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No experimental evidence of an adaptive antioxidative response induced by trace metals exposure in feral pigeons

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12 **Abstract**

13 Trace metals produced by anthropogenic activities in particular in urban environments, such as
14 lead and zinc, can induce oxidative damage in exposed individuals. Therefore, trace metals
15 could act as a selective pressure for higher resistance to oxidative damage by favouring
16 individuals able to plastically produce antioxidants once exposed to metal to counterbalance the
17 oxidative damage production. In this study, we experimentally challenge this hypothesis in 69
18 feral pigeons (*Columba livia*) originating from an urban environment by exposing a subset of
19 them to zinc (n = 18), lead (n = 17) or zinc and lead (n = 16) during 14 weeks to see if exposed
20 individuals showed an increased antioxidant production compared to controls (n = 18). Our
21 results reported that zinc exposure induced oxidative stress by increasing oxidative damage but
22 failed to detect a compensatory production of antioxidants in exposed individuals. However,
23 our results report lower oxidative damage for reproducing individuals, which is consistent with
24 an oxidative shielding phenomenon occurring prior to reproduction in males to protect the
25 sperm, and during egg laying in females to protect offspring. In conclusion, our study does not
26 support an adaptive antioxidative response in individuals exposed to trace metals, but it does
27 partly support the oxidative shielding hypothesis.

28

29

30 **Keywords:** oxidative stress, trace metals, urban pollution, oxidative shielding, feral pigeon,
31 colour polymorphism, eumelanin

32

33 **Introduction**

34 Urbanization constitutes a novel constraint for living organisms that drastically changes the
35 environmental variables and generally has a negative impact on biodiversity (Johnson and
36 Munshi-South 2017). However, several species called urban exploiters are found in urban areas
37 because they can cope with the constraints of urbanization such as pollutions and human
38 proximity. In this context, one of the blooming topics in urban ecology is to understand how
39 species adapt to survive in cities (Johnson and Munshi-South 2017). Trace metal pollutants are
40 known to be linked to urban environments. Concentrations of trace metals are higher in urban
41 environments compared to rural ones, thus urban exploiters have to face high chronic exposure
42 to trace metals (Azimi et al. 2003, Roux and Marra 2007, Sánchez-Virosta et al. 2021). Once
43 absorbed, trace metals often occur as divalent cations that can cause oxidative damage (Nuran
44 et al. 2001, Schilderman et al. 1997, Valko et al. 2005). Indeed, most trace metals are harmful
45 pro-oxidants that can interfere with metabolism by altering antioxidant enzymatic activities and
46 increasing reactive oxygen species (ROS) levels (Koivula et al. 2011). Free radicals are
47 naturally produced during cell metabolism. These ROS can cause molecular and cellular
48 damage, but are necessary for several cellular functions. In consequence, ROS production may
49 entail fitness costs (Alonso-Alvarez et al. 2017). Since antioxidants can reduce internal levels
50 of ROS, keeping up the redox balance, trace metals could act as a selective pressure for higher
51 resistance to oxidative damage. This selective pressure would favour individuals able to
52 plastically produce antioxidants once exposed to metal to counterbalance ROS production.

53 During reproduction, metabolism demands are higher and oxidative stress may occur
54 (Alonso-Alvarez et al. 2004, Costantini et al. 2014). However, several studies failed to observe
55 a link between reproduction and an increase in oxidative damage. A recent idea to explain the
56 absence of a relation between reproduction and oxidative damage is that reproducing
57 individuals can produce higher antioxidants to prevent oxidative damage (the so-called

58 oxidative shielding) and protect their offspring and/or gametes (Birch et al. 2024, Blount et al.
59 2016, Vitikainen et al. 2016). This hypothesis predicts to observe a higher production of
60 antioxidants in reproducing individuals compared to non-reproducing ones. Similarly, the
61 adaptation to cope with metal exposure in an urban environment would involve reducing
62 oxidative damage by plastically increasing antioxidant production once individuals are exposed
63 to trace metals. In this study, we experimentally tested this hypothesis in an urban exploiter: the
64 feral pigeon (*Columba livia*). If this hypothesis is true, we expected to observe, in trace metal
65 exposed individuals, a higher production of antioxidants as compared to non-exposed
66 individuals, maintaining the oxidative damages at similar levels in the two groups.
67 Alternatively, if our hypothesis is wrong, we should observe a higher oxidative damage in trace
68 metal-exposed individuals as compared to non-exposed individuals.

