Female oxidative status in relation to calcium availability, metal pollution and offspring development in a wild passerine

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Abstract

Both Ca deficiency and metal exposure may affect physiological and nutritional condition of breeding females altering their ability to deposit essential resources (e.g. Ca, antioxidants) into the eggs. This effect of the maternal investment into egg quality is not strictly limited to the embryonic period, but may persist after hatching, since nutrient levels in yolks can compromise nestling antioxidant status, growth and fledging success. The goal of this study is to investigate how metal pollution and Ca availability during the breeding season affect oxidative stress biomarkers and plasma biochemistry in adult female pied flycatchers (*Ficedula hypoleuca*). In addition, we aim to evaluate how maternal antioxidant status and body condition relate to breeding parameters and offspring oxidative balance. Females breeding in a metal-polluted area in SW Finland showed higher metal concentrations compared to the control area, although current levels are below the toxic level able to affect female physiology. In addition, female oxidative status and general health are not constrained by insufficient Ca availability in the study area. Interestingly, our results suggest that antioxidant response to metals is better when Ca concentrations are high enough to cover the physiological Ca requirements in breeding females. There seems to be a subtle balance between the concentrations of Ca in the organism and the tolerance to metal-related effects that requires further research. This study supports that offspring oxidative balance and nestling development are affected by maternal body condition and antioxidant status.

Capsule: Female antioxidant response to metals improves when Ca concentrations are high and its oxidative status and body condition affect offspring oxidative balance and development

Keywords: oxidative stress; reproduction; female condition; metal pollution; calcium availability

Introduction

Oxidative processes and the generation of reactive oxygen species (ROS) are natural in the cellular metabolism of organisms (Finkel and Holbrook, 2000). Oxidative stress is the disruption of the prooxidant/antioxidant balance, where the defense is overcome by the radical formation, which may cause oxidative damage (Halliwell and Gutteridge, 2007). Organisms have an antioxidant defense system that inhibits ROS generation and reduces oxidation and cell damage (McGraw, 2011). This antioxidant system is altered by different factors including reproductive investment, immune response activation, environmental constrains such as nutrient availability and food quality, or the contaminant exposure intensity (Costantini, 2008; Espín et al., 2016a, 2017a; Koivula and Eeva, 2010; Koivula et al., 2011; Rainio et al., 2013).

One essential nutrient closely related with reproduction in birds is calcium (Ca), required for eggshell formation during the egg laying, skeletal growth of embryo and calcification during postnatal development, as well as for different biochemical processes (Reynolds et al., 2004; Stanford, 2006). Due to these requirements, small passerines need to consume food rich in Ca (e.g. snail shells) in addition to their normal diet (Graveland and van Gijzen, 1994). Therefore, acidified or polluted environments may pose a problem to their reproduction due to a potential snail abundance reduction (Eeva et al., 2010; Graveland, 1996).

A previous study showed that even though pied flycatcher (*Ficedula hypoleuca*) nestlings suffering from low Ca availability enhanced their reduced glutathione relative to glutathione disulfide (GSH:GSSG), they still showed signs of higher damage to membrane lipids as an indication of physiological stress (Espín et al., 2017a). In addition, Ca deficiency may enhance metal absorption and accumulation for e.g. lead (Pb) and cadmium (Cd) (Dauwe et al., 2006; Scheuhammer, 1996), altering Ca metabolism (Pounds, 1984; Suzuki et al., 2004, 1985). Moreover, exposure to redox-inactive (e.g. Cd, Pb, arsenic, As, mercury, Hg) and redox-active (e.g. zinc, Zn and copper, Cu) elements can deplete/alter cell antioxidants such as glutathione (GSH) and enzymes (glutathione peroxidase, GPx, catalase, CAT, superoxide dismutase, SOD and glutathione-S-transferase, GST) and generate ROS (Ercal et al., 2001; Koivula and Eeva, 2010; Sánchez-Virosta et al., 2015). Different researchers have observed that avian species inhabiting metal-polluted areas may experience enhanced oxidative stress or altered activity of antioxidant enzymes (Berglund et al., 2007; Espín et al., 2014; Martinez-Haro et al., 2011; Stauffer et al., 2016). A recent experiment in wild great tits (*Parus major*) susggest that Ca protects against metal toxicity by modulating oxidative stress (Sánchez-Virosta et al., 2019) and by decreasing the metal absorption in the gastrointestinal tract, as suggested before for other species (e.g. mice and fish; Jamakala and Rani, 2012; Prasanthi et al., 2010).

Reproduction is an energetically demanding activity that increases energy expenditure and metabolic rates, which has been associated with increased prooxidant production (Alonso-Alvarez et al., 2004; Losdat et al., 2011; Wiersma et al., 2004). When combined with other stressful conditions interfering with antioxidant status in birds, consequences for breeding performance can arise. Both Ca deficiency and metal exposure may affect physiological and nutritional condition of breeding females altering their ability to deposit essential resources (e.g. Ca, antioxidants, vitamins and carotenoids) into the eggs (Espín et al., 2016a; Hargitai et al., 2016; Hõrak et al., 2002). The effects of maternal investment on egg quality are not strictly limited to the embryonic period (Hõrak et al., 2002; Saino et al., 2002), but persist after hatching, since nutrient levels in yolks can compromise nestling antioxidant status, growth, survival and fledging success (Espín et al., 2016a; Marri and Richner, 2014; McGraw et al., 2005). In this sense, López-Arrabé et al. (2016) observed that total GSH levels in pied flycatcher nestlings were associated with parental oxidative status, suggesting that parental condition can be determinant for endogenous antioxidants of the offspring.

