order to study some biological parameters among *A. fasciatus* populations, we have focused on the evaluation of the variation of Hepato-somatic (HSI), Gonado-somatic (GSI) indexes and the condition factor (K-factor) between populations collected from the different sites. HSI is defined as the ratio of liver weight to body weight while GSI represents the ratio of gonad weight to body weight. Values of the compiled growth exponent were used for the calculation of the condition factor (K). In fact, the K-factor was determined following the equation: $[BW / (Tl)^3] \times 10$; where BW (g) represents the body weight of the fish and Tl (cm) its total length.

Biochemical assays

Tissue homogenates were obtained in 0.1 M Sodium phosphate buffer (pH 7.0) at a ratio of 1:10 (W/V). Homogenizations were carried out at 4 °C followed by a centrifugation at 12.000 *g* for 30 min at +4 °C. The supernatants were collected and used to determine MTs and PSH levels and to evaluate Thiobarbituric Acid-Reactive Substances (TBARS). The total protein content was measured in the homogenate according to the Bradford method (Bradford 1976). It consisted briefly on measuring the optic density (OD) at 595 nm using the Bovine serum albumin (BSA) as a standard.

Metallothioneins (MTs)

MTs protein levels were measured by a spectrophotometric assay using the Ellman's reagent (0.4 mM, 5'Dithio-Nitro-Benzoate (DTNB) in 100 mM KH_2PO_4) at pH 8.5 in a solution

containing 2 M NaCl and 1 mM EDTA (Viarengo *et al.*, 1997). Aliquots were homogenized in three volumes of 0.5 M sucrose, 20 mM Tris–HCl buffer, pH 8.6, with 0.006 mM leupeptine, 0.5 mM PMSF (phenylmethylsulphonyl_fluoride) as an antiproteolitic and 0.01% 2-mercaptoethanol as a reducing agent. The homogenate was then centrifuged at 15.000 *g* for 30 min at 4 °C. The obtained supernatant was treated with ethanol/chloroform according to Viarengo *et al.* (1997) in order to obtain a MTs enriched pellet. This latter was resuspended in HCl/EDTA in order to remove metal cations still bound to MTs. Finally, NaCl (2M) was added to the reaction to facilitate thiol- interactions with DTNB through the reduction of divalent metals interaction with the apothionein. Absorbance was evaluated at 412 nm and metallothionein concentration was estimated utilizing reduced glutathione (GSH). The calibration curve was established from a stock solution of GSH reagent (SIGMA) with a range of 0-80 g GSH. MTs levels were expressed as µg MTs mg^{-1} protein.

Protein sulfhydryl (PSH)

An aliquot of 10 µl of the homogenate was added to a mixture containing: 150 µl Tris (0.2 M, pH 8.2), 40 µl EDTA (0.02 M), 790 µl Methanol and 10 µl DTNB (0.01M) and the reaction is incubated for 15 min at room temperature. The homogenate was then centrifuged for 10 min at 14.500 *g* (+4 °C). A first, the OD at 412 nm was measured in order to determine the total sulfhydryl content. To precipitate the PSH, 45 µl TCA (5%) were added to the previous mixture. The mixture was then incubated for 3 min at room temperature and a second measurement of OD at the same wavelength was carried out to determine the non-sulfhydryl protein content. Reduced GSH standard solutions were used for calibration (0-400 µg) and data were expressed as µg PSH mg^{-1} of proteins.

Thiobarbituric Acid-Reactive Substances (TBARS)

The extent of lipid peroxidation was measured by the quantification of TBARS according to Persky *et al.* (2000) protocol, which was based on a condensation reaction between Malondialdehyde (MDA) and 2-Thiobarbituric acid (TBA). TEP (1, 1, 3, 3-tetraethoxypropane) was used as a MDA standard, without hydrolysis prior to the TBA reaction. A standard curve was made from TEP diluted in 7.5% TCA solution, at concentrations raising from 0 to 120 µM. An aliquot of 100 µl was used. On the latter, a volume of Thiobarbituric acid / hydrochloric acid ($TBA/HCLO_4$) was added. Afterwards, the mixture was vigorously vortexed and placed in a bath of boiling water for 60 min. After cooling the tubes for 10 min, 2 ml of Butanol were added and the mixture was agitated. Finally, a centrifugation step at 4000 *g* for 10 min at 8 °C was carried out to recover the supernatant. MDA-TBA complex absorbance was measured at 530 nm and results were expressed as micromoles of TBARS present per mg of proteins.

