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Abstract

Aquatic wildlife is exposed through trophic transfer of hazardous substances to several threats inducing physiological impairments. We aimed at assessing the impact of contamination in one of the hot spots of pollution along Mediterranean coasts, the gulf of Gabes in Tunisia, on Common tern *Sterna hirundo*, a piscivorous top predator bird. Firstly, we compared the reproductive effort of breeding adults through clutch size distribution in three sites with different levels of pollution. Then, a battery of genotoxicity and oxidative stress biomarkers was carried out to assess physiological impairments in chicks. Whilst defense mechanisms showed a depletion, lipid peroxidation and genotoxicity increased significantly according to pollution level. The multi-biomarker approach used here, discriminated chicks according to contamination degree of their nesting sites. Increase in genotoxicity and oxidative stress were correlated to a decrease in chick body mass known to lead to long-term impacts on juvenile survival and recruitment in birds.

Keywords: biomarker, antioxidant, marine pollution, oxidative stress, *Sterna hirundo*, genotoxicity, Gulf of Gabes, reproductive impairment, chick body mass.

1. Introduction

Pollution of the marine environment is a global issue owing to the discharge of urban, industrial and agricultural effluents into rivers, estuaries and coastal areas (Cravo *et al.*, 2012). Due to their ubiquity and highly persistent nature, a wide array of pollutants can accumulate in tissues of numerous marine organisms and are transferred across the food chain (Wu *et al.*, 2009). This often leads to rising concentrations (biomagnification) in top predators of marine trophic webs which may cause a prominent threat while their concentration in the environment remains below policy thresholds (Wu *et al.*, 2009; Nendza *et al.*, 1997). Indeed, dietary exposure represents the main source of contamination of predating marine birds and mammals, when direct uptakes of environmental pollutants (water, sediment, air) are of minor contribution (Nendza *et al.*, 1997). Combined with high trophic status and long-life span, life history traits of upper predators may exacerbate their vulnerability to chronic contamination and their biological costs (Rowe, 2008). On the one hand, long-lived species are prone to accumulate high loads of persistent chemicals during their life cycle including early developmental stages and on the other hand, they have a low capacity for genetic adaptation due to slow renewal of generations (Rowe, 2008).

Several biological defects have been associated with high body burdens of chemicals in aquatic wildlife and fish species ultimately affecting their fitness. Reduced eggshell thickness (Cortinovis *et al.*, 2008), offspring developmental defects (e.g., abnormalities, impaired growth, developmental instability) (Hoffman *et al.*, 1986; Jenssen *et al.*, 2010; Letcher *et al.*, 2010) and physiological disturbances of the exposed organisms such as endocrine disruption and altered vitamin homeostasis have been demonstrated (Letcher *et al.*, 2010; Rolland. 2000). There is increasing awareness of the ubiquitous role of oxidative stress inducing physiological disturbances due to to chemicals exposure (Valavanidis *et al.*, 2006). When reactive oxygen species (ROS) production overwhelms antioxidants, a state of physiological imbalance known as “oxidative stress” occurs (Valavanidis *et al.*, 2006). This altered redox homeostasis may significantly alter biomolecules (DNA damage, lipid peroxidation and protein oxidation), affect individual growth, survival and reproduction of the exposed organisms which in turn might lead to population declines (Koivula and Eeva, 2010; Viarengo *et al.*, 2007; Goutte *et al.*, 2014). Nevertheless, studies addressing the relationship between contaminant-induced stress and individual fitness alterations in wild populations remain scarce (Provencher *et al.*, 2016). While oxidative stress related to pollutants (especially metals) has been demonstrated in experimental studies in birds, it has rarely been studied in wild free-ranging bird species (Koivula and Eeva,

2010; Espín *et al*., 2014 but see Costantini *et al*., 2014). Therefore, there is an increasing interest in assessing the fate of chemicals, individual impacts and ecological risks of coastal pollution on bird populations. Expanding our comprehension about tolerance and detoxification ability of birds is particularly important as it may help in predicting how other species respond to chemical stressors in their environment. It is of main interest to identify most sensitive species to chemical pollution and to understand mechanisms that may be responsible of populations decline in polluted areas (Provencher *et al*., 2016; Koivula and Eeva, 2010).

Biomarkers are "biochemical, cellular, physiological or behavioral variation that can be measured in tissue or body-fluid samples or at the level of whole organisms providing evidence of exposure to, and/or effects of, one or more chemical pollutants (and/or radiation)'' (Depledge and Fossi, 1994). They are considered as early warning tools, which used together in combination, investigating several relevant physiological functions, provide an integrated measure of exposure to, and/or effects of, contaminants in the aquatic environment (Cajaraville *et al*., 2000; Van derOost *et al*., 2003). They could be predictive of population dynamic disturbances, and thus allow anticipating detrimental changes at higher levels of biological organization (population, community or ecosystem) (Cajaraville *et al*., 2000; Van derOost *et al*., 2003; Vasseur and Cossu-Leguille, 2003; Amiard-Triquet *et al*., 2013).

Yet, since various antioxidants can be employed differently depending on species, in defense against oxidative damage, one biomarker of oxidative stress is not reliable enough to reflect changes in the complex homeostasis (Koivula and Eeva, 2010; Espín *et al*., 2014). Thus, it is recommended to investigate simultaneously a set of biomarkers of exposure and effect, especially when individuals are exposed to complex mixtures of environmental stressors (Cajaraville *et al*., 2000; Cravo *et al*., 2009). Often used in multi-criteria approaches investigating several relevant biological endpoints, biomarkers are efficient tools to assess the health status and ecological risks of organisms exposed to environmental pollutant mixtures. Such multi-biomarker approaches have been successful in covering various relevant infra-individual responses, including oxidative stress with invertebrates and fish species (Chèvre *et al*., 2003; Viarengo *et al*., 2007; Damasio *et al*., 2011; Benali *et al*., 2015; Santos *et al*., 2016).

The gulf of Gabes (southeastern coasts of Tunisia) is one of the main hotspot of pollution along Mediterranean coasts where high levels of polycyclic aromatic hydrocarbons (PAH) and heavy metals have been detected in water and sediments (Zaghden *et al*., 2014; El Zrelli *et al*., 2015; Rabaoui *et al*., 2015; Fourati *et al*., 2017). In this area, several common tern colonies (*Sterna hirundo*) are established during the breeding season. This seabird used in this work as a model species is common over a wide geographical range with low annual reproductive output. As a

long-lived species and a top-level predator of aquatic ecosystems consuming almost exclusively fish, this bird is prone to bioaccumulate xenobiotics inducing adverse impacts including oxidative stress and potentially changes in reproductive activity (Van derOost *et al*., 2003; Rowe, 2008). In order to achieve a comprehensive evaluation of adverse outcomes of pollution on birds we conducted a comparative study on tern colonies breeding in three sites subjected to different level of pressures. As income breeders, like common terns, depend on locally acquired resources to form eggs rather than on reserves, contamination may be a major constraint for reproduction (Ezard *et al*., 2007). So, our first objective was to detect the impact of pollution on the reproductive potential of tern females. We hypothesized that chemical pressures may prevent females from increasing their reproductive effort through laying reduced clutches compared to their conspecifics breeding in a relatively clean area. Our second objective was to assess the alteration of physiological status and genotoxic damage of chicks by carrying out a multibiomarker approach using a combination of oxidative stress and genotoxicity biomarkers. The use of chicks presents two advantages. First, the effect of age-related bioaccumulation is avoided, since the exposure time is known and similar for all individuals (Quirós *et al*., 2008). Second, because common terns are known to have a limited foraging range around their colony site (maximum foraging range of 9 km; Rock *et al.,* 2007) and all the chicks are provisioned with prey caught by parents in the surroundings of the colony, the results should reflect the impact of local pollution (Quirós *et al*., 2008). Hence, cellular antioxidant defenses, namely activities of two antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)), and two exogenous antioxidants (vitamin A and vitamin E) besides a biomarker of impact (TBARS) were selected as oxidative stress biomarkers. These biomarkers have been widely used in many field studies in order to assess the extent of pollution in different environments (An *et al*., 2012; Cravo *et al*., 2009, 2012). The micronucleus assay (MN) reflecting chromosomal damages was used as a biomarker of genotoxicity. Although investigating genotoxicity is valuable in wildlife risk assessment, a few studies have been conducted on wild birds especially for biomonitoring purposes (e.g., Quirós *et al*., 2008; Skarphedinsdottira *et al*., 2010). In overall, the present work aims at investigating whether contamination affects reproduction of common tern on a pollution gradient impacting parental nesting capacities. We also investigated physiological disturbances and body mass of offspring reared and fed in nesting sites under contrasted chemical pressures.