Based on this background, the present study has as main goal to investigate how Ca availability and metal pollution during the breeding season affect oxidative stress biomarkers and plasma biochemistry in adult female pied flycatchers. In addition, we aim to evaluate how maternal antioxidant status and body condition relate to breeding parameters (i.e. egg mass, nestling size, fledging success and fledglings' number) and offspring oxidative balance. For this purpose, a Ca-supplementation experiment was done in a metal-polluted area in southwestern Finland (Espín et al., 2016b). A battery of parameters was determined in adult females, such as plasma biochemistry (Ca, creatine kinase, uric acid and alkaline phosphatase), body condition, and a set of antioxidants (GSH, GST, GPx, CAT and SOD) and oxidative damage biomarkers in red cells (lipid peroxidation, TBARS, and protein carbonylation, PC). These oxidative stress biomarkers and biochemical parameters in pied flycatcher nestlings and the different breeding parameters have been reported elsewhere (Espín et al., 2016b, 2016a, 2017a). To the best of our knowledge, this is the first study assessing the protective role of Ca on metal-related oxidative stress in wild breeding females.

Material and methods

Experimental set-up

The Ca-supplementation experiment on a pied flycatcher population was conducted in the vicinity of a Cu-Ni smelter in Harjavalta (61°20′ N, 22°10′ E), SW Finland, during the breeding season 2014. A detailed description of the experiment can be found in Supplementary material (Document S1) and in Espín et al. (2016b). Nest boxes (Lambrechts et al., 2010) in the polluted and unpolluted zones (0 − 4 km and 4 − 11 km from the smelter, respectively) were periodically

checked to monitor the nest progress and to record breeding parameters (i.e. laying and hatching date, clutch size, number of hatchlings, brood size on day 7 and 12 after hatching, and number of fledglings). Nests occupied by pied flycatcher were assigned either to the control (30 nests, 15 in each area) or to the Ca-supplemented group (35 nests, 17 in polluted and 18 in unpolluted area), and feeders with crushed mussel shells (Versele Laga; *ad libitum* supplementation in Ca-supplemented nest boxes) or empty feeders (control nest boxes) were put inside the nest boxes. Espín et al. (2016b) provide the Ca consumption during the experiment. All the licenses required were obtained before the experiment.

Sampling and measurements

During the laying period, we collected one egg from each clutch to assess the effect of the experiment on yolk vitamins and egg characteristics (published in Espín et al., 2016a). During the egg incubation period, adult females (n = 76 individuals) were captured from nest boxes and ringed, when necessary, with individually numbered metal rings. Females were aged based on their plumage (Svensson, 1992) in two groups: one year old (hereinafter young) or older (hereinafter old). Females were weighted with a spring scale and the length of the wing was measured with a ruler (Svensson, 1992). The body condition index was calculated using residuals of the linear regression of body mass on wing length (Velando, 2002). When possible, feces were sampled and conserved in tubes at −20°C for metals analyses (n = 26 individuals). Blood collection from the brachial vein was done using heparinized hematocrit tubes that were centrifuged to measure hematocrit. We divided plasma (n = 69 samples) and red blood cells (RBC, n = 65 samples) in different tubes conserved in liquid nitrogen and then stored at −80°C. We could not get blood samples from all females, and only samples with sufficient volume were used for analysis.

Nestlings were ringed on day 7 post-hatching (hereinafter d7) and feces of nestlings from the same brood were combined and kept at −20°C for metals analyses (Espín et al., 2016b). On d7 and on d12, nestling body measurement were collected (weight, wing, head and tarsus length), and blood samples were collected as described for females. In nestlings, plasma collected at d7 was used to analyze vitamins (Ruiz et al., 2017), plasma collected at d12 was used to measure the plasma biochemistry (Espín et al., 2016b), and RBCs were used for oxidative stress measurements (Espín et al., 2017a).

Analysis of metals, plasma biochemistry and oxidative stress biomarkers in red cells

Metal concentrations in feces were measured by inductively coupled plasma optical emission spectrometry (ICP-OES). Further details on metal analyses can be found in Supplementary material (Document S2).

Ca, uric acid (UA), alkaline phosphatase (ALP) and creatine kinase (CK) were measured in plasma in a microplate reader (EnSpire, Perkin-Elmer) using commercial kits from BioSystems S.A. These parameters are considered useful tools to evaluate health status (Hochleithner, 1994). Measurements (in triplicate) were performed using 384-well microplates to minimize the volume of sample, and a mean value was produced from the triplicates.