Statistical analysis

All data related to biological parameters (HSI, GSI and K-factor) and metal concentrations levels were given as mean ± S.D and performed with one way ANOVA *test* using STATVIEW statistical software package. Normality and homogeneity of data were confirmed before test. The means were subsequently compared by Post hoc Fisher's protected least significant difference test (Fisher's PLSD). Values were considered statistically significant at $P < 0.05$.

RESULTS

Metal levels in sampling tissue and index progression

Analysis of metal content in fish tissues revealed that females captured from S2 present the highest levels of Cd, Cu and Zn in liver and gonad tissues (Table 1; $P < 0.05$), whereas, there was no any significant difference between the three other sites (Table 1; $P > 0.05$). During April, indexes progression revealed the existence of a spatial difference between *A. fasciatus* studied populations. For the HSI, we have observed a significant difference between populations with the highest values measured in S3 and S4 specimens (Fig. 2; $P < 0.05$). In addition, GSI measured in females captured from S3 and S4 were significantly the highest obtained values in comparison to S1 and S2 (Fig. 2; $P < 0.05$). Concerning the K-factor, females collected from the S3 presented the highest values relatively to S2 populations which were significantly different (Fig. 2; $P < 0.05$).

Table 1. Heavy metal content in liver and gonad tissue in female *A. fasciatus* from studied sites (µg g^{-1} dry weight)

Sites	Tissues	Cd	Zn	Cu
Luza (S1)	Liver	0,036±0,023	7,84±2,96	1,23±0,68
	Gonad	0,027±0,024	7,24±2,57	1,365±0,73
Sfax (S2)	Liver	1,412±0,508*	15,67±4,08*	4,01±1,63*
	Gonad	0,833±0,348*	11,06±2,77*	2,69±0,98*
Oued Hamdoun (S3)	Liver	0,072±0,054	6,89±3,24	2,29±1,24
	Gonad	0,053±0,04	6,45±2,69	1,58±1,14
Khniss (S4)	Liver	0,146±0,075	7,85±3,07	1,93±0,91
	Gonad	0,0148±0,005	6,41±2,35	0,306±0,75

*: Value significantly higher $P < 0.05$; n=6

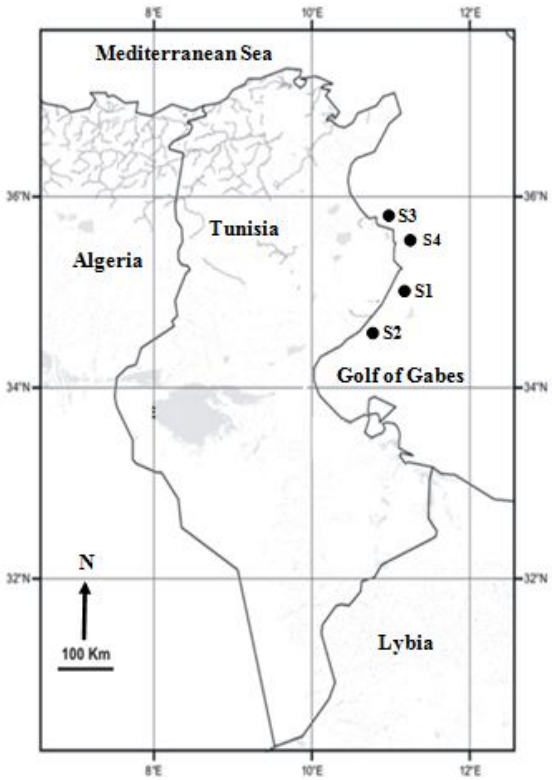

Fig. 1. General overview of sampling sites. S1: Luza; S2: Sfax coast; S3: Oued Hamdoun and S4: Khniss coast

