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# Reproductive effects in hybrid sparrow from a polluted area in Tunisia: Oxidative damage and altered testicular histomorphology

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ABSTRACT

Air pollution is a threat for human health and wildlife. The aim of this study is to assess the pathophysiological changes and the oxidative–antioxidative status in testicular tissues of 40 Hybrid sparrows collected from four areas in Gabès city, one of the most polluted areas in Tunisia. The testis histopathological analysis revealed alterations in birds from Ghannouche, the polluted area. The thiobarbituric acid reactive substance (TBARS) levels were higher in testis of birds from the contaminated site compared to less polluted areas indicating oxidative damage to membrane lipids. Antioxidant enzyme activities (superoxide dismutase and catalase) were lower in testis sparrows from the polluted site compared with the reference site, suggesting deficiency of the antioxidant system to compensate for oxidative stress. Overall, our results suggest that the hybrid sparrow offers a suitable model for biomonitoring programs of atmosphere pollutants and the selected biomarkers could be useful tool to evaluate pollution impacts in living organisms.



## 1. Introduction

Rapid progress in the industrial sector during the last century has resulted in the production of wide range of industrial effluents which can lead to various deleterious effects not only to the living organisms but also to the ecological equilibrium of the biosphere. In fact, it has been shown that exposure to carbon monoxide (CO), sulfur oxides (SOx), nitrogen oxides (NOx), heavy metal particles and other combustion-derived hydrocarbons gases, is associated with numerous harmful effects (Isaksson, 2010).

It has been also reported that the exposure to pollutants can decrease measures of reproductive performance (Marettová et al., 2015). Therefore, their toxic effects on male reproduction system have become a major health concern in the globe (Chowdhury, 2009; Sharma and Garu, 2011).

Wild birds are often used as environmental sentinels for industrial contamination due to their high metabolic rates and sensitivity to xenobiotics (Belskii et al., 2005; Eeva and Lehikoinen, 1996; Morrison, 1986). Particularly, air pollutants, such as heavy metal pollution, are shown to affect different phases of the avian life cycle, from egg development to adult reproduction (Scheuhammer, 1987; Eeva and Lehikoinen, 2000; Janssens et al., 2003a,2003b).

Hybrid sparrow (*Passer domesticus* × *Passer hispaniolensis*) is distributed worldwide. It is sedentary and closely associated with urban environments. These characteristics make it one of the most suitable candidates for urban biomonitoring of industrial contamination in terrestrial ecosystems of atmosphere pollutants (Swaileh and Sansur, 2006).

In recent years, there has been growing concern about the deleterious effects of pollutants on developing male reproductive system in terrestrial free-living birds (Tsipoura et al., 2008; Sánchez-Virosta et al., 2015). The impact of pollutants on reproduction need to be more studied to understand the real ecological impact of contaminants and to complete the evaluation of their toxicological profile.

To evaluate the impact of xenobiotic substances on reproduction performance of birds, numerous ecotoxicological biomarkers

*Abbreviations:* CAT, catalase; Cd, cadmium; CO, carbon monoxide; Cr, chromium; Cu, copper; GSI, gonadosomatic index; IT, interstitial tissue; MDA, malondialdehyde; NOx, nitric oxides; Pb, lead; ROS, reactive oxygen species; SOD, superoxide dismutase; Sox, sulfuric Oxides; ST, seminiferous tubules; TAS, total antioxidant status; TBA, thiobarbituric acid; TBARS, thiobarbituric acid reactive substances; VA, vitamin A; VE, vitamin E; Zn, zinc

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have been employed in the last decades. Some authors propose the use of ecological indexes like gonadosomatic indexes to evaluate the influence of biotic processes or as an additional tool in biomonitoring approaches (Adams and Ryon, 1994). Histopathological changes in gonads have been widely used as biomarkers in the evaluation of the health of animals exposed to contaminants (Badraoui et al., 2010; Marettová et al., 2010; Atef, 2011; Marettová et al., 2015).