Oxidative stress/damage biomarkers (GSH, GST, GPx, CAT, SOD, TBARS and PC) were measured in RBCs. The concentration of proteins was measured according to the Bradford method (Bradford, 1976). SOD was measured with a Fluka kit (Fluka, Germany), while GPx, CAT and GST were determined using Sigma kits (Sigma Chemicals, USA). The ThioStar® glutathione detection kit (Arbor Assays, USA) was used to determine total GSH (tGSH) and the ratio of reduced to oxidised glutathione (GSH:GSSG). PC was measured following Rainio et al. (2015) and peroxidation of lipids measured as thiobarbituric acid-reactive substances or TBARS was determined fluorometrically following Espín et al. (2017b). All measurements were done in triplicates using 96- or 384-well microplates in a microplate reader (Envision and EnSpire, Perkin-Elmer). Additional details are provided in Document S2.

Statistical analysis

The statistical package SAS 9.3 was used for statistical analyses. Linear models (LMs; Glimmix procedure) with Gaussian distribution and identity link function were performed to explore the differences between treatments (Control/Ca-supplement), zones (unpolluted/polluted) and their interaction on hematocrit, biochemistry (Ca, UA, ALP, CK) and oxidative stress/damage biomarkers (tGSH, GSH:GSSG ratio, GST, GPx, CAT, SOD, TBARS and PC). LMs were also done to study the effects of metal concentrations in feces (as a proxy of dietary levels) and plasma Ca levels on biochemistry and oxidative stress/damage biomarkers. Since we only collected feces from 26 females, metal levels in nestling feces were used in the models to describe the general level of metal exposure at breeding territories. Absolute metal concentrations in feces from nestling have been reported earlier (Espín et al., 2016b). Metal concentrations in nestling feces were positively intercorrelated (Espín et al., 2017a) and we performed Principal Components Analysis (PCA, Princomp procedure), using the first principal component (hereinafter $PC1_{metals}$) to provide a single measure describing the variation in pollution levels (for details see Espín et al., 2017a). Thus, $PC1_{metals}$, plasma Ca concentrations, and their interaction were in the model as explanatory variables, together with female condition index, age-class, laying date and clutch size. For PC we performed linear mixed models (LMMs) including microplate number as random factor.

Finally, separate linear or generalized models (GLMs) were performed to study the associations of maternal body condition index and oxidative status with nestlings oxidative balance and

reproductive performance published elsewhere (Espín et al., 2016b, 2016a, 2017a). Therefore, the response variables studied were egg mass, nestling antioxidant levels, nestling lipid damage (TBARS), nestling size, number of fledglings and fledging success (probability of a hatchling to fledge), while maternal oxidative status, body condition index, hatching date and brood size at d7 were included in the models as explanatory variables. In order to reduce the number of variables for further analyses, PCA were performed for the functionally-related antioxidant biomarkers (GSH metabolism) that were closely associated to each other (tGSH, GSH:GSSG ratio, GPx, GST and SOD) in females (the first principal component for female antioxidants − $PC1_{AOF}$ − explained 49% of the variation in our data, eigenvalue 2.47, eigenvectors: tGSH 0.54, GSH:GSSG ratio −0.26, GPx 0.49, GST 0.37 and SOD 0.52) and in nestlings (the first principal component for nestling antioxidants − $PC1_{AON}$ − explained 53% of the variation in our data, eigenvalue 2.65, eigenvectors: tGSH 0.50, GSH:GSSG ratio −0.08, GPx 0.53, GST 0.54 and SOD 0.42). The first principal component for the nestling size parameters ($PC1_{size}$; length of wing, tarsus and head, and body mass on d7) was used to describe the nestling size (the percentage of variation explained by the component and eigenvalue are provided in Espín et al. 2016b). The model residuals were used to check normality of variables, and some of them were log-transformed (Ca, UA, ALP, CK, tGSH, GSH:GSSG ratio, GST, GPx, CAT and TBARS). For fledging success, we used events/trials type of response and binomial error distribution, while Poisson error distribution was used for fledgling number. Model residuals were used as a random factor in these models. Kenward-Roger method was used to adjust the degrees of freedom. All the analyses were done using brood means for nestling measurements. When terms were significant, they were retained in the model, and non-significant variables were dropped starting from interactions. As we did not have all measurements for every bird, degrees of freedom differ between models. Correlation coefficients were used to analyse associations among variables (Pearson, $r_{p,}$ or Spearman, r_s). In all the analyses, the significance level was $p < 0.05$.

Results

Pied flycatcher females showed higher metal concentrations in feces in the polluted zone, although significant differences were not found for Ca, Zn and Hg (Table 1). Fecal concentrations of all metals, except Ca and Zn, correlated positively between females and their nestlings (r_p = 0.45 − 0.72, $p < 0.05$, $n = 21$).

Hematocrit was 6% lower in the Ca-supplemented group compared to the control group (Table 2, Figure 1). The interaction between zone and treatment had a significant effect on the plasma concentrations of UA, with higher levels in females from the polluted control group and unpolluted Ca-supplemented group compared to the other groups (Table 2, Figure 1). No among-

group differences were found in the other physiological parameters and oxidative damage biomarkers.