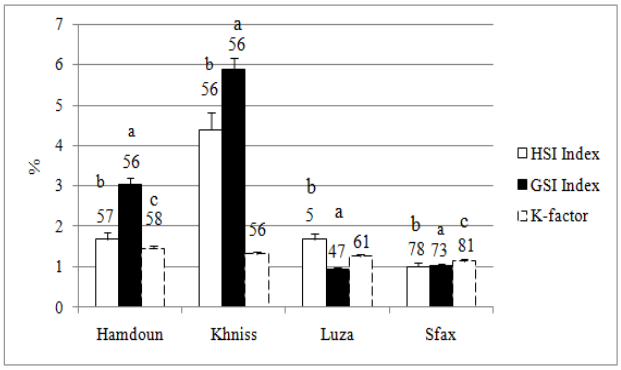

Fig. 2. Progression of HSI, GSI and K-factor index during April month in different studied sites. Letters indicate the significant difference between indexes (P < 0.05)

Biochemical analysis

Biochemical analyses revealed a spatial variation in MTs and PSH contents between *A. fasciatus* studied populations (Figs. 3 and 4). However, no significant difference in TBARS levels was observed (Fig. 5). Indeed, the obtained data showed a significant difference between MTs levels measured in hepatic tissues of fishes collected from S1 in comparison to S3 and S4 (Figs. 3 and 5; P < 0.05).

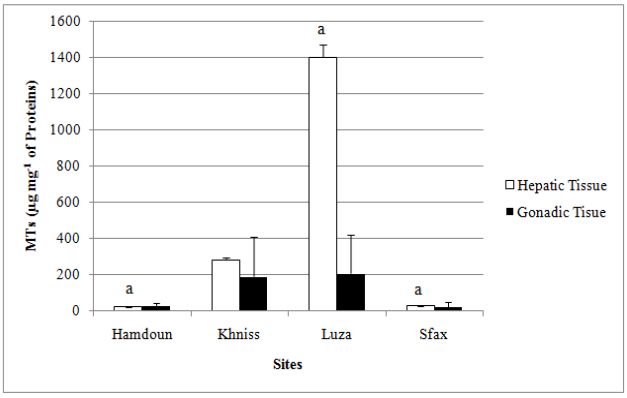

Fig. 3. MTs levels (µg mg^{-1} Protein) in hepatic and gonadic tissue of females captured from the studied sites. Letters indicate the significant difference between MTs levels (P < 0.05); n=6

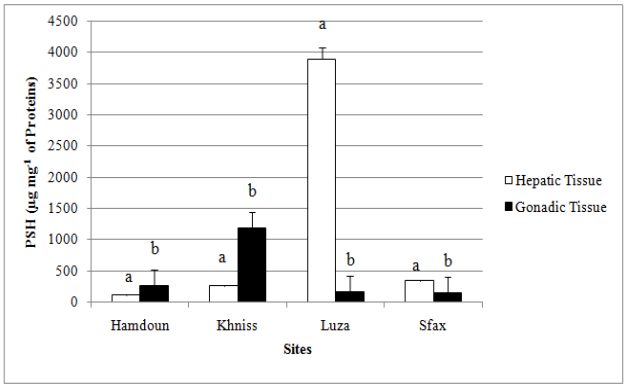

Fig. 4. PSH levels (µg mg^{-1} Protein) in hepatic and gonadic tissue of females from the studied sites. Letters indicate the significant difference between PSH content (P < 0.05); n=6

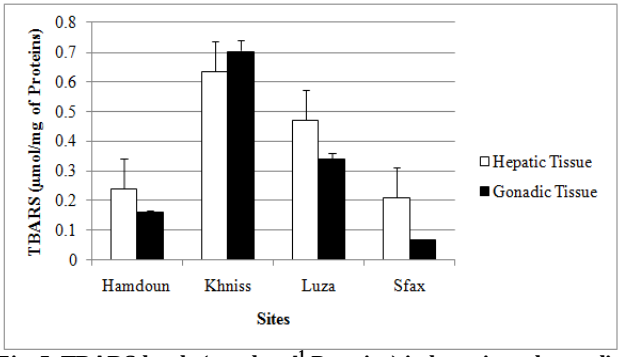

Fig. 5. TBARS levels (µmol mg^{-1} Proteins) in hepatic and gonadic tissue of females from the studied sites

