



Reproduction impairments in metal-polluted environments and parental hormones: No evidence for a causal association in an experimental study in breeding feral pigeons exposed to lead and zinc

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ABSTRACT

Humans are responsible for land-cover changes resulting in the emission of hazardous chemical elements including metallic trace elements i.e. MTEs. As a consequence, urban wildlife is exposed to high concentrations of MTEs, which exposure is linked to reproductive impairments. MTE effects on reproduction outputs might result from MTE exposure disrupting the endocrine pathways involved in reproductive behaviours. In birds, there is strong evidence that prolactin, corticosterone and testosterone are all involved in the regulation of parental effort during incubation and chick rearing. Endocrine-disrupting chemicals might stimulate or inhibit the production of those hormones and consequently alter parental investment and reproductive success outcomes. We measured baseline corticosterone, prolactin and testosterone plasma levels, and the corticosterone stress response of breeding feral pigeons (*Columba livia*) experimentally exposed to ecologically relevant lead and/or zinc concentrations. Independently of lead and/or zinc exposure, male and female plasma levels of corticosterone and prolactin (but not testosterone) showed temporal variations along the reproduction stages (i.e. incubation, early rearing and late rearing). In addition, both hatching and fledging success were slightly correlated with corticosterone, prolactin and testosterone levels. However, our study did not find any influence of lead or zinc exposure on hormone levels, suggesting that MTE effects on reproductive outputs might not be explained by MTE-induced modifications of corticosterone, prolactin and testosterone-linked behaviours during incubation and rearing. Alternatively, MTE-induced reproductive impairments might result from MTE exposure having direct effects on offspring phenotypes or prenatal indirect effects on the embryo (e.g. maternal transfer of MTEs, hormones or immune compounds).

1. Introduction

Across the globe, anthropogenic activities have led to numerous changes in ecosystems through agricultural activities, deforestation, industrial development or urban land expansion. Amongst other environmental modifications, humans are responsible for land-cover changes resulting in the emission of hazardous chemical elements (e.g. CO₂, NO, metallic trace elements, persistent organic pollutants, organohalogenated contaminants, etc.; Crutzen, 2006). Most of metallic trace elements (MTEs; e.g. lead, cadmium, zinc, copper, etc.) present in the environment originate from anthropogenic sources (Azimi et al., 2005; Bilos et al., 2001; Nriagu, 1989), leading to elevated concentrations nearby metallurgic factories (Dauwe et al., 1999; Derome and Nieminen, 1998; Kiikkilä et al., 2003) but also in urban areas, where lead and zinc are two of the most abundant metals (Azimi et al., 2003;

Maas et al., 2010; Manta et al., 2002; Roux and Marra, 2007). In 2030, urban areas are expected to cover 1.5% of the planet's land area (Seto et al., 2012). For this reason, it becomes increasingly essential to better understand MTE effects on urban wildlife.

Nowadays, MTEs are chemical pollutants of prime concern given their implication in several human diseases (reviewed in Jarup, 2003) and their noxious effects on wildlife (Hsu et al., 2006). The relationship between reproductive success and proximity to a metallurgic smelter has been extensively studied in passerine birds from two study regions (in Belgium and in Finland). Birds nesting in the most polluted area consistently exhibited lower reproductive outputs. For instance, proximity to the smelter was associated with higher egg-laying interruptions (in great tits *Parus major* and blue tits *Cyanistes caeruleus*; Janssens et al., 2003; Dauwe et al., 2005), reduced clutch size and hatching success (in great tits and pied flycatchers *Ficedula hypoleuca*; Eeva and

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Lehikoinen, 1995; Janssens et al., 2003; Eeva et al., 2009), lower growth and lower fledging success (in great tits and blue tits; Eeva et al., 2009; Eeva and Lehikoinen, 1996). Moreover, experimental exposure to lead decreased nestling growth, fledgling corpulence and fledging success, while it increased fledgling physiological stress (i.e. heterophile/lymphocyte ratio) (in feral pigeons *Columba livia*; Chatelain et al., 2016a).

Reproductive behaviours including territorial defence, courtship display, incubation and chick rearing are under control of multiple endocrine pathways (Cooke et al., 2004; Eisner, 1960). For instance, there is strong evidence that prolactin, corticosterone and testosterone are all involved in the regulation of parental behaviour in birds (i.e. incubation and rearing, reviewed in Lynn, 2016). First, prolactin secretion is responsible for the initiation and maintenance of incubation (Angelier and Chastel, 2009; Eisner, 1960; Khan et al., 2001; Lormée et al., 2000; Sockman et al., 2000), but also for the expression of parental cares during chick-rearing period (reviewed in Angelier et al., 2016b). For example, experimentally increased prolactin levels can enhance the expression of incubation behaviour, chick-feeding behaviour and chick defense in the rock pigeon (Buntin, 1991; Lea, 1991; Miller et al., 2009; Wang and Buntin, 1999). Importantly, reduced prolactin levels are associated with reproductive failure and abandonment of the chicks in wild birds (Angelier et al., 2015, 2013; Groscolas et al., 2008; Smiley and Adkins-Regan, 2016; Thierry et al., 2013), suggesting that this hormone is linked to reproductive performances. Second, the corticosterone stress response mediates behavioural and physiological adjustments that reduce reproductive activities to promote survival (Breuner et al., 2008; Hau et al., 2010; Landys et al., 2006; Love and Williams, 2008; Wingfield et al., 1998; Wingfield and Sapolsky, 2003). Overall, elevated circulating corticosterone levels are associated with a reduction in parental effort. For instance, experimentally increased corticosterone levels are associated with lower incubation commitment, lower nest attendance, and reduced food provisioning to nestlings in wild birds (Angelier et al., 2009; Silverin, 1986; Spée et al., 2011; Thierry et al., 2014) although the relationship between circulating corticosterone levels and parental behaviour seems context-dependent (Bonier et al., 2009; Ouyang et al., 2015, 2013). Finally, although testosterone is classically involved in the initiation of reproduction (territorial aggression, sexual and courtship behaviour; Wingfield, 1984; Wingfield et al., 1987), it is also known to mediate a trade-off between mating and parental effort in both males and females. For instance, experimental elevation of circulating testosterone levels decreased paternal and maternal care for offspring (Ketterson et al., 1992; Saino and Møller, 1995; Veiga and Polo, 2008, reviewed in Lynn, 2016, 2008) and the time the female spent brooding as well as their nestling defence in wild birds (O'Neal et al., 2008).