69 In feral pigeons, the degree of melanism varies continuously from pure white to full
70 black individuals depending on the level of eumelanin in feathers. Darker individuals are found
71 in higher proportions in urban compared to rural environments (Jacquin et al. 2012, Jacquin et
72 al. 2013). Moreover, melanin level was linked to the capacity to store trace metals in feathers
73 (Chatelain et al. 2014). Taken together, these results suggest a possible mechanism where
74 darker individuals are selected through their capacity to handle trace metals better than paler
75 ones. This potential mechanism could occur via oxidative stress, where darker individuals
76 would show lower oxidative damage when exposed to trace metals compared to paler
77 individuals (Chatelain et al. 2016a). Therefore, the eumelanin-based colouration may indirectly
78 modulate the oxidative damages impaired by trace metal exposure and could also modulate the
79 antioxidative response.

80 To test these predictions, we experimentally exposed urban feral pigeons to trace
81 metals, zinc and lead, alone or in interaction at concentrations observed in urban environments
82 (Chatelain et al. 2016a). Then, 7 and 14 weeks after the beginning of the treatment, the levels

83 of oxidative damage and antioxidants were compared between metal-exposed groups (zinc,
84 lead, zinc+lead) and the control group. We also examined whether the levels of oxidative
85 damage and antioxidants are linked to eumelanin-based colouration (Chatelain et al. 2016a).
86 We expected oxidative damage to be more pronounced in pale individuals than in dark
87 individuals when exposed to trace metals. Therefore, we hypothesized that the antioxidative
88 response would mainly occur in pale individuals. Finally, we investigated whether the oxidative
89 shielding occurred in pigeons by comparing the production of oxidative damages and
90 antioxidants between reproducing and non-reproducing individuals.

91 **Materials and methods**

92 *Capture and housing*

93 We captured a total of 69 adult feral pigeons between January 29th and February 4th, in the
94 center of Paris (France) in 2019. Individuals were first sexed visually based on morphological
95 criteria (size, appearance of the caruncle and iridescent reflection of the neck). We confirmed
96 or corrected sex by behavioural observation specific to each sex (such as "bow-coo" for males,
97 (Fabricius and Jansson 1963)). Birds were transferred to the CEREEP (Centre d'Ecologie
98 Expérimentale et Prédictive-Ecotron Ile-de-France, Saint-Pierre-le-Nemours, France) and
99 placed in eight outdoor aviaries (3.1 × 2.0 × 2.4m). They were identified individually with
100 coloured rings. We distributed individuals among aviaries and treatments to balance sex, mass
101 and level of melanism (see below). With a total of 38 males and 31 females, we placed 9
102 pigeons in 6 aviaries (5 males and 4 females), 8 pigeons in one aviary (4 males and 4 females)
103 and 7 pigeons in one aviary (4 males and 3 females). They were fed *ad libitum* with a mixture
104 of corn, wheat and peas. The aviaries contained a drinking-trough and a basin used as a bath, as
105 well as a nesting area and bamboo perches. Birds were released at capture site at the end of the
106 experiment. All experiments were carried out in strict accordance with the recommendations of
107 the 'European Convention for the Protection of vertebrate Animals used for Experimental and

108 Other Scientific Purposes' and were conducted under the authorizations of the French authority
109 (authorization APAFIS#17554-201811161046635v2).