Many pollutants may exert toxicity through oxidative stress, disturbance of prooxidant and antioxidant balance by generation of reactive oxygen species (ROS) or by the depletion of antioxidant molecules (Ercal et al., 2001; Stohs and Bagchi, 1993). An excess of ROS may cause oxidative damage to membrane lipids, DNA and proteins, and their oxidation can lead to cellular dysfunction and tissue injury (Hoffman et al., 1998; Valavanidis et al., 2006). Therefore, tissue levels of lipid peroxidation (LPO) are proven to be an indicator of oxidative stress (Tandon et al., 2003; Alvarez et al., 2004). A common biomarker for measuring lipid peroxidation is thiobarbituric acid reactive substances (TBARS) level (Oakes and Van Der Kraak, 2003; Almroth et al., 2005).

Antioxidants are a major resource of most living organisms for protection against diverse free radicals and other oxidative stressors (Cross et al., 1987; Griffith, 1999). Because several antioxidants are needed to protect against ROS and antioxidant defense may respond differently depending on species, previous studies have shown that the measure of the levels of antioxidant molecules could be interesting biomarkers of pollutant exposure (Berglund et al., 2007; Koivula and Eeva, 2010). It has also been shown that oxidative stress toxicity caused by xenobiotics intoxication affected fertility (Aruldhas et al., 2005).

The pollution of living environment in Gabès, one of the most remarkable pollution hotspots in North Africa and the Mediterranean (Azri et al., 2002a, 2002b), presents an ecological problem because of the installation in the early 1970s of intense phosphate treatment industries for acid and fertilizer production in the Gabès–Ghannouche factory complex. It has been proposed that the Gabès–Ghannouche factory complex releases 10,000 to 12,000 t of phosphogypsum, containing heavy metals mainly, cadmium (Cd), lead (Pb), zinc (Zn), copper (Cu), and chromium (Cr), per day in the sea (Béjaoui et al., 2004; Ayadi et al., 2015). In addition, the industrial process of phosphate treatment ejects huge quantities of toxic gases, especially SOx and NOx into the air (Azri et al., 2002a, 2002b). The industrial activities in the region may have contributed to the degradation of the biodiversity of the ecosystem. A previous study showed a decreased breeding performance of passerines living near the factory complex (Alaya-Ltifi et al., 2012). However, to our knowledge, no studies have assessed histopathologic alteration and oxidative stress biomarkers in Hybrid sparrow testes captured from Gabès which could of considerable interest.

In this regard, the present study is designed to investigate the impact of pollutants on reproduction performance in Hybrid sparrow using many biomarkers including histological alterations, levels of TBARS, antioxidant enzymes activities of superoxide dismutase (SOD) and catalase (CAT) in testes and plasmatic levels of vitamins E and A (VE and VA respectively) and total antioxidant status (TAS) in the oasis habitat close to Gabès city in south-eastern Tunisia. Our approach was based on the comparison between one oasis situated about five hundred meters from the factory complex, and hence exposed to a high pollution level, (polluted oasis), one oasis situated eight kilometers, one oasis situated eleven kilometers and one oasis situated twenty kilometers apart and less exposed to pollution (control oasis).

## 2. Material and methods

### 2.1. Study area and species

Samples used in this work were collected in four locations in the gulf of Gabès, in the south-east of Tunisia;

(i) Ghannouche oasis (33°56′N–10°04′E), which is situated close to the Gabès–Ghannouche factory complex, (ii) Mettouia oasis (33°58′N–10°0′E), iii) Ouedref (33°59′N–9°58′E) and (iv) Kettena oasis (33°45′N–10°13′E), which is situated 20 km to the Southeast in one industry free area. Kettana was chosen to serve as reference site not only because it's the furthest from the factory complex, but also because it's located on the opposite side of the global direction of the wind in Gabès (west) (Elamouri and Ben Amar, 2007) (Fig. 1).