In 2002 the World Health Organization (WHO) defined endocrine-disrupting chemicals (EDCs) as “an exogenous substance or mixture that alters functions of the endocrine system and consequently causes adverse health effects in an intact organism, or its progeny, or (sub) populations.” (Damstra et al., 2002). EDCs can stimulate (agonists) or inhibit (antagonists) the endocrine system, causing overproduction or underproduction of hormones. Amongst EDCs, endocrine-disrupting metals (EDMs; e.g. arsenic, lead, cadmium, mercury and nickel) have been identified (Georgescu et al., 2011; Lavicoli et al., 2009). For instance, cadmium interferes with the biosynthesis of steroid hormones in rats (Piasek and Laskey, 1994; Sen Gupta et al., 2004). MTEs also induce behavioural impairments including changes in activity level, foraging success, courtship behaviour, nest attentiveness and cognitive performance, which arguably result from endocrine alterations (Clotfelter et al., 2004a, 2004b; Zala and Penn, 2004). However, very few ecological studies have investigated the effects of environmental levels of MTEs on the endocrine system (Baos et al., 2006; Franceschini et al., 2017, 2009; Herring et al., 2012; Meillère et al., 2016; Tartu et al., 2015, 2013; Wada et al., 2009; Wayland, 2002). Most of these studies focused on mercury exposure and demonstrated either negative

(Franceschini et al., 2017, 2009; Herring et al., 2012; Tartu et al., 2015; Wada et al., 2009), positive (Franceschini et al., 2017) or non-significant effects (Franceschini et al., 2017; Tartu et al., 2015; Wayland, 2002) of mercury exposure on baseline corticosterone levels and the corticosterone and prolactin stress responses. Interestingly, feather corticosterone levels (i.e. medium-term baseline corticosterone levels) were positively correlated with lead, cadmium and mercury concentrations in the feathers of blackbirds *Turdus merula* along an urbanization gradient (Meillère et al., 2016), while the corticosterone stress response decreased with increasing lead exposure in white storks *Ciconia ciconia* (Baos et al., 2006). While MTEs' disruptive effects on parental investment-linked hormones might explain the lower reproductive outputs of birds exposed to high pollution levels, this potential underlying mechanism has never been experimentally tested but for mercury exposure and therefore remains largely unexplored.

The feral pigeon is an emblematic species of cities and it is therefore exposed to high levels of MTEs (Chatelain et al., 2014; Frantz et al., 2012; Gasparini et al., 2014). To estimate to which extent MTE-induced reproductive impairments are explained by the endocrine disruption properties of these MTEs, we first measured baseline corticosterone, prolactin and testosterone levels, and the corticosterone stress response of feral pigeons experimentally exposed to ecologically relevant lead and/or zinc concentrations. Hormone profiles of both males and females were measured along three reproduction stages: incubation, early chick-rearing and late chick-rearing period. Second, we investigated the effects of hormone profiles, concurrently with lead and zinc exposure, on two proxies of reproduction success: hatching success and fledging success.

2. Methods

2.1. Subjects and housing

Free-living eumelanin feral pigeons were caught in February and March 2014 from several pigeon flocks in Paris (France). Among these birds, we chose an even number of females and males (72 males and 72 females, sexed using discriminant function analysis on weight, caruncle size and wing size). The proportion of correctly classified individuals was estimated by jackknife cross-validation procedure. The discriminant rate was 83%; Dechaume-Moncharmont et al., 2011. Sex was also validated by observations of courtship behaviour) with various eumelanin-based plumage colourations. Pigeons were kept in 12 outdoor aviaries (3.10 m × 2.00 m × 2.40 m) at the CEREEP field station (Centre d'Ecologie Expérimentale et Prédicative-Ecotron Ile-de-France, UMS 3194, Ecole Normale Supérieure, Saint-Pierre-lès-Nemours, France). They were evenly distributed among aviaries according to their sex, flock, and plumage eumelanin-based colouration in such a way that there were no confounding effects between aviaries and these variables (sex: 6 males and 6 females per aviary, flock: $\text{Chi}^2 = 165.76$, $\text{df} = 165$, $P = 0.469$, and plumage colouration: $F_{1,144} = 0.13$, $P = 0.721$). They were fed *ad libitum* with a mix of maize, wheat, and peas. The aviaries were provided with a bowl of water used for bathing and with branches as perches. Birds were individually identified with a numbered plastic ring. At the end of the experiment (i.e. after 6 months of captivity), birds were released back to the wild at their site of capture.