110

111 *Treatment*

112 Treatment started a week after capture and lasted 14 weeks. We implemented 4 different
113 supplementation treatments (one by aviary): a lead treatment (10 ppm in tap water; Sigma-
114 Aldrich, St. Louis, MO, USA; n = 17); a zinc treatment (100 ppm in tap water; n = 18), a lead
115 and zinc treatment (10 ppm of lead and 100 ppm of zinc in tap water; n = 16) and a non-metal
116 control treatment (tap water only; n=18). We chose these concentrations in order to better
117 mimic the concentrations to which pigeons are exposed in urban areas based on the
118 concentrations found in the city of Paris (Chatelain et al. 2016b). Indeed, the effectiveness of
119 this treatment has been previously demonstrated by increasing lead and zinc concentrations
120 circulating in the blood (Chatelain et al. 2016b). There were 2 aviaries per treatment. Treated
121 water was added to drinking-troughs and baths. Pigeons had unlimited access to water which
122 was replaced twice a week.

123

124 *Oxidative stress*

125 Blood was obtained by puncturing the brachial vein on the left wing. Blood samples were taken
126 three times for each pigeon: just before the beginning of trace metal supplementation in the
127 water (Week 0, January 29th), at week 7 (March 21st, 2019) and at week 14 (May 7th, 2019).
128 Plasma was collected after samples were centrifuged 5 minutes (10 000 rpm), and held in a
129 freezer at -20 °C. We analysed two oxidative stress markers: the plasma reactive oxygen
130 metabolites concentration (ROM) and the total antioxidant capacity of the plasma (OXY).
131 These markers were measured using two kits, which function on colorimetric determination of
132 ROM concentrations or of antioxidant capacity. For both kits, the samples were randomly

133 assigned to 96-well plates, mixing the reagent with 4 μL of individual plasma resulted in a
134 coloured mixture readable with a spectrophotometer. ROM plasma concentration was measured
135 using the d-ROM test (kit MC0001, Diacron International, Grosseto, Italy). The test mainly
136 measures the hydroperoxides that are products of the peroxidation of macromolecules (mainly
137 lipids) with ROS. These markers are the first formed during the chain reaction induced by
138 ROS. Hydroperoxides are relatively stable chemical species but can potentially generate new
139 radicals, they are considered as markers of oxidative stress. The ROMs are expressed in mM of
140 H_2O_2 equivalents (Costantini et al. 2007, Vassalle et al. 2008). The OXY test (OXY-adsorbent
141 test, Diacron, Italy) quantifies the total antioxidant capacity of the plasma against the oxidizing
142 action of hypochlorous acid (HClO). The measurements are expressed in μmol of HClO
143 consumed by 1 mL of sample ($\mu\text{mol HClO} / \text{mL}$).

144

145 *Reproductive status*

146 A total of 18 pigeons (8 from the control group, 6 from the lead treatment, 2 from zinc
147 treatment and 2 from lead and zinc treatment) formed 9 pairs that laid at least one egg. Pigeons
148 are indeterminate egg-laying birds (Kennedy 1991), meaning that females can continue to
149 produce eggs after removing a first clutch. Laying started at week 6. From then, we collected
150 eggs twice a week and parents were identified by observing rings of individuals on nests.
151 During the 14 weeks experiment, two pairs laid 1 egg, three laid 3 eggs, two pairs laid 4 eggs
152 and two pairs laid 5 eggs. An individual was considered as a “reproducing individual” when it
153 produces at least one egg. We then used this binary variable for statistical analyses
154 (reproducing versus non-reproducing individuals).

155

156 *Melanism level*

157 Melanism level was estimated via plumage colouration. The upper surface of the right wing
158 was photographed for each pigeon under the same light source with a colour chart in the picture
159 for calibration. Eumelanin-based colouration was estimated as the percentage of black pixels of
160 a delimited part of the wing, which comprises all feathers of a wing except primaries and
161 secondaries. This score was calculated using the Gimp image software (v 2.10). This
162 measurement is repeatable between individuals and is considered a good relative estimate of
163 eumelanin level (Jacquin et al. 2011).

