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**EVALUATION OF ENVIRONMENTAL IMPACT ON NATURAL  
POPULATIONS OF THE MEDITERRANEAN KILLIFISH *Aphanius fasciatus*  
BY QUANTITATIVE RNA BIOMARKERS.**

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**Abstract**

The Mediterranean killifish, *Aphanius fasciatus* (Valenciennes, 1821) represents an excellent sentinel species for analysis of environmental impact in coastal areas.

Quantitative changes on liver mRNA levels of five stress-related genes - metallothionein (metal exposure), vitellogenin, (estrogenic effects), cytochrome P4501A (CYP1A, dioxin-like compounds), superoxide dismutase (oxidative stress) and HSP70 (general stress)- were tested as markers of exposure to pollutants both in natural populations and in experimental treatments with model effectors. Analysis of fish from a metal-contaminated site in the Tunisian coast (Sfax) indicated that fish presenting spinal deformities (eight times more frequent at this site than in reference sites) showed increased levels of hepatic CYP1A mRNA levels compared to non-deformed fish from the same site or from the reference Louza site, suggesting a combined impact by metals and organic pollutants. Due to the strategy used in their design, these quantitative RNA markers will likely be useful to analyze environmental impacts on other related fish species.

Keywords: Cyprinodontiformes; brackish waters; quantitative Real-Time Polymerase Chain Reaction (qRT-PCR); pollution markers; mRNA quantitation; gene expression; Western Mediterranean Sea; sentinel species.

## 1. Introduction

The Mediterranean killifish, *Aphanius fasciatus* (Valenciennes, 1821) is a small cyprinodont fish inhabiting brackish waters in coastal habitats of the central and eastern Mediterranean Sea. It tolerates a wide range of physico-chemical parameters, such as temperature (5–39°C) and salinity (0–180 g/L). Although migration of individuals from one habitat to another is possible, it is considered a non-migratory species, as populations from relatively close habitats show evidences for genetic isolation ((Triantafyllidis et al., 2007) and references therein). Therefore, *A. fasciatus* represents an excellent sentinel species for analysis of environmental impact in coastal lagoons, estuaries and river deltas in the highly anthropized Mediterranean Sea.

The use of small animals as sentinels for environmental pollution has to overcome the difficulty of obtaining enough biological material for biochemical analyses. In this context, markers based on RNA quantification methods, such qRT-PCR, provide a suitable alternative, as they only require minute amounts of sample (Piña et al., 2007; Rotchell and Ostrander, 2003; Valasek and Repa, 2005). These techniques have proved their usefulness for monitoring of different types of environmental pollutants, like estrogens (Barucca et al., 2006; Garcia-Reyero et al., 2004; George et al., 2004), metals (Cheung et al., 2004; Navarro et al., 2009; Tom et al., 2004), and organic substances (Eljarrat et al., 2008; Quirós et al., 2007a; Quirós et al., 2007b). It is clear that biomarkers based on mRNA levels only reflect the initial steps of a cascade of events linking environmental insults to ecological impact (van der Oost et al., 2003). However, they are very useful when the specific nature of the pollutants (or of their primary effects) is unknown, or when dealing with very small animals or tissue portions (Piña et al., 2007). However, gene expression results should be further supported by parallel biochemical, immunological and/or morphological/pathological data, when possible.

The main disadvantage on the use of mRNA markers for environmental monitoring is the requirement for the sequence of test and reference genes in the impacted species. In this work, we develop mRNA markers for monitoring *A. fasciatus* exposure to different kinds of pollutants, using known DNA sequences from evolutionarily related species (mainly Cyprinodonts and Percomorphs). Genes used in this analysis included the main metal-responsive metallothionein (MT) gene (Cheung et al., 2004; Navarro et al., 2009; Schlenk et al., 1995; Tom et al., 2004; Van Cleef-Toedt

et al., 2001), the egg-forming, liver-expressed vitellogenin (Vtg), a known marker for estrogens (Barucca et al., 2006; Denslow et al., 2001; Garcia-Reyero et al., 2004), the dioxin-like responsive Cytochrome P4501A (CYP1A, Chung-Davidson et al., 2004; Craft et al., 2001; Quirós et al., 2007a; Quirós et al., 2007b), the oxidative stress-related gene Superoxide dismutase (SOD, Cho et al., 2006; Gonzalez et al., 2006; Pandey et al., 2003), and the protein chaperone HSP70, which are involved in the processing of missfolded proteins due to different kinds of stress (Carnevali and Maradonna, 2003; Maradonna and Carnevali, 2007; Schroder et al., 2000; Varo et al., 2002). In addition, probes for two housekeeping genes ( $\beta$  actin and the elongation translation factor EF1) were also designed and tested for suitability as reference genes (Olsvik et al., 2005).