2.2. Treatments

The aviaries were randomly assigned to one of the 4 following metal exposure treatments: exposed to lead only (*lead* treatment; 10 ppm lead acetate, Sigma-Aldrich), exposed to zinc only (*zinc* treatment; 100 ppm zinc sulphate, Prolabo), exposed to both lead and zinc (*lead+zinc* treatment; 10 ppm lead acetate and 100 ppm zinc sulphate) or control (*control* treatment; tap water with no added metal). Consequently, there were 3 aviaries with 12 pigeons each (36 pigeons in total) per

treatment. Metals were diluted in tap water. We chose these concentrations based on both lead blood concentrations measured in urban birds (ranging from 0.053 to 0.264 ppm; (Roux and Marra, 2007)), the gastrointestinal absorption concentration of lead in zebra finches (< 10%) calculated from Dauwe et al. (2002), and previous supplementation experiments in feral pigeons (Chatelain et al., 2016a). Drinking troughs and baths were filled with the corresponding treated water every other day from the 22th of March to the 31st of August 2014 (i.e. for 162 days).

The efficiency of such a supplementation protocol in increasing internal metal levels was validated by measuring lead and zinc concentrations in birds' blood and feathers in a previous study in which birds were exposed to metals concentrations 10 times lower than the ones used in this study (Chatelain et al., 2016a). Other studies exposing birds to higher concentrations of metal, also through the drinking water, consistently measured an increase in metal concentrations in bird faeces, bones and feather (Dauwe et al., 2002; Eeva et al., 2014). Hence, we are confident that metals added to water were indeed incorporated by the birds.

2.3. Reproductive success

Reproductive success was estimated through hatching success and fledging success. Two weeks after the start of the metal treatments, six nest boxes per aviary were opened to allow birds to mate and breed. The feral pigeon is a monogamous bird species. Feral pigeons provide bi-parental care during incubation and rearing, which allows to identify both social parents of each clutch soon after egg laying. Females produced two-egg clutches, 1–3 times during the experiment. For each clutch laid between the 11th of April and the 30th of August, we randomly collected one of the two eggs for another study (Chatelain et al., 2016b). A total of 108 eggs have been laid; 54 eggs of them have been incubated. Overall, incubation time lasted 17 days (mean \pm se: 17.63 \pm 0.36 days). Hatching was considered successful when the chick was completely cleared from its shell and alive. Hatching success was measured for eggs that have been incubated. Twenty-eight eggs successfully hatched out of the 54 that have been incubated. The chick was considered as successfully fledged when it was found outside the parental nest and was able to fly and to feed by itself. Fledging success was measured only for eggs that successfully hatched. There were 16 fledglings over 28 nestlings. Fledglings left the nest at 30 day-olds (mean \pm se: 30.00 \pm 1.28 day-old). Hatching and fledging success were relatively low compared to previous studies carried out in similar conditions (Chatelain et al., 2016a), which likely result from the cold temperatures and high rainfall frequency. However, we believe that any influence the weather played on bird physiology and/or reproductive success was identical between the different treatments.

2.4. Blood sampling

Both parents were captured one to three times per reproduction attempt (i.e. per clutch produced), depending on whether they produced successful hatchlings and fledging: 1) five days after the laying date, 2) five days after the hatching date and 3) twenty-five days after the hatching date of the first egg of the clutch (i.e. at the end of nestling rearing). Birds were captured rapidly in such a way that the first blood sample was achieved not more than 3 min after the capture. Birds were then kept in a small cage for thirty minutes (acute stress procedure), at the end of which a second blood sample was done in order to investigate the corticosterone stress-response (see Wingfield et al., 1992). For each blood sampling, 50 μ L of blood was collected from the brachial vein, immediately centrifuged and the plasma was frozen at -20°C until analyses (i.e. parental hormone measurement).

2.5. Plasma hormone concentrations

Corticosterone concentrations in plasma were measured using a sandwich enzyme-linked immunosorbent assay (ELISA), using the Corticosterone EIA kit (AC-14F1, IDS, Boldon, UK). Each well of a 96-well microplate was filled with 100 μ L of corticosterone standard, control or diluted sample (1:1). The standard curve ranged from 0 to 170 ng mL⁻¹. Then 100 μ L of enzyme conjugate solution were added in all the wells and the microplate was left to incubate for 24 h at 4 $^{\circ}\text{C}$. Then, the microplate was washed and 200 μ L of tetramethylbenzidine (TMB) substrate were added in all the wells and again the microplate was left to incubate 30 min at 20 $^{\circ}\text{C}$. Finally, 100 μ L of the stop solution were added in all the wells and absorbance at 450 nm was recorded. Prolactin levels were determined at the CEBC by using a heterologous radioimmunoassay, as detailed in Cherel et al. (1994). This specific prolactin assay has previously been validated for rock pigeons (see Angelier et al., 2016a), and specifically, pooled plasma samples of feral pigeons produced a dose-response curve that paralleled chicken prolactin standard curves. Testosterone levels were determined at the CEBC by using standard radioimmunoassay techniques, as detailed in Mauget et al. (1994). Testosterone and prolactin levels were quantified using duplicates. Prolactin and testosterone samples were respectively run in two and four assays. The intra-assay and inter-assay CVs were respectively 5.13% and 12.69% for prolactin and 6.61% and 8.97% for testosterone.