The hybrid sparrow, in Tunisia, is a result of hybridization of Spanish sparrow (*P. hispaniolensis*) and the House sparrow (*P. domesticus*) (Johnston, 1969). Our specie has been suggested to be of hybrid origin because the plumage of male individuals which is intermediate to males of the Spanish sparrow and the House sparrow, but it's not the same as the Italian sparrow (*Passer italiae*). Birds, chosen for this study, are residents and often nesting and feeding in urban areas close to humans constructions (Selmi, 2000). The selected specie is primarily granivorous during the breeding season (Alaya-Ltifi and Selmi, 2014). It is a sexually dimorphic bird (Summers-Smith, 1988). There were also some subtle differences between yearlings and male adults. In particular, adults showed new rufous feathers, while yearlings had a mixture of male and female-type plumage (Hammouda et al., 2015). In this study, no phenotypic change was detected between the four populations of sparrows sampled. The identification of the specie has been confirmed by an ornithologist (M.A.C.).

A total number of 40 male sparrows were captured between March 2014 and April 2014, to coincide with the reproduction period. Individuals were trapped in mist nets. Birds were captured and brought alive to the laboratory immediately. Upon arrival at the laboratory, each sparrow was weighed. A 500 µl blood sample was taken from the jugular vein, using a sterile syringe. Then, the sparrow was sacrificed under anesthesia by an intraperitoneal injection of 8% chloral hydrate (400 mg/100 g BW) and the testes were removed and weighed. One testis was fixed in formaldehyde solution for histological examination and the other was strored at −20 °C until analyze for oxidative damage biomarkers. The blood was centrifuged (10 min at 5000 g) and the resulting plasma

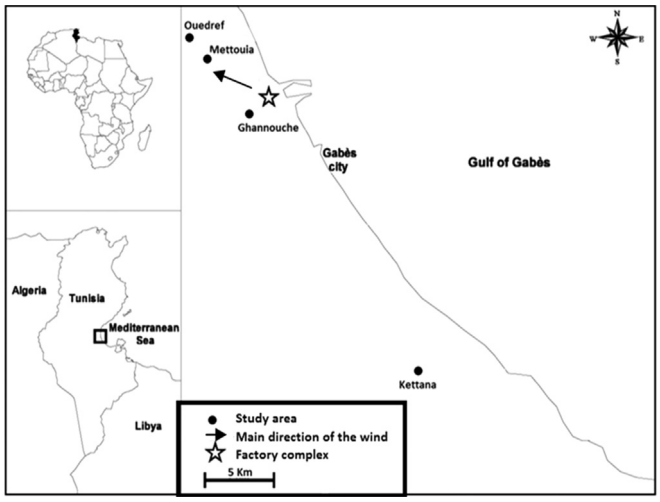


**Fig. 1.** Map of the gulf of Gabès in south-eastern Tunisia showing the locations of Gabès–Ghannouche factory complex and the four studied areas: Ghannouche, Mettouia, Ouedref and Kettana.

samples were stored at −20 °C before testing. Collected plasmatic samples were used to assess the plasmatic levels of VA, VE and TAS.

All experimental procedures, including the sparrow capture and the way they were sacrificed, were conducted in accordance with the guidelines of the local Institute Ethical committee for the care and use of laboratory animals as adopted and promulgated by the United States National Institutes of Health.

### 2.2. Determination of biological indices and histological slides preparation

Gonadosomatic index was calculated as follows: GSI=[testis weight(g)/body weight] × 100 (DeVlaming et al., 1982). Testes, processed for histological examination, were quickly removed, fixed in 10% formalin, dehydrated in ascending grades of ethanol alcohols, cleared in xylol, casted, blocked, sectioned at 4 μm thickness, by using a HM314 microtome, and stained with hematoxylin and eosin (H&E) for microscopic examination (Bancroft, 1975).