164

165 *Statistical analyses*

166 Analyses were performed with JMP[®], version 16. Three individuals died during the
167 experiment, thus we had a total of 66 individuals with oxidative stress measures. ROM were
168 log-transformed to better converge with a normal distribution. To explain variations in
169 oxidative variables using the three repeated measures per individual, the two models used
170 Log₁₀[ROM] and OXY as response variables and tested the effect of zinc coded as unexposed
171 (0) or exposed (1), the effect of lead coded as unexposed (0) or exposed (1), time (three
172 categorical occurrences: Week 0, 7 and 14), sex coded as male (0) or female (1), reproduction
173 (has produced eggs or not) and eumelanin level (continuous variable). We included the
174 following biological meaningful interactions without saturating the models: time*lead,
175 time*zinc, lead*zinc, time*zinc*lead, melanin*zinc, melanin*lead, melanin*lead*time,
176 melanin*zinc*time, time*reproduction time*sex, reproduction*sex and time*reproduction*sex.
177 We also added aviary and pigeon ID as random variables to take into account for
178 pseudoreplication.

179 The two models were linear mixed-model ANOVA with restricted maximum likelihood
180 (REML). The method of best-fitted model selection (based on AIC) was done by removing
181 non-significant interactions in a step-wise manner. Based on AIC, the aviary random factor was

182 not retained in the final models. Statistic outputs were produced using ANOVA type III. Post-
183 hoc t-tests were performed to interpret significant interactions between two factors.

184

185 **Results**

186 *Antioxidative response induced by trace metal exposure*

187 Our results show a significant interaction between zinc exposure and time on ROM plasma
188 concentrations (Table 1). Post-hoc tests revealed that, at week 14, pigeons exposed to zinc had
189 significantly higher ROM concentration than non-exposed pigeons (t-test, $t_{61.7} = 2.25$, $p = 0.03$,
190 Figure 1). Such a difference was not observed in either week 0 (t-test, $t_{61.1} = 0.13$, $p = 0.90$) or
191 week 7 (t-test, $t_{62.7} = 0.16$, $p = 0.87$, Figure 1). Then, lead exposure had no effect on ROM
192 (alone or in interaction, Table 1). Finally, we did not detect any effects of lead and zinc
193 exposure alone or in interaction on the total antioxidant capacity OXY (Table 1).

194 *Oxidative shielding*

195 Our results show a significant three-way interaction between sex, reproduction and time on
196 reactive oxygen species concentration ROM (Table 1). First, post-hoc tests revealed that, at
197 week 0, reproducing male pigeons had significantly lower ROM concentration than non-
198 reproducing male pigeons (t-test, $t_{19.8} = 3.65$, $p = 0.002$, Figure 2A). Such a difference was not
199 observed for males at week 7 (t-test, $t_{12.7} = 0.22$, $p = 0.83$) or at week 14 (t-test, $t_{10.8} = 0.29$, $p =$
200 0.78). Second, post-hoc tests revealed that, at week 14, reproducing female pigeons had
201 significantly lower ROM concentration than non-reproducing female pigeons (t-test, $t_{20.3} =$
202 1.97 , $p = 0.03$, Figure 2B). Such a difference was not observed for females at week 0 (t-test,
203 $t_{16.6} = 0.10$, $p = 0.92$) or at week 7 (t-test, $t_{13.2} = 0.88$, $p = 0.40$). Finally, we did not detect any
204 effect of reproduction alone or in interaction on the total antioxidant capacity OXY (Table 1).

205

206 *Antioxidative response, oxidative shielding and eumelanin-based colouration*

207 We detected no effect of eumelanin-based colouration alone or in interaction on ROM or on the
208 total antioxidant capacity OXY (Table 1).