2.6. Statistical analyses

To distinguish the effects of lead and/or zinc exposure on plasma hormone concentrations, we coded lead and zinc exposure as two binary variables (absence/presence; zinc-exposed (0/1) and lead-exposed (0/1)).

We performed linear mixed-effects models (lmer) with the log-transformation of plasma hormone concentration (i.e. corticosterone, testosterone or prolactin) as the dependent variable, time of blood sampling (for corticosterone only; it allows to investigate both baseline corticosterone level and the stress response, meaning the change in corticosterone plasmatic concentrations between the first and the 30 min later blood sampling), lead and zinc exposure, sex, reproduction stage, and all the 2-way and 3-way interactions with lead and zinc exposure as the explanatory variables; the aviary and the individual (i.e. ID of the bird) were added as nested random intercepts. The linear mixed-effect models were fitted using the restricted maximum likelihood (REML) method. To synthesize hormone profiles along reproduction (i.e. incubation, early rearing and late rearing), we tested the correlation between hormone levels and performed a Partial Least Squares Discriminant Analysis (see Supplementary material; Appendix 1: hormone profiles). All hormone concentrations (i.e. corticosterone, testosterone and prolactin concentrations) have been log-transformed to satisfy normality of the distribution.

To investigate whether plasma hormone concentrations explained reproductive success, we performed generalized linear mixed-effects models (glmer) with hatching success or fledging success as the dependent variable, the log-transformation of baseline corticosterone, testosterone and prolactin concentration of the mother and of the father during incubation (when explaining hatching success) or during early rearing (when explaining fledging success), and the 2-way interaction between the mother and the father hormone levels as the explanatory variables; lead exposure, zinc exposure and their 2-way interaction were added as covariates. The aviary and the mother identity were added as nested random intercepts. Because baseline corticosterone concentration and corticosterone stress response (i.e. the difference between the second and the first blood sampling) are positively correlated (see Supplementary material; Appendix 1: hormone profiles), similar models were performed with the log-transformation of corticosterone stress response replacing baseline corticosterone concentration.

Because of the small sample size of nestlings ($n = 28$; 0–4 nestlings per aviary), taking into account the aviary and the identity of the mother as random factors might partly mask the effects of the fixed factors. For this reason, fledging success was also investigated using generalized linear models (glm) including the same explanatory variables as described for the glmer. The generalized linear models and mixed-effect models were fitted using the Laplace approximation of the maximum likelihood method.

Statistical analyses were performed using R software (version 3.0.2; R Core Team, 2013). For each model, we performed a backward stepwise selection using the AIC. A Type III Wald chisquare test Anova was used to determine the significance of the retained variables in the final models. When discrete explanatory variables were retained in the models, contrasts of hormone concentrations among groups were tested using least-square mean pairwise comparisons (contrast function of the 'lsmeans' package in R; Lenth, 2016). Contrasts of hormone concentrations between metal exposure within reproduction stage and time of blood collection were also tested (see Supplementary material; Appendix 2: contrasts of hormone concentrations).

3. Results

3.1. Plasma hormone concentrations

Neither lead exposure, nor zinc exposure were retained in the models investigating corticosterone, testosterone and prolactin concentrations (Figs. 1–3 and Table 1). Corticosterone concentrations were higher in females than in males ($\text{Chi}^2 = 5.75$, $\text{df}=1$, $P = 0.016$; mean \pm se corticosterone concentration in females and males: 11.55 ± 0.73 and 8.56 ± 0.51 ng/mL) and depended on the interaction between time of blood sampling and reproduction stage ($\text{Chi}^2 = 10.88$, $\text{df} = 2$, $P = 0.004$): plasma corticosterone concentrations were significantly higher after the thirty minutes' isolation than the baseline concentration during incubation ($t = 8.16$, $\text{df} = 235.33$, $P < 0.001$) and during early ($t = 4.00$, $\text{df} = 234.82$, $P = 0.001$) but not during late chick-rearing ($t = 1.11$, $\text{df} = 235.04$, $P = 0.878$; Fig. 1). Moreover, baseline corticosterone concentrations significantly differed between reproduction stages ($\text{Chi}^2 = 38.78$, $\text{df} = 2$, $P < 0.001$): they were lower during incubation than during early ($t = -4.96$, $\text{df}=257.89$, $P < 0.001$) and late rearing ($t = 4.79$, $\text{df} = 264.28$,

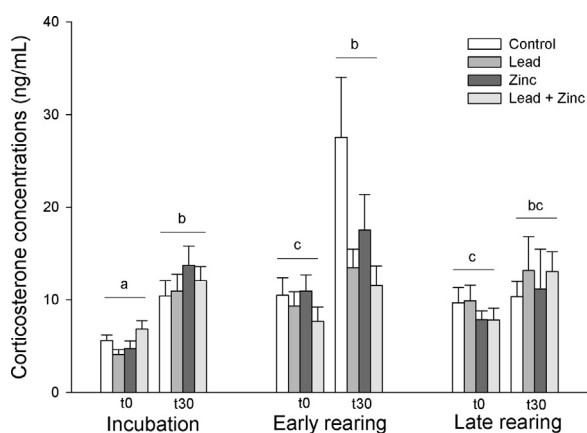


Fig. 1. Plasma corticosterone concentrations (mean \pm se) according to the reproduction stage, the time of blood sampling and metal exposure. Concentrations at t0 and t30 are baseline corticosterone concentrations and corticosterone concentrations after exposure to an acute stress procedure, respectively. Corticosterone concentrations were not different between treatments (see Appendix 2 for detailed statistical values). Significant differences of corticosterone concentrations ($\alpha < 0.05$) between time of blood sampling and reproduction stages are indicated by different letters (a, b, c).