### 2.3. Biochemical study of the oxidative stress

#### 2.3.1. Testes thiobarbituric acid-reactive substances (TBARS) level

Lipid peroxidation was estimated by the determination of thiobarbituric acid-reactive substance (TBARS) level, according to a method based on the reaction of malondialdehyde (MDA) with thiobarbituric acid (TBA) at 95–100 °C (Esterbauer and Cheeseman, 1990). In the TBA test reaction malondialdehyde (MDA) or MDA-like substances and TBA react to produce a pink pigment with an absorption maximum at 532 nm (Esterbauer and Cheeseman, 1990). The reaction was performed at pH 2–3 and 95 °C for 15 min. The samples were mixed with 2 volume of cold 10% (*w*/*v*) trichloroacetic acid (TCA) to precipitate the protein, then homogenated using an Ultra Turax homogenizer. The precipitate was pelleted by centrifugation and an aliquot of the supernatant was reacted with an equal volume of 0.67% (*w*/*v*) TBA in water-bath at 95 °C, for 15 min. After cooling, the absorbance was determined at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3-tetramethoxypropane). Results were expressed as nmol/mg protein.

#### 2.3.2. SOD and CAT antioxidant assays

SOD activity was measured in testes by the method of McCord and Fridovich (1969). This method assay depends on the SOD activity to inhibit cytochrome C reduction mediated by the $O_2^{\bullet -}$ generated. SOD activity was monitored spectrophotometrically at 505 nm and SOD activity expressed as U/mg of protein.

CAT activity was assayed in testes following the methodology described by Aebi (1984), in which the disappearance of peroxide is monitored spectrophotometrically at 240 nm. The assay mixture consisted of 950 μl of potassium phosphate buffer (0.05 M, pH 7.0), 500 μl of $H_2O_2$ (0.03 M) and 50 μl of sample. One unit of CAT is equivalent to the amount of enzyme necessary to decompose 1 μmol of $H_2O_2$ per minute. The extinction coefficient of $H_2O_2$ used was 43.6 $M^{-1}$ $cm^{-1}$.

As enzyme activities were expressed in relation to grams of protein in the homogenates, total protein contents were measured in the homogenates using a spectrophotometer at 595 nm following the Bradford (1976) method, using bovine serum albumin as standard protein.

### 2.4. Measurement of plasmatic antioxidant parameters

Plasmatic VA and VE were measured by High-performance Liquid Chromatography (HPLC) as described by Ferns et al. (2000). Briefly 50 μl of plasma were taken for analysis using a C18 (15 × 4.6 mm) column. Samples were analyzed on an isocratic system using a mixture of methanol *n*-butanol and water as mobile phase (v/v/v; 98, 5:5:5) at a flow rate of 1.7 ml/min, the detection was at 330 nm for the retinol acetate and at 292 nm for the α-tocopherol. Vitamins A and E standard and quality control material were obtained from Bio-Rad Laboratories.

Total antioxidant status (TAS) was measured by a commercial Kit (Randox Laboratories, Crumlin, UK, ref: NX2332). This parameter (TAS) includes the chain breaking antioxidants (ascorbate, urate, and bilirubin) and the membrane preventive antioxidants (β-carotene and vitamin E).

### 2.5. Statistical analysis

Statistical analysis was carried out using the Statistical Package for Social Sciences (SPSS) for Windows version 12.0 software. All data were represented as mean ± standard deviation (SD). The one-way analysis of variance (ANOVA) and the Student–Newman–Keuls *post hoc* test were performed to investigate whether morphological and biochemical parameters varied significantly among sites. In all analyses, data normality and variance homogeneity were verified prior to statistical analysis. Statistical probability of $p \leq 0.05$ was considered to be significant.