Regarding the gonad tissue, the highest values were observed in females of the Luza site (S1). Nevertheless, there were no significant differences in MTs levels between the studied sites (Fig. 3). For the PSH, the higher concentration determined in the hepatic tissue was observed in females from S1 which differ significantly with those captured from S2 (Fig. 4). In addition, analysis of the PSH content in the gonad tissue revealed a significant difference between the studied sites with a highest content observed in females of S4 (Fig. 4).

DISCUSSION

The impact of aquatic pollution on human and animal life has become a matter of a great concern. Fish responses have been used as biomarkers of aquatic pollution. The use of a suitable biomarker with different degrees of specificity is an important aspect of the environmental monitoring. Metals emanating either from natural sources or from anthropogenic activities interact with aquatic organisms as a mixture of more than one metal or in combination with organic pollutants. However, laboratory investigations based on single metal exposure do not offer a realistic empirical model. Therefore, biochemical interactions would determine the outcome of the toxic response. In this context, the present study has been carried out in order to investigate biomarker responses as an early warning signal in pollution assessment. Natural populations of *A. fasciatus* are widely distributed in coastal and brackish-water habitats along the South-Eastern coast of Tunisia and exposed to a mixture of pollutants. Our work was conducted in different sites of Tunisian coast in order to assess and compare the pollution profile.

A specific attribute of *A. fasciatus* is that it has no economic use, and thus populations are not manipulated, but rather reflect natural responses to the environmental disturbance. Interactions between abiotic and biotic factors are more common in the aquatic environment. Among abiotic factors, oxydant stress is a physiological disorder in animals, which directly affects the metabolism, resulting in the accumulation of ROS (Halliwell and Gutteridge, 1999). Changes in the metabolism of Zn and Cu can also lead to disorders in the antioxidant defence system (Kulikowska-Karpinska and Moniuszko-Jakoniuk, 2001). Although many studies emphasize the affinity of heavy metals such as Cu, Zn and Cd with respect to the liver, storage capacity and regulation of these metals have been widely described in fish (De Boeck *et al.*, 2004; Oliveira Ribeiro *et al.*, 2005). Our present work revealed that the oxidative responses, as well as the antioxidant

potential of fish, differed according to tissues (liver and gonad) and habitat behavior. Levels of metals (Cd, Zn and Cu) and PAH were also found to be variable between sites. The comparative analysis among the different studied sites revealed that S2 was the most polluted. This site was precisely the one from which a high proportion of deformed *A. fasciatus* specimens were captured; a circumstance attributed to the dual effects of both organic and inorganic pollution (Kessabi *et al.,* 2010). In a recent paper, researchers reported that fishes captured from this area presented physiological alterations (Messaoudi *et al.,* 2009*a*, 2009*b*). In our study, biochemical analyses suggest that changes could be in response to oxydative stress. Our results provide the presence of a significant difference between MTs and PSH contents in studied populations as well as in the biological indexes progression (HSI, GSI and K-factor). The significantly highest values of both HSI and GSI noted in S4 and S3 relatively to S1 and S2, while we have not investigated the reproductive cycle in the studied *A. fasciatus* populations; tends to suggest a lag in the gonad maturity during the sampling period.

Female's *A. fasciatus* were collected in April that Leonardos and Sinis (1998) consider the period of the higher production of gametes. Furthermore, during this period (gametogenesis), fishes are expected to have an elevated metabolic activity and energy reserves are mobilized for the egg production. The oscillations in oxidative stress markers observed in *A. fasciatus* gonads may be associated with the biotic parameters (e.g. degree of maturation and the reproductive cycle (Annabi *et al.,* in process); synthesis of some hormones might be involved in the expression of antioxidant markers. In addition, high values of MTs noticed in the liver tissue were observed in females captured from S1 which is characterized by the lowest contents of Cd and PAHs. Indeed, during this period of sampling (as mentioned above), females were supplied with nutrients and therefore store their necessary reserves to their metabolic requirements for reproduction (essential trace elements, e.g Calcium). In this direction, Cosson and Amiard (1998) reported that MTs, for example, can also be induced by certain biotic factors (e.g vitamins, hormones). Metallothioneins have high affinity for such metals (mercury (Hg), Zn, Cu and Cd), but their expression may be regulated by many different factors, both extrinsic (metals, temperature) and intrinsic (glucocorticoids, cytokines) (Coyle *et al.,* 2002, Haq *et al.,* 2003).