These quantitative RNA markers were used to monitor environmental impact in two natural *A. fasciatus* populations from the coast of Tunisia, one close to the industrialized coast of Sfax and a second one in the undisturbed Luza area (Kessabi et al., 2009; Messaoudi et al., 2009b). The Sfax population presents eight times higher incidence of spinal deformities (including scoliosis, lordosis, and kyphosis from the head to the caudal fin) than reference sites (Kessabi et al., 2009; Messaoudi et al., 2009b). Deformed fish from Sfax accumulate more Cd than the non-deformed ones, suggesting a direct correlation between Cd accumulation and susceptibility to spinal deformations.

The purpose of this work is to develop and analyze mRNA markers of pollutant exposure in *A. fasciatus*, and use them to explain the high proportion of deformed fish in the Sfax population. We consider that this may be a general approach to study environmental impacts in natural populations of nonstandard species for which no appropriate DNA sequences are available.

## 2. Materials and Methods

### 2.1 Fish sampling

Sampling sites were selected in the Gulf of Gabès, in the South Eastern coast of Tunisia (Fig. 1). The industrialized coast of Sfax (S1), surrounded by important industrial activities, mainly crude phosphate treatments and chemical industries, and contaminated with Cd and other heavy metals, was chosen as a polluted site. The coast of Luza (S2), located  $\approx$  50 km north of S1 and apparently unaffected by human activities, was used as

reference site.

*A. fasciatus* specimens were captured in coastal waters (0.5–1m depth) by hand nets during May 2009, anesthetized in ice and its length and weight measured. Condition factor was calculated as the ratio of weight in grams to the cube of the length in cm X 100. Sex determination was performed by visual inspection of the bands along the body (colour pattern). As the number of male specimens captured in the current sampling effort was very low (a total of 3 fish), the study was performed using only females. Both normal (8 animals from Luza and 8 animals from Sfax) and deformed fish (10 animals from Sfax) were dissected and liver samples were placed in a cryogenic vial with 1 mL RNAlater (Sigma-Aldrich, St. Louis, MO), transported to the laboratory on ice, and stored at -80°C for mRNA analysis.

## 2.2 Induction experiments

Normal *Aphanius* specimens (females, mean total length  $3.61 \pm 0.61$  cm; mean body weight  $0.54 \pm 0.42$  g) were captured from the reference Luza area (S2). The specimens were placed in 15L fish tanks in groups of 10, and acclimatized for 2 weeks in clean seawater under natural conditions of photoperiod and temperature and fed once per day with tetramin. Treatments with cadmium (10mg/L), estradiol (100nM) and beta naphthoflavone (1 $\mu$ M) were performed by adding appropriate amounts of each compound in water. No mortality occurred during exposure. Animals (10 fish per time and treatment) were sacrificed after 0h, 24h and 48 h of exposure, and liver samples were removed and stored in RNA later.

## 2.3 Sequence analysis and primer design

DNA sequences from  $\beta$  Actin, elongation factor 1 (EF1), metallothionein (MT), Vitellogenin (Vtg) Cytochrome P4501A (CYP1A), Superoxide dismutase (SOD), and heat-shock protein (HSP70) were obtained using the SRS tool from the European Bioinformatics Institute (<http://www.ebi.ac.uk/>). Conserved sequence regions were derived from the alignment of known Cyprinodonta sequences when available, and supplemented with Percomorpha sequences when necessary, using the EMBOSS EMMA tool (Rice et al., 2000). Gene specific primer sets were designed from consensus sequences using the Primer Express 2.0 software (Applied Biosystems,

Foster City, CA)

