**Canaries or miners? A meta-analysis of the pollution impact on the oxidative stress machinery of amphibians across life stages**

**The impact of anthropogenic pollution across major life transitions: a meta-analysis on oxidative stress in amphibians**

**The impact of anthropogenic pollution on the oxidative stress machinery of amphibians varies across their major life transitions, a meta-analysis**

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**Introduction**

Human actions are not only resulting in species extinction but also in demographic declines across the globe (Ceballos et al. 2020). In particular, anthropogenic chemical pollution is considered a major human-related cause of biodiversity loss (REF). Anthropogenic chemical pollutants such as fungicides, pesticides, herbicides, or heavy metals are released daily into ecosystems through multiple sources, often creating novel and stressful conditions for wildlife. The susceptibility of organisms to these pollutants could change across a lifetime and be of particular relevance for species with a life cycle that includes abrupt life transitions such as metamorphosis (Lowe et al. 2021). However, no study to date has generally and systematically evaluated to what extent anthropogenic chemical pollutants impact the physiology of species with complex-life cycles. Such assessment would increase our understanding of how pollution affect population dynamics and might help explain population declines under a number of scenarios.

Exposure to pollutants can induce a broad range of phenotypic and physiological changes that are metabolically demanding (REFS). Enhanced metabolism requires the generation of energy through the breakdown of ATP and can lead to the production of reactive oxygen species (ROS). In a homoeostatic scenario, ROS act as a physiological signals; however, if ROS production overpasses the capacity of organisms to counteract them, a cellular oxidative stress state is induced. Oxidative stress can damage essential biomolecules such as lipids, proteins, or DNA, and lead to reductions in organismal health and life expectancy. The physiological mechanisms that neutralise ROS (ie., redox machinery) are hence thought to have a central systemic role (Sohal et al. 2002; Costantini et al. 2010; Costantini et al. 2019). The impact of anthropogenic chemical pollution on the oxidative machinery of organisms can be linked to the mechanism action of a given pollutant, and could potentially change across an individual’s ontogeny. The latter is expected to be particularly marked across the life stages of species undergoing metamorphosis. These species show at least three remarkably different life stages (embryo, larva (or pupa) and adult) that show very different behavioural, phenotypical and physiological characteristics (REF?). Since the life cycle of more than three-quarters of the existing animal species includes a metamorphosis, investigating whether anthropogenic chemical pollutants effects vary across life-stages can have major eco-evolutionary and conservation implications.

Among vertebrates, amphibians are the ideal group to study whether the impact of pollution on the redox machinery vary across life transitions in species undergoing metamorphosis. The life cycle of most amphibians, and particularly of anurans, includes an embryo that hatches into a fish-like larva which abruptly develops to a tetrapod juvenile through metamorphosis. Both embryos and larvae often have a highly permeable external surface/skin, and their habitat is commonly restricted to the aquatic environment. In contrast, post-metamorphic individuals normally develop a less permeable skin and, although they often rely on waterbodies for breeding, juveniles and adults of most anuran species can inhabit the terrestrial environment. Therefore, the impact of chemical pollutants released to water bodies is expected to vary across amphibian life stages, with the embryonic and larval stages potentially being the most vulnerable. Indeed, amphibians are the most threatened vertebrate group and chemical pollution is thought to be an important cause behind their decline. Therefore, investigating how pollutants impact physiological mechanisms linked to individual health and performance, such as oxidative stress, could dramatically improve our understanding of amphibian conservation.

Likely due to the potential susceptibility of amphibians to environmental risks, a considerable number of studies have investigated the role of pollutants in shaping the oxidative stress machinery of amphibians either at the pre- or post-metamorphic stages (REF?). As in other animals, the oxidative stress pathways of amphibians can be divided into the antioxidant system and markers of oxidative damage. The antioxidant system consists of a wide range of enzymatic and non-enzymatic components that work synergistically to control ROS production and thus achieve redox homeostasis (Peng et al. 2014). The first line of defence in response to oxidative damage involves endogenously produced enzymatic scavengers such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) (Matés & Sánchez-Jiménez 1999). The second line of defence involves scavenging non-enzymatic antioxidants with a low molecular weight that allow detoxifying ROS located in cellular areas where large enzymes cannot reach ROS (Pamplona & Costantini, 2011). The tripeptide reduced glutathione GSH is the most abundant thiol in animal cells and can directly scavenge ROS, or work in conjunction with antioxidant enzymes. Finally, some substances are indicators of oxidative damage at different levels, such as oxidative damages in lipids of the cellular membrane which are normally quantified through estimates of malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids (Mateos, & Bravo, 2007).