## 3. Results

### 3.1. Morphological parameters

The pollutants effects on morphological parameters of testes are represented in Table 1. Testes weights are expressed as the mean of the left and right specimens for each bird. There was no significant difference in weights of testes in the four populations of sparrows. However, the GSI was significantly higher in sparrows from Ghannouche (2.40 ± 0.59) than in sparrows from Kettana (1.83 ± 0.55). No significant difference between GSI values was found between sparrows living in Ghannouche and sparrows living in Mettouia and Ouedref ($p > 0.05$).

**Table 1**
Morphometric data of sparrow's testes of the 4 different sites.

| | Ghannouche | Mettouia | Ouedref | Kettana | ANOVA | |
|---|---|---|---|---|---|---|
| | | | | | *F* | *p*-value |
| Testes weight (g) | 0.61 ± 0.13 | 0.53 ± 0.09 | 0.54 ± 0.12 | 0.51 ± 0.06 | 1.62 | 0.201 |
| GSI (%) | 2.40 ± 0.59* | 1.97 ± 0.26 | 2.26 ± 0.48 | 1.83 ± 0.55 | 3.42 | 0.027 |

All values are expressed in mean ± SD; sample size ($n=10$).

* $p \leq 0.05$ significant statistical difference with Kettana by the Newman–Keuls *post hoc* test.

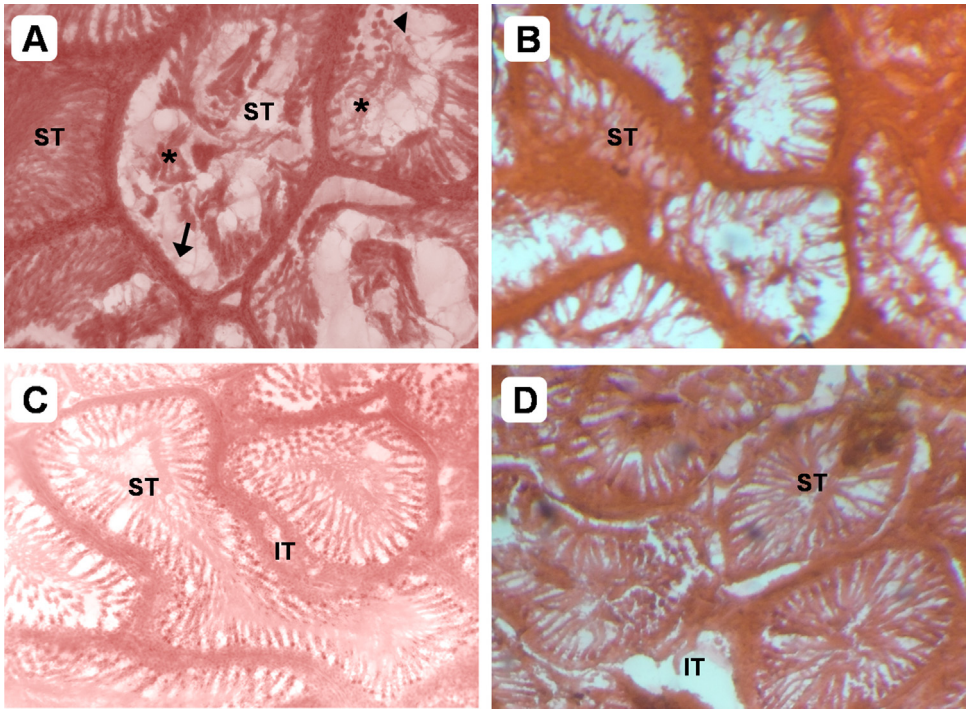


**Fig. 2.** Morphology of testes of Hybrid sparrow. A: Ghannouche ( × 200), B: Mettouia ( × 200), C: Ouedref ( × 200) and D: kettana ( × 200). Pictures show seminiferous tubules and interstitial tissue (ST and IT respectively). Note histopathological alteration in TS: epithelial detachment (arrow), immature germ cells (arrowheads) together with disorganized morphology (*) in bird living in Ghannouche (the polluted area).