#### 2.4 RNA extraction, sequencing, and qRT-PCR analysis

Total RNA was isolated from the tissues using Trizol reagent<sup>®</sup> protocol (Invitrogen). RNA concentration was measured by spectrophotometric absorption at 260nm in a NanoDrop ND-8000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) and the quality checked by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara CA). RNA was treated with DNaseI to remove genomic DNA contamination. Primers for seven genes were deduced from existing sequences in other cyprinodonts ( $\beta$  Actin, EF1, MT, Vtg, CYP1A, SOD and HSP70) and used to amplify the corresponding cDNA fragments. The PCR products (amplicons) were sequenced in an Applied Biosystems 3730 DNA Analyzer (Applied Biosystems), and searched for homologous genes in GenBank by the BLAST algorithm at the NCBI server (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>, see primer sequences in Table 1). The sequences obtained in this process (minimum, three independent clones) were deposited in GenBank (see Table 1 for accession numbers). Quantities from 1 $\mu$ g to 100ng of DNaseI-treated RNA were reverse transcribed to cDNA using First Strand cDNA Synthesis Kit from Roche (F Hoffmann-La Roche, Basel, Switzerland) and stored at -20°C. Aliquots of 100 ng of total RNA were used to quantify specific transcripts in LightCycler<sup>®</sup> 480 Real-Time PCR System (Roche) using SYBR<sup>®</sup> Green Mix (Takara Bio INC, Siga, Japan) and the primer pairs listed in Table 1. Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves ( $C_p$ , calculated by triplicates).  $C_p$  values for stress-related target genes (tg) were compared to the corresponding values for a reference gene (ref) to obtain  $\Delta C_p$  values ( $\Delta C_p = C_{p\text{ref}} - C_{p\text{tg}}$ ).  $\beta$  Actin was selected as reference gene after examination of its variability among all available samples (section 3.1). PCR efficiency values for reference and tested genes were calculated using at least five serial 1:3 dilutions for each gene (Pfaffl, 2001). When necessary, primers were redesigned to obtain efficiency values better than 95% for all tested genes. To facilitate reading of tables and graphs, mRNA abundance values are represented as mRNA copies of target gene per 1000 copies of the reference gene mRNA (% of reference gene,  $1000 \times 2^{\Delta C_p}$ ).



## 2.5 Statistical analysis

Statistical tests were performed using the SPSS 17 (SPSS Inc., Chicago, Ill) package. All statistical calculations were performed using  $\Delta C_p$  values, as this parameter followed normal distributions, as assessed by the Kolmogorov–Smirnov test.

## 3. Results

### 3.1.- Cloning and Sequence of stress-related genes from *A. fasciatus*

DNA fragments from putative  $\beta$  actin, EF1, Vtg, CYP1A, SOD and HSP70 genes were amplified by PCR from *A. fasciatus* liver cDNA (Table 1). BLAST analysis confirmed the close sequence homology of these amplicons with the corresponding genes in different fish species, including representatives from different Cyprinodontiformes families (Cyprinodontidae, Fundulidae, Poeciliidae, etc.) and some Perciformes (Sparida, among others). This result indicates the high evolutionary conservation of the amplified fragments.

### 3.2.- Validation of stress genes as exposure markers.

Translation elongation factor EF1 and  $\beta$ -Actin were tested for mRNA abundance stability in a large set of *A. fasciatus* RNA samples (84 independent qRT-PCR reactions).  $\beta$ -Actin (average  $C_p = 19.31 \pm 0.72$ ) showed a significantly lower variation than EF1 (average  $C_p = 17.82 \pm 1.22$ ), and it was therefore selected as reference gene for all subsequent experiments.

Exposure of control *A. fasciatus* individuals to model substances (Cd for metals, 17- $\beta$  estradiol for estrogens, and  $\beta$ -naphthoflavone as a surrogate for dioxin-like substances) resulted in an increment of liver mRNA abundance of the target genes for each substance. MT mRNA levels were increased approximately 200-fold upon Cd exposure after 24 h of exposure, but this response faded by 80% after a further 24 h (Figure 2A). In contrast, Vtg mRNA levels remained essentially unchanged in liver during the first 24 h of treatment with estradiol, whereas a 48 h-length exposure resulted in a more than 100x induction of the transcript (Figure 2B).  $\beta$ -naphthoflavone acted as a mild CYP1A inducer, increasing mRNA levels of this gene twofold in 24h and by an

extra twofold after a further 48 h of exposure (Figure 2C). These behaviours are typical for these inducers in fish species (Piña et al., 2007) and corroborate the cloned fragments as encompassing *bona fide* MT, Vtg and CYP1A gene sequences from *A. fasciatus*. The different induction kinetics for the tested mRNAs is likely related to regulatory processes affecting transcription of the corresponding genes; it is also likely that the protein products codified by those genes remain active for longer periods of time than mRNA.