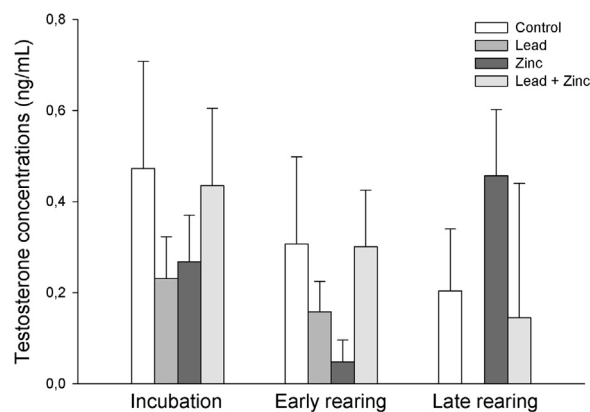


Fig. 2. Plasma testosterone concentrations (mean \pm se) according to the reproduction stage and metal exposure. There were no significant differences between testosterone concentrations (see Appendix 2 for detailed statistical values).

$P < 0.001$) although they did not differ between early and late rearing ($t = 0.53$, $\text{df} = 250.58$, $P = 0.995$; Fig. 1).

Testosterone concentrations were higher in males than in females ($\text{Chi}^2 = 9.40$, $\text{df} = 1$, $P = 0.002$; mean \pm se testosterone concentration in females and males: 0.13 ± 0.04 and 0.42 ± 0.08 ng/mL). Reproduction stage was not retained in the model investigating testosterone concentrations (Fig. 2).

Prolactin concentrations differed between reproduction stages ($\text{Chi}^2 = 90.31$, $\text{df}=2$, $P < 0.001$): they were higher during early rearing than during incubation ($t = 8.99$, $\text{df}=109.42$, $P < 0.001$) and late rearing ($t = 6.88$, $\text{df} = 106.46$, $P < 0.001$) but they were not significantly different between incubation and late rearing ($t = -0.08$, $\text{df} = 122.13$, $P = 0.996$; Fig. 3).

The Partial Least Squares Discriminant Analysis shows that hormone profiles differed along reproduction (i.e. incubation, early rearing and late rearing), independently of metal exposure. While pigeons exhibited low baseline corticosterone and prolactin levels during incubation, they had high baseline corticosterone and prolactin levels during early chick-rearing and high baseline corticosterone but low prolactin levels during late chick-rearing (see Supplementary material; Appendix 1: hormone profiles).

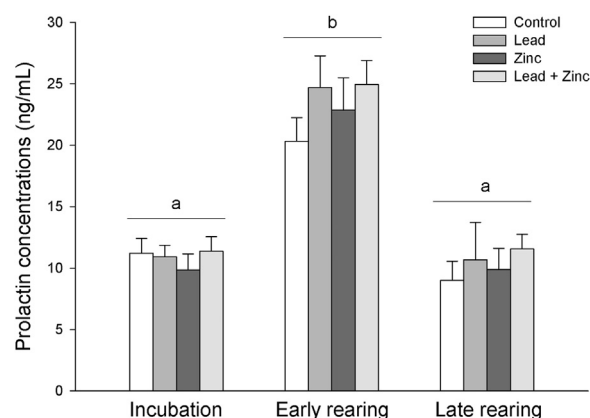


Fig. 3. Plasma prolactin concentrations (mean \pm se) according to the reproduction stage and metal exposure. Prolactin concentrations were not different between treatments (see Appendix 2 for detailed statistical values). Significant differences of prolactin concentrations ($\alpha < 0.05$) between reproduction stages are indicated by different letters (a, b).

Table 1

AIC of the full, final and null models testing for the effects of time of blood sampling (for corticosterone only), lead and zinc exposure, sex, reproduction stage, and all the 2-way and 3-way interactions with lead and zinc exposure on corticosterone, testosterone and prolactin levels. For the final models, the retained variables are detailed between brackets).

	AIC Full model	AIC Final model (<i>Retained explanatory variables</i>)	AIC Null model
Corticosterone	671.52	638.90 (<i>Sex + Time × Reproduction stage</i>)	713.55
Testosterone	127.92	80.57 (<i>Sex</i>)	82.86
Prolactin	241.11	202.17 (<i>Reproduction stage</i>)	262.46