$PC1_{metals}$ and Ca concentration in plasma showed an interactive effect on tGSH levels, GSH:GSSG ratio, GST and GPx activities, and TBARS levels (Figure 2, Table S1 in Supplementary material). As shown in Figure 2, at plasma Ca levels of 10 mg/dl or below the horizontal line does not change the gray-scale color much, reflecting a small response of antioxidants or TBARS levels to metal exposure ($PC1_{metals}$). On the other hand, at higher Ca levels (>10 mg/dl), the graph changes from light (lower values) to dark (higher values) on the horizontal axis (from dark to light for GSH:GSSG ratio), reflecting increased antioxidants or TBARS levels (decreased for GSH:GSSG ratio) with increasing $PC1_{metals}$. In addition, $PC1_{metals}$ and body condition were negatively associated with CK levels in plasma (Table S1). Finally, old females showed higher GST activity and TBARS levels than young females (Table S1).

Correlation analyses (Supplementary material, Table S2) reflected that some antioxidants were correlated with each other and with PC and TBARS levels.

The female body condition index was associated with nestling oxidative status ($PC1_{AON}$) and TBARS levels, both increasing with decreasing female body condition (Table 3). The antioxidant level in females ($PC1_{AOF}$) was positively associated with nestling size ($PC1_{size}$) (Table 3).

Discussion

Avian populations can experience oxidative stress due to a combination of different factors including metal exposure and nutrient constrains (Espín et al., 2016a, 2017a; Koivula and Eeva, 2010; Koivula et al., 2011; Rainio et al., 2013). Metal levels in feces are considered useful indicators of recent metal exposure at a local site, reflecting the concentrations in the diet (Berglund et al., 2015; Sánchez-Virosta et al., 2015). As expected, concentrations of most metals in feces from females were higher in the polluted area as observed for internal tissues or feces of nestlings in previous studies in the same area (Berglund et al., 2011, 2015; Eeva and Lehikoinen, 1996; Espín et al., 2016b). In addition, fecal concentrations of most elements were positively correlated between females and their offspring, supporting feces as a good matrix to reflect metal exposure in passerines. Positive correlations also indicate that, at least for some toxic elements, the levels in nestling feces can be used as a proxy for female exposure, which is practical since nestling feces are easier to collect and it is not always possible to measure both.

When evaluating the differences between treatments (Ca-supplement/control) and zones (polluted/unpolluted) in female physiology, few significant effects were found. This result is consistent with our previous findings, suggesting that metal exposure of pied flycatcher is currently below the toxic level (Espín et al., 2016b, 2017a; Koivula et al., 2011; Rainio et al.,

2013) and that pied flycatcher oxidative status and general health are not constrained by insufficient Ca availability in our study area (Espín et al., 2016b, 2017a).

Hematocrit was lower in the Ca-supplemented group compared to the control group. It has been suggested that Ca supplementation may reduce iron absorption (Cook et al., 1991), which could affect hematocrit levels. Hatton et al. (1991) also reported that supplemental dietary Ca in rats produced a decrease in hematocrit. In addition, vitamin D, an essential factor regulating intestinal Ca absorption (Bar, 2008), has been suggested to have an effect on erythropoiesis increasing the prevalence of anemia (Sim et al., 2010). We could not analyse vitamin D_3 in female plasma due to the limited volume of sample available. However, we analysed yolk vitamins in eggs laid by the females of the present study (Espín et al., 2016a), which could reflect the levels in females during the laying period. In this sense, vitamin D_3 levels tended to be higher in yolks from Ca-supplemented nests compared to control nests, being the effect almost significant ($p = 0.054$) (Espín et al., 2016a). Thus, Ca supplementation and/or vitamin D_3 levels could explain the lower hematocrit in the Ca-supplemented group.

Females from the polluted control group and unpolluted Ca-supplemented group showed higher uric acid levels compared to the other groups. Uric acid is an important and abundant antioxidant, and it is also the main form of avian nitrogen excretion (Koivula and Eeva, 2010). Plasma concentrations of UA may increase because of renal damage (Hochleithner, 1994) and catabolism of proteins associated with starvation, so it is a suitable indicator of nutritional status (Alonso-Alvarez and Ferrer, 2001; Ferrer, 1994). Thus, the highest levels found in the polluted control group may be related to the higher metal exposure and poorer food quality and quantity in this zone. On the other hand, previous studies suggest that Ca availability in the unpolluted zone is lower than in the polluted area (Eeva and Lehikoinen, 2004; Eeva et al., 2010; Espín et al., 2016b), and we observed that Ca provisioning produced eggs with a slightly higher eggshell index (Espín et al., 2016a), so the intake of Ca may be close to a deficient level in the unpolluted area. Thus, Ca supplementation in this unpolluted zone could have suppressed parathyroid hormone (PTH) and vitamin D_3 formation (de Matos, 2008), which could finally elevate uric acid (Dalbeth et al., 2009; Peng et al., 2013; Thakkinstian et al., 2015). However, we did not find a significant difference in plasma Ca levels, so the implication of Ca, PTH and/or vitamin D_3 metabolism in the regulation of uric acid in birds requires further research.