2. Materials and methods

2.1.Study sites

Field work was carried out in three sites on the Eastern coasts of Tunisia which reflect different levels of pollution (Figure 1). The first nesting site (33°54'N 10°06'E) is located in the Gulf of Gabes close to an industrial complex in the region of Chatt-Essalem known to be one of the most contaminated area in Gabes (Zaghden *et al*., 2014; El Zrelli *et al*. 2015; Rabaoui *et al*., 2015). Local industrial activities in the area led to the contamination of the aquatic ecosystems by pollutant mixtures from multiple effluents rejected by the phosphate treatment complex and the Fluor Chemical Industries (ICF) (Rabaoui *et al*., 2015) as well as those from the wastewater treatment plant of Chatt-Essalem and urban activities. Chatt-Essalem is located between the fishing harbor and the commercial harbor, making the water renewal low due to very weak currents and hydrodynamics (Rabaoui *et al*., 2015) which minimize chemicals dilution and promote local accumulation. The second site, in northern Gabes gulf (34°39'N 10°42 E), is located about 12 km south Sfax city. The southern coastal area of Sfax is subjected to industrial pollution due to the discharge of chemical effluents from the phosphoric acid plant (SIAPE) besides a municipal wastewater treatment plant. The approximate quantities of phosphogypsum dumped from the phosphate treatment plants are 135 million tons in Chatt-Essalem and 30 million tons in Sfax (Ben Amor and Jomaa, 2012).

The Monastir salina (35°45'N 10°42'E) is in the gulf of Hammamet. This area was considered as a reference site because industrial or urban effluents are much lower than the other sites.

To describe the level of anthropogenic pressures on these sites, we investigated the landscape use in the area. A radius of 10 km around each nesting area was chosen to measure the surface of agricultural and natural habitats, of urban areas and the surface of industrial activities. Maps were originally produced from a Landsat TM image with 30 m resolution taken in 2013. Image processing included atmospheric and geometric corrections was performed using ENVI Flash. Identification and digitization of geographic features were performed through ArcCatalog and ArcMap modules incorporated in ARCGIS 9.0.The surface of each area was determined using the calculate geometry tool of ARCGIS.

2.2.Data collection and blood sampling

Colonies of common tern were visited in the three sites throughout the nesting period from end of April to early July 2015. However, only synchronous nests and chicks from these sites were considered in our study and blood sampled. Each nest was marked with a small numbered wooden stick 40 to 50 cm apart from the nest. All nests were visited every 2-3 days in Chatt-Essalem, every 2-7 days in Sfax salina and every 2-12 days in Monastir Salina to check nest

content. Visits were more frequent during laying eggs to determine laying date and clutch size of nests. Clutch size was considered as completed when the number of eggs did not change between at least two visits. Since some chicks could have been predated between our field sessions, we could not rigorously assess hatching success for several clutches. In both Monastir and Sfax salina, chicks were easily detected because they hide between rocks of a narrow dike formed by soil and small rocks and surrounded from both sides by water. However, to prevent wandering of chicks or their escape after hatching in Chatt-Essalem site, nests were fenced individually with fishing nets (about 0.30×0.75m). The enclosures included natural or added cover so that chicks can hide and find shade and were open at the top to allow free access by adult birds which continued normal care of chicks even in presence of fences.

Three weeks after hatching, chicks were weighed by a fixed spring scale (Model Kern HDB 5k5) or a Pesola and blood samples (volume equivalent to 1% of body weight) were taken from the brachial vein. Samples were then transported to the laboratory. A droplet of whole blood was taken to prepare smears (2 smears per chick) for the micronucleus assay while the rest of blood was centrifuged at 3500 rpm for 10 min. Both plasma and cell pellets were stored at -20°C until analyzed. Samples were taken from 25, 21 and 19 chicks respectively in Chatt-Essalem, Sfax salina and Monastir salina nesting sites.

2.3. Biomarker analyses

2.3.1. Thiobarbituric acid reactive substances (TBARS)

The TBARS assay is commonly used to quantify oxidative stress by measuring peroxidative damages to lipids that result from free radical generation (Valavanidis *et al*., 2006). The intensity of lipid peroxidation can be approximated as the level of endproducts such as malondialdehyde (MDA) and other aldehydes which are appraised with thiobarbituric acid hence the name of the assay, thiobarbituric acid-reactive substances (TBARS) (Valavanidis *et al*., 2006). Biological samples were combined with 1.5 ml TBA 0.8%, 1.5 ml trichloroacetic acid (TCA) 20% and 800 µl distilled water in the presence of 200 µl of sodium dodecyl sulfate (SDS) 8.1%. The incubation mixture was heated for 30 min at 90°C. After cooling, it was centrifuged (4000 rpm, 15 min) and the TBARS concentration was determined based on the absorbance at 532 nm. Results were expressed as nmol/ml.

2.3.2. Antioxidant enzymes activities

The Superoxide dismutase (SOD) activity was assessed, spectrophotometrically at 505 nm according to McCord and Fridovich (1969), by determining its ability to inhibit the photoreduction of cytochrome C by the superoxide anion. One unit of SOD represents the amount that inhibits the photoreduction of cytochrome C by 50%. SOD activity was expressed as U/ml.

Catalase (CAT) activity was measured spectrophotometrically in ultraviolet accordingly to the protocol from Aebi (1984) by following the consumption of H_2O_2 at 240 nm. Decays in absorbance were recorded in the reaction mixture consisted of 500 µl of H_2O_2 (0.03M), 950 µl of potassium phosphate buffer (0.05M; pH = 7.0) and 50 µl of sample. One unit of CAT was defined as the amount of enzyme that decomposes 1 µmol of H_2O_2 per minute.

2.3.3. Plasmatic antioxidant vitamins

Plasmatic vitamins A (Vit A) and E (Vit E) were estimated by High Performance Liquid Chromotography (HPLC) in isocratic mode using the methodology reported by Ferns *et al* (2000). Plasma was firstly deproteinized with ethanol containing internal standards (retinyl acetate and α-tocopheryl acetate) and then it underwent lipid extraction with hexane. After evaporation, the dry residue was dissolved and diluted with methanol. 50 µl of sample was injected onto a C18 (15 cm; 4.6 mm) column 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. Detection was carried out at 330 nm for the retinol acetate and at 292 nm for the α-tocopherol. Results were expressed in mg/l.

2.3.4. Micronucleus assay

Micronucleus assays were performed as described by Skarphedinsdottir *et al* (2010). Briefly, the blood smears were stained with 4 % Giemsa (> 10 min). The slides were rinsed in distilled water, air-dried, and coded for blind scoring under optical microscope at 100× magnification. Specimens of 25, 20 and 19 chicks respectively from Chatt-Essalem, Sfax and Monastir were randomly analyzed by a single observer. The frequency of micronucleated erythrocytes as well as several erythrocytes nuclear abnormalities described in Figure 2 were scored for 5,000 erythrocytes in each investigated specimen following the zig-zag model (to avoid crossing the same field more than once). The criteria for identifying micronucleus were: (1) same color and intensity as the cell nucleus (2) size smaller than or equal to 1/5 of the main nucleus (3) rounded shape with a nuclear membrane (4) clearly detached from the cell nucleus with intra-cytoplasmatic location.

2.4.Statistical analysis

The first objective was to compare clutch size distribution between sites. A chi-square test was conducted comparing proportion of nests with 3 eggs, 2 eggs and 1 egg among sampled sites. For biomarkers data, inter-site differences of biomarkers responses and chick mass were tested using non-parametric Kruskal-Wallis and Wilcoxon pairwise comparison tests. We investigated the micronucleus probability of detection between sites using Generalized Linear Mixed Models (GLMM) with a binomial error. All erythrocytes without micronucleus were coded as "0" while erythrocytes with micronucleus were coded "1" so that we modeled the probability of exhibiting a micronucleated erythrocyte. An 'individual' random effect was added to the model linking the 5,000 erythrocytes analyzed to the same individual. The fitted model was compared to the null model based on an Analysis of Deviance (ANODEV). To summarize biomarker responses, we implemented a principal component analysis (PCA) to analyze correlations between biomarker responses of the six biomarkers measured in chicks. The site location was added as a supplementary information which has no influence on the principal components but add supplementary information for a better interpretation of the inertia. Finally, the first axis of the PCA discriminating chicks according to their biomarker responses was used to assess the relationship between chick biomarker responses hereafter considered as "chick physiological status" and their masses three-weeks post-hatching using a linear regression.