Several studies demonstrated that Mt levels in a given tissue and species may depend not only on the environmental conditions, but also on the particular physiological status of the analyzed organ (Bourdineaud *et al.,* 2006, Quirós *et al.,* 2007b, Navarro *et al.,* 2009). In our previous study (Annabi *et al.,* 2012), we reported that hepatic vitellogenin (VgA) mRNA levels were 1000 to 100 000 times higher in females than in males, consistent with the mature stage of *A. fasciatus* females in spring. In addition, Mt isoforms (MtA and MtB mRNA) did show significant variation among studied populations and MtA mRNA was more abundant in the ovary than in the testis. Smaoui-Damak *et al.* (2009) revealed that, unlike Cd, sex does affect MT concentrations. The influence of sex on MTs could be explained by the role played by these proteins in the homeostasis of the essential metals (Cosson, 2000; Isani *et al.,* 2000; Roesijadi, 2000). Additionally, the increase in protein oxidation, detected by the decreased levels of PSH in the hepatic tissue of populations from S2, S3 and S4, could be involved in protein redox regulation (Ghezzi, 2005). The absence of difference among TBARS contents among *A. fasciatus* populations for both tissues (liver and gonad) are perfectly in agreement with previously published research works.

However, Machreki-Ajmi *et al.* (2008) revealed a significantly spatial variation of MDA concentrations in the digestive gland of cockles *Cerastoderma glaucum* captured from different sites in the Golf of Gabes. Jurczuk *et al.* (2004) reported that in rats the increases in MDA concentration in the liver and kidney following metal exposure (Cadmium) indicates an escalation of lipid peroxidation whereas Pascual *et al.* (2003) showed that a continuous increase in MDA levels from 21st day of experiment in gilthead seabream (*Sparus aurata*) did not correlate with variations of specific hepatic activities of the antioxidant enzymes analyzed (superoxide dismutase, catalase, glutathione S-transferase, total glutathione peroxidase, selenium-dependent glutathione peroxidase and glutathione reductase). *Channa punctata* exposed to a mixture of metal caused, in gills tissue, a time-dependent significant increase in lipid peroxidation (LPO) with the highest increase on day 30 (Pandey *et al.*, 2008). These data were in accordance with the conclusions of Oakes and Kraak (2003) who reported that the TBARS method could overestimate the actual levels of MDA due to potential reactions with β-unsaturated aldehydes, cyclic peroxides, and other contaminants. However, it is considered as an effective measure of the oxidative stress since several other thiobarbituric acid reactive aldehydes are also the product of LPO (Hermes-Lima and Story, 1995).

Conclusion

To our knowledge, this study is unique in reporting variation of MTs, PSH and TBARS contents in hepatic and gonadic tissues of *A. fasciatus* collected in Tunisian coast. We could hypothesize that the variation on these antioxidant defenses might be associated to a pro-oxidative condition. The inclusion of bio-indicator species, such as the Mediterranean killifish, in areas undergoing urban and industrial development is an important part of conservation studies and ecological monitoring. It will enable us to predict the pollution profile of an aquatic habitat viable for fishery activities. It will also contribute to the development of a battery of mechanism-based on biochemical assays useful to characterize the complex mixture of chemicals in different potentially toxic environments and thus enhance our ability to assess the long-term risk of environmental contaminants to species perenity and human health. Field studies that evaluate several biomarkers, including enzymatic oxidative stress biomarkers, EROD and vitellogenin, are under progress to determine the potential of Mediterranean killifish as an indicator species of sublethal stress in multipollution contexts.

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