

Assessment of reproductive stress in natural populations of the fish *Aphanius fasciatus* using quantitative mRNA markers

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ABSTRACT: Four populations of the Mediterranean killifish *Aphanius fasciatus* were studied to evaluate the biological effects of environmental pollution. These effects were measured using RNA quantification-based markers in the gonads and liver. Metallothionein genes in both testes and ovaries showed elevated expression in males and females from the more contaminated areas. In addition, males from these areas showed elevated levels of vitellogenin synthesis in the liver. These results were correlated with heavy metals and polycyclic aromatic hydrocarbon levels in water and sediment samples from the sampling sites. They represent the first evidence of an incipient feminization of marine fish along the coast of Tunisia and confirm the utility of RNA quantification-based markers for monitoring environmental impact on small animals and in species for which very little biological information is available.

KEY WORDS: Environmental pollution · Brackish water · Coastal pollution · Endocrine disruption · Quantitative real-time polymerase chain reaction · qRT-PCR

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INTRODUCTION

The Mediterranean killifish *Aphanius fasciatus* (Valenciennes, 1821) is a small cyprinodont fish inhabiting brackish ponds and lagoons along the central and eastern Mediterranean coast. It tolerates a wide range of temperatures (5 to 39°C) and salinities (0 to 180 ppt). *A. fasciatus* spawns in batches from April to July, laying up to 500 eggs in each successive spawning, and reaches sexual maturity within a few months, at a total length of less than 2 cm (Leonardos & Sinis 1998). Due to its strong territoriality, it is considered a non-migratory species, as revealed by genetic analyses of populations from relatively close habitats (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.

Specific RNA quantification has become one of the most sensitive tools in molecular biology, especially since the introduction of quantitative real-time polymerase chain reaction (qRT-PCR), which allows detection and quantification of minimal amounts of RNA molecules. This high sensitivity allows both the application to small animals (such as *Aphanius fasciatus*) and the use of dispensable parts of the body (scales, blood and blubber), avoiding the killing of larger animals (Oleksiak et al. 2002, Rotchell & Ostrander 2003, Stefanelli et al. 2004, Valasek & Repa 2005, Piña et al. 2007, Arukwe & Re 2008, Oleksiak 2008, Pelayo et al. 2011). Analyzing changes of expression levels of known target genes in exposed individuals and populations is a convenient method to monitor exposure to different types of environmental pollutants, including estrogens (García-Reyero et al. 2004, George et al. 2004, Barucca et al. 2006), metals (Cheung et al. 2004, Tom et al. 2004,

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Navarro et al. 2009), and organic substances (Sarkar et al. 2006, Quirós et al. 2007a,b, Eljarrat et al. 2008).

The eastern coast of Tunisia hosts a mosaic of *Aphanius fasciatus* populations, which can be used to monitor the environmental impact on the area. This coastal region presents a blend of industrial, residential, seafaring, and leisure activities carried out at relatively proximal, if not coincident, areas. The Gulf of Gabès is under the influence of medium-sized towns such as Sousse and Monastir, several tourism resorts, fisheries, and industrial areas. The coast of Sfax is known for being polluted with high levels of heavy metals and other compounds, and fish from this area present physiological alterations, including spinal deformations (Kessabi et al. 2009, Messaoudi et al. 2009a,b). In recent papers, we developed different mRNA-based pollution markers for *A. fasciatus*, and demonstrated that a combination of metals and dioxin-like pollutants is the likely etiological cause of the very high prevalence of skeletal deformities in the Sfax killifish population (Kessabi et al. 2010, 2012).