All data are represented as the mean ± standard error (SE) and statistical significance was defined at $p \leq 0.05$. The entire statistical analysis was carried out using R.3.2.2 software (R Development Core Team, 2011) with the packages "lme4" for GLMM, "PMCMRplus" for pairwise multiple comparisons (Pohlert *et al.*, 2018) and "FactoMineR" for multivariate analysis (Husson *et al.*, 2012).

3. Results

3.1.Evaluation of landscape use around the monitored sites

Data showing land use and the ratio of different types of pressures present 10 km around each site are presented in Table 1 and Supplementary materials 1. Land cover results showed that Chatt-Essalem nesting site has less natural and agricultural zone (77.17%) than Sfax (84.34%) and Monastir salina (88.35%). Additionally, there is a slight gradual increase in urban area from Monastir (11.39%) to Sfax (13.16%) and Chatt-Essalem (18.59%). The site of Chatt-Essalem (4.24% of industrial area) is conspicuously closer (distance = 0 km) and 1.70 times more

industrialized than Sfax (distance = 1.8 km; 2.5% of industrial area) and 16.31 times more industrialized than Monastir salina (distance = 4.7 km; 0.26% of industrial area).

3.2.Clutch size distribution

Clutch size ranged from 1 to 3 eggs but there was a predominance of nests with 3 eggs in all areas (Table 2). Frequencies of clutches of 1, 2 and 3 eggs were 4.9%, 9.7% and 85.4% in Monastir, 6.3%, 16.3% and 77.4% in Sfax and 5.8%, 10.6% and 83.6% in Chatt-Essalem respectively. No significant difference was detected on clutch size distribution among the 3 sites ($\chi^2 = 9.1$; df = 4; p = 0.06).

3.3.Biomarker analyses

3.3.1. Biomarkers of impacts: Lipid peroxidation and DNA damage

Peroxidative damage measured as TBARS in chicks showed significant differences between the three sites (KW-χ^2 = 32.65; df = 2; $p < 0.001$). The level of lipid peroxidation was significantly higher in chicks from Chatt-Essalem and Sfax (103.99 ± 4.52 and 74.89 ± 2.55 $nmol.ml^{-1}$ respectively) compared to chicks from the reference site (64.31 ± 2.64 $nmol.ml^{-1}$; W = 331, $p < 0.001$ and W = 316.5, $p < 0.01$ respectively; Figure 3). Concerning micronucleus, the probability to detect micronucleus between sites was significantly recorded ($p < 0.01$). The between sites fitted model was significantly different from the null model (AIC = 373.12 and 383.51), confirmed by ANODEV comparison of the fitted model to the null model ($\chi^2 = 14.38$; df = 2; $p < 0.01$). Coefficients of the fitted model highlighted significant lower probability of observing a micronucleated erythrocyte in the reference site (0.13 ± 0.03 ‰; Figure 3) than in Sfax salina (0.50 ± 0.15 ‰; $p < 0.01$) and Chatt-Essalem (0.36 ± 0.11 ‰; $p < 0.01$).

3.3.2. Biomarkers of defense: antioxidant enzymes (SOD and CAT)

Highly significant differences between sites were recorded for both SOD (KW-χ^2 = 11.39; df = 2; $p < 0.01$) and CAT activities (KW-χ^2 = 11.26; df = 2; $p < 0.01$). These two enzymes exhibited lower activities at Chatt-Essalem (SOD = 7.62 ± 0.52 $U.ml^{-1}$; CAT = 68.98 ± 3.82 $U.ml^{-1}$; Figure 4) and Sfax (SOD = 8.71 ± 0.28 $U.ml^{-1}$; CAT = 76.95 ± 3.55 $U.ml^{-1}$) in comparison with the Monastir reference site (SOD = 9.74 ± 0.37 $U.ml^{-1}$; CAT = 88.45 ± 4.04 $U.ml^{-1}$). However, this decrease was significant only between Chatt-Essalem and Monastir (W = 274, $p < 0.01$ for SOD and W = 275, $p < 0.01$ for CAT; Figure 4).

3.3.3. Biomarkers of defense: plasmatic antioxidant vitamins (Vit.A and Vit.E)

Significant differences between sites were observed in Vitamin A (KW-χ^2 = 9.03; df = 2; p = 0.01) and Vitamin E (KW-χ^2 = 9.68; df = 2; p < 0.001). The level of Vit.A demonstrated that the content in chicks from Chatt-Essalem (0.154 ± 0.010 mg.L^{-1}) was significantly depleted compared to Monastir (0.188 ± 0.009 mg.L^{-1}; W = 287.5, p < 0.01). In Sfax salina, although a lower value was reported (0.179 ± 0.007 mg.L^{-1}), no significant difference was observed neither with the Monastir reference site nor with Chatt-Essalem (Figure 5). The level of Vit. E demonstrated that the content in chicks from Sfax salina (1.84 ± 0.06 mg.L^{-1}) was significantly depleted compared with Monastir (2.19 ± 0.07 mg.L^{-1}; W = 311, p < 0.01). In Chatt-Essalem although a lower value was reported (1.93 ± 0.08 mg.L^{-1}), no significant difference was observed neither with the Monastir reference site nor with the Sfax salina (Figure 5).

3.3.4. Three-week-old chick mass

Significant differences between sites were observed in chick masses (KW-χ^2 = 6.87; df = 2; p = 0.03). The pairwise comparison demonstrated that three-week-old chick masses from Chatt-Essalem (117.54 ± 13.53 g) were significantly lower compared to Monastir (126.55 ± 9.20 g; W = 101.5, p = 0.05). Masses of three-week-old chicks from Sfax (117.19 ± 10.47 g) were significantly lower compared to Monastir (W = 98.5, p = 0.01) but no significant differences in chick mass were observed between Chatt-Essalem and Sfax sites.

3.4.Correlations between biomarker responses in chicks and their body mass

The first two components of the PCA accounted for 55.13% of the total dataset (Figure 6). The first component explained 34.64% of the variance and pointed out that SOD and CAT were positively correlated and negatively correlated with TBARS. The second component explained 20.48% of the overall data variance, mainly explained by the level of Vit. A and Vit. E which were positively correlated and inversely correlated to the level of MN. Based on the biomarker responses, PCA allowed a clear discrimination of chicks from Monastir to Chatt-Essalem according to their physiological status in a gradual increase of anthropogenic pressure (Figure 6). Finally, an increase of the PCA-Axis 1 values representing a decrease in physiological stress detected in chicks was significantly correlated to an increase in three week-old chick mass (R^2 = 0.20; F-value = 13.85; df = 55; p < 0.01; Figure 7).

4. Discussion

To our knowledge, this study is the first to provide evidences of physiological and genotoxic disturbances in wild common tern (*Sterna hirundo*) under natural conditions using a multi-biomarker approach. No significant difference was found in the reproductive investment of breeding adults, reported as the number of eggs laid per nest and their distribution among sites with contrasted level of contamination. We carried out a battery of relevant biomarkers investigating antioxidant enzymes, dietary exogenous vitamins (A and E), a biomarker of lipid peroxidation and a biomarker of genotoxicity. This multi-biomarker approach outlined physiological redox homeostasis disturbances and genotoxicity in *Sterna hirundo* chicks reared and fed in polluted areas which could imperil in the long-term their development. We demonstrated that chicks with disturbed physiological status also presented a decrease in body mass compared to reference conditions. As chick body mass is a useful fitness-related trait known as the predictor most commonly associated with post-fledging survival besides hatching date in birds, this work outline that the measured biomarker responses could affect their fitness over the course of their life span and lead to long-term changes in demographic traits and thereby in population growth and dynamics.

