



University
of Glasgow

The impact of pollutants on oxidative stress in anurans across development: a meta-analysis

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BSc Honours Zoology

BIOL4246P Investigative Honours
Project

Date of submission: 31/03/21

Word Count: 5877



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ABSTRACT

Environmental pollution is one of the main stressors driving the global decline of anurans. To avoid or reduce damage, anurans activate physiological or behavioural plastic responses, a metabolically demanding process that can cause oxidative stress. This can cause long-term fitness trade-offs, such as reduced lifespan, low immune system, and reduced locomotion. This meta-analysis synthesised research from 2015-2020 to evaluate whether pollution can affect the antioxidant responses or cause oxidative stress, and to understand which life stage antioxidant machinery is most sensitive at. Pollutants included in this study were pesticides, herbicides, heavy metals, wastewater contaminants and fungicides. A meta-analysis was conducted and 523 effect sizes were calculated from 30 studies, then the meta-regression revealed that pollution increases oxidative stress and oxidative damage in anurans. This study also suggested that antioxidant machinery was most sensitive at the tadpole life stage. These results highlight the importance of understanding the capacity at which anurans can withstand pollutant stress to enforce management practices and regulations to help conserve anurans.

ABBREVIATIONS

SOD: Superoxide dismutase

CAT: Catalase

GPx: Glutathione peroxidase

GR: Glutathione reductase

GSH: Glutathione

MDA: Malondialdehyde

TBARS: Thiobarbituric acid reactive substances

SMD: Standard Mean Difference

ROS: Reactive oxygen species

CI: Confidence Interval

PI: Prediction Interval

EEC: Expected Environmental Concentration

1. INTRODUCTION

Over the past 30 years, amphibians have declined at an unprecedented rate and are now classed as the most threatened vertebrate group in the world (Hayes et al. 2010). This global decline is driven by multiple interacting stressors: habitat modification, invasive species, climate, and environmental pollution (Grant, Miller, and Muths, 2020; Green et al., 2020). The complex lifecycle of anurans, with both terrestrial and marine phases, are exposed to environmental pollutants on land and in water and can be used as bioindicators (Blaustein and Belden, 2003; Strong et al., 2017). They are extremely vulnerable to environmental pollution because their eggs are shell-less and they have exposed (no hair/scales), permeable skin, which functions as a respiratory organ and regulates water uptake (Brühl, Pieper and Weber, 2011). In addition, anurans are considered to be most vulnerable to environmental stressors during their early stages of development, and several studies have suggested that the earlier the development stage that is exposed, the more detrimental the long-term fitness effects will be (Greulich and Pflugmacher, 2003; Hopkins et al., 2014; Wagner et al., 2017).

1.1 Pollution

The physiological responses of anurans to pollution have been largely reviewed in recent literature, exploring the impact of fungicides, pesticides, herbicides and metal contaminants at sublethal doses (Isaksson, 2010; Slaby et al., 2019). The results of many studies indicate that exposure to low doses of pollutants can activate physiological, behavioural and morphological plastic responses in amphibians to avoid or reduce damage (Burraco et al. 2020; Strong et al., 2017). For example, exposure of the pesticide, lambda-

cyhalothrin, disrupts the development of *Xenopus tropicalis* embryos, causing long-term malformations, including a bent notochord and hypopigmentation (Jiang et al. 2019). However, environmentally induced phenotypic modifications are metabolically demanding and can cause oxidative stress that results in long-term fitness trade-offs, negatively impacting aspects of an amphibian's life, including lifespan, reproductive output, immune function and locomotion (caused by growth malformities and delays in larval growth) (Charbonnier and Vonesh 2015; Lee, Monaghan and Metcalfe, 2013; Metcalfe and Alonso-Alvarez 2010; Smith, Nager and Costantini, 2016). Therefore, understanding the impacts of pollution on the antioxidant status and oxidative stress in amphibians is critical for conservation biology.

1.2 Oxidative Stress

During normal physiological activities, animals produce excess reactive oxygen species (ROS) including highly reactive and unstable free-radicals, superoxide anion (O_2^-) and hydroxyl radicals ($OH\cdot$), and the less reactive non-radical, hydrogen peroxide (H_2O_2) (Finkel & Holbrook 2000). ROS are a toxic metabolic by-product of oxygen-reduction (REDOX) reactions and, to prevent damaging effects, the antioxidant system releases enzymatic and non-enzymatic antioxidant enzymes to regulate the redox status (Balaban, Nemoto and Finkel, 2005). Although most ROS molecules are produced endogenously during metabolism in the mitochondria and peroxisomes, they can also be produced exogenously after exposure to ultraviolet radiation, ozone, diet or environmental toxins (Balaban, Nemoto and Finkel, 2005). In addition, low ROS molecule concentrations are critical for vital biological processes and are involved in immune responses, detoxification and intracellular signalling (Dröge 2002). However, while ROS can have beneficial

effects, 90% of ROS molecules cause damage and, under stressful environmental conditions, an insufficient removal of ROS molecules by antioxidants can damage essential biomolecules, causing lipid peroxidation, protein oxidation and DNA damage, consequently causing oxidative stress (Balaban, Nemoto and Finkel, 2005; Ježek & Hlavatá 2005).

1.3 Antioxidant System

The antioxidant system consists of a wide range of enzymatic and non-enzymatic components that work synergistically to control the level of ROS molecules and achieve redox homeostasis (Peng et al. 2014). The first line of defence in response to oxidative damage involves the endogenously produced enzymatic scavengers, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR), which collectively control the intracellular production of ROS molecules (Matés & Sánchez-Jiménez 1999). SOD is a vital antioxidant that catalyses the breakdown of reactive superoxide anion radicals into the less reactive H_2O_2 and oxygen (Figure 1.1). The H_2O_2 produced by SOD is then detoxified by CAT and GPx (Figure 1.1) by converting the reactive species to water (H_2O) (Matés & Sánchez-Jiménez 1999). As shown in Figure 1.1, GPx detoxifies H_2O_2 by removing hydrogen from 2 Glutathione (GSH) molecules, producing H_2O and Glutathione disulfide (GSSG) and then Glutathione reductase (GR) catalyses the reduction of GSSG to GSH. CAT rapidly converts H_2O_2 to water despite having a low affinity for the reactive species, whereas GPx has a high affinity and converts H_2O_2 at a slower rate (Pamplona & Costantini 2011). Therefore, CAT is suitable for controlling high influxes of H_2O_2 production, whereas GPx can be used to control H_2O_2 that is produced constantly at low levels.

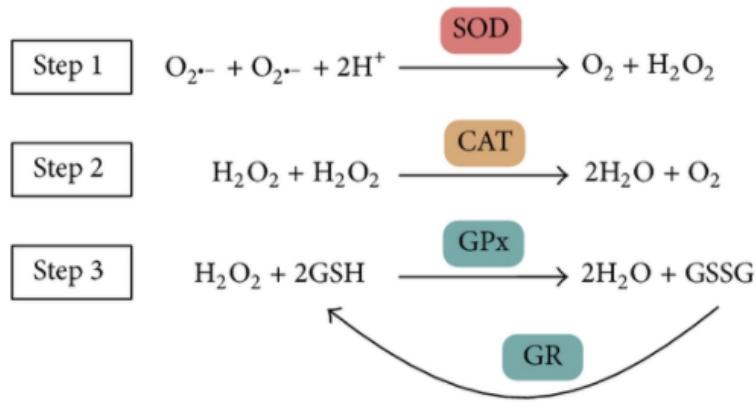


Figure 1.1: The enzymatic antioxidant system reactions. The reactions demonstrate the enzymatic antioxidants, superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR), scavenging free radicals (from Peng et al. 2014).

The second line of defence involves scavenging non-enzymatic antioxidants that can be produced endogenously (GSH, thioredoxin and ascorbate) or obtained through the diet (carotenoids and Vitamin E) (Sies, Stahl, & Sundquist, 1992). The low molecular mass of non-enzymatic antioxidants allows them to access parts of the body that large enzymes cannot reach to eliminate ROS (Pamplona & Costantini, 2011). The thiol tripeptide GSH (glutamyl-cysteinyl-glycine) is a major multifactorial antioxidant found abundantly within animal cells (cytosol, mitochondria and nuclei) in its reduced form with cellular concentrations ranging from 1-10 millimoles (mM) (Smith et al., 1996). Cysteine residue contains an extremely reactive sulphhydryl group (SH) which, in an oxidised state, exists as GSSG in the reduced form (McBean, 2017). GSH can directly scavenge ROS molecules or, as mentioned previously, work in conjunction with the three enzymatic antioxidants, SOD, CAT and GPx, to decompose the hydrogen peroxide and, after this process is complete, GSH can be regenerated to its reduced form by GR (this requires NADPH). GSH is also involved with the protection of cells from ROS through the recycling of Vitamin C and Vitamin E molecules (Janciauskiene, 2020).

1.4 Measuring Oxidative Stress

To detect the impact of pollution on anurans' redox status, a combination of biomarkers, including the antioxidant status and oxidative damage, can be measured and quantified. The end products of lipid peroxidation are commonly used as an indicator of oxidative damage in amphibians. Malondialdehyde (MDA), an end product of the peroxidation of polyunsaturated fatty acids, is a suitable biomarker that can be measured using the thiobarbituric acid reactive substances (TBARS) assay (Sestini, Carlson, & Allsopp, 1991; Mateos, & Bravo, 2007). Although, MDA is also found in the diet and is absorbed into the gastrointestinal tract, which can alter the background levels of MDA within the tissue, disrupting the reliability of this test (Mateos, & Bravo, 2007). In addition, the specificity of the test is easily disrupted because TBARS reacts with other aldehydes in the organism and, therefore, the results of this test must be interpreted with caution and other tests, including determining the antioxidant activity, can be carried out in conjunction with this test (Giustarini et al. 2009). The activity of both enzymatic (SOD, CAT and GPx) and non-enzymatic (GSH) antioxidants can be measured using indirect assays with spectrophotometry (Monaghan, Metcalfe & Torres, 2009).

1.5 Meta-analysis

Meta-analyses help to establish a consensus across literature, which is done by combining and integrating the results of several independent studies that study the same treatment to identify a common effect (Lee, 2019). It can also overcome the issue of small sample sizes of individual studies as many individual studies can be too small to detect small effects.

Combining studies increases the sample size and, therefore, the chance of detecting an effect. Increasing the sample size also increases the statistical power and helps to determine small but significant effects across the studies.