Interestingly, in spite of the very limited effects of the Ca availability on the antioxidant status, blood antioxidant levels and TBARS changed over the range of metal concentrations depending on the Ca concentrations in plasma. When plasma Ca levels were below ca. 10 mg/dl, antioxidants or TBARS levels were not affected by metals, while at higher Ca levels (>10 mg/dl), antioxidants and TBARS increased and GSH:GSSG ratio decreased with increasing fecal metal

concentrations. It is possible that when Ca concentrations are high enough for the physiological Ca requirements, this essential element can modulate the antioxidant status, increasing antioxidant capacity with increasing metal exposure to protect against metal-related lipid damage. These results suggest that there may be a subtle balance between Ca levels in the organism and the tolerance to metal-related effects in breeding females.

The oxidative stress biomarkers correlated among them, reflecting that antioxidant molecules work coordinately to ensure a balanced antioxidant defense, as shown in several wild avian species (Espín et al., 2014, 2016c; Koivula et al., 2011). It is well known that SOD and GPx work in collaboration to catalyse the decomposition of O_2^- and H_2O_2 (Halliwell and Gutteridge, 1999), explaining the positive correlations observed between these enzymes. GST also removes ROS from the cells through the GSH oxidation (Valko et al., 2006), and it was positively correlated with SOD, CAT and GPx. Finally, the antioxidant GSH acts as a cofactor of different enzymes and it also directly scavenges radicals, detoxifying H_2O_2 and lipid peroxides (Valko et al., 2006), which may explain the correlations found with GPx, CAT, SOD, TBARS and PC.

We found that body condition and the state of antioxidant system of a female may affect positively on oxidative balance and development of nestling. These results are difficult to interpret due to the complexity of the antioxidant system. First, antioxidant levels in incubating females were positively related to nestling size. It is possible that females with higher reproductive effort produce bigger nestlings but at the same time need to upregulate their enzymatic antioxidant levels as a response to increased production of free radicals. In other words, females would invest in the quality of the offspring at the expense of their own oxidative balance. Second, the female body condition index was negatively associated with antioxidant and TBARS levels in chicks. This result could mean that females in good condition invest more resources in their offspring (e.g. by supplying more nutritive food or in higher quantities), which then would suffer lower oxidative damage to lipids. Positive association between female condition and nestling health could arise via female and/or male quality, territory quality, or both. Such life-history trade-offs would be in accordance with the fast pace-of-life concept, which outlines that behavioral and physical traits covary (e.g. Réale et al., 2010), so that high reproductive activity may, for example, expose the female to higher oxidative stress with possible negative consequences to future reproduction.

Conclusions

This study shows that pied flycatcher females breeding in a metal-polluted area in SW Finland are exposed to higher metal concentrations compared to the control area, although current levels are below the toxic level that would have detrimental effects in female physiology and reproduction. In addition, their oxidative status and general health are not constrained by

insufficient Ca availability in the study area. Interestingly, our results suggest that antioxidant response to metals is better when Ca concentrations are high enough for the physiological Ca requirements in breeding females. This may be related to Ca modulating the antioxidant status, but the mechanism triggering this process requires further research. Finally, this study supports that offspring oxidative balance and nestling development are not only affected by environmental factors, but also by maternal body condition and antioxidant status.

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Table 1. Mean (± SD) metal concentrations in feces of pied flycatcher females according to zone (polluted and unpolluted) in Harjavalta, Finland

	Polluted		**Unpolluted**		Models[2]		
Element[1]	N	Mean ± SD	N	Mean ± SD	F	$_{ndf,ddf}$	p
As*	8	5.37 ± 3.37	18	0.67 ± 0.73	29.1	$_{1,24}$	<0.0001
Ca[3]*	8	16.2 ± 36.7	18	3.64 ± 3.50	1.56	$_{1,24}$	0.224
Cd*	8	8.68 ± 5.73	18	3.76 ± 1.74	10.4	$_{1,24}$	0.004
Cu*	8	208 ± 88.5	18	106 ± 72.7	11.3	$_{1,24}$	0.003
Ni	8	22.4 ± 7.30	18	5.66 ± 2.50	77.6	$_{1,24}$	<0.0001
Pb	8	8.13 ± 2.22	18	3.23 ± 2.57	21.7	$_{1,24}$	<0.0001
Zn	8	661 ± 261	18	632 ± 290	0.06	$_{1,24}$	0.815
Hg*	8	0.47 ± 0.37	15	0.31 ± 0.11	1.92	$_{1,21}$	0.181

[1]Element concentrations in micrograms per gram, dw except for Ca (mg/g, dw). All metal concentrations were determined with an inductively coupled plasma optical emission spectrometer (ICP-OES) except for total mercury that was analyzed in a Milestone DMA-80 direct Hg analyzer by atomic absorption spectrophotometry

[2]LM with normal error distribution

[3]Since Ca concentrations in feces did not differ among treatments, all individuals (Ca-supplemented and control) were used for the analysis

*Concentration was log-transformed before analysis.