4.1.Clutch size

As a significant part of the energy of birds under chemical pressure would be allocated in xenobiotic metabolization and costly defenses, and since reproduction is physiologically an energy demanding process, one may expect that increased energy expenditure and chemically-induced stress might divert resources away from reproductive investment in tern populations which in turn reduce chances for fueling laying efforts. In our study, the reproductive output of common tern, investigated in term of number of eggs laid per nest was similar with predominance of 3-egg clutches in all monitored areas. This may suggest that pollution does not represent a constraint for reproduction considering the number of eggs in polluted areas and that females seem to be resistant or balancing their energy requirements between these critical biological traits. Mateo *et al* (2004) highlighted that one of two common tern colonies they studied in a contaminated area from the Ebro delta (Spain) tend to have smaller clutch size, lower hatching success and higher yolk organochlorine compounds. These authors suspected that this is due to differences in fish consumed as terns from Banya's colony feed on demersal and benthic fish species that are more exposed to contaminants from sediments whereas terns from Fangar's colony would feed mostly on pelagic species of small clupeiformes that are less exposed to contaminants. Wiersma *et al* (2004) have demonstrated the negative compromise between reproduction and oxidative protection in zebra finches. They showed that birds trading-

off their oxidative protection against the reproductive output, suffer decreased antioxidant enzymes activities when having experimental increased clutch size. However, Markó *et al* (2011) did not find a significant correlation between clutch size (number of eggs) and the oxidative status of collared flycatcher (*Ficedula albicollis*). In our work, we did not assess the oxidative status of females but it would be of great interest to further analyze correlations between clutch sizes and parental physiological status. An alternative explanation that may explain no differences in clutch sizes could be that even if the clutch size was not affected in polluted areas, a trade off may be mirrored in other functions (Markó *et al*., 2011) such as egg volume or the quantity of maternal antioxidants and xenobiotic defenses invested into eggs. Thus, further work will be needed to investigate these points. Furthermore, one limitation to our work is that it was restricted to the progeny during early life stages, so that it might be biased toward good quality individuals that succeed in hatching and chick rearing. Yet this potential bias is conservative, it should reduce the differences between our study sites if only individuals with lower level of contamination succeed in breeding. Future works, studying the entire population, notably adults including non-breeding ones or those which fail breeding early in the season, and potentially over several years for instance using Capture-Recapture approach are definitely needed to further elucidate this point.

4.2.Biomarker analyses

A significant rise in the level of TBARS was measured according to the degree of pressures surrounding nesting sites. The highest value was observed in chicks from Chatt-Essalem and Sfax in a lesser extent while those from Monastir showed the lowest value. An increase in TBARS levels mean an excessive production of free radicals damaging polyunsaturated lipids. After hatching, common tern parents feed their progeny with fish caught locally in the feeding habitats (Burger and Gochfeld, 1993). Thus, overgeneration of free radicals might be promoted both directly or indirectly by xenobiotic metabolization entering the organisms mostly due to food intakes in aquatic bird top predators (Limón-Pacheco and Gonsebatt, 2009; Valavanidis *et al*., 2006; Quirós *et al*., 2008). In order to make accurate inferences about the level of oxidative stress experienced by chicks, we measured the TBARS as a biomarker of impact which can reflect the level of oxidative stress and biomarkers of defenses that allow organisms to cope with the presence of pollutants in their environment (Valavanidis *et al*., 2006; Amiard-Triquet, 2013). Thus, we took in account the cooperative tasks of the antioxidant system, choosing enzymatic biomarkers and non-enzymatic ROS scavenger antioxidants (Espín *et al*., 2014). Antioxidant molecules play a key role in defense mechanisms by counteracting free radicals

formation and their harmful effects. In this study, both enzymatic and non-enzymatic antioxidants exhibited a significant decline at contaminated sites in comparison with the reference site. They revealed a balanced and coordinated work between each other in their biological role of detoxifying. For instance, SOD and CAT were positively correlated between each other. Indeed, SOD-CAT is the first line of defense against free radicals proliferation that causes damages to lipid membrane (Van der Oost *et al*., 2003). They act synergistically, firstly SOD catalyzes superoxide (O_2•−) to hydrogen peroxide (H_2O_2), which is then transformed by CAT to H_2O and O_2 (Vijayavel *et al*., 2004). An excess of H_2O_2 related to accumulation of reactive oxygen species (ROS), can inhibit SOD activity (Jodynis-Liebert *et al*., 2005) and CAT activity can be likewise inhibited by an excess of superoxide radicals (Jodynis-Liebert *et al*., 2005). This was conspicuous in our data where negative correlation was found between the level of reactive species presented here as TBARS and the inhibition of SOD-CAT in contaminated sites. This inhibition of enzymes can be explained by the high level of contamination previously highlighted in the gulf of Gabes by toxic metals and polycyclic aromatic hydrocarbons inhibiting SOD and/or CAT activities (Atli and Canli, 2010; Romeo *et al*., 2000; Vijayavel *et al*., 2004). More interestingly, in some cases, contaminants can even inhibit the gene expression of antioxidants in chicks which can therefore promote reactive oxygen species propagation (Limón-Pacheco and Gonsebatt, 2009).

Similar to antioxidant enzymes pattern, vitamins A and E were positively correlated between each other. This was not surprising since both vitamins are lipophilic chain breaking antioxidants, concentrated in cellular membranes and acting in tandem to inhibit lipoperoxidation by scavenging ROS (Koivula and Eeva, 2010). Their decreased level suggests their intense mobilization as an antioxidizing agent consequent to an abnormal production of free radicals as it is displayed by the level of lipid peroxidation. As a consequence of this depleted stock, chicks from Gabes gulf (Chatt-Essalem and Sfax sites) will have exacerbated lipid radicals spread, inducing further disturbances in coherence with other reports indicating repercussions of vitamins A and E deficiency (neurological abnormalities, abnormal erythrocyte membrane morphology, oxidative stress, altered immune system, malformations; Rolland, 2000; Clarke *et al.,* 2008). For instance, in the work of Fernie *et al* (2005) a lack of vitamin A in American kestrel was associated with oxidative stress and immunopathological effects.

Unlike all antioxidants measured in the present work, concentration of vitamin E was found higher in the heavily contaminated site of Chatt-Essalem than in Sfax and was not significantly different from the reference site. We suspect that this discrepancy may be due to

436 disparity in the exogenous intake of vitamin E. In fact, according to prey items found in nests,
437 parents in Sfax salina and Monastir salina feed their chicks with several fish preys such as
438 annular seabream, sardines and sand steenbras but in Chatt-Essalem, parents bring to their
439 chicks mostly sardines (unpublished results) which are well known to be highly rich in
440 polyunsaturated lipids and omega 3 fatty acids (Bandarra *et al.,* 1997). Consequently, we
441 hypothesize that this richness involves an increase in serum content of vitamin E to prevent
442 their oxidation. A similar pattern of increased vitamin E was observed in eggs of four
443 piscivorous bird species consuming fish rich in polyunsaturated fatty acids (Surai *et al.,* 2001).
444 As a final point, biomarker responses of *Sterna hirundo* chicks integrated in multivariate
445 analysis differed considerably between Gabes gulf sites and the reference site as we detected
446 inter-site differences. Chicks were discriminated according to their physiological status in a
447 way reflecting chemical pollution gradient and landscape use in each nesting area even if we
448 cannot exclude that chicks from the three studied sites could have been exposed to other
449 environmental stressors such as traffic noise, light and human presence. To monitor chicks and
450 prevent their escape after hatching, nests were fenced individually with fishing nets in Chatt-
451 Essalem site until we sampled chick blood. According to our observation, we did not notice that
452 fences affected parents or chicks' behaviors. Nevertheless, we cannot exclude the high level of
453 genotoxicity or oxidative stress could have been partially caused by a lack of freedom even if
454 chicks had spaces, could hide and find shade. Regarding the high level of genotoxicity and
455 oxidative stress also detected in chicks from Sfax, where chicks were not fenced, we concluded
456 that fences we designed had a limited impact on chick physiological status. This field work
457 pointed Monastir salina as a reference site as chicks had the "best health status", whilst in Sfax
458 salina and Chatt-Essalem previously described as highly polluted sites, chicks had altered
459 physiological status. Therefore, we consider that the level of genotoxicity and oxidative stress
460 detected in chicks from these sites was related to local pollution exposure.
461
462 The measured oxidative damages observed here are not restricted to lipids. Lipid peroxidation,
463 as a process of chain reactions alter membrane structure, permeability and fluidity (Almroth *et*
464 *al*., 2005; Lushchak and Bagnyukova, 2006) but most of end products promoted by lipid
465 deterioration have also genotoxic and mutagenic potential. Chicks from contaminated sites
466 suffer from genotoxic effects triggered by free radicals or other genotoxicants such as PAH
467 known to be highly reactive and own mutagenic and cancerogenic properties (Lushchak and
468 Bagnyukova. 2006; Valavanidis *et al*., 2006). Indeed, previous studies highlighted moderate to
469 high hydrocarbons contamination of surface coastal waters in the gulf of Gabes compared to