### 3.2. Histopathological analysis

Testicular morphology changes in testes specimens stained with hematoxylin–eosin are shown in Figs. 2 and 3. Overall, testes histological sections of birds living in Kettana showed normal testes structure. Capsule (tunica albuginea) surrounds the testes and emits conjonctive trabeculae. Seminiferous tubules (ST) of testes are located throughout the medullary area, and germ cells are located within the seminiferous tubules. ST of kettana sparrow contains healthy gonads which contain developing spermatogonia, spermatocytes and spermatids, whereas sparrow from the polluted area exhibited several histopathological lesions. Histopathological features include leukocyte infiltration, architectural disorganization, epithelial detachments… Some seminiferous tubules of testes of Hybrid sparrow living in Ghannouche showed absence of germinal cells and loss of spermatogenesis process (Figs. 2A and 3) in comparison with normal structure of seminiferous tubules in birds living in Mettouia, Ouedref and Kettana (Fig. 2 B–D).

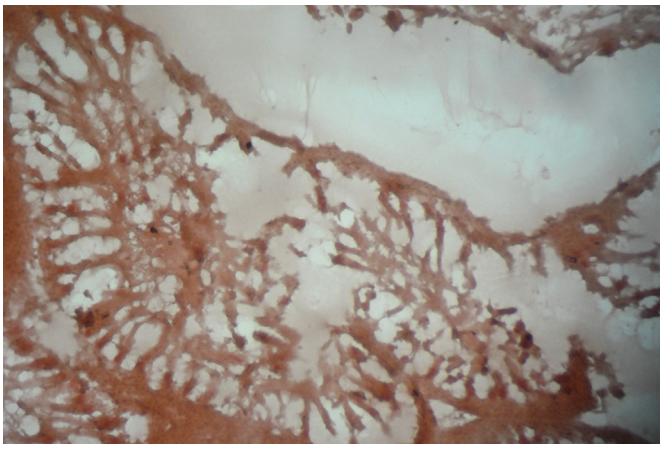

**Fig. 3.** Seminiferous tubules structure of birds from Ghannouche ( × 400). Note the disappearance of almost all the seminiferous cells in the upper tubule.

**Table 2**
Mean values of each sparrow population of TBARS, SOD and CAT of testes and plasmatic levels of VA and VE.

| | Ghannouche | Mettouia | Ouedref | Kettana |
|---|---|---|---|---|
| TBARS (nmol/mg protein) | 73.2 ± 9.28** | 64.5 ± 13 | 59 ± 9.29 | 60.2 ± 7.9 |
| SOD (U/mg protein) | 5.18 ± 0.73*** | 5.38 ± 1 | 6.08 ± 1.18 | 7.15 ± 1.26 |
| CAT (μmol/min/mg protein) | 45.7 ± 7.17** | 51.3 ± 10.8 | 55.3 ± 10.6 | 63.67 ± 9.46 |
| VA (mg/L) | 0.21 ± 0.04** | 0.22 ± 0.03 | 0.24 ± 0.07 | 0.28 ± 0.04 |
| VE (mg/L) | 2.88 ± 0.42* | 3.14 ± 0.49 | 3.28 ± 0.49 | 3.55 ± 0.39 |

All values are expressed in mean ± SD; sample size ($n = 10$).
* $p < 0.05$ Significantly different with Kettana.
** $p < 0.01$ high significantly different with Kettana.
*** $p < 0.001$ high significantly different with Kettana.

### 3.3. Biochemical findings

As shown in Table 2, Hybrid sparrows from Ghannouche displayed significant TBARS induction in testes when compared to those living in Kettana ($p < 0.01$). However, there was no significant difference in the levels of TBARS between Ghannouche sparrows and Mettouia and Ouedref sparrows ($p > 0.05$). We detected also a significantly lower CAT ($p < 0.01$) and SOD ($p < 0.01$) activities in testes of sparrows sampled in the polluted area.

