Pilot study to determine optimal diquat concentration to generate oxidative stress within physiological levels

METHODS

We carried out a pilot study in order to determine the appropriate dosage of diquat that would expose nestlings to an increased oxidative stress but avoiding pharmacological or critical situations. The pilot study was performed in 2015 and in the same study site as the main study. We selected 48 nestlings from the 9 first broods of the season, in order to obtain the results in sufficient time to perform the main experiment. Nestlings were exposed to one of the following doses of diquat (in mg/kg) diluted in sterile PBS and injected intraperitoneally: 0, 0.35, 0.5, 0.7, 1, 1.4, 2.8, 5.6 and 11.2. On day 5 of age, birds were weighed (±0.01 g) and injected one of the mentioned doses. Each nestling was marked with a non-toxic, waterproof permanent marker to allow recognition in the following days. On day 6, they were weighed again and we collected a 30 µl blood sample from the jugular vein using a heparinised syringe. Blood samples were transported immediately to the laboratory in cooled containers and centrifuged at 5000 g. Plasma was separated from the cellular fraction and stored at -80°C until analysis. Nests were visited again on days 7 and 8 to record nestling survival.

In order to evaluate the effect of diquat dosage on nestling oxidative status we used two different biomarkers. Quantification of malondialdehide (MDA) levels is the most common approach for the assessment of lipid peroxidation in biological and medical sciences (Halliwell and Gutteridge, 2007; Mateos and Bravo, 2007). For the quantification of MDA levels in plasma we used a well-established method based on the reaction of MDA with thiobarbituric acid (TBA) at high temperature followed by a detection of MDA-TBA adducts by fluorescence via high performance liquid chromatography (a detailed protocol has been described in Perez-Rodriguez et al., 2015). Results are reported as mM MDA per litre of plasma. Also, as a measure of status of antioxidant defences we used the OXY-adsorbent assay (Diacron, Grosetto, Italy). This colorimetric test evaluates the non-enzymatic antioxidant capacity of plasma by quantifying the ability of plasma samples to cope with the oxidant action of hypochlorous acid (HOCl; an oxidant of pathologic relevance in biological systems) using N,N-diethyl-p-phenylenediamine as chromogen). Analyses were run blind with respect to treatment, following manufacturer instructions with some modifications described in (Costantini,

2011). Measurements are expressed as µmol of HOCl neutralized per ml of plasma. Two samples with clear signs of hemolysis were excluded from the analyses of MDA and OXY as this may affect levels of these biomarkers.

Data were analysed in R 3.5.0 using *lme4* package (Bates et al., 2015). Brood identity was entered as a random effect. OXY and body mass were log transformed. For the analysis of body mass, mass at day 5 was included as a covariate in the analysis.

RESULTS AND CONCLUSIONS

Mortality rate was 20% (1/5) at dose 2.8 mg/kg, 80% (4/5) at dose 5.6 mg/kg and 100% (3/3) at dose 11.2mg/kg. No mortality was observed at doses 1.4 mg/kg or below (0/35). Body mass at day 6 of age (controlled for body mass at day 5) after a single diquat injection was negatively related to the dose injected ($F_{1, 35.8}$=7.16, p=0.01; Figure 1Sa). Also, high diquat doses increased oxidative damage to lipids, as revealed by MDA levels in plasma ($F_{1, 34.0}$=8.89, p<0.01, Figure S1b). This was paralleled by an increase in plasma OXY levels ($F_{1, 33.3}$=23.3, p<0.001, Figure S1c), probably as a result of mobilization of antioxidant compounds from body stores to blood stream to fight the oxidative challenge (Costantini and Verhulst, 2009).

Considering the above results of a single injection of diquat, we decided that a dose of 1.2 mg/kg, administered at days 5, 7, 10 and 12, would satisfy our aim to challenge developing nestlings with an oxidative insult, but causing no mortality and keeping our manipulation close to the natural range of oxidative stress suffered by developing nestlings.