1.6 Aims and Hypothesis

This study performed a meta-analysis to determine the effects of pollution on oxidative stress levels in anurans. The aims of this study are to summarise recent literature investigating the effects of pollution on anurans to: (1) evaluate the evidence based on whether pollution affects antioxidant responses (SOD, CAT, GPx and GSH) or cause oxidative damage (MDA) to amphibians (2) determine whether the antioxidant machinery of anurans is more sensitive to environmental changes in early life (larval development) or later in life (juveniles or adults). It was hypothesised that environmental conditions will alter the antioxidant status and, by studying a number of biomarkers of oxidative stress, this investigation will provide an in-depth understanding of the sensitivity of individual antioxidants to different environmental stressors. In addition, it is hypothesised that the altered antioxidant status will cause oxidative stress, and thus be likely to negatively impact the fitness and future survival of the anurans. It is also hypothesised that anurans are more sensitive to environmental changes during early development.

2. METHODOLOGY

2.1 Data Collection

Relevant papers were identified and recorded using the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (Moher et al., 2015). Database searches were conducted between the 9th of October and the 20th of November 2020 on the Web of Science, PubMed, and the University of Glasgow Library using the following string: ('Oxidative Stress') AND ('Amphibians'). 'Amphibians' was chosen as a keyword because this study first aimed to study oxidative stress in amphibians, but all papers included only contained anurans, and the study's focus was adjusted accordingly. Articles published between 2015-2020 were reviewed and, as shown in the PRISMA Flow Diagram (Figure 2.1), 553 studies were generated through the database search and 3 were identified through searching the reference sections of the papers reviewed. After duplicates were removed, the titles and abstracts were screened and 85 articles were identified as potentially suitable for the meta-analysis. From these, studies were only then included in the meta-analysis if they met the following criteria:

1. The study reported the means, variability, i.e. standard deviation (SD) or standard error (SE), and the sample sizes for the control group, i.e. non-exposed to pollutant and the treatment group, i.e. group(s) exposed to pollutant.
2. The study used an appropriate biomarker including the enzymatic biomarkers (SOD, GPx, CAT, GR), non-enzymatic biomarker (GSH) and indicator of oxidative damage (MDA)
3. The study reported the anuran's development stage (embryo, tadpole, adult)
4. The duration of the experiment was reported
5. The study did not test the effect of a pollutant in combination with another factor.

A total of 30 studies met the criteria for inclusion and from these, a dataset was compiled consisting of 523 estimates. From each study, the mean, SD and sample size were derived from both control and treatment groups, and results reported as SE in the original paper were transformed into SD using the formula:

$$SD = SE * \sqrt{sample\ size}$$

Most of the data in the papers were presented graphically. Therefore, numerical data was obtained using digitalising software, WebPlotDigitiser Version 4.4, which has been shown to be a valid and reliable method of data extraction for meta-analyses (Drevon et al., 2017; Rohatgi, 2020). From each study, additional data were collected, including the species' family, development stage, pollutant class (Herbicide, Pesticide, Wastewater contaminant, Fungicide, Heavy Metal) and the specific biomarker measured. To account for the variation in the pollutant concentrations across the 30 studies, the concentrations used in the papers were compared to reported ranges found in the environment, also known as expected environmental concentrations (EEC), that were extracted from relevant research papers and inserted into a table (see Appendix B: Table 2). Pollutant concentrations were classed into 'Low', 'Medium', and 'High' and Low was the lower half of the range of the EEC, Medium was in the upper half of the range of the EEC, and High included concentrations that were outside the range found in the environment, and so are not considered ecologically relevant.

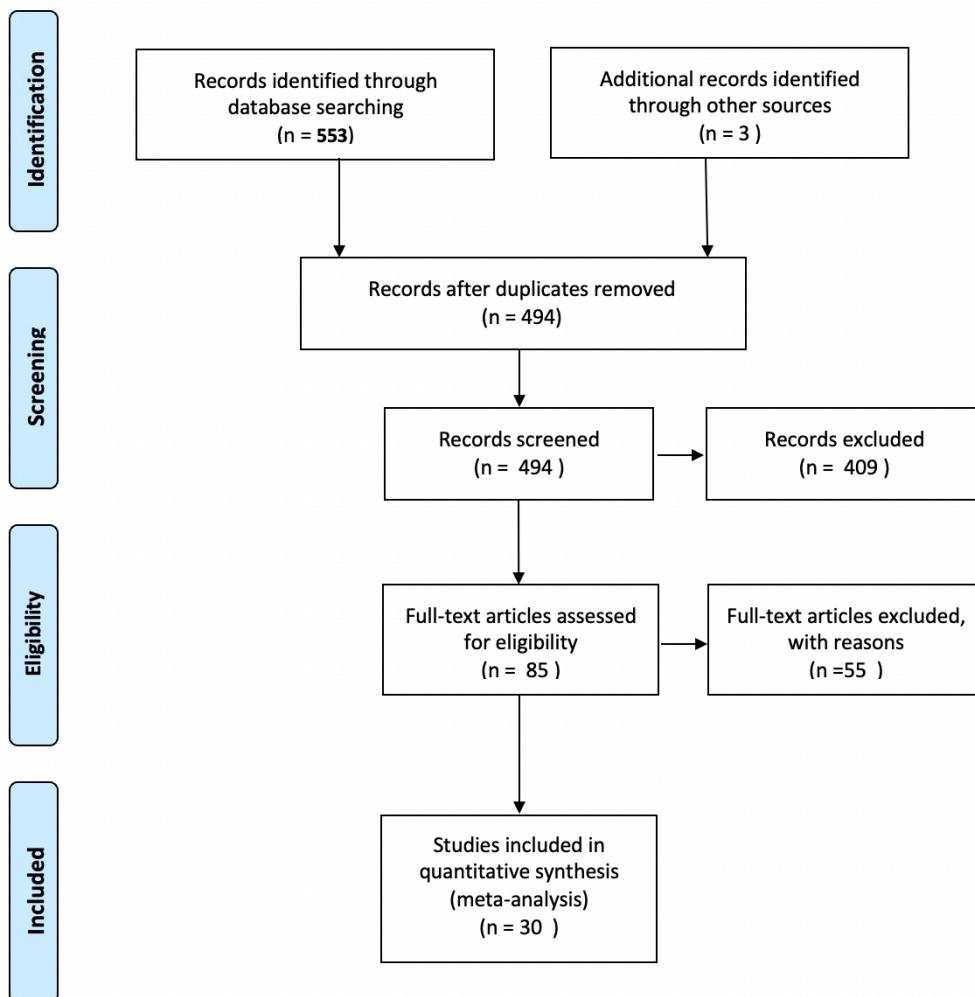


Figure 2.1: PRISMA Flow Diagram outlining literature review process (adapted from Moher et al., 2015).

2.2 Meta-Analysis

The data collected were entered into a excel spreadsheet and then all tests were completed using R Version 4.0.4 of 2021-02-15 (R Core Team, 2021). The ‘*metafor*’ (Version 2.4-0), ‘*ggplot2*’ (Version 3.3.3) and ‘*orcharD*’ (Version 0.0.0.900) packages were used for the meta-analysis (Nakagawa et al., 2020; Viechtbauer, 2010; Villanueva and Chen 2019). The ‘*metafor*’ package calculated effect sizes (see below 2.3) and sampling variances using the ‘*escalc*’ function and a random effect size model was generated using the ‘*rma.mv*’ function. The ‘*ggplot2*’ and ‘*orcharD*’

packages were used to visualise the results in a forest-like/orchard plot, which shows the overall mean effects, 95% confidence intervals (CIs) and 95% prediction intervals, (PIs) (Nakagawa et al., 2020). Meta-analyses regularly use forest plots, which display individual point estimates, CIs and an overall mean of effect sizes at the bottom of the graph. However, this study has >500 effect sizes, therefore the orchard plot was used to clearly visualise the results by displaying the average effect size. Also, unlike forest plots, orchard plots include PIs, which incorporate heterogeneity and predict the range in which the effect size is expected to fall in future studies, and effect sizes scaled by their precision are displayed in the graph.

2.3 Calculating effect sizes

Effect sizes are a standardised measure of the magnitude of observed effect and, therefore, studies that have measured different pollutants or used different scales of measurement can be directly compared. The means, SDs and sample sizes were used to calculate standard mean differences (SMD), also known as Hedges' g, using the 'escalc' function in the '*metafor*' package (Hedges and Vevea, 1998). Hedges' g corrects for possible bias in studies with a small sample size (<20) and, as described by Cohen (1988), an effect size of 0.2, 0.5 and 0.8 can be classed as 'small', 'medium' and 'large', respectively. Most studies reported more than one biomarker of oxidative stress, therefore, a total of 523 effect sizes were computed from the 30 studies. In this study, the control group was defined as the group not exposed to any form of pollutants. Therefore, a positive Hedges' g means that in anurans exposed to pollutants, the oxidative biomarker level has decreased and a negative Hedges' g means that the oxidative biomarker level has increased.

2.4 Heterogeneity and random-effects model

The publications reviewed in this study are prone to sources of heterogeneity, including pollutant concentration levels, the type of pollutant, the development stage, species studied, the biological matrix studied and the biomarkers of oxidative stress. Statistical heterogeneity between studies was evaluated using the I^2 Index, which describes percentage of variance across summary effect sizes that is due to heterogeneity, and Cohen's Q, a statistic that measures magnitude of heterogeneity (Huedo-Medina et al., 2006). I^2 values of 25%, 50%, and 75% were considered as low, moderate, and high heterogeneity, respectively. Significant heterogeneity was detected in this study ($I^2 > 75\%$). Therefore, a random effects model was created using the 'rma.mv' function in the '*metafor*' package. This model was chosen over a fixed-effects model because the true effect sizes in this study are affected by both within-study and between-study heterogeneity. However, fixed-effects models only consider within-study variation (Viechtbauer, 2010). A multivariate linear mixed effects model with restricted maximum likelihood estimation (REML) was used to test whether the fixed factors were significantly different from zero, and in this study the fixed factors were biomarker type (SOD, CAT, GPx, GR, GSH, MDA) and development stage (Embryo, Tadpole, Adult). Study ID was included as a random effect in all models conducted during this study. Overall effect sizes were visually assessed using an orchard plot, which shows overall mean effects, CIs and PIs. Where the 95% CIs did not cross over zero, the effect was significant ($p < 0.05$).

