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Condition and Health of Rufous Bush Robin (*Cercotrichas galactotes*) Nestlings in a Polluted Oasis Habitat in Southern Tunisia

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Abstract We investigated whether the proximity to the Gabès-Ghannouche factory complex of phosphate treatment, in south-eastern Tunisia, was associated with notable changes in the condition and health of Rufous bush robin (*Cercotrichas galactotes*) nestlings hatched in the neighbouring oasis habitat. Results demonstrated that excrements of nestlings hatched in one oasis close to the factory complex contained higher concentrations of lead and zinc than the excrements of nestlings from one oasis situated 20 km away. Furthermore, when effects of age and nestling number in the nest were controlled, nestlings hatched near the factory complex showed reduced tarsus length, rectrix length, haematocrit level and haemoglobin concentration, but higher levels of fluctuating asymmetry compared to those from the more distant oasis. Overall, results suggest that the proximity to the factory complex was associated with increased exposure to metals and deterioration in nestling condition and development.

Keywords Cadmium · Biomarkers · North Africa · Passerines · Lead · Zinc

Because of their great sensitivity to xenobiotics, passerine nestlings have potential as reliable biomonitors of polluted terrestrial ecosystems (Belskii et al. 2005; Janssens et al. 2003). Numerous studies have shown air pollution by metals and toxic gases generally result in a remarkable deterioration of the condition and health of passerine nestlings, as assessed by different morphological, biochemical and physiological biomarkers (Eeva et al. 2000; Janssens et al. 2003). These studies were almost exclusively concerned with passerines inhabiting polluted European forests (Janssens et al. 2003; Dauwe et al. 2006; Eeva et al. 2000), and there is a need for data from different habitat systems and species to draw more general conclusions about this issue.

Gabès region, in southeast Tunisia, has been classified as a pollution hotspot with potential risk of trans-boundary effect, mainly due to the installation in the early 1970s of the Gabès-Ghannouche factory complex of phosphate treatment for the production of acids and fertilizers (UNEP/WHO 1999; UNEP/MAP 2012). In addition to the phosphogypsum and associated metals, like cadmium, copper, zinc and lead, the industrial process of phosphate treatment ejects huge quantities of toxic gases, especially sulfuric oxides (SO_x) and nitric oxides (NO_x) into the air (Azri et al. 2002a, b). During the first 20 years following the installation of this factory complex, 50 million tons of phosphogypsum have been discharged in the environment (Guillaumont et al. 1995). Unfortunately, detailed information on the amount of toxic gases and particles and their patterns of dispersion in the surroundings are lacking because of the sensitivity of this issue.

Currently, there is widespread opinion among locals and NGOs that the terrestrial biodiversity inhabiting the oasis habitat close to the Gabès-Ghannouche factory complex has been negatively affected by pollution. Due to the lack of detailed studies, this claim has remained unverified. A recent work investigated clutch size, nest survival and chick productivity of insectivorous passerines inhabiting Gabès oases, in relation to habitat pollution (Alaya-Ltifi et al. 2012). Results showed evidence of decreased breeding performance of the studied species near the factory complex (Alaya-Ltifi et al. 2012). The current study aimed to go further and check whether the proximity to the

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factory complex was associated with notable deterioration in the condition status of passerine nestlings, by using the Rufous bush robin (*Cercotrichas galactotes*) as a model species. This trans-Saharan migrant is a regular and abundant breeder in southern Tunisian oasis habitat (Selmi 2000). Nests are generally built on bushes, small palm trees and fruit trees, often near the trunk and at low heights (Boukhriss and Selmi 2010). Because this insectivorous passerine is relatively abundant in the two oases of study, it was supposed to provide a suitable candidate for investigating the effects of pollution on nestling condition and health. A series of simple morphological and haematological parameters were measured in Rufous bush robin nestlings hatched in two oases situated at different distances from the factory complex. These data were used these to assess the relationship between the proximity of the factory complex and nestling condition parameters.

Methods and Material

Data used in this work were collected in two oases in southeastern Tunisia: (1) Ghannouche oasis (33°56′N, 10°03′E) which extends from 600 to 4500 m northwest of the Gabès-Ghannouche factory complex and (2) Kettena oasis (33°45′N, 10°13′E), which is situated 20 km to southeast in an industry-free area.