### *3.3.-Biometric parameters and mRNA abundance of stress genes in natural A. fasciatus populations.*

*A. fasciatus* females were captured in Luza (control site,  $n=9$ ) and in Sfax (polluted site,  $n=18$ ). Ten out of the 18 animals captured in Sfax showed apparent spinal deformities, a phenotype long associated to this site (Messaoudi et al., 2009b). Animals from Luza were longer ( $6.0\pm0.7$  cm versus  $4.5\pm0.7$  cm) and heavier ( $3.0\pm1.2$  g versus  $1.4\pm0.7$  g) than animals from Sfax, either normal or deformed (Figure 3A). No difference in condition factor (CF) was observed between normal fish of both sites, suggesting that the size variability did not alter the animal proportions. The increased CF value observed for deformed animals ( $1.6\pm0.2$  versus  $1.3\pm0.2$ , Figure 3A) is likely related to the deformation itself, which shortens the animal without changing its weight.

Two of the five analyzed genes showed significant variations in mRNA abundance among the three fish populations. Animals from Luza showed elevated (about 4-fold, Figure 3B) levels of MT transcript relative to normal or deformed fish from Sfax, whereas the CYP1A transcript was 10 times more abundant in deformed fish for Sfax than in normal animals from either site (Figure 3B). Very high and uniform levels of Vtg was observed in all three sites, which is consistent with the period of capture, close to the spawning season (June-July), and suggests that differences in size and ecological impact between the two sites did not affect the female reproductive cycle (Figure 3B), although more extensive studies are required to corroborate this hypothesis.

Bivariate correlation analyses between mRNA transcript levels for different genes and biometric parameters showed that SOD and CYP1A were the only two genes whose transcript levels appeared mutually correlated (Figure 4A). In addition, CYP1A mRNA levels in different fish correlated with the corresponding CF values (Figure 4B).

This correlation applied especially to samples from Sfax, either deformed and normal (grey and black circles in Figure 4B), suggesting a link between the extent of deformity of impacted fish and their exposure to CYP1A-inducing pollutants (dioxins, dioxin-like PCBs, PAHs, etc.) in the environment.

The strong correlation between MT transcript abundance and size and weight of the animal shown in Figure 4C was surprising, but consistent with the high MT mRNA levels found in the Luza population (Figure 3A). Figure 3C shows estimates of mean transcript levels for each gene and population after suppressing the effect of the differences in weight, calculated by ANCOVA. This calculation indicates that all the differences in MT transcript abundance between fish groups can be explained by differences in fish size. Although statistical correlation cannot be taken as an indicator of cause-effect relationship, the results do suggest that MT transcript levels in this population were related to some unknown physiological parameter linked to size and weight rather than to the presence of Cd or other metals in the environment. In contrast, the 10-fold higher CYP1A transcript abundance in deformed fish appeared unrelated to fish weight (Figure 3C).

#### 4. Discussion

Use of markers based on mRNA quantification techniques has many advantages for environmental monitoring of natural populations. These techniques require very small amounts of samples (less than 100 mg of tissue is often enough for performing hundreds of tests (Piña et al., 2007)), permitting the use of small species or small pieces of tissue (Fossi et al., 2000; Quiros et al., 2007). In combination with RNA stabilizing agents, samples can be stored and shipped with minimal or no refrigeration, allowing sampling in remote sites. Total mRNA contains information of the whole transcriptome of the sample; as it can be stored indefinitely at -80°C, this allows re-evaluation of old samples if a new potentially interesting marker is developed. And last, comparison of multiple markers (theoretically, the whole transcriptome) from a single sample allows a complete analysis of the effects of environmental changes at the transcriptional level to define regulatory networks of genes involved in different stress responses.