other Mediterranean regions (PAH concentrations were 15.48-56.79 ng.L^{-1} and 8.94-197.83 ng.L^{-1} in Gabes and Sfax coasts respectively; Fourati *et al.*, 2017). The probability to detect micronucleated erythrocytes was more than three times lower in the reference site than in contaminated sites demonstrating that chicks were exposed to genotoxicants. This difference is similar to that found in purple heron nestlings from polluted and reference sites (three- to six-fold difference) in the Ebro basin in Spain and which exhibited also reduced blood antioxidant defenses (Quirós *et al.*, 2008).

It is worthy to report that, in addition to micronuclei, chicks revealed other analogous of abnormal nuclear structures as shown in Figure 2. Although their frequency was not determined in this study, erythrocyte nuclear abnormalities (ENA) has already been successfully performed in genotoxic surveys with fish and amphibians but only more recently with birds (Santos *et al.*, 2017). Therefore, the ENA assay could presumably be an additional tool for assessing genotoxic damage in *sterna hirundo.*

Thus, the overall physiological status of chicks from contaminated areas might expose them to further defects at different level of organization. Despite the high level of contamination around Chatt-Essalem, this site is still an attractive area for nesting for years which could be suitable in the short term but could lead to long term impacts on progeny survival and reproduction.

In fish, the exposure of the brown bullhead *Ameiurus nebulosus* to carcinogens in contaminated hydrosystems induced age-selective mortality and population structure impairment (lack of the oldest age classes in contaminated sites; Baumann *et al.*, 1990). Further delayed biological impacts, through trans-generational effects or early life-stage exposure, leading to decreases in juvenile survival and population growth rates, have also been highlighted in other fish species *Oncorhynchus gorbuscha*, *Pimephales promelas* and *Chondrostoma nasus* (Heintz *et al.*, 2000; Heintz, 2007; White *et al.*, 1999; Santos *et al.*, 2013a,b; Devaux *et al.*, 2011, 2015). Hence, chicks may ultimately pay the outcome of oxidative and genotoxic damages observed in this study in terms of impaired growth, developmental abnormalities, reproduction or survival (Koivula and Eeva, 2010). As a first evidence of potential long term-effects, we outlined in the present work a significant correlation between an increase in physiological redox homeostasis disturbances and a decrease in chick mass three weeks post-hatching known to lead to a decrease in juvenile survival. Indeed, it is hypothesized that heavier individuals survive better than lighter ones because lightweight birds are more vulnerable to diseases, predation and parasites during their early life (Van der Jeugd and Larsson, 1998). Previous studies have found that offspring of higher body mass prior to or at fledging may have higher survival rates than

lighter ones (e.g Naef-Daenzer *et al*., 2001 and Monrós *et al*., 2002 for great tit; Monticelli and Ramos, 2012 for roseate tern; Mougin *et al*., 2000 for Cory's Shearwater *Calonectris diomeda*; Gaston, 1997 for ancient Murrelet; Van der Jeugd and Larsson, 1998 for barnacle geese; Fear and Bristol, 2013 for sooty tern). In a four-year study on free-living great tits, a strong positive relationship between pre-fledging body mass and fledging success and recruitment probability was reported while pre-fledging resistance to oxidative stress significantly predicted fledging success (Losdat *et al*., 2012). In another study, Noguera *et al.* (2011) demonstrated that chicks of European shags *Phalacrocorax aristotelis* suffering of elevated oxidative damage have decreased recruitment success probability in the next years. In spite of these evidences, there is still a dearth of studies that relate changes in demographic components such as juvenile survival and adult fertility to markers of oxidative stress and genotoxicity (Costantini *et al*., 2015).

5. Conclusion

This work investigates ecotoxicological risks of contamination on populations of costal bird breeding in one of the main polluted areas in the Mediterranean region, the Gulf of Gabes. Our results highlighted that wild chicks from polluted sites suffer of genotoxicity and oxidative perturbations subsequent to excessive ROS generation and impoverished antioxidant defenses. The selected battery of biomarkers was effective to monitor biological impacts of contamination in *Sterna hirundo* in different locations discriminating chicks from sites under high chemical pressures. It is noteworthy to mention that such physiological impacts might not be costly only to exposed individuals but also to future generations. Physiological disruptions may potentially give rise to further disorders which could affect demographic traits and lead to long-term changes in population dynamics. As a first line of evidence, this work underlined a relationship between an increase of chick redox homeostasis disturbances and a decrease in body mass three weeks post-hatching, well-known to impact juvenile survival. Further analyses will be implemented to assess the relationship between chick redox status, chick body condition and the level of morphological abnormalities. Finally, this work highlighted that it is of major interest to incorporate more ecotoxicological approaches to population dynamic research programs and vice-versa to better investigate the impact of chemical pressures on aquatic wildlife.

6. References

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Table 1. Total surface and percentage of anthropogenic pressures in the surrounding 10 km of the three nesting sites of common tern in 2015. *Differences in total surface investigated is linked to variations in sea surface areas around investigated sites (see Figure S1).*

Site	Monastir	Sfax	Chatt-Essalem
Total surface (Km^2)	228.43	170.23	146.77
Natural habitat and agricultural zone (%)	88.35	84.34	77.17
Urban zone (%)	11.39	13.16	18.59
Industrial zone (%)	0.26	2.5	4.24
	100	100	100
Distance from industrial zone (Km)	4.7	1.8	0

766 **Table 2.** Early nests clutch size distribution of common tern among monitored areas in 2015

	Monastir salina	Sfax salina	Chatt-Essalem
1 egg	6	33	26
2 eggs	12	85	47
3 eggs	105	403	372
Total	123	521	445
Mean	2.80 ± 0.05	2.71 ± 0.03	2.78 ± 0.03

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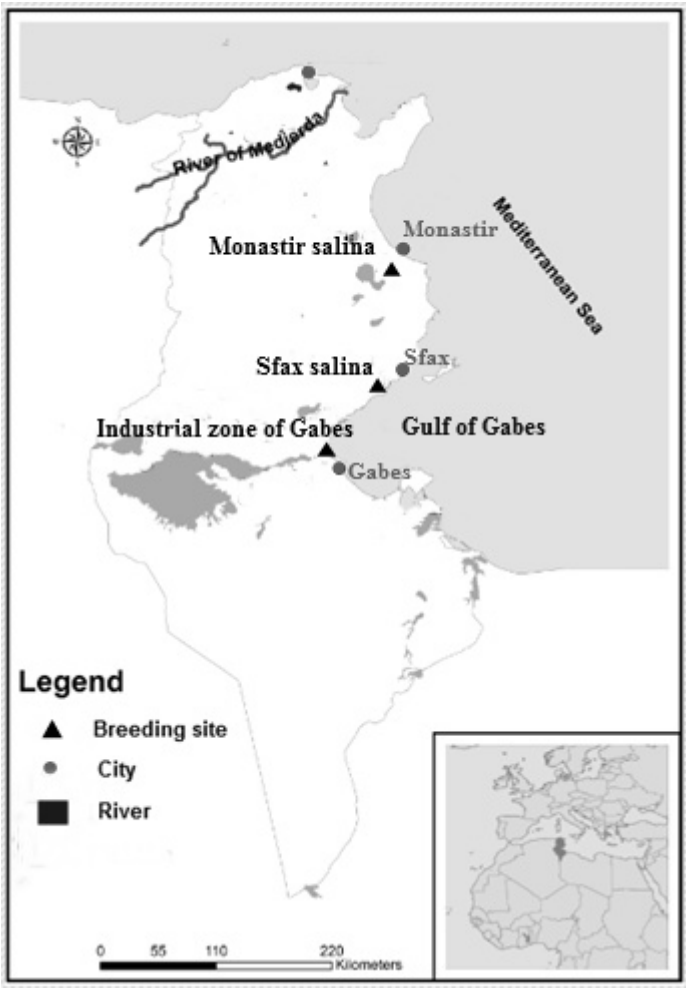

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773 **Figure 1.** Geographical location of selected sites in Tunisia for studying pollution effects on *Sterna*
774 *hirundo* in 2015.