209

210 **Discussion**

211 *Antioxidative response induced by trace metal exposure*

212 In the context of an antioxidative response, a higher production of antioxidants is expected in
213 individuals exposed to trace metal than in non-exposed individuals. The antioxidative
214 production would thus help to maintain oxidative damages at similar levels in both groups.
215 Contrary to these predictions, we observed no effect of zinc or lead exposure on antioxidant
216 production, but also an increase of oxidative damage in pigeons when exposed to zinc (Figure
217 1). Therefore, our results suggest no antioxidative response in pigeons towards trace metal
218 exposure, and confirm previous studies on the toxicity of zinc via the oxidative stress (Koivula
219 and Eeva 2010) and the weak toxicity of lead via the oxidative stress (Rainio et al. 2015). As
220 zinc is a redox-inactive metal, it has been suggested that this compound depletes the major
221 antioxidants of cells, contributing to higher stress levels (Koivula and Eeva 2010). Our results
222 are not in agreement with this mechanism as we did not detect any negative effect of zinc on
223 antioxidants production. Nevertheless, one can imagine that the depletion may occur by
224 preventing the *de novo* synthesis of antioxidants leading to the temporary stabilization of their
225 concentration over our experiment. Alternatively, as oxidative damage and antioxidant
226 response may differ among tissues, our measures in blood plasma may not be generalized to the
227 entire organism (Costantini 2019). The effect of zinc exposure on oxidative damage could also
228 be indirect. Individuals exposed to zinc would invest more in the immune system (Chatelain et
229 al. 2016b) which is known to induce oxidative damages (Costantini 2022, Costantini et al.

230 2014). Finally, the failure to detect the antioxidative response might be due to the duration of
231 metal exposure which would be too short to create sufficient oxidative stress to induce
232 antioxidants production. It is possible that short-term chronic metal exposure may be
233 detoxified by the pigeons by accumulating metals into several tissues such as the feathers
234 (Frantz et al. 2012). A longer-term experimental study, which would lead to potential saturation
235 of detoxifying tissues with metals, would be necessary to truly falsify the antioxidative
236 response hypothesis.

237 *Oxidative shielding*

238 At first glance, no evidence of oxidative shielding was observed in response to reproduction in
239 the feral pigeon. Specifically, there was no effect on the production of antioxidants that would
240 maintain oxidative damage at consistent levels over time in reproducing individuals. However,
241 we found contrasting results regarding oxidative damage in reproducing individuals between
242 males and females (Figure 2). Reproducing males have significantly lower levels of oxidative
243 damage at the beginning of the experiment (week 0) compared to non-reproducing males
244 (Figure 2A). Conversely, non-reproducing females displayed elevated levels of oxidative
245 damage at the end of the experiment compared to reproducing females (Figure 2B). These
246 results are consistent with the oxidative shielding hypothesis, as reproducing individuals
247 exhibited lower levels of oxidative damage than non-reproducing individuals during the
248 relevant period of reproduction. In males, the relevant period for oxidative shielding could be at
249 the beginning of the reproduction to protect sperm before the fertilization (Birch et al. 2024). In
250 contrast, in females, the relevant period for oxidative shielding could be during eggs laying to
251 protect offspring from intergenerational transmission of damage via the egg (Blount et al.
252 2016). As reported before and in disagreement with this hypothesis, we did not detect any
253 increase in antioxidant production. Our inability to detect antioxidant production in reproducing
254 individuals may have been because the antioxidants we measured were not good markers of

255 antioxidant production in that particular case (to be measured in another tissue, see before).
256 Alternatively to the oxidative shielding hypothesis, only better individuals can invest in the
257 reproduction, which are the females that better cope with oxidative stress or that are less
258 impacted by environmental factors across the time of the experiment that might have induced
259 the oxidative damages observed in the non-reproducing females (such as parasites for instance
260 through immune responses (Costantini 2022)). For males, as females started to lay eggs 6
261 weeks after the beginning of the experiment, the difference observed between reproducing and
262 non-reproducing males may be explained by the mate choice occurring a few weeks before egg
263 laying. As we caught pigeons from the natural conditions, pigeons had to create new mating
264 pairs in the aviaries and the period of mate pairing could therefore have taken place at week 0.
265 According to the sexual selection theory (Andersson and Simmons 2006), females would have
266 chosen better males, which might be males with lower oxidative damage (Schantz et al. 1999).