2.5 Publication Bias

Furthermore, studies with significant results are more likely to be published than those with no significant results, resulting in publication bias (Rosenthal, 1979). Therefore, the possibility of

publication bias across the entire study was visually assessed using a funnel plot and quantitively assessed using an Egger's test. If publication bias was detected by the Egger's test ($p<0.05$), the trim-and-fill method was used to calculate the missing studies and adjust the summary effect size (Duval and Tweedie, 2000; Sterne and Egger, 2001). Also, publication bias is not the only factor that can create funnel plot asymmetry because factors associated with both sample size and the effect size, such as poor study design can cause asymmetry (Peters et al., 2008). Therefore, an additional extension known as the contour enhanced funnel plot was used because it displays areas of statistical significance on the plot for each individual study.

3. RESULTS

3.1 Overall dataset

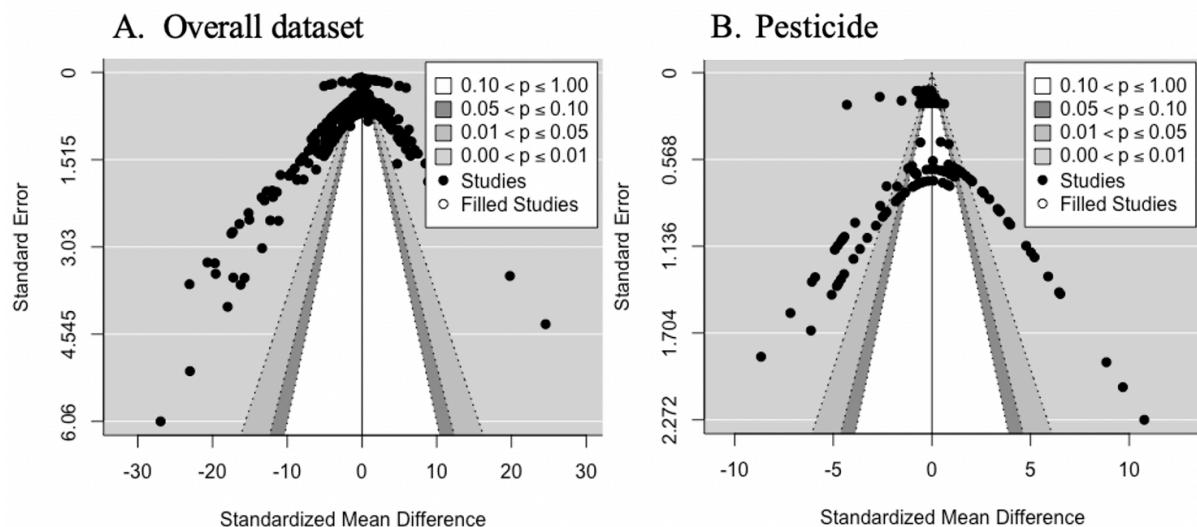
There were 21 different species of anurans included in this study (from 8 taxonomic Families) and the tadpole stage of development was most frequently used in the experiments (416/523 effect sizes) compared to embryos and adults (30 and 77/523 effect sizes, respectively). Most studies used herbicides as the treatment group (168 effect sizes), compared to heavy metal (135 effect sizes) and pesticide (117 effect sizes) exposure. A lower number of studies exposed amphibians to water contaminants (49 effect sizes) and fungicides (54 effect sizes). Enzymatic biomarkers of oxidative stress accounted for 353 effect sizes compared to 82 and 93 effect sizes for GSH (non-enzymatic biomarker of oxidative stress) and MDA (indicator of oxidative damage), respectively. In this study “Low” and “High” pollutant concentrations each accounted for 232 effect sizes and the “Medium” pollutant concentration included 64 effect sizes (See Appendix A: Table 1).

3.2 Publication Bias and heterogeneity

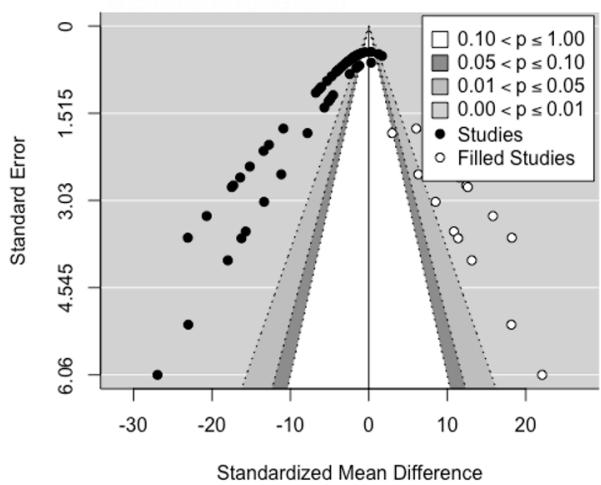
A visual assessment of the contoured funnel plot (Figure 3.7A) determined that the data points were symmetrical, however, the studies were located within the light grey areas of statistical significance, which indicates that there is publication bias for the whole dataset. Egger’s multivariate regression was significantly different from zero ($z= -12.5$, $p<0.0001$), confirming the publication bias of the whole dataset (See Appendix A: Table 1). A Trim-and-fill analyses was conducted to correct for this bias; however, no missing studies were found at either side of the plot ($p=0.5$). In addition, the contoured funnel plots from the pesticide, heavy metal and herbicide datasets (Figure 3.7 B,D,E) were symmetrical and the

plots from fungicide and wastewater contaminants were asymmetrical (Figure 3.7 C,F). However, significant publication bias was found in the fungicide ($z = -18.8$, $p < 0.0001$), heavy metal ($z = -3.2$, $p = 0.001$) and water contaminant datasets ($z = -8.08$, $p < 0.0001$) (See Appendix A: Table 1). There was no significant publication bias found in pesticide ($z = -0.11$, $p = 0.9$) or herbicide ($z = 0.3$, $p = 0.7$) datasets (See Appendix A: Table 1). A trim-and-fill analyses on the fungicide dataset estimated that (± 17) effect sizes were missing from the right side of the mean ($p < 0.0001$). Also, an analysis on the wastewater contaminant dataset estimated (± 5) effect sizes were missing from the right side of the mean ($p = 0.01$).

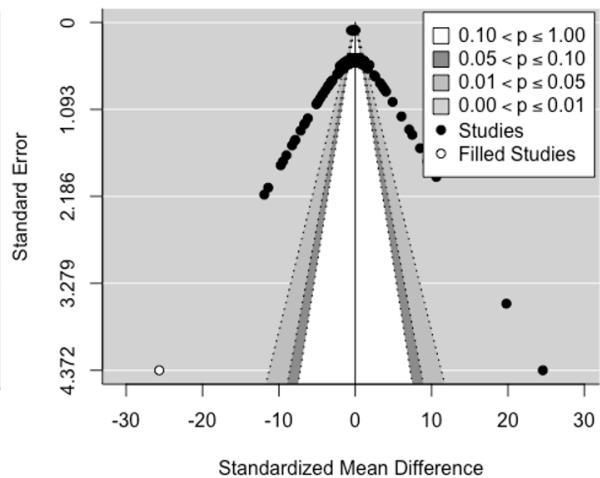
Meta-analysis regression revealed that the total heterogeneity across the overall dataset was statistically significant ($I^2 = 98.82\%$, $Q_{523} = 10472$, $p < 0.0001$) (See Appendix A: Table 1). There was also evidence of considerable statistical heterogeneity across the pesticide ($I^2 = 98.35\%$, $Q_{116} = 1544$, $p < 0.0001$), fungicide ($I^2 = 98.37\%$, $Q_{53} = 663$, $p < 0.0001$), heavy metal ($I^2 = 98.75\%$, $Q_{134} = 1368$, $p < 0.0001$), herbicide ($I^2 = 80.67\%$, $Q_{167} = 757$, $p < 0.0001$), and wastewater contaminants ($I^2 = 99.67\%$, $Q_{48} = 3949$, $p < 0.0001$) datasets.



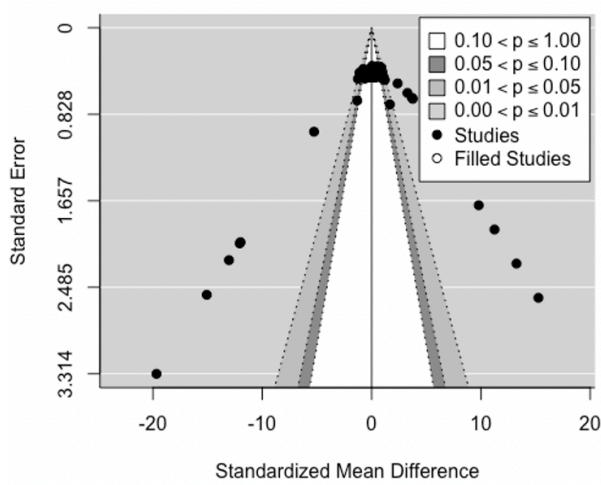
C. Fungicide



D. Heavy Metals



E. Herbicide



F. Wastewater contaminants

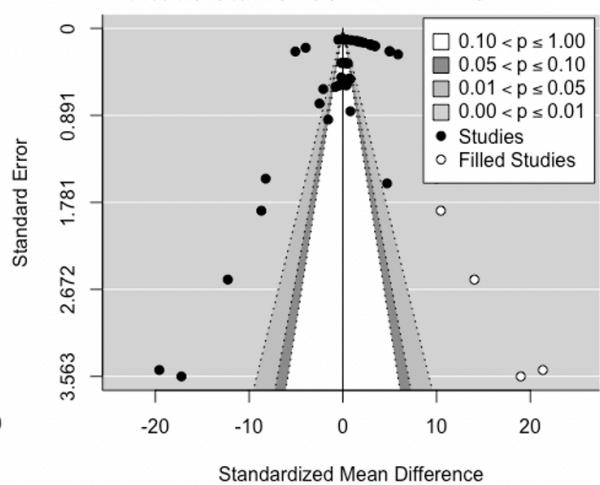


Figure 3.7: Contour enhanced, trim-and-fill funnel plots of publication bias for the relationship between pollutants and oxidative stress. Funnel plot with Standard Mean Difference plotted against Standard error on a reverse scale. White and dark grey show areas of statistical non-significance, with white areas corresponding to p-values between 0.1-1 and dark grey areas corresponding to p-values between 0.05-0.1. The two areas of lighter grey show areas of statistical significance corresponding to p-values ranging between 0-0.05. Effect sizes of observed studies are represented by dark circles and white circles represent the missing studies imputed by trim-and-fill analyses. A- overall dataset; B- Pesticides; C- Fungicides; D- Heavy Metals; E- Herbicides; F- Wastewater contaminants.