3.2. Reproductive success

Neither lead exposure, nor zinc exposure were retained in the models investigating hatching and fledging success (Table 2). Hatching success significantly increased with decreasing maternal baseline corticosterone concentration measured during incubation ($\text{Chi}^2 = 4.56$, $\text{df} = 1$, $P = 0.033$; Table 2). The model also retained maternal prolactin concentration during incubation ($\text{Chi}^2 = 2.60$, $\text{df} = 1$, $P = 0.107$). When considering corticosterone stress response (instead of baseline corticosterone concentration), both maternal and paternal corticosterone stress response and maternal prolactin concentration during incubation were retained in the model ($\text{Chi}^2 = 2.36$, $\text{df} = 1$, $P = 0.125$, $\text{Chi}^2 = 0.33$, $\text{df} = 1$, $P = 0.563$ and $\text{Chi}^2 = 2.96$, $\text{df} = 1$, $P = 0.085$, respectively; Table 2). Maternal baseline corticosterone and testosterone concentrations, and paternal prolactin concentration during early rearing were retained in the final models investigating fledging success ($\text{Chi}^2 = 1.18$, $\text{df} = 1$, $P = 0.278$, $\text{Chi}^2 = 1.06$, $\text{df} = 1$, $P = 0.304$ and $\text{Chi}^2 = 1.16$, $\text{df} = 1$, $P = 0.282$, respectively; Table 2). When removing the random effects from the model (i.e. the aviary and the identity of the female), fledging success increased with decreasing maternal baseline corticosterone and paternal prolactin concentrations during early rearing ($\text{Chi}^2 = 5.26$, $\text{df} = 1$, $P = 0.022$ and $\text{Chi}^2 = 4.56$, $\text{df} = 1$, $P = 0.033$, respectively). It tended to increase with increasing maternal testosterone concentration during early rearing ($\text{Chi}^2 = 3.26$, $\text{df} = 1$, $P = 0.071$). The model failed to converge when considering corticosterone stress response; the mean corticosterone stress response during early rearing was higher in the parents of the offspring that successfully fledged relative to the parents of the offspring that died before fledging but concentration ranges were strongly overlapping (Table 2).

Table 2

A Mean ± se hormone plasmatic concentrations (i.e. baseline corticosterone, testosterone and prolactin concentration, and corticosterone stress response, in ng/mL) of females and males that produced successful (1) or unsuccessful (0) hatchlings (hormone levels during incubation) and fledglings (hormone levels during early rearing). Corticosterone stress response was calculated as the difference of corticosterone concentration between the second and the first blood sampling. Explanatory variables retained in the final glmer models explaining hatching and fledging success are highlighted in bold. Significant differences between hormone concentrations in successful and unsuccessful parents ($\alpha < 0.05$) are indicated by different letters (a, b). B Mean ± se hatching and fledging success in control, lead and/or zinc exposed treatments. Hatching and fledging success did not significantly differ between the four metal treatments.

			Hatching success		Fledging success	
			0	1	0	1
A	Females	Baseline corticosterone	5.49 ± 0.57^a	3.92 ± 0.59^b	7.65 ± 0.49^a	7.22 ± 1.73^a
		Testosterone	0.02 ± 0.02	0.08 ± 0.05	0.04 ± 0.10^a	0.16 ± 0.08^a
		Prolactin	11.49 ± 1.55^a	12.85 ± 1.05^a	22.24 ± 2.39	23.01 ± 2.15
	Males	Corticosterone stress response	1.01 ± 0.33^a	4.20 ± 1.52^a	1.03 ± 0.70	2.24 ± 0.82
		Baseline corticosterone	5.36 ± 0.97	5.85 ± 0.86	11.82 ± 1.83	10.15 ± 1.57
		Testosterone	0.68 ± 0.22	0.37 ± 0.12	0.22 ± 0.18	0.36 ± 0.15
B	Metal treatment	Control		0.40 ± 0.16		0.50 ± 0.29
		Lead exposure		0.41 ± 0.12		0.57 ± 0.20
		Zinc exposure		0.57 ± 0.14		0.25 ± 0.39
		Lead + Zinc exposure		0.46 ± 0.14		0.83 ± 0.43
		Corticosterone stress response	2.17 ± 0.88^a	2.94 ± 1.28^a	0.71 ± 0.25	1.40 ± 0.52
		Prolactin	8.92 ± 1.05	10.04 ± 1.02	28.45 ± 2.32^a	21.28 ± 2.87^a

4. Discussion

Alteration of reproductive outputs in wild bird populations has been associated with exposure to metallic trace elements (MTEs) (Dauwe et al., 2005; Eeva et al., 2009; Eeva and Lehikoinen, 1996, 1995; Janssens et al., 2003). In feral pigeons, offspring quality (i.e. mass at hatching, nestling growth, fledging corpulence and physiological stress) was either reduced or increased depending on whether birds were experimentally exposed to urban concentrations of lead or zinc (Chatelain et al., 2016a). Because several MTEs, including lead, have been identified to be endocrine-disrupting chemicals (EDCs) (Georgescu et al., 2011; Lavicoli et al., 2009), MTE effects on the regulation of parental hormones (e.g. corticosterone, prolactin or testosterone) may stem from reproduction impairments in MTE-polluted environments. However, our study does not support this hypothesis because corticosterone, prolactin and testosterone concentrations were not affected by exposure of feral pigeons to ecologically relevant lead and/or zinc concentrations. Nonetheless, the high variability in bird response to MTE exposure stresses the need to conduct studies involving high sample sizes.