Whereas spinal deformities are an obvious sign of environmental stress, the impact of pollution on the reproductive system may be considered as a key factor in the health status of the killifish populations (van der Oost et al. 2003, Kessabi et al. 2010). Expression of the egg-yolk protein vitellogenin in male liver is a well-known marker of exposure to feminizing substances in different fish species (Cajaraville et al. 2000, Matthiessen et al. 2002, García-Reyero et al. 2004). This protein is normally produced in female liver during oogenesis and it is transported to the ovaries through the bloodstream. Generally regarded

as unrelated to the reproductive system, metallothioneins constitute a family of proteins whose expression has been linked to exposure to heavy metals and, secondarily, to other forms of stress in essentially all animal species (Tom et al. 1999, Cajaraville et al. 2000, Bourdineaud et al. 2006, Sarkar et al. 2006). In the present study, we analyzed vitellogenin, metallothionein and other molecular markers using mRNA quantification techniques to describe the environmental impact on 4 *Aphanius fasciatus* populations from the Gulf of Gabès. Our data provide the first evidence of gonadal stress and endocrine disruption in these marine fish populations from the Tunisian coast.

MATERIALS AND METHODS

Study sites

Aphanius fasciatus (Cyprinodontidae; Cyprinodontiformes) were collected along the southeastern coast of Tunisia (Fig. 1). Sites were selected to cover different levels of human impact. Site S1 (Louza) is relatively free from industrial and human activities, although it cannot be properly regarded as a reference site (Kessabi et al. 2009, Messaoudi et al. 2009a,b). Site S2 lies close to the industrialized town of Sfax in the Gulf of Gabès. Shallow waters, weak currents, high salinity, strong tides, and temperature characterize this area. This ecosystem is chronically polluted by cadmium (Cd); its accumulation and the subsequent biological effects in fish local populations have only recently been evaluated (Annabi et al.

2009, Barhoumi et al. 2009, Kessabi et al. 2009, Messaoudi et al. 2009a,b). Site S3 is located at the mouth of Oued Hamdoun, an irregular freshwater course feeding the southern coastal region close to Sousse (170 000 inhabitants), and is considered an impacted site (Afli et al. 2008). A power station located close to S3 uses seawater for refrigeration, expelling heated water into the Oued. The last site (S4), located in the south of Monastir (Khniss zone), receives the impact of many industrialized dumps (especially from the textile industry) as well as urban discharges, which may constitute a significant source of pollution. Site location and water parameters of the 4 sampling sites are listed in Table 1.

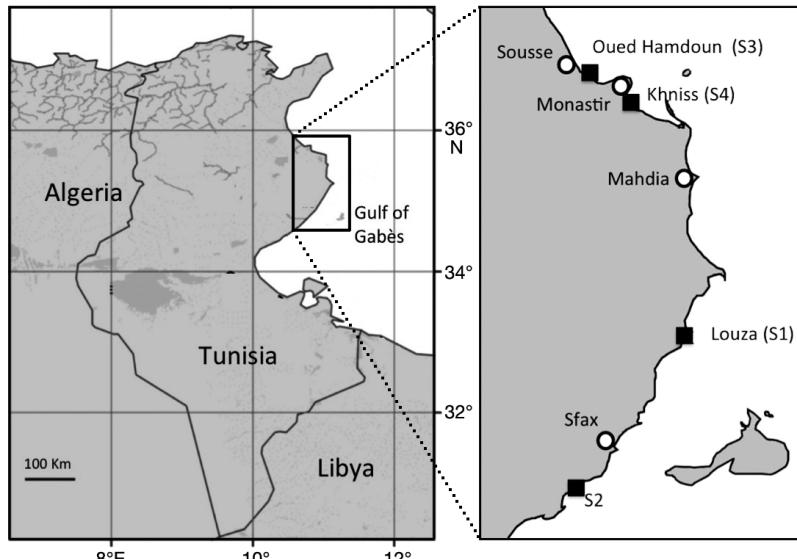


Fig. 1. Sampling sites (■)

Table 1. Description of sites and water parameters at the time of sampling

Site	Code	Latitude (°N)	Longitude (°E)	pH	Conductivity (mS cm ⁻¹)	Temperature (°C)	Dissolved O ₂ (ppm)
Luza	S1	35° 02' 63"	11° 01' 35"	8.1	63.7	24	8.4
Sfax	S2	34° 38' 08"	10° 39' 08"	7.98	60.7	23	8.5
Oued Hamdoun	S3	35° 47' 20"	10° 41' 00"	7.94	56.3	29	8.4
Khniss	S4	35° 42' 30"	10° 49' 30"	8.16	56	24	8.5