3.3 Overall responses to pollution exposure

The first aim of this study was to determine whether exposure to pollution alters the antioxidant status or cause oxidative damage to anurans. An overall increase in enzymatic biomarkers (SOD, CAT, GPx, GR) was observed in anurans following exposure to pollution (Figure 3.1A). Firstly, SOD activity was significantly increased in response to pollution ($H_g = -0.67$, 95% CI [-1.28-(-0.06)], $p=0.029$) since the effect size is negative and the CIs did not overlap zero (See Appendix A.Table1). Overall, GPx ($H_g = -1.03$, 95% CI [-1.92 – (-0.14)], $p=0.023$) activity was also significantly increased after exposure to pollutants which indicates that pollution has triggered the antioxidant response and oxidative stress was high. Additionally, the overall effect of pollution on the activity of enzymatic biomarkers, CAT and GR, and the concentration of non-enzymatic biomarker, GSH, was non-significant since the CI's overlapped zero and, because $p>0.05$. MDA activity ($H_g = -0.79$, 95% CI [-1.47-(-0.10)], $p=0.023$) also significantly increased which indicates that oxidative damage increased. The second aim of this study was to determine if the antioxidant machinery of amphibians is more sensitive to environmental changes at early life or later in life. As shown in Figure (3.1B), exposure to pollutants significantly increased biomarker activity in tadpoles ($H_g = -0.88$, 95% CI [-1.20-(-0.56)], $p<0.0001$). However, anurans exposed to pollutants during embryo ($p=0.5$) or adults ($p=0.1$) life stages were not significantly impacted by exposure to pollutants which suggests tadpoles are more vulnerable to pollutant stressors.

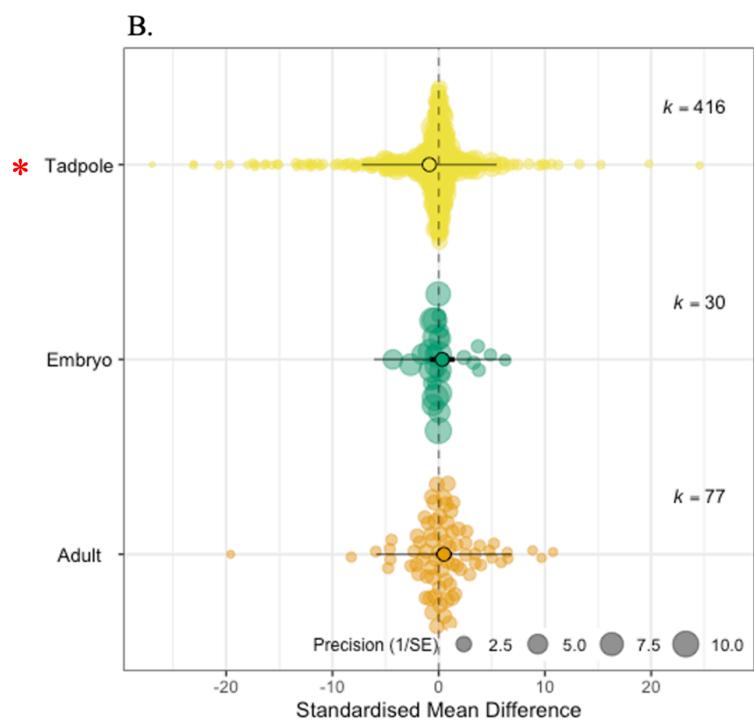
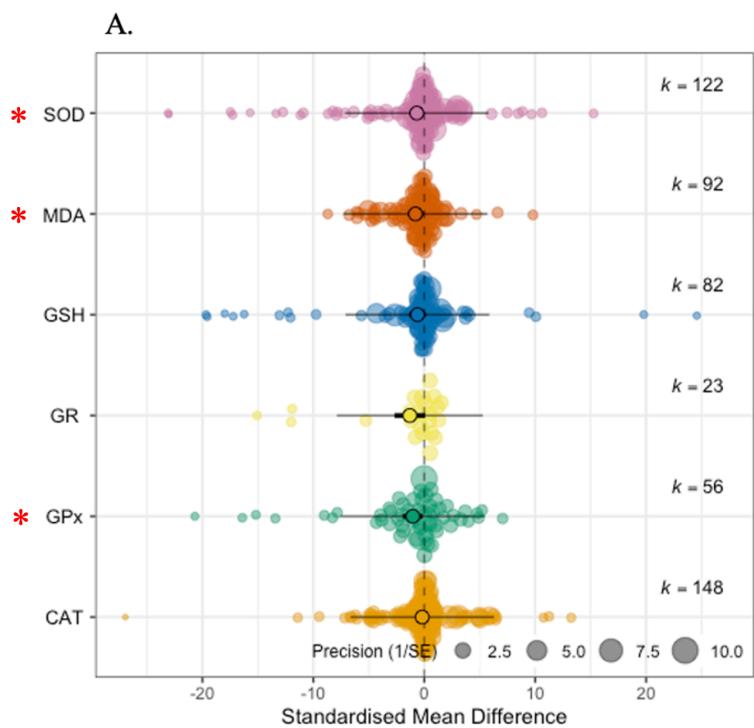


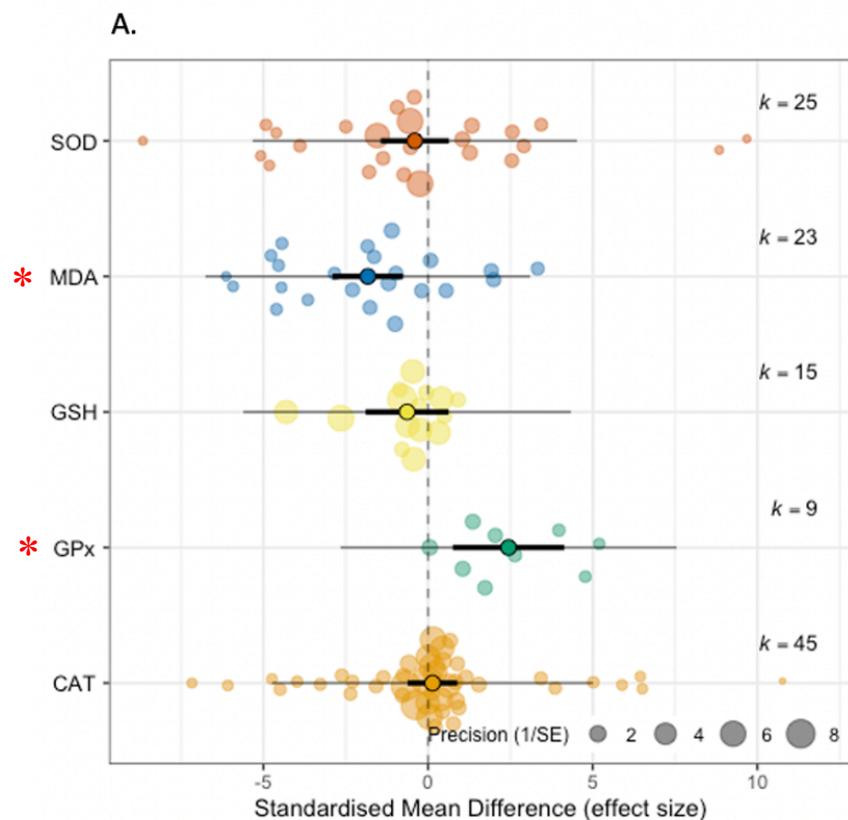
Figure 3.1: Orchard plot of the overall effect of pollution on the oxidative stress biomarkers. The orchard plot shows the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p < 0.05$). Significant effects on biomarkers or at developmental

stages is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. A demonstrates the overall effect of pollutants on different biomarkers of oxidative stress and oxidative damage. (Superoxide dismutase -pink, Malondialdehyde -red, Glutathione - blue, Glutathione reductase - yellow, Glutathione peroxidase - green, Catalase - orange); B demonstrates the effect of pollutants on oxidative stress and oxidative damage at different developmental stages. (tadpole- yellow, embryo- green, adult- orange).

3.4 Responses to pesticide exposure

As demonstrated in Figure 3.2A, the exposure of anurans to pesticides significantly decreased GPx activity ($H_g = 2.44$, 95% CI [0.75-4.13], $p=0.004$) and significantly increased MDA activity ($H_g = -1.82$, 95% CI [-2.90-(-0.75)], $p=0.0008$) (see Appendix A.Table1). SOD ($p=0.44$) and GSH ($p=0.32$) activity increased after exposure to the treatment group, although this increase is not significant. Also, CAT ($p=0.72$) activity reduced after exposure to pesticides although not significantly. In addition, to account for the variation in pesticide concentration levels amongst the studies in this study, the effect of the different concentrations (low, medium and high) on biomarker levels of oxidative stress were observed (Appendix C: Figure 1A.). The low pesticide concentration levels significantly increased biomarker activity ($H_g = -1.22$, 95% CI [-2.016-(-0.43)], $p=0.0023$), and medium ($p=0.84$) and high ($p=0.27$) concentrations did not significantly affect the biomarker levels in anurans (Appendix C: Table 3). Further analyses indicated that MDA ($H_g = -1.65$, 95% CI [-2.56-(-0.74), $p=0.0004$] and SOD activity ($H_g = -1.64$, 95% CI [-2.48-(-0.80)], $p=0.0001$) significantly increased in response to exposure to low concentration levels of pesticides (Appendix C: Table 4). As shown in Figure 3.2B, pesticide exposure significantly increased the biomarker activity in tadpoles ($H_g = -1.11$, 95% CI [-1.73-(-0.49)], $p=0.0004$) and significantly reduced biomarker activity in adults ($H_g = 1.41$, 95%

CI [0.59-2.23], p=0.0007). However, pesticide exposure had no significant effect on the biomarker activity in embryos (p=0.1). Investigating the effect of pesticides on biomarker activity in tadpoles found a significant increase in SOD ($H_g=-2.69$, 95% CI [-3.76-(-1.62)], p<0.0001) and CAT ($H_g=-0.77$, 95% CI [-1.45-(-0.10)], p=0.024], a trend that is not observed in the overall pesticide dataset (see Appendix D: Figure 3,Table 4). Conversely, SOD ($H_g=3.22$, 95% CI [1.58-4.86], p=0.0001), GPx ($H_g=2.43$, 95% CI [0.84-4.02], p=0.0028), and CAT activity ($H_g=3.22$, 95% CI [1.71-4.73], p<.0001) significantly decreased in adults and MDA levels significantly increased ($H_g=-3.03$, 95% CI [-4.56-(-1.49)], p=0.0001).