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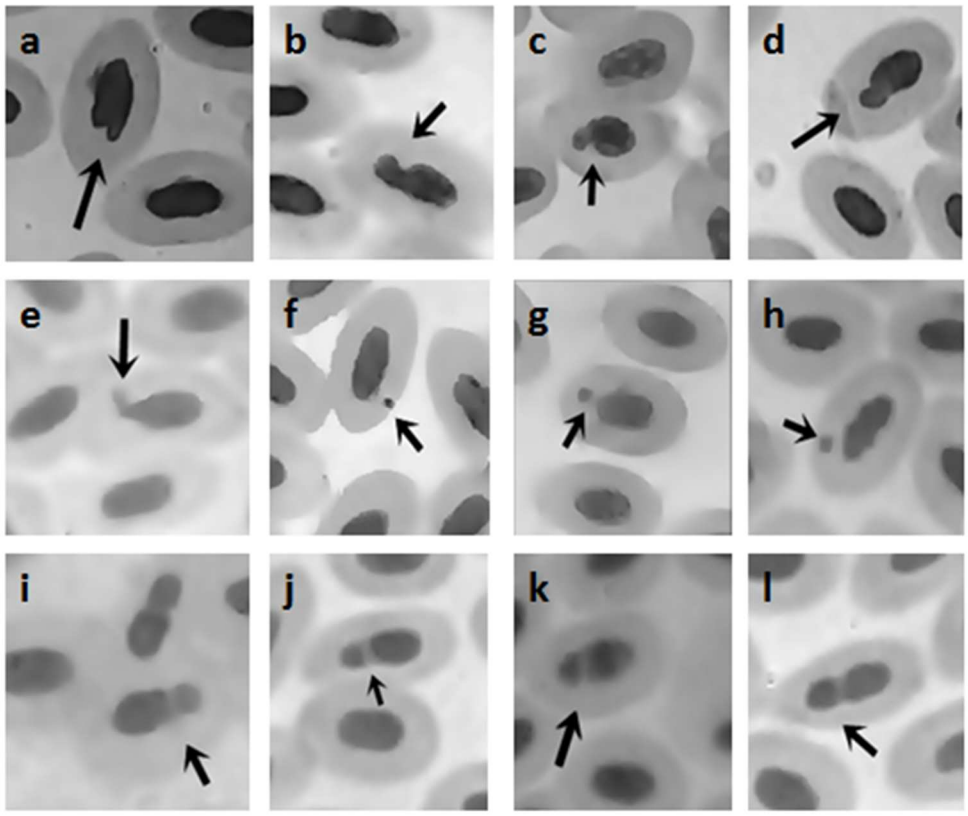

Figure 2. Erythrocyte nuclear abnormalities observed in *Sterna hirundo* chicks in 2015: a-b: notched nuclei, c-d: nuclear buds, e: nuclear tail, f-g: micronuclei, h: polymorphic nuclei+MN, i-l: segmented nuclei

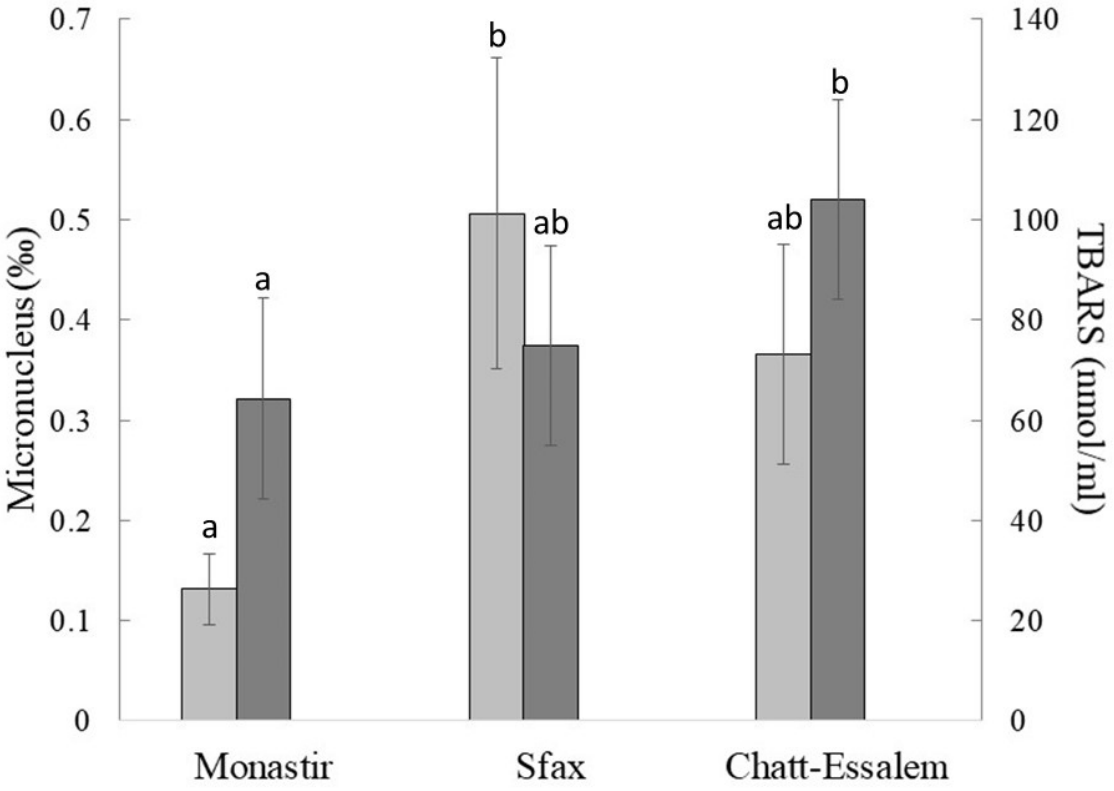

Figure 3. Micronucleus frequencies (‰) and TBARS levels (nmol/ml) in blood of common tern chicks from the three nesting sites in 2015. Values are expressed as the mean (±SE). For each biomarker, different letters (a-b) denote significant differences of means ($p < 0.05$) between sites according to pair-wise non-parametric tests.