Field sampling

Adult male (>3.5 cm fork length) and female (>4 cm fork length) *Aphanius fasciatus* were captured with a hand-held net at the beginning of the spawning cycle in April 2009. Fish were euthanized, measured, and weighed, avoiding undue suffering. Fish sampling and studies were authorized by the Tunisian committee of Agriculture and Veterinary healthcare. Condition factor, an indicator of fish health, was calculated as $K = (W/L^3) \times 100$, where W is wet weight (g) and L is fork length (cm). Liver and gonad samples (50 mg) were placed in a cryogenic vial with 1 ml RNAlater (Sigma-Aldrich), transported to the laboratory on ice, and stored at -80°C for mRNA analysis.

For heavy metal analysis, water samples were collected from the same area at a depth of 50 cm in clean amber-colored bottles. Sampling bottles were previously cleaned by soaking in 10% nitric acid and rinsed with ultra pure water. Sediment samples (the upper 5 cm) were obtained as cores from each site and transported to the laboratory in a thermos flask with dry ice. Sediment samples for polycyclic aromatic hydrocarbon (PAH) analyses were collected in glass containers previously cleaned with distilled water, washed, dried with tetrachloride carbon, and stored at -20°C.

Chemical analysis

Metal determination

Sediment samples were oven-dried for 48 h at 100°C and 100 mg of each sample were mineralized at 250°C with a set of acids composed of 1 ml nitric acid, 2 ml fluorhydric acid, and 0.5 ml perchloric acid, and then adjusted to 10 ml with deionized water.

All samples were analyzed to determine Cd, copper (Cu), and zinc (Zn) concentrations using a graphite furnace atomic absorption spectrometry technique (ZEEnit 700). Samples were analyzed in

triplicate. The variation coefficient was usually less than 10%. Metal concentrations are reported as µg g⁻¹ dry weight for sediments and as µg l⁻¹ for water samples.

Polycyclic aromatic hydrocarbons

The content of PAHs in the sediment was determined by GC-MS in an HP-GC (Hewlett-Packard) equipped with a split/splitless injector as described previously (Baumard & Budzinski 1997, Mzoughi et al. 2002). PAHs were quantified relative to perdeuterated PAHs (Quilliam et al. 1994, Baumard & Budzinski 1997). The response factors of the different compounds were measured by injecting a standard reference material (SRM 2260 24 aromatic hydrocarbons in toluene, NIST) spiked with the same solution containing the perdeuterated PAHs as that used to spike the sediment samples. Analyzed compounds and their respective detection limits are listed in Table S1 in the supplement (available at www.int-res.com/articles/suppl/b017p285_supp.pdf). The following compounds were analyzed: acenaphthylene, acenaphthene, naphthalene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(a)pyrene, benzo(e)pyrene, benzo(g,h,i)perylene, and dibenzo[a,h]anthracene. Data are reported as µg PAH g⁻¹ dry weight.

Sequence analysis and primer design

Conserved sequence regions were derived from the alignment of known Cyprinodonta sequences when available, supplemented with Percomorpha sequences when necessary (Kessabi et al. 2010), using the EMBOSS EMMA tool (Rice et al. 2000). DNA sequences from β-actin, elongation factor 1 (EF1), metallothionein (MT), vitellogenin A (VgA) cytochrome P4501A (CYP1A), superoxide dismutase (SOD), and heat-shock protein (HSP70) were obtained using the

SRS tool from the European Bioinformatics Institute (www.ebi.ac.uk/). Gene-specific primer sets were designed from consensus sequences using Primer Express 2.0 software (Applied Biosystems).