267

268 *Melanism and oxidative stress*

269 Dark eumelanin pigeons are better able to detoxify trace metals as compared to paler ones
270 (Chatelain et al. 2016a) and we therefore expected to detect the strongest effects of trace metals
271 exposure on oxidative damages or shielding in paler individuals. Contrary to our expectations,
272 eumelanin-based colouration did not affect oxidative damage or antioxidant capacity. The
273 detoxication mechanism proposed here should mainly occur during moulting or juvenile
274 growth in pigeons when feathers are growing (Chatelain et al. 2016a). It is possible that during
275 the time lapse of our experiment, the accumulation of metals in feathers was not enough
276 important to create different levels of metal detoxication between dark and pale eumelanic
277 pigeons because pigeons were not moulting. However, alternative mechanisms would have
278 expected correlations between melanin-based colouration and oxidative status, as reported

279 earlier (Emaresi et al. 2016, Leclaire et al. 2019), such as (i) the biochemical role of
280 antioxidants in melanogenesis (Galván and Alonso-Alvarez 2009, Galván and Solano 2009) or
281 (ii) through pleiotropic effects of the melanocortin system (Ducrest et al. 2008, Roulin and
282 Ducrest 2011). However, we did not detect such relationships in pigeons.

283 **Conclusion**

284 In conclusion, our study provides no support for an adaptive antioxidative response induced by
285 trace metals exposure in feral pigeons. More longer-term experimental studies, which would
286 lead to the potential saturation of detoxifying tissues with metals, involving measurements of
287 antioxidant production in several tissues would be necessary to truly falsify this hypothesis. In
288 addition, our results partly support the oxidative shielding hypothesis, suggesting that it occurs
289 at different periods for males and females: during sperm production in males and during egg
290 laying in females.

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297 **Data Availability**

298 The data used in this study are available on request from the corresponding author.

299 **Conflict of interest**

300 The authors declare that they have no conflict of interest with the content of this article.

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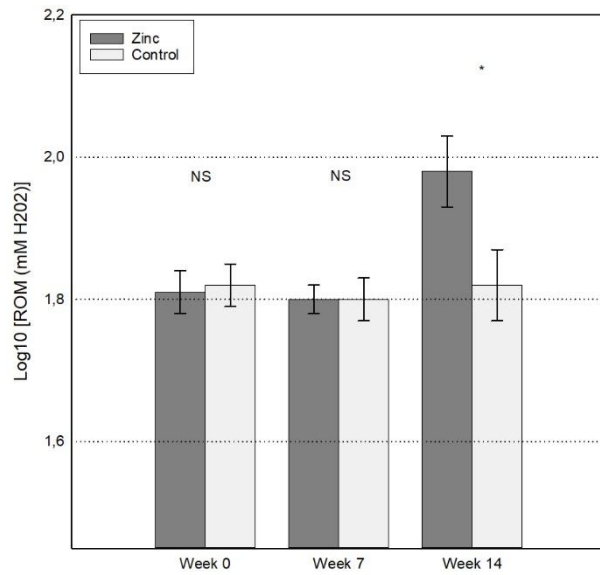
413 **Table 1:** Output of the final linear mixed models retained after model selection on Log10
 414 [ROM] and OXY using three repeated measures (Week 0, 7 and 14) on each adult feral pigeon.
 415 Aviary and pigeon ID were initially included as random factors, then, aviary ID was finally
 416 removed based on AIC. ANOVA type III was applied. Non-significant interactions that **were**
 417 **removed** are not shown. P-value < 0.05 are highlighted in bold.