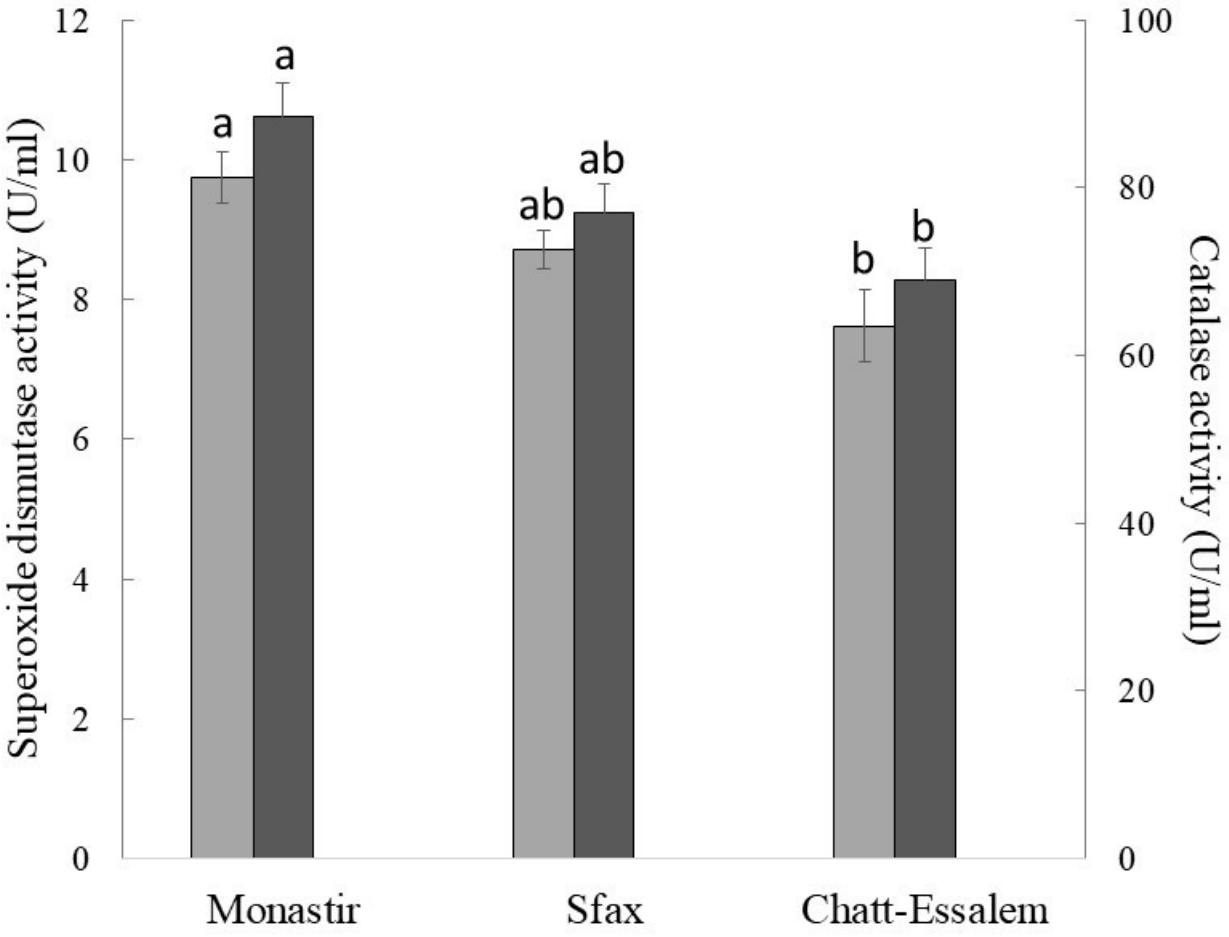

Figure 4. Superoxide Dismutase (SOD; U/ml) and Catalase (CAT; U/ml) levels in blood of common tern chicks from the three nesting sites in 2015. Values are expressed as the mean (±SE). For each biomarker, different letters (a-b) denote significant differences of means ($p < 0.05$) between sites according to pair-wise non-parametric tests.

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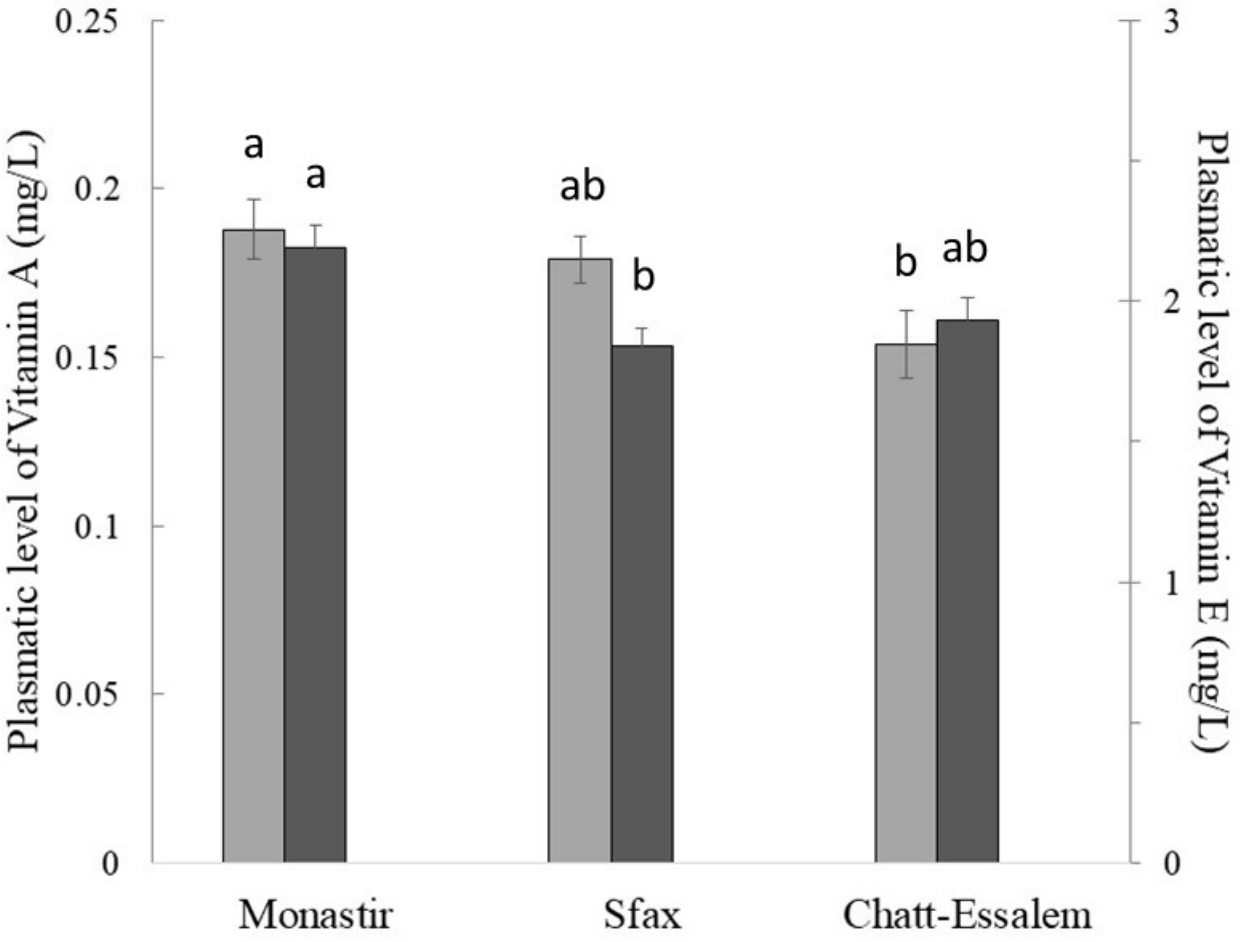


801

802 **Figure 5.** Vitamin A (mg/L) and Vitamin E (mg/L) levels in blood of common tern chicks from the
803 three nesting sites in 2015. Values are expressed as the mean (±SE). For each biomarker, different
804 letters (a-b) denote significant differences of means ($p < 0.05$) between sites according to pair-wise
805 non-parametric tests.