It sounds that exposure to the factory complex of Ghannouche pollutants altered antioxidant plasmatic biochemical parameters. In fact, we have noted significant decreases in the content of VA, VE (Table 2) and TAS (Fig. 4) in birds living in Ghannouche.

## 4. Discussion

Wild bird populations are susceptible to dangers derived from

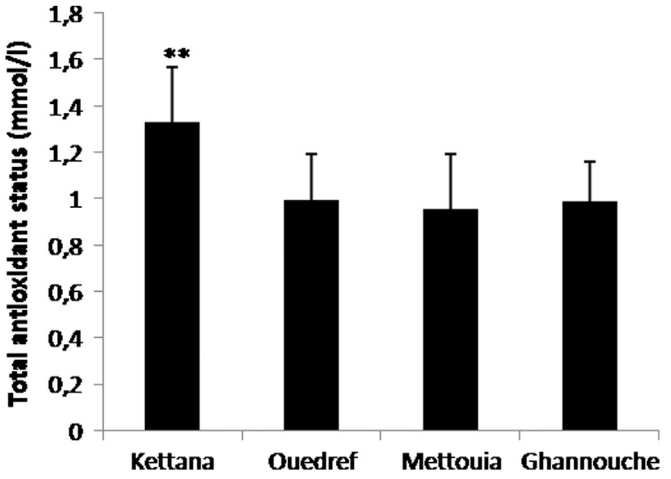


**Fig. 4.** Plasmatic level of total antioxidant status (TAS). Sparrows from Ghannouche vs sparrows from Kettana: $^{**}p < 0.01$.

the environmental presence of toxic elements, particularly those that are non-degradable and that tend to concentrate through the food chain (Guitart et al., 1994). Here, we report evidence that the exposure to pollutants exhibits negative effects on reproduction performance of male Hybrid sparrow.

Overall, our results showed that Hybrid sparrows living in Ghannouche, polluted area, display a pronounced impairment in gonadic function which is confirmed by disruptions of morphometric and histological parameters of testes. Moreover, we reported that the proximity to the factory complex has a negative effect on both enzymatic and non enzymatic anti-oxidative system.

Several recent studies showed that the exposure to pollutants exhibits negative effects on reproduction by producing testicular damage (Marettová et al., 2010; Atef, 2011; Marettová et al., 2015). The present study indicated that the exposure to xenobiotic substances induced morphological and histopathological alterations in testes; leukocyte infiltration, architectural disorganization, epithelial detachments…, as well as loss of spermatogenesis process and absence of germinal cells in some seminiferous tubules. In accordance with our results, Marettová et al. (2013) showed that necrosis and degeneration of seminiferous tubules were frequently reported in fowl exposed to Cd. Atef (2011) noted that the exposure to heavy metals is proven to provoke testicular damage, which can lead to spermatogenic arrest. More recently, it was also highlighted that the exposure to pollutants can decrease measures of reproductive performance including fertility, abnormal embryonic development, prenatal death, and sexual dysfunction (Marettová et al., 2015).

Testes damage depends also upon the toxicity of pollutants which is related with the production of free radicals associated pathophysiology of many diseases. Lipid peroxidation is known to be the first step of cellular membrane damage caused by xenobiotics (Viarengo, 1989). It has been used as a biomarker of oxidative stress damages, which may be defined as the disequilibrium between the prooxidants and antioxidants in biological systems (Kelly et al., 1998). In our study, the levels of TBARS were found to be significantly higher in sparrow testes from Ghannouche when compared to those from Kettana. In agreement with our results, it has been showed that different doses of Cd increase organ lipid peroxidation in many organs including male sex organs (Stajn et al., 1997; Patra et al., 1999). Mateo and Hoffman (2001) reported that Pb-induced lipid peroxidation has been associated with several mechanisms, such as the inhibition of antioxidant enzymes involved in the protection of cells. Fadillioglu et al. (2004) showed that the exposure to pollutants seems to engender membrane lipoperoxidation indices of oxidant injury on cellular structures which could be the consequence and/or the cause of an important antioxidant depletion that confer protection against the oxidative damage.