B.

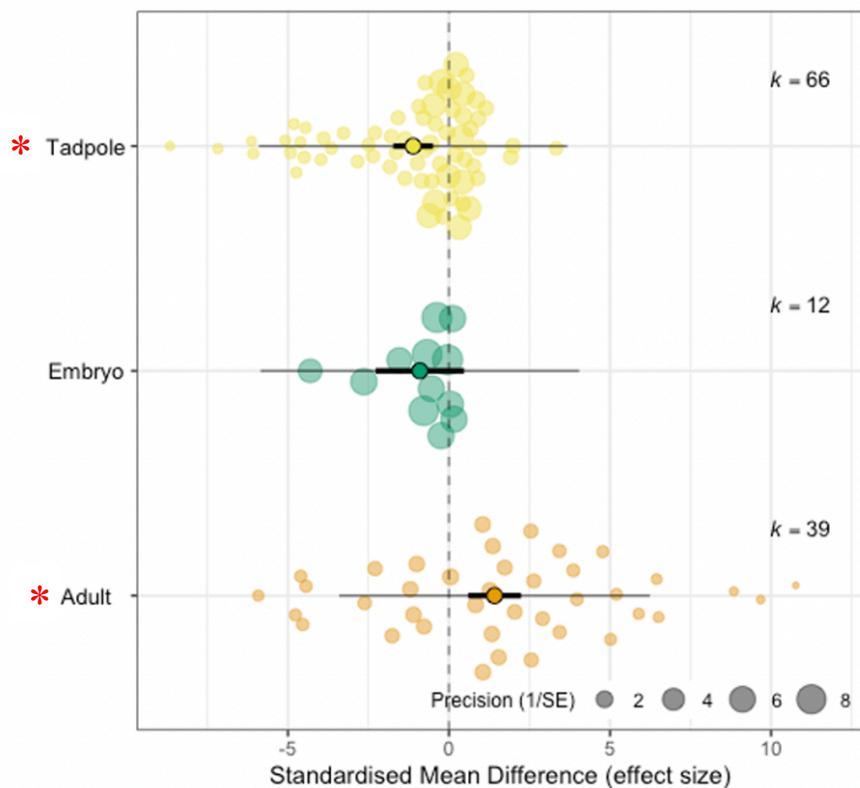


Figure 3.2: Orchard plot of the overall effect of pesticides on the oxidative stress biomarkers. The orchard plot shows the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p < 0.05$). Significant effects on biomarkers or at developmental stages is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. A demonstrates the overall effect of pesticides on different biomarkers of oxidative stress and oxidative damage. (Superoxide dismutase- red, Malondialdehyde- blue, Glutathione - yellow, Glutathione peroxidase- green, Catalase - orange); B demonstrates the effect of pesticides on oxidative stress and oxidative damage at different developmental stages (tadpole- yellow, embryo- green, adult- orange).

3.5 Responses to fungicide exposure

Fungicide exposure (Figure 3.3) significantly increased levels of SOD ($H_g = -8.20$, 95% CI [-10.82-5.57], $p < 0.0001$), GSH ($H_g = -5.77$, 95% CI [-10.32-1.23], $p = 0.01$), GPx ($H_g =$

11.55, 95% CI [-16.11-7], $p<0.0001$), and CAT ($H_g = -3.85$, 95% CI [-6.95-0.75], $p=0.01$) in anurans. Fungicide exposure increased MDA activity, although not significantly ($p=0.08$). The effect of fungicides on oxidative stress at different developmental stages could not be investigated during this study because all the samples were from tadpoles.

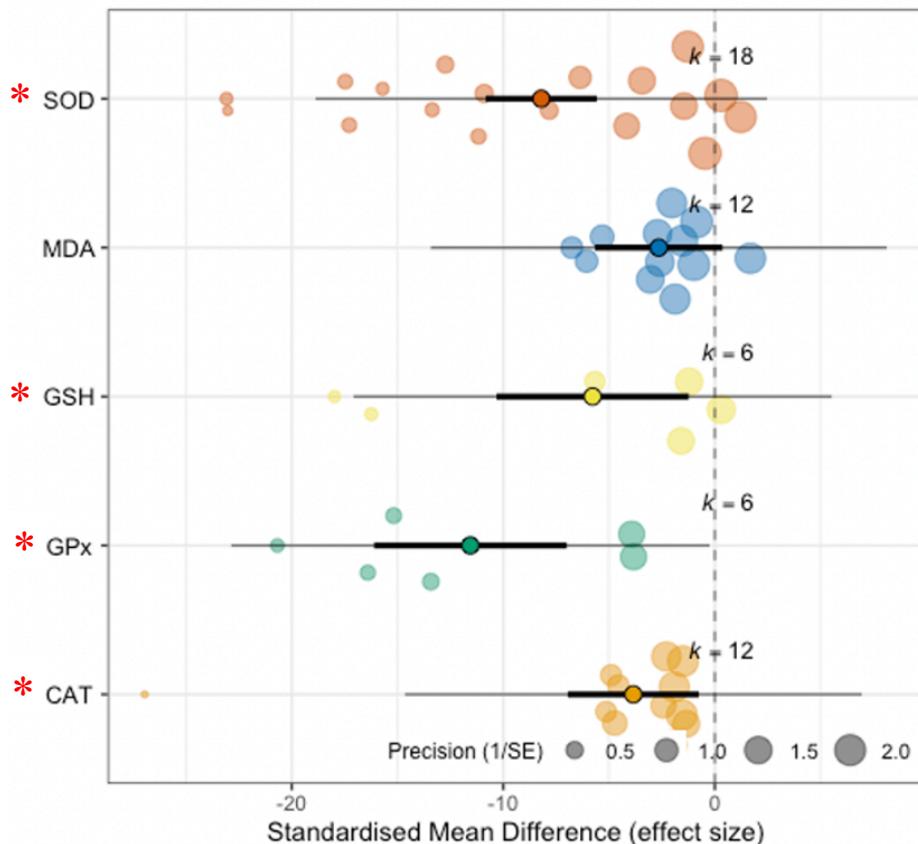
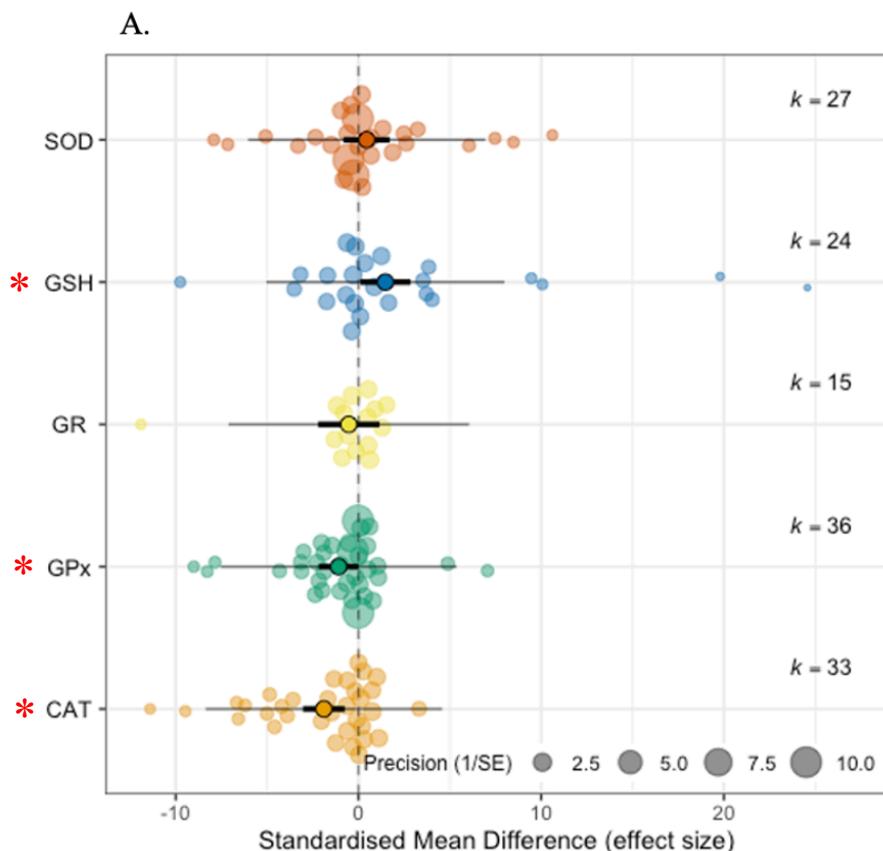


Figure 3.3: Orchard plot of the overall effect of fungicides on the oxidative stress biomarkers. The orchard plot shows the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p<0.05$). Significant effects on biomarkers or at developmental stages is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. (Superoxide dismutase - red, Malondialdehyde- blue, Glutathione- yellow, Glutathione peroxidase- green, Catalase- orange).

3.6 Responses to heavy metal exposure

Exposure to heavy metals (Figure 3.4A) significantly increased the activity of CAT ($H_g = -1.89$, 95% CI [-3.03-(-0.75)], $p=0.001$), GPx ($H_g = -1.08$, 95% CI [-2.16-0.003], $p=0.05$), GSH ($H_g = 1.47$, 95% CI [0.09-2.84], $p=0.03$) in anurans. However, biomarkers GR ($p=0.53$) and SOD (0.48) were not significantly impacted by exposure to heavy metals. The effect of the different concentration levels in the studies were also analysed and the results revealed no significant relationship between the pollutant concentration and biomarker levels (see Appendix C: Figure 1C, Table 3). Biomarker levels in adults ($H_g = -0.06$, 95% CI [-1.28-1.16], $p=0.9$), tadpoles ($H_g = -0.62$, 95% CI [-1.32-0.069], $p=0.07$) and embryos ($H_g = -0.19$, 95% CI [-2.92-2.52], $p=0.8$) were not significantly affected by exposure to heavy metals (Figure 3.4B) (see Appendix A: Table 1).



B.