806

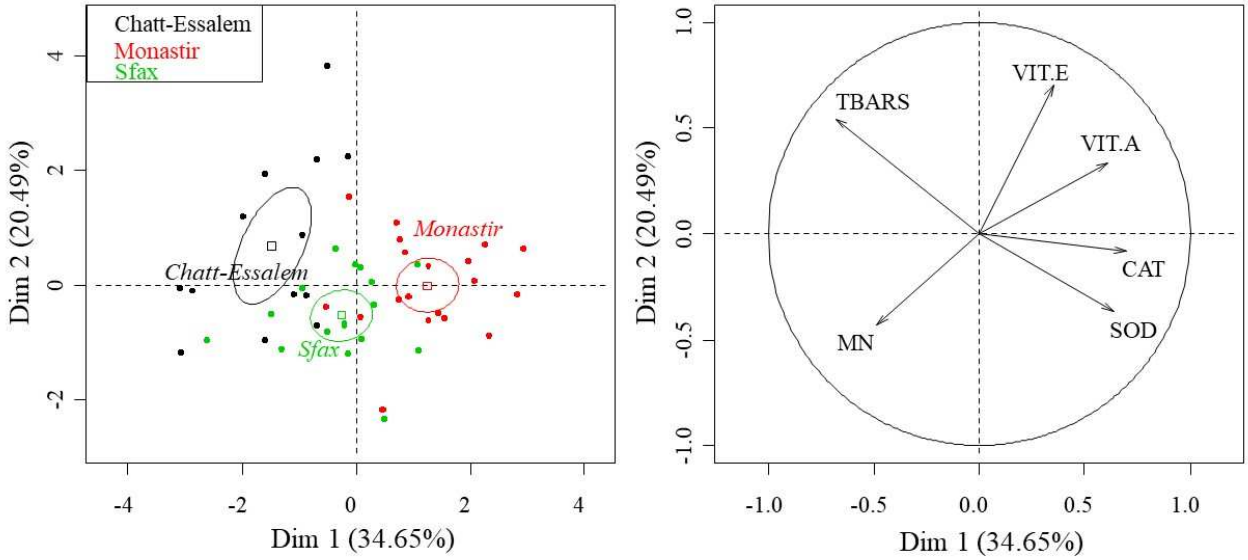
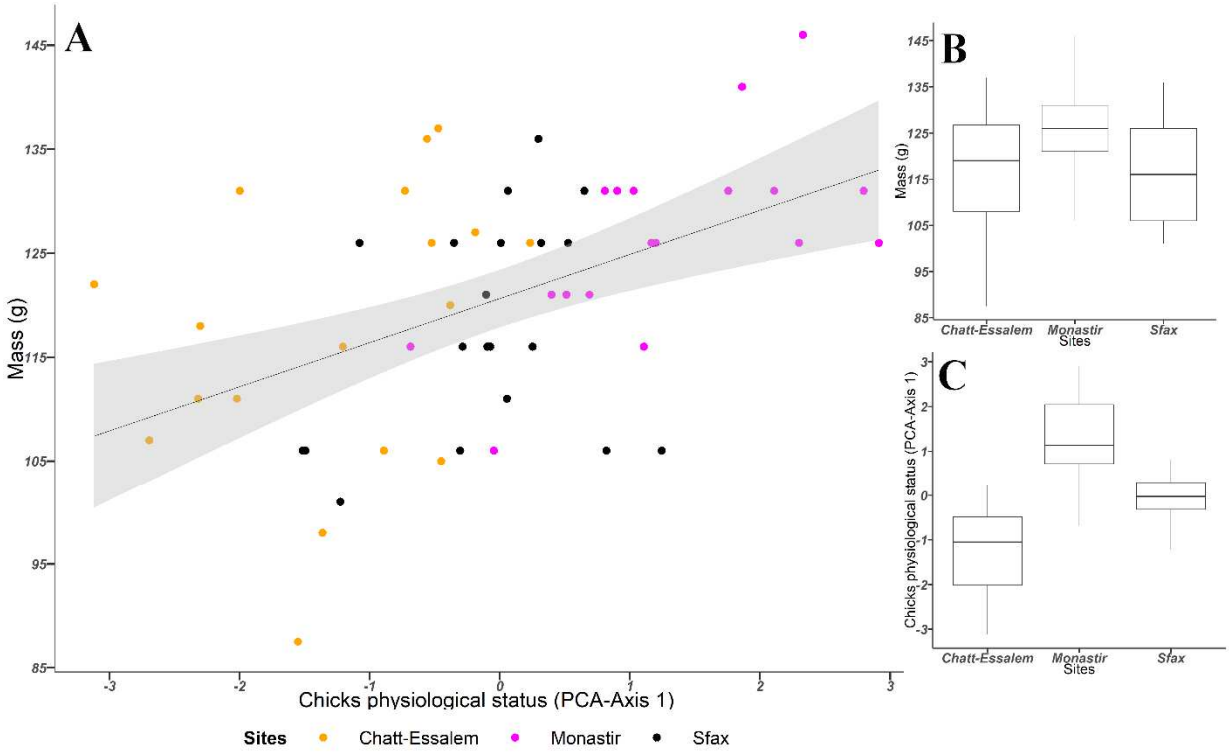


Figure 6. Principal component analysis performed on biomarker responses in *Sterna hirundo* blood samples of chicks from the three nesting sites (Monastir, Sfax and Chatt-Essalem) in 2015. *CAT: Catalase activity (U/ml); MN: number of micronucleus (‰); SOD: Superoxide dismutase activity (U/ml); TBARS: level of lipid peroxidation in equivalent TBARS (nmol/ml); Vit.A: level of plasmatic vitamin A (mg/L); Vit.E: level of plasmatic vitamin E (mg/L).*



816

817 **Figure 7.** Relationship between chicks body mass three weeks post-hatching and their physiological
818 status (A) representing chick redox homeostasis disturbances and genotoxicity (PCA-Axis 1: highest
819 values represent chicks with a "good physiological status"). Solid line corresponds to the linear model
820 with 0.95 confidence intervals of this slope in grey ($R^2 = 0.20$; $p < 0.01$). Body mass (B) and
821 physiological status values (PCA-Axis 1) (C) of common tern chicks from the three nesting sites in
822 2015.

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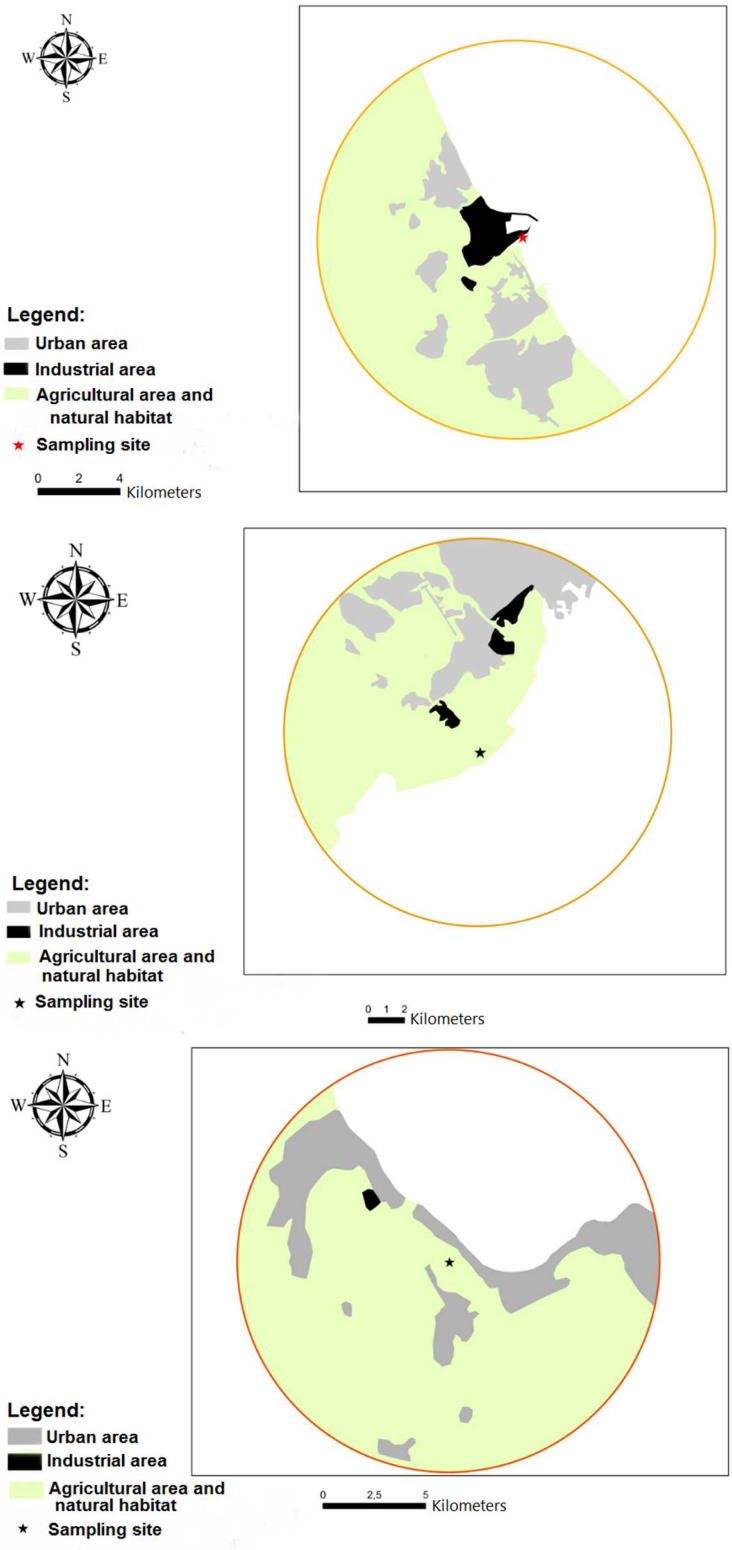

Supplementary material S1: Landuse map in 10 Km around nesting sites (Chatt-Essalem, Sfax and Monastir) of common tern in Tunisia in 2015.