Table 2. Mean (± SD) biomarker levels in blood of female pied flycatchers for Ca-supplemented and control groups in polluted and unpolluted zone, and linear models[1] of the effects of zone and Ca treatment on biomarkers

Zone	Treatment		Hematocrit (%)	Ca in plasma* (mg/dl)	Uric acid* (mg/dl)	ALP* (U/l)	CK* (U/l)	tGSH* (nmol/mg)	GSH:GSSG Ratio*	GST* (µmol/min/mg)	GPx* (µmol/min/mg)	CAT* (µmol/min/mg)	SOD (% inhibition)	TBARS* (nmol/mg)	PC (nmol/mg)
Polluted	Ca-supplemented	n	19	19	19	18	16	17	17	17	17	17	17	17	16
		Mean ± SD	45.9 ± 5.91	8.61 ± 1.57	13.7 ± 3.96	264 ± 130	1080 ± 855	37.9 ± 9.9	0.85 ± 1.02	0.004 ± 0.002	0.015 ± 0.004	1.12 ± 0.64	83.4 ± 4.81	0.027 ± 0.009	1.22 ± 0.31
	Control	n	14	14	14	14	13	13	13	13	13	12	12	13	12
		Mean ± SD	46.6 ± 3.16	9.14 ± 3.02	15.5 ± 4.6	269 ± 91.0	961 ± 568	41.9 ± 19.2	0.56 ± 0.22	0.004 ± 0.003	0.017 ± 0.005	0.88 ± 0.42	83.0 ± 4.31	0.035 ± 0.026	1.02 ± 0.38
Unpolluted	Ca-supplemented	n	17	19	19	17	14	18	18	18	18	15	15	17	15
		Mean ± SD	45.6 ± 4.19	8.46 ± 1.14	14.8 ± 4.29	249 ± 66.7	1140 ± 686	46.0 ± 26.5	0.59 ± 0.46	0.004 ± 0.003	0.017 ± 0.005	1.16 ± 0.70	83.0 ± 4.33	0.030 ± 0.009	1.10 ± 0.47
	Control	n	18	17	17	15	12	16	16	16	16	15	15	16	15
		Mean ± SD	50.2 ± 3.55	7.77 ± 1.08	12.7 ± 5.69	303 ± 202	1210 ± 1210	40.3 ± 14.8	0.60 ± 0.26	0.004 ± 0.002	0.015 ± 0.004	1.36 ± 1.19	82.2 ± 4.90	0.028 ± 0.008	1.25 ± 0.30
LM[1]		n	68	69	69	64	55	64	64	63	64	59	59	63	58
	Zone	$F_{ndf,ddf}$	2.08 $_{1,65}$	2.16 $_{1,67}$	**1.11 $_{1,65}$**	0.03 $_{1,61}$	0.72 $_{1,53}$	0.33 $_{1,62}$	0.33 $_{1,62}$	1.17 $_{1,61}$	0.06 $_{1,62}$	0.99 $_{1,57}$	0.24 $_{1,56}$	0 $_{1,60}$	0.12 $_{1,52.99}$
		p	0.153	0.146	**0.296**	0.870	0.399	0.565	0.569	0.283	0.808	0.325	0.625	0.966	0.728
	Treatment	$F_{ndf,ddf}$	**6.83 $_{1,66}$**	0.44 $_{1,66}$	**0.34 $_{1,65}$**	0.62 $_{1,62}$	0.05 $_{1,52}$	0.03 $_{1,61}$	0.02 $_{1,61}$	0.08 $_{1,60}$	0.01 $_{1,61}$	0.20 $_{1,56}$	0.31 $_{1,57}$	0.24 $_{1,61}$	0.03 $_{1,52.05}$
		p	**0.011**	0.509	**0.561**	0.433	0.819	0.859	0.898	0.778	0.929	0.659	0.579	0.626	0.853
	Zone x Treatment	$F_{ndf,ddf}$	3.20 $_{1,64}$	1.73 $_{1,65}$	**4.02 $_{1,65}$**	0.01 $_{1,60}$	0.26 $_{1,51}$	0.84 $_{1,60}$	0.83 $_{1,60}$	1.07 $_{1,59}$	3.51 $_{1,60}$	0.87 $_{1,55}$	0.05 $_{1,55}$	1.75 $_{1,59}$	0.03 $_{1,51.23}$
		p	0.078	0.192	**0.049**	0.918	0.612	0.362	0.365	0.305	0.065	0.356	0.830	0.190	0.857

[1]LM with normal error distribution *The variables were log-transformed before GLM analysis. Terms left in the final model are shown in bold. n = number of samples. For PC we performed GLMMs including microplate number as random factor. ALP = alkaline phosphatase in plasma, CK = creatine kinase in plasma, tGSH = total glutathione in red cells, GSH:GSSG ratio = ratio of reduced glutathione to glutathione disulfide in red cells, GST = glutathione-S-transferase in red cells, GPx = glutathione peroxidase in red cells, CAT = catalase in red cells, SOD = superoxide dismutase in red cells, TBARS = lipid peroxidation in red cells, estimated as thiobarbituric acid-reactive substances, PC = protein carbonylation in red cells.

Table 3. Results of linear or generalized linear models[1] testing for the effect of female condition index and oxidative status on breeding parameters and offspring oxidative status.