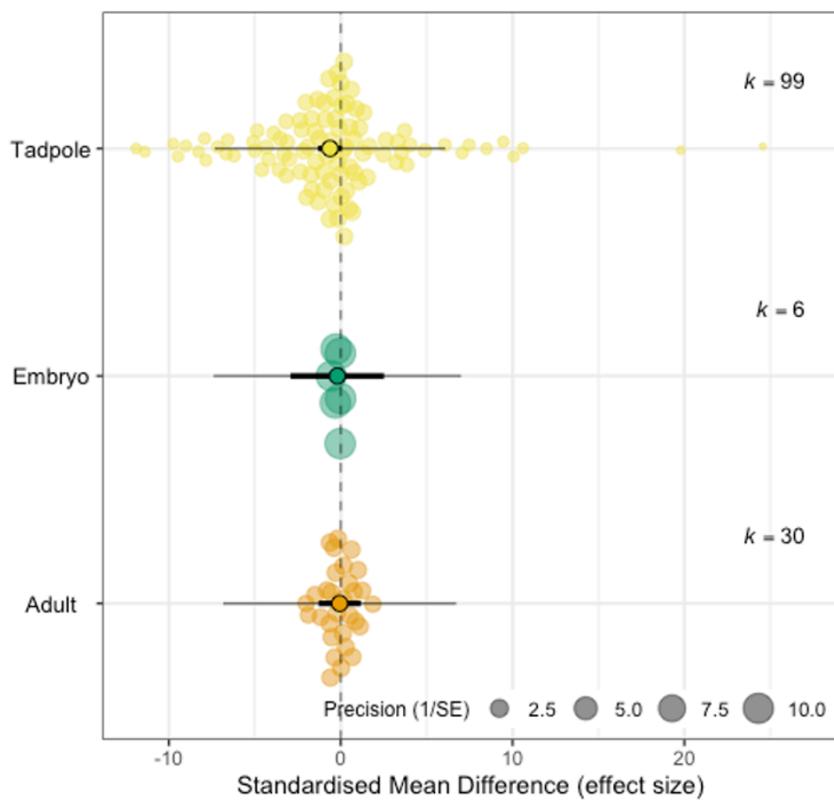
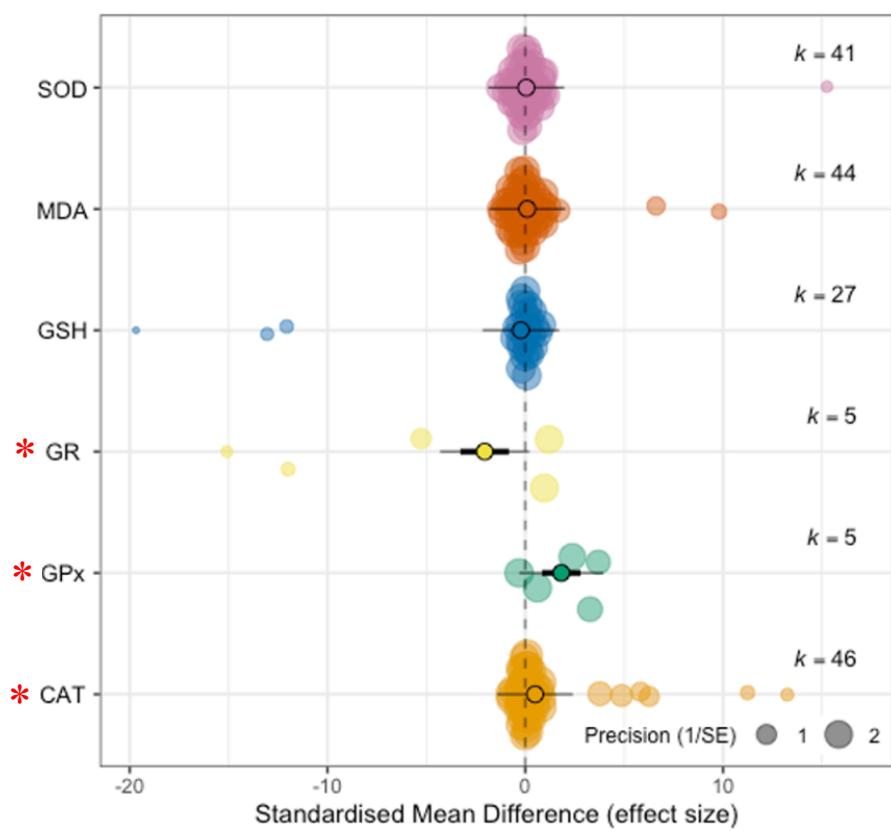


Figure 3.4: Orchard plot of the overall effect of heavy metals on the oxidative stress biomarkers. The orchard plot shows the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p<0.05$). Significant effects on biomarkers or at developmental stages is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. A demonstrates the overall effect of heavy metals on different biomarkers of oxidative stress and oxidative damage (Superoxide dismutase- red, Glutathione- blue, Glutathione reductase- yellow, Glutathione peroxidase- blue, Catalase-orange); B demonstrates the effect of heavy metals on oxidative stress and oxidative damage at different developmental stages (tadpole- yellow, embryo- green, adult- orange).

3.7 Responses to herbicide exposure

Exposure to herbicides (Figure 3.5A) significantly decreased levels of CAT ($H_g = 0.49$, 95% CI [0.17-0.81], $p=0.002$) and GPx ($H_g = 1.82$, 95% CI [0.84-2.79], $p=0.0002$) (see Appendix A. Table1.). Conversely, GR levels were significantly increased in anurans exposed to herbicide treatments ($H_g = -2.05$, 95% CI [-3.27-0.82], $p=0.001$). Exposure to the herbicides did not significantly impact GSH ($p=0.3$), MDA ($p=0.5$) and SOD ($p=0.7$). In addition, the varying herbicide concentration exposure levels were taken into account and high pollutant concentrations were found to significantly affect the oxidative stress biomarker levels ($H_g = -0.30$, 95% CI [0.026-0.59], $p=0.032$) and low ($p=0.82$) and medium ($p=0.17$) herbicide concentrations had no significant effect (see Appendix C: Figure 1, Table 3). High herbicide concentrations, significantly reduced GSH ($H_g = -3.94$, 95% CI [-7.09-(-0.79)], $p=<0.014$) and GR levels ($H_g = -5.05$, 95% CI [8.95-(-1.14)], $p=<0.011$). Furthermore, in comparison to the tadpoles (160 effect sizes), embryos (6) and adults (2) are very underrepresented in this sample (Figure 3.5B). Although, as shown in Figure 3.5B, exposure to herbicides increased the biomarker levels in tadpoles, although this effect is not significant ($H_g = 0.04$, 95% CI [-0.04-0.13], $p=0.3$). Also, there was no significant effect on biomarker levels in adults after exposure to herbicides ($H_g = 0.11$, 95% CI [-1.0006-1.23], $p=0.8$). However, there was a significant decrease in biomarker levels in embryos in response to herbicide treatment ($H_g = 3.69$, 95% CI [3.06-4.32], $p=<0.0001$). Further analyses on this significant relationship found that GPx ($H_g = 3.06$, 95% CI [2.11-4.0], $p=<0.0001$) and CAT ($H_g = 4.76$, 95% CI [3.63-5.89], $p=<0.0001$) levels significantly decreased in embryos (see Appendix D: Figure 3C, Table 5). However, the number of effect sizes studying the effect of herbicides on embryos is small (6), therefore, the statistical power is low and this result must be interpreted with caution.

A.



B.

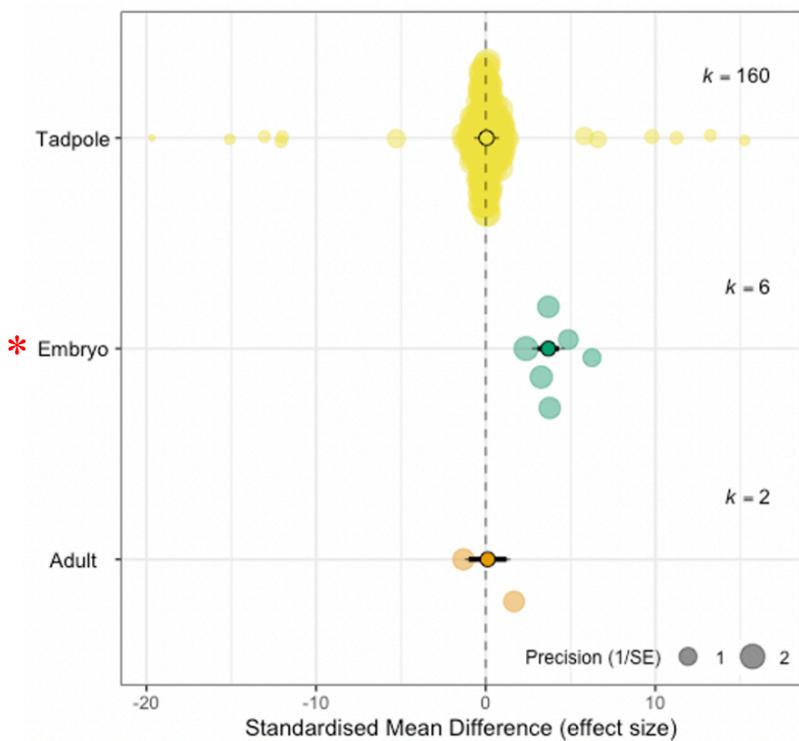


Figure 3.5: Orchard plot of the overall effect of herbicides on the oxidative stress biomarkers. The orchard plot shows the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p<0.05$). Significant effects on biomarkers or at developmental stages is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. A demonstrates the overall effect of herbicides on different biomarkers of oxidative stress and oxidative damage (Superoxide dismutase- pink, Malondialdehyde- red, Glutathione- blue, Glutathione reductase- yellow, Glutathione peroxidase- green, Catalase- orange); B demonstrates the effect of herbicides on oxidative stress and oxidative damage at different developmental stages (tadpole- yellow, embryo- green, adult- orange).