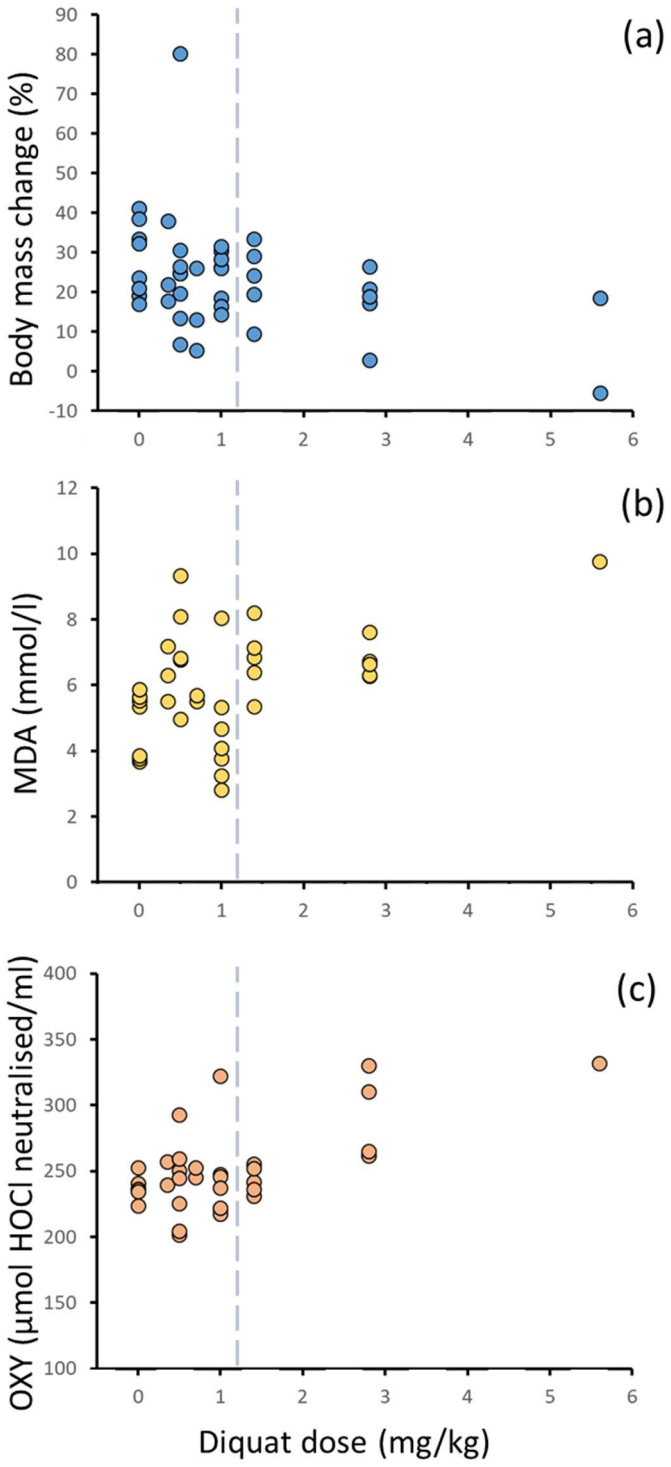

Figure 1S. Effect of a single intraperitoneal injection of different doses of diquat on body mass changes, plasma MDA and plasma OXY in pied flycatcher nestlings. Dashed grey line indicates the dose finally selected for the experiment based on this pilot assay (1.2 mg/kg).

References

Bates, D., Machler, M., Bolker, B.M., and Walker, S.C. (2015). Fitting linear mixed-effects models using *lme4*. *J. Stat. Softw.* 67**,** 1-48.

Costantini, D. (2011). On the measurement of circulating antioxidant capacity and the nightmare of uric acid. *Methods Ecol. Evol.* 2**,** 321-325. doi: 10.1111/j.2041-210X.2010.00080.x.

Costantini, D., and Verhulst, S. (2009). Does high antioxidant capacity indicate low oxidative stress? *Funct. Ecol.* 23**,** 506-509. doi: 10.1111/j.1365-2435.2009.01546.x.

Halliwell, B., and Gutteridge, J.M.C. (2007). *Free Radicals in Biology and Medicine.* Oxford: Oxford University Press.

Mateos, R., and Bravo, L. (2007). Chromatographic and electrophoretic methods for the analysis of biomarkers of oxidative damage to macromolecules (DNA, lipids, and proteins). *J. Sep. Sci. 30, 175-191. doi: 10.1002/jssc.200600314.*

Pérez-Rodríguez, L., Romero-Haro, A.A., Sternalski, A., Muriel, J., Mougeot, F., Gil, D., et al. (2015). Measuring oxidative stress: the confounding effect of lipid concentration in measures of lipid peroxidation. *Physiol. Biochem. Zool.* 88**,** 345-351. doi: 10.1086/680688.