The main disadvantage on the use of mRNA markers for environmental monitoring is the requirement for the exact DNA sequences of test and reference genes in the impacted species. In most cases, species abundant in interesting sites are poorly

known, and our current genetic knowledge of many species of high environmental relevance is still scarce. To apply these techniques to *A. fasciatus*, we used known DNA sequences from evolutionarily related species (mainly Cyprinodontiformes and Perciformes) to find conserved regions of the desired genes and to derive qRT-PCR primers out of them. Due to the high conservation of the gene regions used in the process, these primers are likely useful for other related Cyprinodont species.

The response of the putative *A. fasciatus* MT, Vtg and CYP1A genes to the presence of model effectors was similar to the one observed for these genes in other better known fish systems (Piña et al., 2007). Estradiol induced Vtg mRNA some 100-fold after a lag period, whereas MT transcript peaked at 24 h of Cd exposure and reduced its abundance afterwards.  $\beta$ -naphthoflavone appeared as a weak, but steady inducer of CYP1A. The transient effect of Cd (and other heavy metals) in liver MT transcript abundance has been observed in different systems (Gonzalez et al., 2005; Quirós et al., 2007b). In natural fish populations, metal chronic exposure usually results in elevated MT mRNA levels in kidney and other tissues, but not necessarily in the liver (Hayes et al., 2004; Navarro et al., 2009; Quirós et al., 2007b; Tom et al., 1999). This may well be the case for *A. fasciatus*, as we did not observe an increment of MT mRNA levels in the Cd chronically exposed Sfax fish population. Rather, we found a direct correlation between MT transcript levels and fish size and weight, for which we do not have any explanation. The smaller size and weight of Sfax animals could in principle be related to pollution, although some aspects of our data indicate otherwise. A surprisingly conclusion of our data is the relatively good health status of fish in Sfax, including deformed ones. They showed CF values similar to, if not higher than (see below) those from Luza sites, they seem to spawn normally, and HSP70 transcript levels, a marker for general stress (Schroder et al., 2000), were identical in both sites. These data suggest a specific etiology for spinal deformities, rather than a massive toxic effect.

The fish sampling coincided with the pre-spawning period, a circumstance reflected in the very high levels of Vtg transcript present in all three fish groups. These levels were not affected by the approximately two-fold differences in weight between Luza and Sfax fish populations. This suggests that the smaller fish in the impacted area, including deformed ones, were spawning synchronously with the larger Luza fish. The likely conclusion is that, whatever the pollution impact that determines the deformed phenotype may be, it did not affect the Vtg synthesis in liver. Possible effects in other

reproductive functions in the impacted animals remain to be elucidated.

The only mRNA biomarker clearly differentiating deformed from normal fish is their 10 times higher CYP1A mRNA levels. This gene, and the enzymatic activity associated to its protein product, EROD, are classic markers of exposure to dioxin-like pollutants, like dioxins, other organochlorine compounds and polycyclic aromatic hydrocarbons (van der Oost et al., 2003; Whyte et al., 2000). Hepatic CYP1A mRNA abundance has been directly correlated to the presence of organic pollutants in different fish species (Jarque et al., 2010; Quirós et al., 2007b; Roberts et al., 2005; Roling et al., 2004). In contrast, metals tend to suppress, rather than induce, CYP1A expression (Bruschweiler et al., 1996). Therefore, our data suggest that the elevated CYP1A transcript levels were not related to the known metal pollution in Sfax, but to still uncharacterised organic pollutant(s). The correlation between individual levels of CYP1A and SOD transcripts may also be related to this putative environmental impact, as SOD is a classical marker for oxidative stress affected by many pollutants, both organic and inorganic (Bainy et al., 1996; Hansen et al., 2007; Peters et al., 1994; Porte et al., 2002).

High CF values are usually associated to healthy, well-fed fish, under the assumption that heavier fish of a given length are in better condition than leaner ones (Bervoets and Blust, 2003; Fulton, 1904). However, spinal deformation decreases the animal length; therefore, deformed fish would have higher CF than normal ones, provided deformity does not impair feeding capacity. Surprisingly enough, this seems to be the case, as normal and deformed fish from the same area were very similar in weight. Under these circumstances, CF can be considered as a measure of the extent of the deformity, and its correlation with CYP1A transcript is consistent with a possible link between abnormal animal shape and exposure organic pollutants.