To protect against oxidative damage, cells possess defense mechanisms that include antioxidants. Here, some antioxidant parameters were assessed as biomarkers of oxidative stress. Our results suggest a prooxidative role for environmental pollutant-induced oxidative stress via the alteration of redox ratio values, characterized by a significant decrease in the levels of antioxidant enzymes (SOD and CAT) in testicular tissue of sparrows living near the factory complex compared with those living in Kettana. It's well known that SOD and CAT serve many vital physiological functions including protection of cells from reactive oxygen species (ROS). Their function is based on enzymatically detoxifying peroxides $H_2O_2$ and $O_2^-$ (Gurer and Ercal, 2000). SOD operates as a transformer of superoxide anion ($O_2^-$) to hydrogen peroxide ($H_2O_2$), which is then catalyzed further by CAT to $H_2O$ and molecular oxygen (Finkel and Holbrook, 2000; Pinto et al., 2003).

Some xenobiotics are demonstrated to induce oxidative stress in birds (Somashekariah et al., 1992; Ji et al., 2006), but antioxidant defense responds differently depending on pollution levels and species (Berglund et al., 2007; Ji et al., 2006; Martínez-Haro et al., 2011). In our case, significant inhibitions in CAT and SOD activities have been noted in hybrid sparrows living in the polluted area compared to sparrows from more distant areas. Similarly, many authors reported that the exposure to pollutants can reduce activities of several enzymatic and non enzymatic antioxidants; most of them were associated with histological damages (Ercal et al., 2001; Cao et al., 2010; Atef, 2011). In this study, several histopathological disorders have been outlined including leukocyte infiltration, architectural disorganization, epithelial detachments… Espín et al. (2014) showed that concentrations of Pb above 15 mg/dl in blood produced an inhibition of 11.3% in CAT activity in Griffon vulture (*Gyps fulvus*). To add, significant decrease in the levels of SOD in testes and kidney tissues were observed in mice treated with heavy metals (Atef, 2011). The low levels of CAT could be attributed to high production of superoxide anion radical, which has been reported to inhibit CAT activity in case of excess production of superoxide anion (Pandey et al., 2003).

In this study, not only enzymatic antioxidant (SOD and CAT) were depleted, but also non enzymatic antioxidants (vitamins A and E). Moreover, TAS level was significantly reduced in Ghannouche sparrows when compared to sparrows from Kettana. Vitamins, as antioxidants, are known to have been proven beneficial in some disease processes. Several studies reported that the decline of the report vitamin E to lipids was considered as an indicator of the anti-oxidizing status in several pathological processes (Pusztai, 1984; Kardinaal et al., 1998) including genotoxicity and cardiovascular diseases (Badraoui et al., 2007). In our work, the depletion of non enzymatic antioxidants and the total antioxidant status (TAS) was associated to the increase of TBARS in testicular samples.

## 5. Conclusion

In conclusion, our study indicates that the proximity to Gabès–Ghannouche factory complex of phosphate treatment was associated with testicular injury, TBARS induction, inhibition of SOD and CAT activities in testicular tissue and depletion of plasmatic levels of non enzymatic antioxidants (VA and VE) and TAS. Overall, the histological injury, outlined in this study, could be the cause and/or the consequence of the severe antioxidant depletions

conditioned by the factory complex. It also could indicate that Hybrid sparrow seems to be a reliable bioindicator for possible biomonitoring programs and that the selected biomarkers could be useful to evaluate the pollution impacts in living organisms. Further studies need to be realized to assess the mechanisms of the gonadic toxicity in sparrow and other animals. That would certainly help in the control of the reproductive toxicity and propose some therapeutic approaches.

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