RNA extraction, sequencing, and qRT-PCR analysis

Total RNA was isolated from the tissues using the Trizol reagent protocol (Invitrogen). RNA concentration was measured by spectrophotometric absorption at 260 nm in a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and the quality was checked with an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was treated with DNaseI to remove genomic DNA contamination. Quantities from 1 µg to 100 ng of DNase I-treated RNA were reverse transcribed to cDNA using the First Strand cDNA Synthesis Kit (F Hoffmann-La Roche) and stored at -20°C. Aliquots of 100 ng of total RNA were used to quantify specific transcript in a LightCycler 480 Real-Time PCR System (Roche) using SYBR Green Mix (Takara Bio). Seven out of the 8 primer pairs used in the present study have been described previously (β -actin, EF1, metallothionein A, vitellogenin, cytochrome P4501A, SOD, HSP70; Kessabi et al. 2010; see short names and amplicons in Table 2). A 124 bp long amplicon from a new *Aphanius fasciatus* metallothionein gene (MTB) was amplified and sequenced, and suitable primers were designed (Table 2). The responsiveness of this new MT gene to Cd was tested as described previously (Kessabi et al. 2010). Relative mRNA abundances of different genes were calculated from the second derivative maximum of their respective amplification curves (quantitative cycle or C_p ; calculated by triplicate), as defined by the manufacturer (Roche). C_p values for stress-related target genes (TG) were compared with the corresponding values for a reference gene (ref) to obtain ΔC_p values ($\Delta C_p = C_{p\text{ref}} - C_{p\text{TG}}$) (Pfaffl 2001). β -Actin was selected as a reference gene after examination of its variability among all available samples, as described previously (Kessabi et al. 2010). PCR efficiency values for reference and tested genes were calculated as close to 100% following the standard procedures (Pfaffl 2001, Kessabi et al. 2010), and appropriated RT-minus (no reverse transcriptase) controls were preformed to ensure that putatively remaining genomic DNA did not influence the results. 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, $1000 \times 2^{\Delta C_p}$).

Table 2. *Aphanianus fasciatus* DNA sequences sequenced and analyzed in the present study (including sequences from Kessabi et al. 2010). Upper case letters indicate the primers used for amplification

Statistical analysis

Statistical tests were performed using the SPSS 19 package. All statistical calculations were performed using ΔCp values. This parameter followed normal distributions, as assessed by the Kolmogorov-Smirnov test. Significant differences between populations were analyzed by ANOVA followed by a Tukey's B post hoc test, using 0.05 as significance limit.

RESULTS AND DISCUSSION

Pollution levels in sampled areas

Analytical data showed a different pattern of distribution for inorganic and organic pollutants. PAHs were more than 10 times lower in sediments from Louza (S1) than in sediments from the other 3 sites (Fig. 2; see a complete description of results in Table S1). These data, along with the low contents of Cd and Zn in water and sediments from S1, are in keeping with the consideration of site S1 as relatively free from human impact (Kessabi et al. 2009, Messaoudi et al. 2009a,b). However, Cu levels were significantly elevated in water and sediment samples from the 2 southern sites (S1 and S2) relative to the northern sites (S3 and S4; Fig. 2). Despite the high Cu levels found at site S1, there was no evidence of any phenotypic effect linked to Cu toxicity in the corresponding *Aphanius fasciatus* population. Site S2 (Sfax) appeared to be the most polluted site, showing the maximal concentrations of all pollutants analyzed (Cu,

Cd, Zn, and PAHs; Fig. 2). Fish captured at this site present a high incidence of spinal deformities (Messaoudi et al. 2009b, Kessabi et al. 2010). Our previous results related these high incidences of deformed animals to the dual effects of both organic and inorganic pollution (Kessabi et al. 2010, 2012). Table 1 shows the variations in pH, conductivity, temperature, and dissolved O₂ between sites, with conductivity higher at northern sites. S3 showed the highest temperature recorded, which is a likely consequence of the use of the water for cooling by a power plant.