Spinal deformities have been reported in natural fish populations (Ayed et al., 2008; Kessabi et al., 2009; Messaoudi et al., 2009a; Messaoudi et al., 2009b; Sun et al., 2009; Tutman et al., 2000) as well as in farmed fish (Aunsmo et al., 2008; Aunsmo et al., 2009; Bardon et al., 2009; Silverstone and Hammell, 2002). Whereas skeletal malformations in aquaculture appear to be related to a complex interaction of genetic, procedural and infectious/immunological factors (Aunsmo et al., 2008; Aunsmo et al., 2009; Bardon et al., 2009; Lall and Lewis-McCrea, 2007), the presence of deformities in natural populations are most likely related to pollution (Kessabi et al., 2009; Lemly, 2002; Messaoudi et al., 2009a). Spinal deformities can be experimentally induced in

fish upon exposure to different organic and inorganic substances, including metals, metalloids and organic substances like PAHs, OC and dioxins (Cachot et al., 2007; Gagnon and Rawson, 2009; Holth et al., 2008; Miller et al., 2007; Yamauchi et al., 2006). In the case of *A. fasciatus* Tunisian populations, deformed animals show a high content in different heavy metals, especially Cd (Kessabi et al., 2009). Whereas the effect of heavy metals in fish bone density and malformations is well reported (Bengtsson and Larsson, 1986; Cheng et al., 2000), our data suggest that the observed high incidence of adult, deformed fish in the Sfax area results from a combination of organic and metal pollution. We are aware that the present study is only a first approach on the characterization of the physiological processes linked to the appearance of spinal deformities in the examined populations; additional information on either activity and or protein levels would further support and possibly clarify RT-PCR data.

We consider that the interest of the analysis performed here is not restricted to a specific problem in the Tunisian coast. Brackish water bodies, like deltas, estuaries, marshes and coastal lagoons are severely endangered in the Mediterranean Sea and elsewhere, both by the increasing population pressure and by the impending raise of sea levels. Cyprinodontiformes are often found in these environments, and constitute therefore excellent sentinel species of environmental impact. With qRT-PCR probes likely adaptable to any Ciprinodontiform species and RNA transport and quantification procedures that allow the preservation and precise analysis of milligram-size tissue samples, the markers and the techniques shown here cope with the three main problems intrinsically present in this type of studies: largely unknown fish species of very small size and captured in remote areas.

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## Figure legends

Figure 1.

Localization of sampling sites in the Gulf of Gabès (Tunisia).

Figure 2

Transcript level response to the presence of cognate effectors for metallothionein (MT, Cd, left), vitellogenin (Vtg, 17- $\beta$ -estradiol, center) and Cytochrome P4501A (CYP1A,  $\beta$ -naphthoflavone, right). Data are presented as mRNA copies for 1000 copies of  $\beta$  Actin mRNA. Asterisks indicate significant differences from controls (Student's T-test): \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

Figure 3

Biometric parameters (A) and mRNA levels of stress genes (B) in natural *A. fasciatus* populations from Luza (empty bars) and Sfax sites (grey bars, normal animals, solid bars, deformed ones). Transcript level data are presented as number of mRNA copies for 1000 copies of  $\beta$  Actin mRNA. Low case "a" and "b" letters correspond to statistically homogeneous groups (Tukey's), only shown for those data with significant variations ( $p < 0.05$ ) among the three groups of animals (ANOVA). Panel C shows estimates of mean (columns) and 95% confidence intervals (bars, only the upper limit is shown) of mRNA copy number values after adjusting weight to the central value (2.0523g, ANCOVA). In this case, "a" and "b" letters indicated differences on the estimated values for each population (F test with Bonferroni correction,  $p < 0.05$ )

Figure 4

Graphic representation of significant correlations between biometric parameters and/or mRNA abundance values, evaluated by bivariate correlation tests. Dots represent individual fish from Luza (open triangles), Sfax normal animals (grey circles) and deformed ones (solid circles). Statistical significance of the correlations is indicated for each graph.