Nests were extensively searched from April to July 2009. Each discovered nest was monitored until the end of the breeding attempt (fledging or failure) by means of regular visits (one visit per 3 days). Hatched chicks were color-marked so that hatching order was determined. Approximately 10 days after hatching, a series of measurements were carried out on the second hatched nestling. Chicks were first weighed and then measured bilaterally for the lengths of tarsus, third primary and outermost rectrix. All measurements were carried out by the same observer (L. Alaya-Ltifi) and repeated three times in random order. Measurement repeatability, following the procedure described by Lessells and Boag (1987), was high ($p < 0.05$ for all traits). Before putting the chick in the nest, a blood sample (approximately 100 µL) was taken from the brachial vein with a sterile needle and transferred into a heparinised microhaematocrit capillary. The blood sample was placed in a cooled transport box and transported within 3 h to the laboratory where haematocrit level was determined according to Merila and Svensson (1995) and the haemoglobin concentration using the cyanmethaemoglobin method (Drabkin and Austin 1935).

Excrements were collected from the second nestlings of a sub-sample of 14 nests (7 from Ghannouche and 7 from Kettana) to determine metal concentration. Only nestlings that defecated during handling were included in our study sample, which explains the low number of excrement samples (see "Results and Discussion"). Excrements were placed in metal free vials to avoid external contamination. In the laboratory excrement samples were briefly washed with deionised water. Dry weight of the excrements was determined after they were put in an oven (60°C) for 24 h. Excrements were digested in a 1:1 mixture of HNO_3 (70 %) and H_2O_2 (30 %). Digestion was completed with the microwave procedure described by Blust et al. (1988). After digestion, samples were diluted by adding 4 mL deionized water and stored at 20 °C until analysis. Cadmium, lead and zinc levels were measured with an atomic absorption spectrophotometer (Avanta, GBC spectrometer, Australia), using an air-acetylene flame. Concentrations were expressed in mg g^{-1} based on dry weights. Equipment was calibrated using working standards (0, 1, 3, 5 and 10 µg mL^{-1}), with satisfactory recoveries (more than 80 %). Due to small sample sizes, significant differences between the two studied oases were assessed using the non-parametric Mann–Whitney test.

With regard to nestlings, data on tarsus length (T), third primary length (P) and outermost rectrix length (R) of both right and left sides were used to obtain one composite measure of fluctuating asymmetry (FA). For each trait, the signed difference between the left and right sides ($l - r$); the absolute difference between left and right ($|l - r|$); and the average size [$(l + r)/2$] were calculated. To evaluate the possibility of anti-symmetry (i.e. a tendency away from bilateral symmetry), departures from normality of the distribution of the signed differences ($l - r$) were assessed using the Shapiro–Wilk test. To test for any directional asymmetry (i.e. biased to one side), a Student's t test was used to determine whether the mean of signed differences between left and right sides ($l - r$) was significantly different from zero (Palmer 1994). If no directional asymmetry was present and the distribution of signed differences was normal, then the variation in these differences represented classical fluctuating asymmetry (Palmer 1994). For all three traits (T, P and R), a size-corrected index of fluctuating asymmetry was calculated: $FA_i = [(|l - r|)/((l + r)/2)]$. A composite fluctuating asymmetry index (FA) for each sampled nestling was calculated by summing the FA_i values across the three traits: $FA = FA_T + FA_P + FA_R$.

Given that nestling age and the number of nestlings at the moment of sampling varied among nests, the current study had to account for their possible effects when comparing nestling condition parameters between the two oases. Measured condition parameters (mass, tarsus length, third primary length, outermost rectrix length, FA, hematocrit and haemoglobin concentration) were first regressed to nestling age (in days) and the number of nestlings in the nest at the moment of sampling. Regression residuals were then used, as corrected condition parameters, to investigate whether nestling condition varied between the two studied oases by means of multivariate analysis of variance (MANOVA). Data collected in the polluted oasis were also used to investigate relationships between nestling condition parameters and nest distance to the factory complex by means of Spearman correlation coefficient. All statistical tests and analyses were conducted using SAS software (SAS Statistical Institute 1998).