Response variable	Explanatory variables	$F_{ndf,ddf}$	p	Est ± SE
Egg mass (N = 71)	Body condition females	$2.76_{1,69}$	0.101	0.027 ± 0.016
	$PC1_{AOF}$	$1.14_{1,51}$	0.291	0.010 ± 0.009
	Laying date	$0.51_{1,50}$	0.479	−0.003 ± 0.005
	Clutch size	$0.42_{1,49}$	0.521	−0.012 ± 0.018
$PC1_{AON}$ (N = 45)	**Body condition females**	**$5.64_{1,43}$**	**0.022**	**−0.648 ± 0.273**
	$PC1_{AOF}$	$0.03_{1,31}$	0.866	−0.029 ± 0.168
	Hatching date	$0.27_{1,41}$	0.607	0.039 ± 0.075
	Brood size	$1.08_{1,42}$	0.304	−0.256 ± 0.246
TBARS nestling (N = 45)	**Body condition females**	**$5.66_{1,43}$**	**0.022**	**−0.004 ± 0.002**
	$PC1_{AOF}$	$0.14_{1,32}$	0.707	0.0004 ± 0.0010
	Hatching date	$0.00_{1,31}$	0.968	0.00002 ± 0.0005
	Brood size	$0.64_{1,42}$	0.430	0.001 ± 0.002
$PC1_{size}$ nestlings (N = 51)	Body condition females	$0.15_{1,45}$	0.700	0.123 ± 0.318
	$PC1_{AOF}$	**$8.86_{1,48}$**	**0.005**	**0.453 ± 0.152**
	Hatching date	**$5.76_{1,48}$**	**0.020**	**−0.190 ± 0.079**
	Brood size	$1.71_{1,47}$	0.198	−0.291 ± 0.223
Number of fledglings (N = 59)	Body condition females	$0.15_{1,49}$	0.702	0.052 ± 0.134
	$PC1_{AOF}$	$0.60_{1,57}$	0.440	0.054 ± 0.069
	Hatching date	$0.09_{1,51}$	0.767	0.010 ± 0.033
Fledging success (N = 54)	Body condition females	$0.06_{1,48}$	0.811	0.073 ± 0.30
	$PC1_{AOF}$	$1.78_{1,52}$	0.188	0.195 ± 0.146
	Hatching date	$0.04_{1,50}$	0.850	0.014 ± 0.075
	Number of hatchlings	$0.98_{1,51}$	0.327	−0.258 ± 0.261

[1]LM with normal error distribution except for number of fledglings (Poisson distribution) and fledging success (events/trials type response with binomial distribution). Terms left in the final model are shown in bold. Sample size varies according to the model because of missing values in some of the explanatory variables.

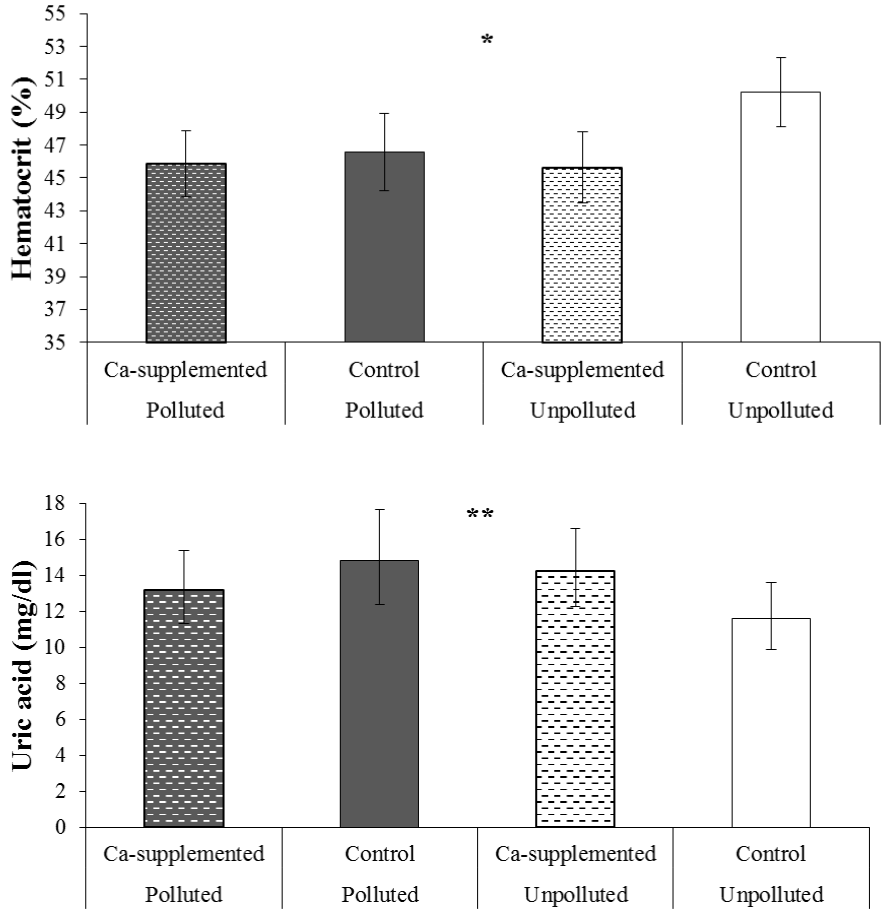

Figure 1. Mean (± 95% CIs) hematocrit (%) and uric acid in plasma (mg/dl) of pied flycatcher females according to zone (polluted and unpolluted) and treatment (Ca-supplemented and control) in Harjavalta, Finland. The data are back-transformed least-squares means from the linear models shown in Table 2. Effects of zone and treatment on the different parameters are shown in Table 2. *Significant differences between treatments. **Significant effect of the interaction Zone x Treatment.