The different levels of pollution between sites did not seem to affect the phenotype of the respective *Aphanius fasciatus* populations, besides the already mentioned high prevalence of deformed fish at site S2. Within a given population, males were significantly smaller than females, and fish from the Louza population (S1) were on average larger and heavier than the rest (Table 3). This is consistent with previous data from these populations (Kessabi et al. 2010). Animals from the 2 northern populations (S3 and S4) were on average significantly smaller than the rest, especially males (Table 3). Condition factor was similar for all male populations, whereas females showed some variability, with the S1 population having the highest average condition factors and the S3 population having the lowest values (Table 3). Deformed fish from site S2, which showed abnormally high condition factor values (Kessabi et al. 2010), were not included in this study.

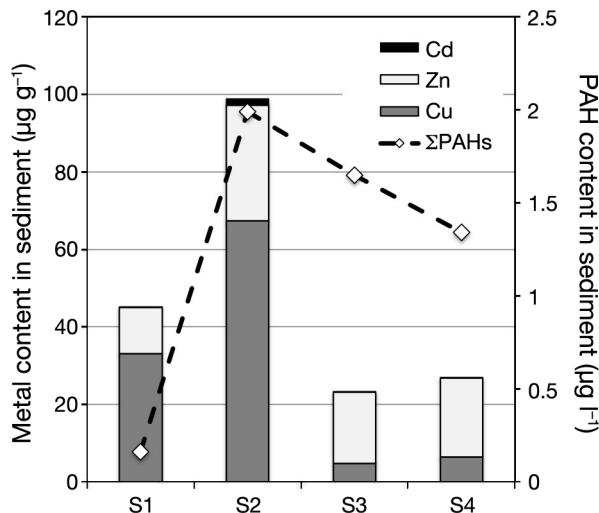


Fig. 2. Sediment levels of organic (polycyclic aromatic hydrocarbons [PAHs]) and inorganic (metals) pollutants at the 4 sampling sites

Stress genes in gonad tissue

Steady-state levels of mRNA for several stress-related genes (MTA and MTB, SOD, HSP70, and CYP1A) and for the egg-yolk protein VgA were measured in ovaries and testes of fish from the 4 studied populations. CYP1A mRNA levels were too low in both tissues to allow its precise quantitation, whereas SOD and HSP70 mRNA levels did not show any significant variation related to either population or sex (data not shown). VgA mRNA showed very low and similar levels in both sexes, reflecting its almost exclusively hepatic synthesis in Cyprinodonts (Fig. 3, see below) (Lattier et al. 2001, García-Reyero et al. 2004). In contrast, MTA and MTB mRNA did show significant variation among populations. MTA mRNA was more abundant in the ovary than in the testis, and its expression was maximal in populations from S2 (only ovaries) and S4 (both gonads). MTB mRNA showed a similar pattern, with significant peaks in S2 (both tissues) and S4 (only testes) populations. Compared with

Table 3. Mean (\pm SD) size, weight, and condition factor values for *Aphanius fasciatus* males and females captured at the 4 sampling sites. Different lower case letters (Tukey's) indicate significant differences (Tukey's B-test, $p < 0.05$) between sites

Site	n	Size (cm)	Tukey's	Weight (g)	Tukey's	Condition factor	Tukey's
Females							
S1	8	5.33 \pm 0.87	a	2.19 \pm 1.13	a	1.35 \pm 0.13	a
S2	8	4.53 \pm 0.66	b	1.26 \pm 0.52	b	1.27 \pm 0.13	a,b
S3	10	3.77 \pm 0.42	b	0.60 \pm 0.25	b	1.06 \pm 0.20	b
S4	7	4.10 \pm 0.28	b	0.83 \pm 0.32	b	1.15 \pm 0.21	a,b
Males							
S1	8	4.59 \pm 0.84	a	1.28 \pm 0.83	a	1.17 \pm 0.22	a
S2	8	3.46 \pm 0.41	b	0.49 \pm 0.21	b	1.12 \pm 0.10	a
S3	10	3.26 \pm 0.29	b	0.34 \pm 0.12	b	0.97 \pm 0.29	a
S4	7	3.59 \pm 0.35	b	0.55 \pm 0.16	b	1.16 \pm 0.11	a