Table 1. qRT-PCR Primers and GenBank accession numbers of the corresponding *A. fasciatus* sequences developed in t

Gene	Acronym	Accession #	Forward primer (5'-3')	Reverse primer (5'-3')
Metallothionein	MT	HM017068	GCGAGTGCTCCAAGACTGG	TGGGCAGCATGAGCA
Vitellogenin	Vtg	HM017070	GACTGGATGAAAGGAAAGACCTGT	ACGCGTCCGTTGGGA
Heat-shock protein 70	HSP70	HM017074	TTTTATCCTGGTGGTGGC	GCCCTCTTGTTGTCTC
Superoxide dismutase	SOD	HM017073	AGAGCATGGTTTCCATGTCCAT	CCAACTCACCAGGTCT
Cytochrome P4501A	CYP1A	HM017071	ACATCTCTGAATGGCTACTT	TTCTCTCCATCTAGCT
$\beta$ -Actin	$\beta$ -actin	HM017072	AGATCATTGCCCCACCAGAG	ACTCATCGTACTCCTG
Translation elongation factor 1EF1		HM017075	AGGAAATCCGTCGTGGATACG	TGACCTGAGCGTTGA

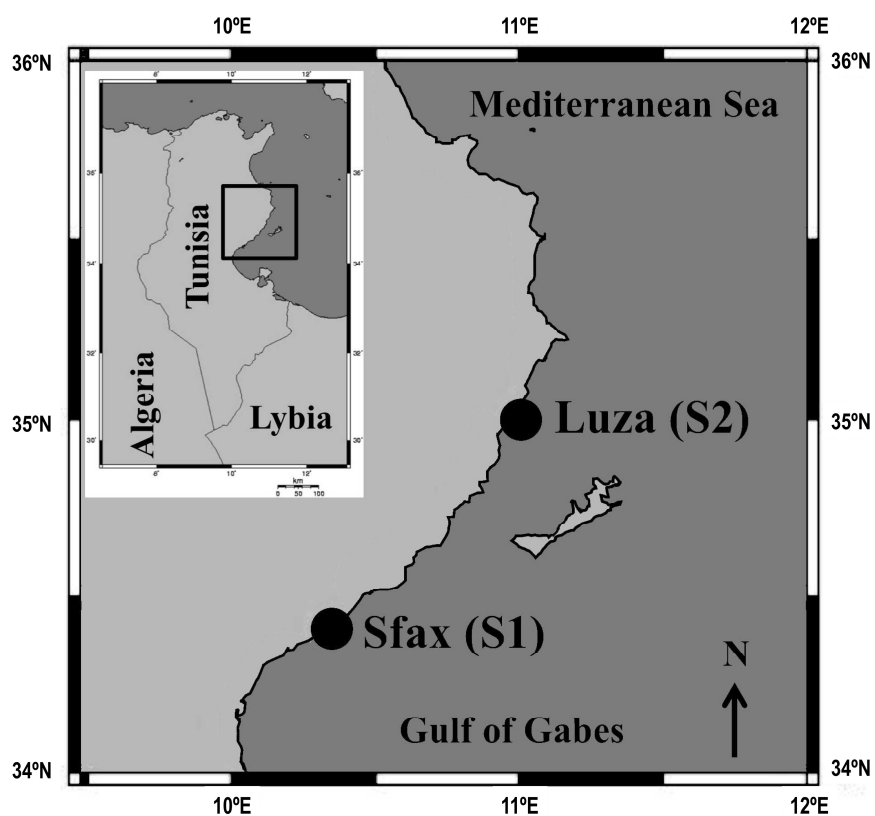


Figure 1

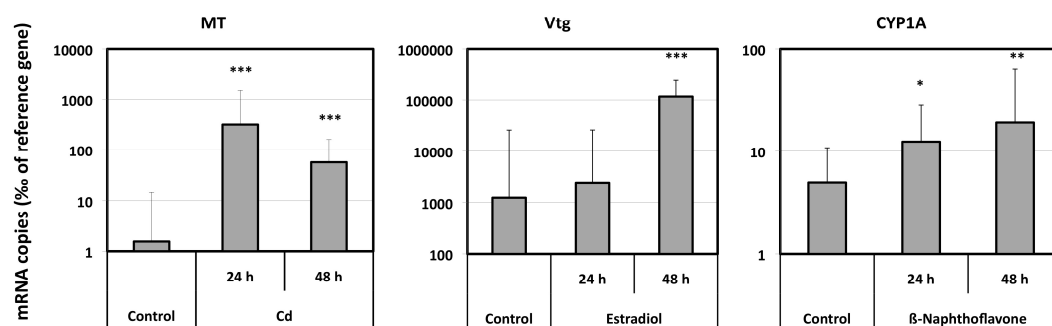


Figure 2

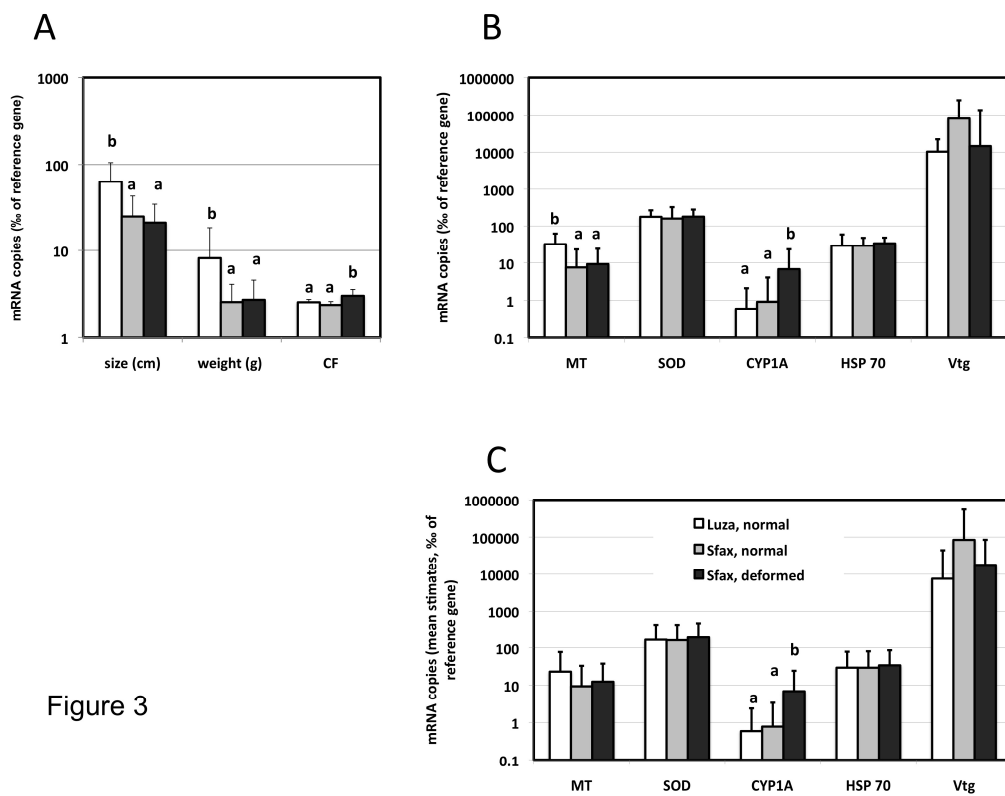


Figure 3



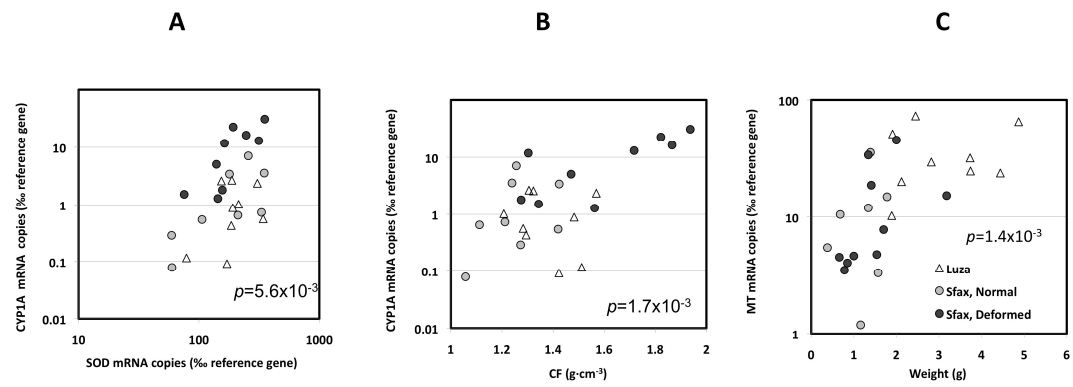


Figure 4