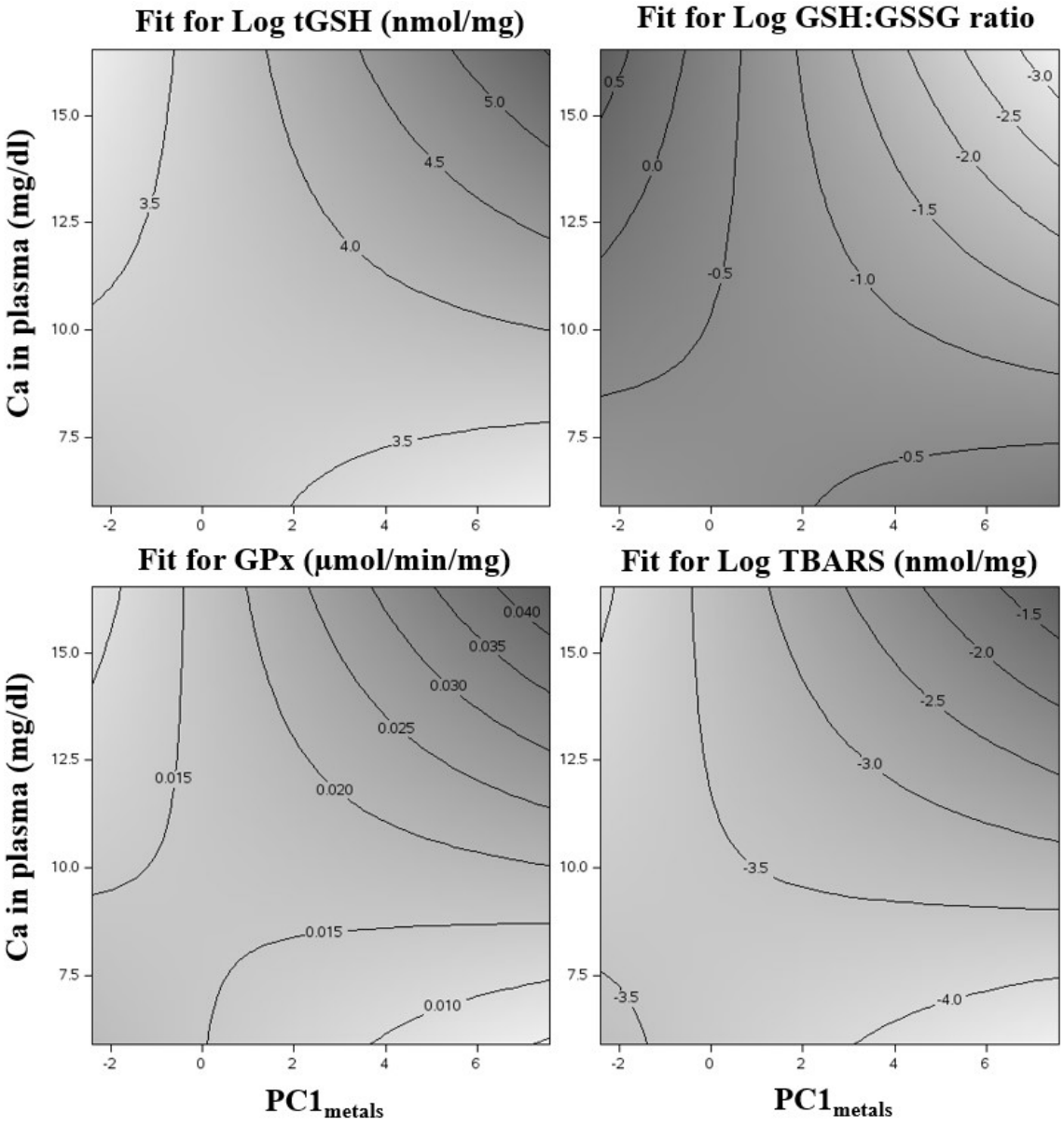

Figure 2. Contour plots for tGSH, GSH:GSSG ratio, GPx activity and TBARS levels in red cells of pied flycatcher females displaying the interaction between $PC1_{metals}$ (first principal component of As, Cd, Cu, Ni, Pb and Hg in feces) and Ca in plasma (mg/dl). The lighter areas denote lower values of the contour variables (antioxidants and TBARS levels), while darker areas denote higher values (some contours are labeled with the corresponding values). The GLM models are shown in Table S1.