3.8 Responses to wastewater contaminant exposure

Furthermore, as demonstrated in Figure 3.6A, GSH levels significantly increased after exposure to wastewater contaminants ($H_g = -2.62$, 95% CI [-4.93-0.31], $p < 0.05$). On the other hand, CAT levels significantly decreased in response to exposure to wastewater contaminants ($H_g = 1.94$, 95% CI [-0.01-3.8], $p = 0.04$) (see Appendix A.Table1.). GR ($p = 0.9$), MDA ($p = 0.3$) and SOD ($p = 0.4$) levels were not significantly impacted by treatment exposure. The effect of the different wastewater contaminant concentrations was taken into account during this analyses, and no significant relationship was detected between low ($p = 0.16$), medium ($p = 0.056$) or high ($p = 0.19$) wastewater contaminant concentrations and the biomarker levels (see Appendix C: Figure 2D, Table 3). Similarly to herbicide exposure, embryos (6 effect sizes) and adults (6 effect sizes) are underrepresented in this sample in comparison to tadpoles (37 effect sizes). As demonstrated in Figure 3.6B, biomarker levels significantly increased in adults after exposure to wastewater contaminants ($H_g = -3.28$, 95% CI [-6.21-(-0.35)], $p = 0.02$), although there are only 6 effect sizes in this analysis, therefore, this result must be interpreted with caution (See AppendixD.Table5). Biomarker levels were not significantly affected by wastewater contaminant treatment in tadpoles ($p = 4$) or embryos ($p = 0.9$).

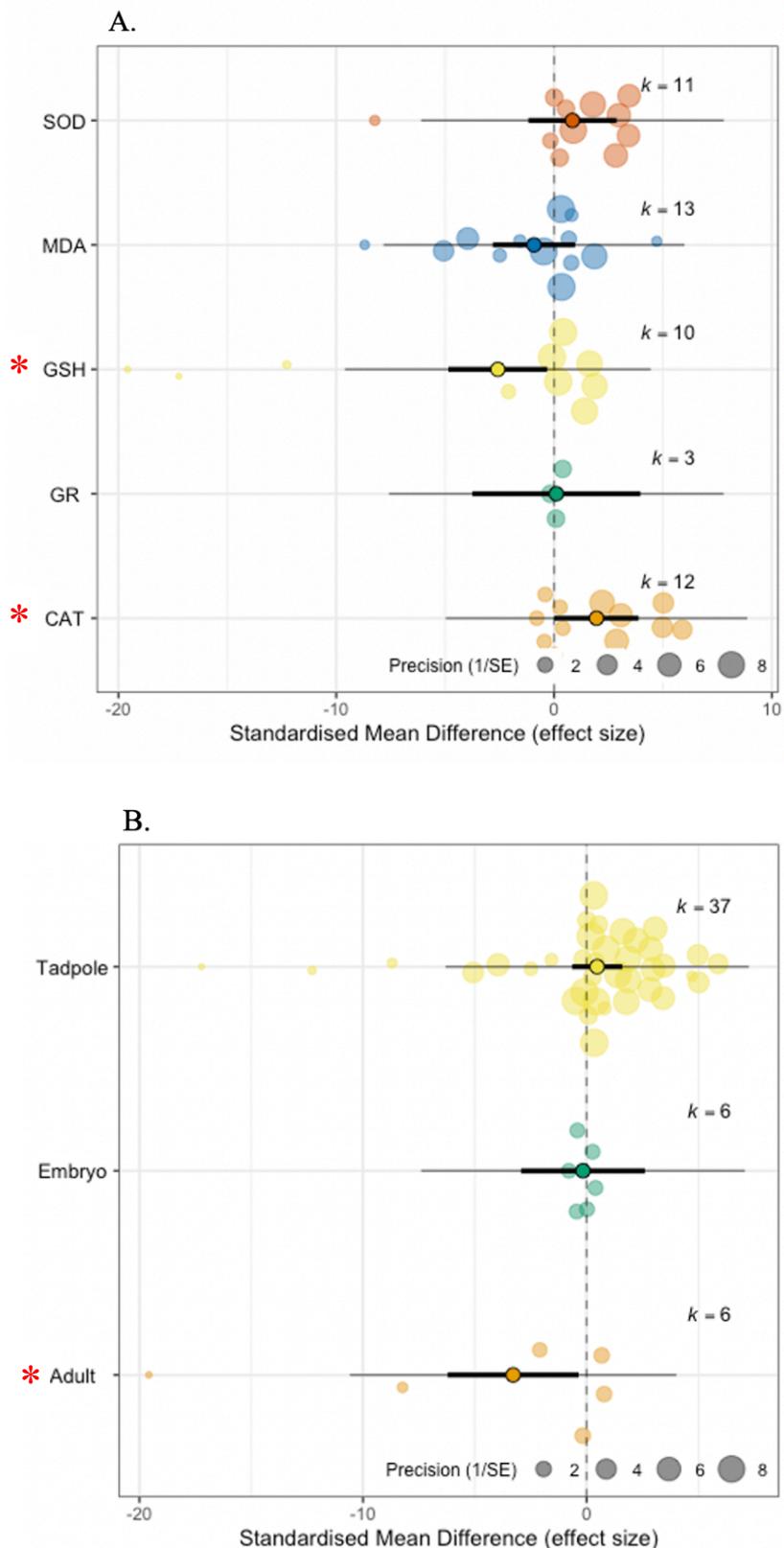


Figure 3.6: Orchard plot of the effect of wastewater contaminants on the oxidative stress biomarkers. These orchard plots show the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was

significant ($p<0.05$). Significant effects on biomarkers or at developmental stages is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. A demonstrates the overall effect of wastewater contaminants on different biomarkers of oxidative stress and oxidative damage (Superoxide dismutase- red, Malondialdehyde- blue, Glutathione- yellow, Glutathione reductase- green, Catalase- orange); B demonstrates the effect of wastewater contaminants on oxidative stress and oxidative damage at different developmental stages (tadpole- yellow, embryo- green, adult- orange).

4. DISCUSSION

Previous meta-analyses have investigated the effects of anthropogenic pollutants on factors, including oxidative stress (Isaksson, 2010), survival, mass, development time and reproduction on vertebrates (Baker, Bancroft and Garcia, 2013; Egea-Serrano et al., 2012; Slaby et al., 2019). This present study directly focuses on the effects of anthropogenic pollution on the antioxidant capacity and oxidative damage in anurans at different developmental stages. Exposure to environmental pollutants has resulted in observed physiological, behavioural and morphological plastic responses to avoid or reduce damage, which are metabolically demanding and result in the release of ROS. Therefore, it is important to consider the effects that pollutants have on the redox machinery in anurans as it will help us to understand the internal impact of pollution on the antioxidant system and the impact this will have on the health, fitness and lifespan of anurans.

The results from this meta-analysis suggest that exposure of anurans to pollutants altered the antioxidant machinery and also caused oxidative damage, and that each pollutant type altered the enzymatic and non-enzymatic antioxidants differently. Pesticide exposure significantly reduced the enzymatic antioxidant, GPx, and increased MDA levels in anurans. GPx detoxifies the H₂O₂, and a reduction in GPx activity will negatively impact anurans as it will not be able to remove H₂O₂ from the body (Matés and Sánchez-Jiménez, 1999). In addition, MDA, the end product of the peroxidation of polyunsaturated fatty acids, can indirectly determine the extent of oxidative damage caused by pollution. Therefore, an increase in MDA levels after pesticide exposure suggests that pesticides cause oxidative damage in anurans. Lipid peroxidation increases the level of polyunsaturated fatty acids in cells, and this results in loss of cell function and damage to

cell structures, including telomere sequences (Al-Gubory, 2014). Telomeres are non-coding regions located at the end of chromosomes, and although they protect chromosomes from damage, telomeres shorten with age caused by oxidative damage that accumulates over an organism's lifetime (Capper et al. 2007). Therefore, an increase in oxidative damage caused by pesticides may reduce the long-term survival of anurans (Burraco, Díaz-Paniagua, and Gomez-Mestre, 2017; Smith, Nager, and Costantini, 2016).

Furthermore, herbicides reduced GPx and CAT activity and increased GR activity. Exposure to wastewater contaminants has a similar response and reduced the activity of CAT and increased the concentration of GSH. The reduced enzymatic antioxidant activity might be due to a surge of lipid peroxidation or excessive ROS generation as these can both inactivate CAT or GPx activity (Halliwell and Gutteridge, 1984; Sun et al., 2007). Therefore, the reduction in CAT and GPx may reflect oxidative stress. In addition, GSH is a major multifactorial antioxidant as it can directly scavenge ROS molecules, or it can work in conjunction with enzymatic antioxidants to prevent oxidative stress. Therefore, herbicide and wastewater contaminant exposure caused oxidative stress. In addition, fungicide exposure increased the activity of enzymatic biomarkers, SOD, GPx, and CAT, and the concentration of non-enzymatic biomarker, GSH. Similarly, heavy metals exposure increased GPx, CAT and GSH levels and, therefore, both pollutants increased the activity of the antioxidant system. Anurans continually exposed to pollutants in the environment need to constantly upregulate their antioxidant system to prevent or reduce oxidative damage, which can be detrimental to their fitness (Pamplona and Costantini, 2011). Diverting energy towards the antioxidant system can result in long-term trade-offs, including shortened lifespan, low reproductive output, low immune function and reduced locomotion (caused by growth malformities and delays in larval growth) (Charbonnier &

Vonesh 2015; Lee, Monaghan, & Metcalfe, 2013; Metcalfe & Alonso-Alvarez 2010; Smith, Nager & Costantini, 2016). A meta-analysis has shown the fitness costs of exposure to chemical pollution on amphibians (Egea-Serra et al. 2012). The study showed exposure to pollutants increased the risk of physical abnormalities, and malformities can reduce locomotion making amphibians more vulnerable to predators. Another trade-off the meta-analysis showed was a decrease in mass which could be a result of impaired foraging ability.

The effect of fungicides on the antioxidant capacity of amphibians at different developmental stages could not be included in this study because the dataset was limited to adults, therefore a comparison could not be made. In addition, tadpoles represented a large proportion of the dataset, therefore the results must be interpreted with caution. Also, although this study reported a significant decrease in biomarker activity in embryos exposed to herbicides and a significant increase in biomarker activity in adults exposed to wastewater contaminants, the small sample sizes made the statistical tests uninformative. The overall dataset suggested that biomarker levels significantly increased in tadpoles, although the sensitivity of the antioxidant machinery at different stages varied across the different pollutants.