Here, we investigate the impact of anthropogenic chemical pollution (e.g., pesticides, herbicides, heavy metals) on the oxidative stress machinery (enzymatic and non-enzymatic antioxidant responses, and oxidative damage in lipids) of amphibians across life transitions (embryo, larva, adult). Specifically, we carry out a systematic literature review and meta-analysis to test the impact of anthropogenic chemical pollution on the redox system of amphibian and assess whether these impacts vary across life stages. In our meta-analysis, we only include experimental studies that directly tested the effects of different pollutants on the redox system of embryo, larva and adult amphibian. As amphibian embryos and larvae have very permeable skins (i.e., they can easily absorb pollutants) and low vagility, we predicted higher antioxidant responses after exposure of chemical pollutants at these pre-metamorphic stages than later in life,. We predicted higher oxidative damage in post-metamorphic amphibians facing pollution, as the higher vagility of individuals after metamorphosis may have favoured a behavioural avoidance response rather than a physiological response to neutralise ROS. Finally, we expected that the type of pollutant will have different consequences on the amphibian redox machinery, and we investigated whether oxidative responses are tissue-specific.

**Results**

We systematically inspected 361 unique studies published between 1998 and 2021 on pollution and oxidative stress in amphibians (Figure X). After assessing for inclusion (see methods; Figure XX), we extracted 1966 effect sizes from 83 studies, including 33 amphibian species (see Figure XX). All these species are anurans that have a life cycle including an embryo, larva, and post-metamorphic stage. The dataset included 1419, 305 and 337 effect sizes for enzymatic markers of oxidative stress, non-enzymatic markers of oxidative stress and indirect indicators of oxidative stress respectively (see Table XX).

*Overall effects of pollution on the redox machinery of amphibians*

An initial overall model including all the oxidative stress parameters and life stages showed that experimental exposure to pollutants increased by 16% the levels of the studied redox machinery as compared to control conditions (model intercept [95% confidence interval; ‘95%CI’ hereafter] = 0.152 [0.032, 0.271]; Figure XX; Table XX). The total heterogeneity of that overall model was high (I2total = 99.89), with 1.44% and 2.19% of it respectively explained by species and phylogeny, and 27.69% explained by among-study differences.

E*ffects of pollution on different components of the redox machinery of amphibians*

Exposure to pollutants increased the levels of all studied redox markers, with 95% CI for model estimates not overlapping zero for enzymatic antioxidants (estimate [95%CI] = 0.120 [0.001, 0.232]) and lipid damage (estimate [95%CI] = 0.297 [0.175, 0.418]) and slightly overlapping zero in the non-enzymatic antioxidants (estimate [95%CI] = 0.121 [-0.002, 0.244]).

*Effect of pollution across amphibian life stages*

Pollutants had a contrasting effect on the redox machinery on embryos, larvae, and adults. In embryos, pollutants increased the levels of the non-enzymatic antioxidants (estimate [95%CI] = 0.383 [0.031, 0.735]; Figure X) but did not have a remarkable effect on the enzymatic antioxidants (estimate [95%CI] = 0.039 [-0.231, 0.309]; Figure X) or lipid peroxidation (estimate [95% CI] = 0.221 [-0.223, 0.645]; Figure X). Redox marker explained 7.96% of the overall variation in redox response to pollutants in embryos (i.e., r2marginal = 7.96%). In tadpoles, pollutants increased to a similar extent the levels of the enzymatic and non-enzymatic antioxidants although 95%CI slightly overlapped zero in both cases (estimate ‘enzymatic’ [95%CI] = 0.178 [-0.014, 0.371] and estimate ‘non-enzymatic’ [95%CI] = 0.201 [-0.014, 0.415]; Figure X). In contrast, the effect of pollutants on lipid peroxidation was unnoticeable in tadpoles (estimate [95%CI] = 0.061 [-0.150, 0.271]; Figure X). Redox marker explained 0.59% of the overall variation in redox response to pollutants in tadpoles (i.e., r2marginal = 0.59%). In adults, while pollutants had a weak effect both on the enzymatic (estimate [95%CI] = 0.112 [-0.123, 0.347]; Figure X) and non-enzymatic antioxidants (estimate [95%CI] = 0.110 [-0.130, 0.350]; Figure X), they remarkably increased lipid peroxidation levels (estimate [95%CI] = 0.506 [0.266, 0.746]; ; Figure X). Redox marker explained 9.76% of the overall variation in redox response to pollutants in adults (i.e., r2marginal = 9.76%).