Effects	Log10 [ROM]			OXY		
	F	DF	P	F	DF	P
Lead	0.52	1,61.8	0.47	0.28	1,61.4	0.60
Zinc	1.99	1,61.0	0.16	2.52	1,60.2	0.12
Time (T0, T1 and T2)	5.16	2,122	0.007	1.63	2,128.5	0.20
Melanin based coloration	0.31	1,60.8	0.58	0.04	1,59.9	0.84
Sex	15.1	1,63.4	0.0002	0.00	1,61.8	0.97
Reproduction (yes or no)	1.43	1,62.5	0.24	0.44	1,61.9	0.51
Zinc * Time	3.36	2,121.2	0.04	–	–	–
Sex * Time	1.11	2,122.8	0.33	–	–	–
Sex * reproduction	0.05	1,62.7	0.82	–	–	–
Time * Reproduction	1.34	2,122.9	0.27	–	–	–
Sex*reproduction*Time	3.30	2,122.9	0.04	–	–	–

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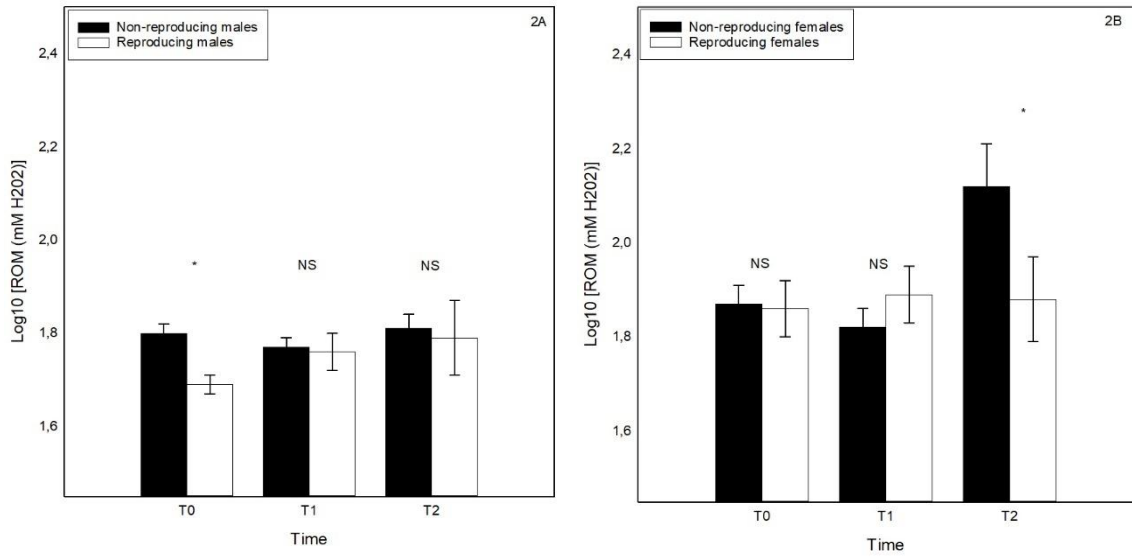
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422 **Figure 1.** Oxidative damage (i.e Reactive Oxygen Metabolites concentrations, ROM expressed
 423 in Log10 [mM of H₂O₂ equivalent]) for pigeons supplemented with zinc (dark grey, **N=34**) and
 424 control pigeons (**pale grey, N = 35**, mean ± SE; NS above bars indicate a non-significant
 425 difference and * a significant difference) across time (Week 0 (before experiment), Week 7 and
 426 Week 14).

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436 **Figure 2:** Oxidative damage (i.e Reactive Oxygen Metabolites concentrations, ROM expressed
437 in Log10 [mM of H₂O₂ equivalent]) for reproducing pigeons (dark grey) and non-reproducing
438 pigeons (mean \pm SE; NS above bars indicate a non-significant difference and * a significant
439 difference between reproducing and non-reproducing pigeons) across time (Week 0 (before
440 experiment), Week 7 and Week 14) for **2A.** males and **2B.** females.