4.1. MTEs as endocrine disruptors in breeding feral pigeons?

This study did not highlight any significant effect of lead or zinc exposure on baseline corticosterone levels, the corticosterone stress response, prolactin and testosterone levels in feral pigeons. While this study was the first one to experimentally test the effects of lead and zinc exposure on plasma prolactin and testosterone levels, previous studies have reported contrasted effects of lead exposure on plasma corticosterone levels. Specifically, feather corticosterone levels increased with increasing lead exposure in blackbirds (Meillère et al., 2016) while plasma corticosterone levels were not correlated with lead exposure in great tit nestlings (Eeva et al., 2003). Furthermore, the corticosterone stress response increased with increasing lead exposure in white stork nestlings (Baos et al., 2006). Two experimental studies failed to detect any impact of lead exposure on fecal corticosterone levels (great tit nestlings; Eeva et al., 2014) or the corticosterone stress response (zebra finches, *Taeniopygia guttata*; Snoeijs et al., 2005). The discrepancy between those studies may result from the dose-dependence effects of lead. For instance, the shape of the response curve might be complex (for instance, one might imagine a minimal threshold level of safe exposure followed by a linear or a non-linear dosage effect, a U-shaped or even an inverted U-shaped relationship (Zala and Penn, 2004)). Environmental concentrations of MTEs are highly variable depending on urbanization rate (Azimi et al., 2003; Maas et al., 2010; Manta et al.,

2002; Meillère et al., 2016; Roux and Marra, 2007). While our study suggests that the exposure to lead and zinc concentrations that may occur in cities does not disrupt the endocrine function involving corticosterone, prolactin and testosterone, we might still expect noxious effects of MTEs in more or even in less polluted environments. Such effects might also depend on the assemblage of pollutants in the environment. Indeed, wild animal populations are exposed to multiple potential EDCs (e.g. various MTEs including cadmium, mercury or arsenic, PCBs or Bisphenols A) that may have additive, synergetic or antagonistic effects (Lavicoli et al., 2009). Moreover, while this study investigated the response of three major hormones involved in parental care in birds (corticosterone, prolactin and testosterone; Lynn, 2016), we cannot exclude that MTE could affect other hormones that has not been tested in this study (e.g. progesterone, oestrogen, oxytocin; Buntin, 1996). Alternatively, animal sensitivity to MTE exposure might be taxon-specific and might depend on the environment the species or population originates from. The feral pigeon has evolved in natural environments in close proximity with human for centuries (Johnston and Janiga, 1995). For this reason, this species might be the best example of synurbization (Luniak, 2004). Therefore, we can expect urban populations of feral pigeons to have adjusted to the specific conditions of urban environments such as high MTE levels. For instance, corticosterone stress response was higher in darker feral pigeons from a rural area than from an urban centre (Corbel et al., 2016), which may result from adaptive or habituation mechanisms to human presence resulting in the downregulation of the stress response (Cyr and Romero, 2007; Rich, 2005). In the same way, feral pigeon endocrine system might be less responsive to MTE exposure in urban populations. The complexity of EDCs adverse effects stresses the necessity to investigate the endocrine disruptive effects of MTEs in populations from different environments but also in species with different timing of urbanization (i.e. colonization date of urban environments). Finally, although our study did not demonstrate any effect of lead and zinc exposure on corticosterone, testosterone and prolactin levels, hormonal response variability between individuals within a same metal treatment might have masked low amplitude effects. For instance, the mean concentration of corticosterone during early rearing was lower in birds exposed to lead (i.e. lead and lead+zinc) compared to controls and birds exposed to zinc only, while this pattern was reversed for prolactin levels. Moreover, testosterone levels were highly variable within and between metal treatments, but its mean was consistently lower in birds exposed to lead only compared to controls. Those non-significant differences raise the interest in conducting similar but broader studies to disentangle the effects of real physiological processes from a potential lack of statistical power.

4.2. MTEs and reproductive success in feral pigeons

Consistently with our previous experiment in feral pigeons (Chatelain et al., 2016a), lead and zinc exposure did not have any significant effect on hatching and fledging success, suggesting that lead and zinc exposure in urban areas are unlikely to affect offspring short time survival while it might impair their quality (i.e. nestling growth, fledging body condition and physiological stress; Chatelain et al., 2016a). The absence of relationship between lead or zinc exposure and corticosterone, prolactin and testosterone levels suggests that the effects of MTEs on reproductive outputs highlighted in previous studies (Chatelain et al., 2016a; Dauwe et al., 2005; Eeva and Lehikoinen, 1995, 1996, Janssens, 2003) might not be explained by MTE-induced modifications of corticosterone, prolactin and testosterone-linked behaviours during incubation and rearing. While MTE-induced parental behaviour alterations (e.g. through other endocrine pathways or non-hormonal mechanisms) cannot be excluded (Stolzenberg and Champagne, 2016), most effects of MTEs on reproductive outputs may be explained either by direct effects of MTE on offspring phenotypes (e.g. growth) or by prenatal indirect effects of MTE on the embryo. For

instance, we previously demonstrated that lead and zinc exposure may affect maternal investment during egg formation in the feral pigeon. Specifically, we showed that lead-exposed females laid eggs with thinner eggshells and lower amounts of specific antibodies, while zinc-exposed ones laid eggs with thicker eggshells, heavier yolks and higher amounts of lysozymes (i.e. antimicrobial proteins) (Chatelain et al., 2016a, 2016b). In addition, female exposure to MTEs may also influence maternal transfer of hormones, including corticosterone (in Japanese quails *Coturnix japonica* and great tits; Okuliarová et al., 2010; Pitk et al., 2012). Corticosterone levels in eggs have been associated with reproduction impairments including higher embryonic mortality, lower egg hatchability, slower nestling growth or increased juvenile oxidative stress (Eriksen et al., 2003; Haussmann et al., 2012; Hayward and Wingfield, 2004; Heiblum et al., 2001; Mashaly, 1991; Rubolini et al., 2005; Saino et al., 2005). Alternatively, MTEs can be transferred from the mother into the eggs (Dauwe et al., 1999; personal data in feral pigeons), which may alter embryo development (Buerger et al., 1986; Kertész et al., 2006; Kertész and FánCSI, 2003). Finally, we may expect direct effects of MTE exposure on nestlings through water provisioning by parents (e.g. in crop-milk). Because maternal transfers may have carry-over effects, future studies should try to disentangle MTE-induced changes in parental investment during egg formation, incubation and rearing from direct effects of MTE exposure on nestlings. The effects of EDCs on endocrine function during pre-breeding (e.g. initiation of sexual behaviour including courtship behaviour or nest building) and on sex-ratio (Pike and Petrie, 2006, 2005) also constitute interesting avenues to better understand how MTE exposure may impact reproductive outputs in wild bird populations.