Results and Discussion

We determined cadmium, lead and zinc concentrations in excrement samples from 14 nests (7 from Ghannouche oasis and 7 from Kettana oasis). Lead concentration ranged from 3.25 to 20.25 $\mu g\ g^{-1}$, with a median value of 8.75 $\mu g\ g^{-1}$, in Ghannouche samples and from 0 to 9.2 $\mu g\ g^{-1}$, with a median of 0 $\mu g\ g^{-1}$, in Kettana samples (Mann–Whitney test: $Z = 2.45$, $p = 0.0141$). Zinc concentration ranged from 21.25 to 54.75 $\mu g\ g^{-1}$, with a median of 30.25 $\mu g\ g^{-1}$, in Ghannouche samples and from 5.75 to 37.25 $\mu g\ g^{-1}$, with a median of 11 $\mu g\ g^{-1}$, in Kettana samples (Mann–Whitney test: $Z = 2.30, p = 0.0213$). Cadmium concentration ranged from 1.5 to 3.5 $\mu g\ g^{-1}$, with a median value of 2.5 $\mu g\ g^{-1}$, in Ghannouche samples and from 1 to 4.75 $\mu g\ g^{-1}$, with a median of 2.5 $\mu g\ g^{-1}$, in Kettana samples (Mann–Whitney test: $Z = 0, p = 1.000$).

Results show that excrements of nestlings from the oasis close to the factory complex contained higher concentrations of lead and zinc than the excrements of nestlings from the more distant oasis, while no significant difference was observed for cadmium concentration. Overall, these results support the assumption that nestlings from the oasis close to the factory complex suffer higher exposure to lead and zinc than those from the more distant oasis. They are also consistent with findings of previous studies on other passerine species living in different polluted habitats, in that metal concentrations were higher in the sites close to the pollution source compared to those situated faraway (Eeva et al. 2000; Janssens et al. 2001, 2003).

With regard to birds, data on a total of 26 nestlings from 26 different nests, 15 nestlings from Ghannouche and 11 from Kettana, were collected. Nestling age at the moment of sampling ranged between 8 and 13 days (mean $\pm$ SE $=$ 10.40 $\pm$ 0.40) in Ghannouche and from 8 to 12 days (mean $\pm$ SE $=$ 9.73 $\pm$ 0.33) in Kettana, but the difference was not statistically significant (t test: $t_{24} = 1.23$, $p = 0.2317$). The number of nestlings at the moment of sampling ranged from 1 to 4 (mean $\pm$ SE $=$ 2.73 $\pm$ 0.23) in Ghannouche and from 2 to 4 (mean $\pm$ SE $=$ 3.27 $\pm$ 0.24) in Kettana. Range and mean values for the measured morphological and haematological parameters are shown in Table 1. The average signed $(l - r)$ values for tarsus, third primary and outermost rectrix length did not deviate significantly from a normal distribution (Shapiro–Wilk test: $p > 0.05$ for all traits), and their means did not significantly depart from zero (t test: $p > 0.05$ for all comparisons). This would suggest that in all traits, observed asymmetry was fluctuating and that no problem of directional asymmetry or antisymmetry occurred in the data. The composite FA, obtained by summing the calculated size-corrected fluctuating asymmetry values across the three traits, ranged from 0.004 to 0.070 (Table 1).

The MANOVA of condition parameters (controlling for effects of age and number of nestlings) as functions of site showed an overall significant difference between the two studied oases (Wilks' $\lambda = 0.37$, $F_{7,18} = 4.30$, $p = 0.0058$). Tarsus length, outermost rectrix length, haematocrit value and haemoglobin concentration were significantly lower in nestlings from Ghannouche compared to those from Kettana, but an opposite trend was found for FA level (Fig. 1). For body mass and primary length, no significant difference was detected, although there was a tendency toward decreased values in nestlings from Ghannouche compared to those from Kettana (Fig. 1).

At the scale of the Ghannouche oasis, only primary length was positively correlated with nest distance to the factory complex ($r = 0.521$, $p = 0.0462$). No significant correlation was found for nestling mass ($r = 0.129$, $p = 0.6479$), tarsus length ($r = 0.360$, $p = 0.1866$), rectrix length ($r = -0.032$, $p = 0.9095$), FA ($r = 0.054$, $p = 0.8496$), haematocrit ($r = 0.236$, $p = 0.3977$) and heamoglobin ($r = 0.507, p = 0.0537$).