the S1 site, MTB mRNA levels showed a particularly high increase in testes (some 100-fold) at sites S2 and S4, suggesting that this probe may be a good marker for pollution in gonads. Whereas mRNA levels of MTA and MTB at S2 can be easily explained by the high levels of metal pollution at this site, their equally high levels at S4 remain unexplained by the chemical data (see below). Metallothioneins have high affinity for metals such as mercury (Hg), Zn, Cu, and Cd, but their expression may be regulated by many different factors, both extrinsic (metals, temperature) and intrinsic (glucocorticoids, cytokines) (Kägi & Schäffer 1988, Coyle et al. 2002, Haq et al. 2003). Therefore,

their 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)

The meaning of the presence of (at least) 2 metallothionein genes with relatively different patterns of expression in *Aphanius fasciatus* is presently unclear. Several studies on fishes have shown 2 metallothionein isoforms in salmonids, carp, gudgeon *Gobio gobio*, and other species, whereas other fishes possess a single metallothionein isoform (Bonham et al. 1987, Bargelloni et al. 1999, Knapen et al. 2005). Where present, the 2 metallothionein isoforms may show different sensibility to exogenous inputs, such as metals and oxidative stress (Zafarullah et al. 1990, Scudiero et al. 2001). In the case of *A. fasciatus*, the 2 sequenced amplicons present a 70 bp overlap, with 5 base differences between them (Fig. S1 in the supplement at www.int-res.com/articles/suppl/b017p285_supp.pdf). We consider our data as indicative of the presence of 2 metallothionein genes in *A. fasciatus*, and that their expression is regulated differently in liver and gonads.

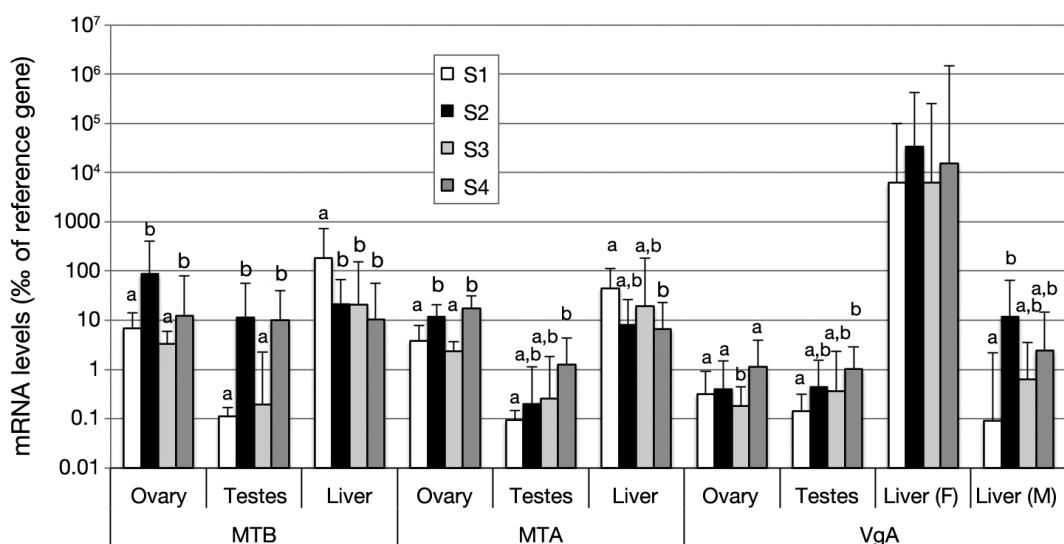


Fig. 3. Steady-state mRNA levels of metallothionein A and B (MTA, MTB) and vitellogenin A (VgA) genes in ovary, testes and liver for each fish population. Note that VgA values in liver are shown separately for males and females (M and F). Letters indicate homogenous data groups determined by Tukey's post hoc test