4.3. Hormones, reproductive stages, and reproductive success

Independent of lead and/or zinc exposure, our study allowed to better understand the variation in corticosterone, prolactin and testosterone plasma levels along the reproductive period in both female and male feral pigeons. Male and female plasma levels of corticosterone and prolactin (but not testosterone) showed temporal variations, suggesting that both corticosterone and prolactin would be relevant measure of parental investment in pigeons. Prolactin levels were higher during early rearing than during incubation and late rearing. This pattern arguably stems from the role of prolactin in crop-milk production that occurs during the first days after hatching (Buntin, 1991; Eisner, 1960). Elevated prolactin levels have been associated with higher parental cares and better reproductive success in birds (reviewed in Angelier et al., 2016b; Angelier and Chastel, 2009), and especially with increased crop-milk production, squab feeding and squab condition in doves (Buntin, 1991; Miller et al., 2009; Wang and Buntin, 1999). Surprisingly, our study did not show a strong relation between prolactin levels and reproductive success (i.e. hatching and fledging success). Yet, hatching success tended to be positively related to maternal prolactin levels during incubation which may stem from prolactin positive effects on nest attendance during incubation and parents' unwillingness to abandon their eggs (reviewed in Angelier et al., 2016b; Angelier and Chastel, 2009). Counter-intuitively, fledging success was, however, negatively related to paternal prolactin levels during early rearing. While a reduced paternal effort may be compensated by an increased maternal effort, there was no significant relation between paternal and maternal prolactin levels during early rearing (see Supplementary material; Appendix 3: hormone level covariation between parents). Alternatively, the males who invested a lot in early chick rearing may have suffered a strong reproduction cost which may have prevented them to invest in late chick rearing, potentially explaining this counter-intuitive result.

Baseline corticosterone concentrations increased from incubation to rearing period in male and female parents. Because moderate elevated corticosterone levels allow the parents to sustain the energetic demands of reproduction (reviewed in Romero, 2002, Love et al., 2004, Landys

et al., 2006, Bonier et al., 2009), the higher baseline corticosterone levels during early and late rearing than during incubation supports the idea that rearing/feeding a chick is more demanding than incubating an egg, probably because the production of crop milk is energetically costly in feral pigeons. In addition, we also found that elevated maternal corticosterone levels during incubation and early rearing were associated with breeding failure. Elevated corticosterone levels have often been associated with lower incubation commitment and nest desertion in incubating birds (Angelier et al., 2007; Bonier et al., 2009; Groscolas et al., 2008; Ouyang et al., 2011; Spée et al., 2011; Thierry et al., 2013) and lower food provisioning to nestlings (Silverin, 1986), explaining potentially this link between circulating corticosterone levels and hatching and fledging success in our study.

Finally, testosterone levels did not show significant variation between the three reproduction stages, suggesting that this hormone does not play a major role in parental investment in the feral pigeon. Moreover, this study did not demonstrate any significant relation between testosterone levels and reproductive success outcomes (i.e. hatching and fledging success). Plasma testosterone levels were highly variable between individuals. A potential lack of statistical power might have prevented to detect such a relationship. However, testosterone levels tended to be higher in mothers of successful fledglings. Moreover, although not significant, the mean testosterone levels were higher in mothers and lower in fathers of successful hatchlings. While previous studies demonstrated negative effects of experimental testosterone injections on paternal and maternal care for offspring (Ketterson et al., 1992; Saino and Møller, 1995; Veiga and Polo, 2008, reviewed in Lynn, 2016, 2008), to the best of our knowledge, this is the first study suggesting a potential positive effect of maternal testosterone levels on hatching and fledging success. This positive correlation arguably stems from the effect of testosterone on embryonic development. Testosterone deposition into the eggs, which is correlated to maternal testosterone levels during yolk formation (Schwabl, 1996), stimulates muscle growth of the embryo and consequently influences the hatching process (Lipar and Ketterson, 2000). Maternal testosterone levels remain poorly studied in wild bird populations and future studies should further investigate the link between natural testosterone levels and reproductive success.

While MTEs are responsible for reproduction impairments in wild bird populations, the underlying physiological mechanisms remain unclear. Our study, by finding no evidence that lead and zinc exposure alters three of the main hormones linked to parental investment (i.e. corticosterone, prolactin and testosterone) in urban feral pigeons, stresses the necessity to investigate other avenues, including other endocrine pathways, bird behaviour, maternal transfers of MTEs and of hormones into the eggs, to explain the effects of MTEs on reproductive outputs demonstrated in previous studies.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.ecoenv.2018.06.043>.

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