Overall, current results suggest the proximity to the factory complex was associated with increased exposure to metals and decreased nestling condition. This relationship was clearly visible when investigated at the regional scale, i.e. when comparing one oasis close to the factory complex (polluted oasis) with an oasis situated twenty kilometers faraway. However, it was less apparent when investigated at the scale of the polluted oasis, where distance to the factory complex varied between sampled nests but was always <5 km. A similar pattern was observed when investigating the breeding performance of passerines inhabiting the same area (Alaya-Ltifi et al. 2012).

With regard to morphological parameters, current results showed reduced growth and abnormal development in nestlings hatched close to the factory complex, a pattern that has repeatedly been observed in different passerine species and polluted habitat systems (Eeva and Lehikoinen 1996; Eeva et al. 2000). Different hypotheses could nonetheless be proposed to explain the observed trend. It could be that pollution decreased the availability of suitable food items, which may have caused food shortage and reduced energy intake, leading to nutritional stress and abnormal growth and development. It could also be that

Table 1 Statistics for the measured condition parameters in the sampled nestlings

Parameter	Ghannouche (n = 15)		Kettana (n = 11)	
	Range	Mean ± SE	Range	Mean ± SE
Mass (g)	14.50–25.00	21.43 ± 0.71	20.00–27.00	22.25 ± 0.59
Tarsus length (mm)	21.80–28.60	25.50 ± 0.55	25.12–30.62	27.60 ± 0.50
Third primary length (mm)	20.00–45.00	33.50 ± 1.62	30.00–39.25	34.81 ± 1.01
Outermost rectrix length (mm)	38.50–58.33	47.30 ± 1.53	48.50–58.00	51.59 ± 0.87
FA	0.018–0.070	0.042 ± 0.004	0.004–0.060	0.025 ± 0.006
Haematocrit (%)	17.57–46.67	32.69 ± 2.10	32.50–65.22	48.38 ± 2.78
Haemoglobin concentration (g mL^{-1})	0.14–0.26	0.21 ± 0.01	0.23–0.31	0.26 ± 0.01