*Effect of individual pollutants on the redox machinery of embryos, tadpoles, and adults*

The exposure to herbicides increased the non-enzymatic antioxidants in tadpoles (estimate [95%CI] = 0.5279 [0.193 – 0.862]; Figure X), whereas it did not involve noticeably changes in their enzymatic antioxidants (estimate [95% CI] = 0.092 [-0.111, 0.295]; Figure X) nor lipid peroxidation (estimate [95% CI] = 0.182 [-0.061 – 0.422]; Figure X). Herbicides did not alter the enzymatic response or the lipid peroxidation of adults (k = 2 and 10 effect sizes from one and two studies, respectively; Figure X). There were no data available for meta-analysing the effect of herbicides on the redox status of embryos.

The effect of pesticides on the studied parameters was life-stage dependent. In embryos, pesticide exposure did not alter any of the three studied components of the redox machinery (Figure X, Table X). In tadpoles, pesticides increased the levels of the enzymatic and non-enzymatic antioxidants to a similar extent (estimate ‘enzymatic’ [95%CI] = 0.393 [0.047, 0.740] and estimate ‘non-enzymatic’ [95%CI] = 0.374 [0.006, 0.742]) but not their lipid peroxidation (Figure X, Table X). Finally, pesticides remarkably increased the levels of lipid peroxidation in adults (estimate [95% CI] = 0.567 [0.152, 0.983] but it did not involve noticeable antioxidant responses (Figure X, Table X).

Metallic elements did not alter the oxidative stress status of tadpoles (only k = 3 effect sizes available for lipid peroxidation; see Figure X and Table X). In contrast, adults exposed to metals increased the levels of their non-enzymatic antioxidants and lipid peroxidation (estimate ‘non-enzymatic’ [95% CI] = 0.489 [0.226, 0.752] and estimate ‘lipid peroxidation’ [95% CI] = 0.382 [0.094, 0.670]), whereas the levels of the antioxidant enzymes did not remarkably change (k = 8 effect sizes; Figure X). Not enough data were available for meta-analysing the effect of metallic elements on all redox markers of embryos (k = 16 from two studies including enzymatic data only).

The exposure to inorganic elements did not alter the oxidative stress parameters of embryos or tadpoles (the model did not converge for adults as we only had a total of k = 16 effect sizes; Figure X, Table X). Finally, organic elements did not involve changes in the studied parameters of tadpoles and adults (no data was available for embryos; Figure X, Table X).

*Effect of tissue, life mode and climate*

An overall model combining all pollutants and developmental stages but assessing redox responses across different tisuses showed a similar redox response among them(estimate [95%CI]: whole body = 0.124 [0.013, 0.235]), liver = 0.157 [0.017, 0.297], muscle = 0.148 [-0.012, 0.308], kidney = 0.162 [-0.008, 0.332], brain = 0.147 [-0.041, 0.334], and heart = 0.279 [0.087, 0.472]; Figure X). Tissue type only explained 0.36% of the overall variation in redox response to pollutants.

Pollution was associated with increased redox markers in aquatic species (estimate [95% CI] = 0.228 [0.124, 0.332]) but not in semi-aquatic species (estimate [95% CI] = 0.082 [-0.018, 0.182]. Life mode explained 1.76% of the overall variation in redox response to pollutants.

Tropical species showed increased levels of redox markers in response to pollution (estimate [95% CI] = 0.311 [0.068, 0.554]). In, subtropical and temperate species pollution did not increase redox markers (Figure XX). Climate explained 1.48% of the overall variation in redox response to pollutants.

*Publication bias*

We did not detect small-study effects in our dataset (estimate for the square-root of the inverse of the effective sample size [95% CI] = -0.109 [-0.403, 0.185]), with the overall model intercept after correcting for effective sample size being very similar to the one without the correction (unbiased estimate [95% CI] = 0.214 [0.000, 0.429]). We did not detect time-lag effects (estimate [95% CI] = 0.004 [-0.010, 0.018]).