Expression of VgA and MT genes in liver

Fish from S1 showed hepatic MTA and MTB mRNA levels higher than those from the other sampling sites (Fig. 3). This result is in keeping with previous results from *Aphanius fasciatus* and other species, indicating that MT mRNA levels in liver are sensitive to acute, but not to chronic, heavy metal pollution (Quirós et al. 2007b, Navarro et al. 2009). Hepatic 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. Male VgA mRNA levels showed a significant (100-fold) increase in the S2 population (Fig. 3) relative to the other populations. This indicates an impact by estrogenic pollutants at this site, likely related to the high urban influence. This result exemplifies the utility of hepatic Vg mRNA levels as a marker of pollution-induced feminization in natural populations of marine fish (Matthiessen et al. 2002, Barucca et al. 2006).

General discussion

The reproductive system has been recognized as a major target for environmental pollutants in different ecosystems, including marine coastal areas (Cajaraville et al. 2000, Matthiessen et al. 2002, Barucca et al. 2006, Sarkar et al. 2006). Malformations in reproductive organs in fish and mollusks, and formation of ovotestis and ectopic expression of sex-specific proteins, such as vitellogenin in fish liver or the androgen-responsive spiggin in female *Gasterosteus aculeatus* kidney, have been considered evidence for estrogenic or androgenic impact in different water bodies, including marine coastal zones (Cajaraville et al. 2000, Matthiessen et al. 2002, Barucca et al. 2006). The identification of the pollutants putatively responsible for these observed effects is still unclear, especially in marine areas, due to the variety of possible impacts. Synthetic and natural estrogens, nonyl phenol and related compounds, some organochlorinated compounds, Cd and different pesticides and biocides have been correlated with an estrogenic response in fish (Karels et al. 2003, Scholz & Mayer 2008). Although we have evidence for heavy metal and organic compound pollution (including Cd) in the examined Tunisian coastal areas, our limited knowledge does not allow us to establish a direct correlation between a particular compound or compound families and the observed physiological effects. Our current data suggest that the observed effects on the skeletal system are brought about by a combination

of different types of pollutants (Kessabi et al. 2010, 2012).

Monitoring of the marine environment requires the identification of adequate sentinel species, to ensure that the eventually observed impacts correspond to the local pollution and are not a result of the vital history of the examined individual, which could have been exposed to multiple environments in the essentially boundless marine environment. Therefore, marine pollution sentinels are typically sessile or non-migrating species, such as mollusks (Cajaraville et al. 2000) or sand-dwelling fishes (Matthiessen et al. 2002, Barucca et al. 2006). In this regard, the high territoriality of *Aphanius fasciatus* makes this species very adequate for pollution monitoring in coastal areas of the Mediterranean basin.

Markers based on levels of mRNA for genes known to respond to different forms of pollution (metals, aromatics, and estrogens) have the advantage of requiring very small amounts of sample, which is particularly important for small fish. This methodology requires previous knowledge of the sequence of the targeted gene, but, as has been shown previously, this can be solved *in silico*, by comparing sequences from related species (Piña et al. 2007). The application of these techniques to *Aphanius fasciatus* allowed the use of this small fish as a sentinel of coastal pollution (Kessabi et al. 2010, 2012). Although there is no evidence for a decreased reproductive success in any of the studied populations, we consider that our data show a putative impact of pollution in the reproductive system of *A. fasciatus*, mainly through overexpression of the MT gene in the gonads and an incipient feminization of males, detected by the expression of the egg-yolk protein VgA genes in liver (Matthiessen et al. 2002, Barucca et al. 2006). This can be regarded as an early warning of the exposure of the S2 (and perhaps also S4) population to anthropogenic pollution, as we observed phenotypic effects in the absence of truly deleterious consequences in the fish. Preliminary data suggest that gonadosomatic and hepatosomatic indices are altered in S2 and S4 *A. fasciatus* populations (A. Annabi, I. Messaoudi, K. Saïd unpubl. data). Therefore, we consider this methodology to be a useful tool for monitoring the health status of fish populations in areas of known or suspected environmental impacts.

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