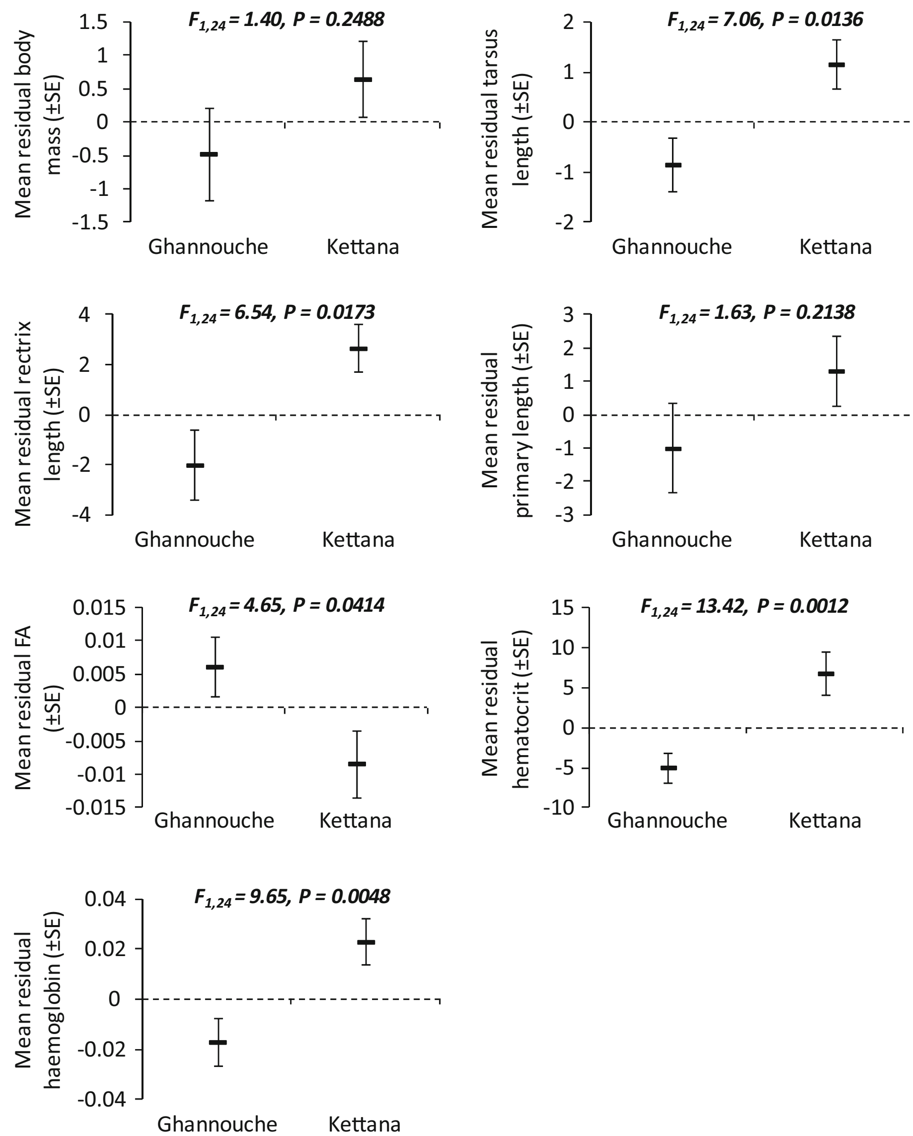

Fig. 1 Comparison between nestlings from Ghannouche (n = 15) and those from Kettana (n = 11) regarding the seven measured biomarkers

because of direct or/and indirect intoxication, energy reserves were channelled into energy-consuming detoxication processes, which may have reduced energy allocation to tissue production, resulting in abnormal development and growth (Miroslava 2007). Reduced growth in tarsus and feathers of nestlings living close to the factory complex could also be related to decreased calcium intake. Indeed, calcium metabolism is known to be detrimentally affected by habitat pollution (Scheuhammer 1987, 1996). Acidifying emissions (mainly SO_2) are known to decrease the number of calcium-rich food items, such as snails, in the vicinity of the pollution source, resulting in decreased calcium intake and skeletal development (Eeva and Lehikoinen 1996; Belskii and Grebennikov 2014).

With regard to haematological parameters, the significant decrease in the haematocrit level and haemoglobin concentration observed in nestlings from the polluted oasis compared to those from the less polluted one agrees with the results of further studies on different species and habitat systems (Hoffman et al. 1985; Nyholm 1998; Henny et al. 2000). Haematocrit and haemoglobin level are indicators of the amount of red blood cells and reflect the extent and efficiency of oxygen uptake and transfer to tissues (Ots et al. 1998). Metals are known to cause anemia, which might be reflected as lowered haemoglobin and haematocrit values (Grasman and Scanlon 1995; Nyholm 1998). For example, lead depresses the activity of the blood enzyme delta aminolevulinic acid, which is essential for haemoglobin production (Grasman and Scanlon 1995). However, some studies did not detect any effects of heavy metals on haematocrit and haemoglobin values (Llacuna et al. 1996; Eeva et al. 2000; Fair and Ricklefs 2002; Janssens et al. 2003; Dauwe et al. 2006). These conflicting results might be related to interspecific differences in sensitivity (Henny et al. 2000).

One possible problem associated with our study is that the approach we followed may be critical for statistical reasons, as it was correlative and based on only one comparison (polluted oasis *vs* unpolluted one). It might have been more appropriate to compare several oases situated at different distances from the factory complex and exposed at different levels of pollution. However, the latter approach was unlikely because of time limitations, especially as the current work was carried out on natural nests. Indeed, locating and monitoring natural nests was a time-consuming and uncertain task. Sampling more oases would result in reduced sampling effort per oasis, and consequently on small nest sample sizes.

In conclusion, although the current study approach was correlative and did not permit establishing causal links, obtained results would suggest that stressful conditions suffered by Rufous bush robin nestlings hatched close to the Gabès-Ghannouche factory complex of phosphate treatment negatively affected their condition and development. These results, together with previously published results on passerine breeding performance (Alaya-Ltifi et al. 2012) and abundance (Alaya-Ltifi and Selmi 2014), suggest that habitat pollution is likely to represent a serious threat against the avifauna and probably additional wildlife inhabiting the oasis habitat close to the Gabès-Ghannouche factory complex. Results also indicate the usefulness of passerine nestlings as reliable biomonitors of polluted terrestrial ecosystems.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study complied with the Tunisian laws regarding wild animal sampling. Authorizations were obtained from the forest service in the Tunisian Ministry of Agriculture (Permit No. 1047-DGF/DGP). All applicable international and national guidelines for the care and use of animals were followed.

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