Pesticide exposure increased biomarker activity in tadpoles, and reduced the activity in adults. SOD and CAT activity significantly increased in tadpoles which suggests that oxidative stress has occurred. On the other hand, adults exposed to pesticides increased MDA activity and reduced SOD, CAT and GPx levels which indicates that the pesticides inactivated the antioxidant machinery allowing lipid peroxidation to increase. Tadpole development is highly plastic and so when exposed to pollutant stressors, they can

prioritise the regulation of the antioxidant system, although as mentioned previously, prioritising the antioxidant system can have long-term fitness trade-offs (Burraco, Díaz-Paniagua, and Gomez-Mestre, 2017; Charbonnier and Vonesh, 2015). Adults on the other hand must prioritise investing energy into energetically demanding activities including reproduction, therefore, this will make them more vulnerable to oxidative stress and damage (Metcalfe and Alonso-Alvarez, 2010). A study investigating the antioxidant system in subadult and adult *Bufo viridis* toads found that the life stages differ in their antioxidant defence, with subadults increasing the activity of CAT and SOD and the adults increasing GPx/GSH system (Prokić et al., 2018).

The meta-analysis provided here had limitations and the results from this study should be interpreted with caution. One limitation of this study is that when searching for eligible articles, only English written articles were considered, therefore, the database search for papers written between 2015-2020 may have missed out eligible papers. Secondly, studies were heterogenous in terms of the type of species, location of study, pollutant concentration, and days of exposure. Despite being included in the test for heterogeneity, due to the small sample size of some levels of the moderators, the statistical power was not high enough to test for significant relationships. Additionally, studies investigating herbicide and pesticide exposure on oxidative stress in anurans dominates the overall dataset, while exposure to wastewater contaminants and fungicides were less represented in the overall dataset. Therefore, as indicated by the results, both stressors impact the antioxidant capacity of anurans and should be investigated further. Third, there was significant publication bias within this study in most datasets, and although a trim-and-fill analysis was conducted to compensate for this bias, this analysis does not perform well in studies with large between-study heterogeneity (Moreno et al. 2009). Therefore, the

adjustments to the estimates and coverage possibilities can be biased, and so the results must be interpreted with caution.

Overall, the results of this study show that exposure to pollutants alter the antioxidant capacity and causes oxidative damage in anurans. Pollutants present in the environment threaten the health, fitness and survival of anurans because to prevent oxidative stress and damage, anurans allocate energy to upregulate the antioxidant defence system. This results in long-term fitness trade-offs which can be detrimental to the future survival of anurans. In addition, the results suggest that the antioxidant machinery is most sensitive during the tadpole stage. The developmental plasticity of tadpoles allow them to divert energy toward upregulating the antioxidant system. The antioxidant machinery is less sensitive during the adult stage because they must divert energy towards demands, including reproduction. Further research is required to understand the capacity at which anurans can withstand pollutant stress and also the long-term effects on anurans to enforce management practices and regulations to help conserve anurans. Also many studies don't combine direct effects of pollution, such as behavioural and physiological changes (e.g. oxidative stress, reproductive behaviour, predator-avoidance), and indirect effects (e.g. reduced prey abundance). A combination of these effects may be a reason for the mass decline of anurans in response to pollutants.

5. ACKNOWLEDGEMENTS

I would like to thank my supervisors, Professor Pat Monaghan and Dr Pablo Burraco for their support and guidance throughout the production of my project. I would also like to thank Dr Pablo Capilla and Dr Jan Lindstrom for their advice on statistical analyses.

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7. APPENDICES

Appendix A: Meta-regression estimates

Table 1: Meta-regression estimates for each biomarker of oxidative stress and oxidative damage. The number of effect sizes, the main effect, heterogeneity and publication bias. P-values significant at (<0.05).

Oxidative Stress Biomarkers	No. of effect sizes	Main Effect		Heterogeneity				Publication Bias	
		Hedges g' (95% CI)	P-value	I ² Statistic(%)	Q Statistic	Df	P-value	Egger's Test	P-value
Overall dataset	523			98.82%	10317.1901	522	< .0001	-12.5	<0.0001
SOD	122	0.67 (-1.28--(-0.06))	0.029						
MDA	92	0.79 (1.47--(-0.10))	0.023						
GSH	82	-0.61 (-1.36-0.13))	0.10						
GR	23	-1.30 (-2.68-0.086))	0.06						
GPx	56	-1.03 (-1.92-0.14))	0.023						
CAT	148	-0.18 (-0.72-0.35))	0.50						
Embryo	416	0.33 (-0.82-1.50))	0.56						
Tadpole	30	-0.88 (-1.20--(-0.56)))	<.0001						
Adult	77	0.49 (-0.25-1.23))	0.19						
Pesticide	117			98.35%	1544.0997	116	< .0001	-0.1142	0.9090
SOD	25	-0.40 (-1.43-0.63))	0.44						
MDA	23	-1.82 (-2.90--(-0.75)))	0.0008						
GSH	15	-0.63 (-1.89- 0.62))	0.32						
GPx	9	2.44 (0.75-4.13))	0.004						
CAT	45	0.13 (-0.62-0.89))	0.72						
Embryo	66	-0.90 (-2.27-0.47))	0.19						
Tadpole	12	-1.11 (-1.7300--(-0.49)))	0.0004						
Adult	39	1.41 (0.59-2.23))	0.0007						
Fungicide	54			98.37%	663.0885	53	< .0001	-18.8897	< .0001
SOD	18	-8.20 (-10.83--(-5.57)))	<.0001						
MDA	12	-2.65 (-5.66-0.36))	0.081						
GSH	6	-5.77 (-10.32--(-1.23)))	0.012						
GPx	6	-11.55 (-16.11--(-7)))	<.0001						
CAT	12	-3.85 (-6.95--(-0.75)))	0.014						
Embryo	n/a	n/a	n/a						
Tadpole	n/a	n/a	n/a						
Adult	54	n/a	n/a						
Heavy Metal	135			98.75%	1368.4826	134	< .0001	-3.2897	0.0010
SOD	27	0.45 (-0.81-1.71))	0.48						
GSH	24	1.47 (0.09-2.82))	0.035						

GR	15	-0.52 (-2.20-1.15)	0.53								
GPx	36	-1.08 (-2.16-0.0039)	0.050								
CAT	33	-1.89 (-3.035-(-0.75))	0.0012								
Embryo	99	-0.19 (-2.92-2.52)	0.88								
Tadpole	6	-0.62 (-1.32-0.069)	0.077								
Adult	30	-0.06 (-1.28-1.16)	0.92								
Herbicide	168			80.67%	757.6570	167	<.0001	0.3105	0.7562		
SOD	41	0.053 (-0.27-0.38)	0.74								
MDA	44	0.1 (-0.21-0.41)	0.53								
GSH	27	-0.22 (-0.64-0.20)	0.30								
GR	5	-2.05 (-3.27-(-0.82))	0.0010								
GPx	5	1.82 (0.84-2.79)	0.0002								
CAT	46	0.49 (0.17- 0.81)	0.002								
Embryo	160	3.69 (3.06-4.32)	<.0001								
Tadpole	6	0.046 (-0.045-0.13)	0.31								
Adult	2	0.11 (-1.00-1.23)	0.83								
Wastewater Contaminants	49			99.67%	3949.967	48	<.0001	-8.0824	<.0001		
SOD	11	0.84 (-1.18-2.87)	0.41								
MDA	13	-0.92 (-2.81-0.96)	0.33								
GSH	10	-2.58 (-4.85-(-0.31))	0.025								
GR	3	0.10 (-3.75-3.96)	0.95								
CAT	12	1.94 (0.012-3.88)	0.048								
Embryo	37	-0.16 (-2.93-2.61)	0.90								
Tadpole	6	0.47 (-0.65-1.59)	0.40								
Adult	6	-3.28 (-6.21-0.35)	0.027								

Appendix B: Toxicity Classification

Table 2: Pollutant toxicity classifications

Reference	Location	Treatment	Treatment Concentration	Expected Environmental Concentration (EEC) Range	Level of Exposure	EEC Journal Reference
Burraco & Gomez-Mestre 2016	Spain	glyphosate	1 mg/L	0.0001-0.165 mg/L	High	Bruggen et al. 2018
Burraco & Gomez-Mestre 2016	Spain	glyphosate	2 mg/L	0.0001-0.165 mg/L	High	Bruggen et al. 2018
Bhuyan et al 2020	India	phenanthrene	0.61 mg/L	0.000887-1.46 mg/L	Low	Peng et al. 2019
Bhuyan et al 2020	India	phenanthrene	1.22 mg/L	0.000887-1.46 mg/L	Medium	Peng et al. 2019
Li et al 2018	China	4-Octylphenol	2.063 ng/ml	0.006- 0.828 ng/ml	High	Cheng et al 2018
Li et al 2018	China	4-Octylphenol	20.632388 ng/ml	0.006- 0.828 ng/ml	High	Cheng et al 2018
Li et al 2018	China	4-Octylphenol	206.32388 ng/ml	0.006- 0.828 ng/ml	High	Cheng et al 2018
Jiang et al 2019	China	lambda-cyhalothrin	0.4 µg/L	0.21-29.72 µg/L	Low	Tang et al 2018
Jiang et al 2019	China	lambda-cyhalothrin	10 µg/L	0.21-29.72 µg/L	Medium	Tang et al 2018
Jiang et al 2019	China	lambda-cyhalothrin	250 µg/L	0.21-29.72 µg/L	High	Tang et al 2018
Xua & Huang 2017	China	α-cypermethrin enantiomers	0.05 µg/L	0.01-9.8 µg/L	Low	Xu & Huang 2017
Wu et al 2017	China	cadmium	5 µg/L	4-167 µg/L	Low	Wu et al 2017
Wu et al 2017	China	cadmium	100 µg/L	4-167 µg/L	Medium	Wu et al 2017
Wu et al 2017	China	cadmium	500 µg/L	4-167 µg/L	High	Wu et al 2017
Melvin 2016	Australia	diclofenac, naproxen, atenolol and gemfibrozil mixture	0.1 µg/L	Naproxen and diclofenac: 0.4-1.2 µg/L, atenolol and gemfibrozil: 0.3-0.5 µg/L	Low	Melvin 2016
Melvin 2016	Australia	diclofenac, naproxen, atenolol and gemfibrozil mixture	1 µg/L	Naproxen and diclofenac: 0.4-1.2 µg/L, atenolol and gemfibrozil: 0.3-0.5 µg/L	Medium	Melvin 2016
Melvin 2016	Australia	diclofenac, naproxen, atenolol and gemfibrozil mixture	10 µg/L	Naproxen and diclofenac: 0.4-1.2 µg/L, atenolol and gemfibrozil: 0.3-0.5 µg/L	High	Melvin 2016
Lajmanovich et al 2018	Argentina	chlorpyrifos	5 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Lajmanovich et al 2018	Argentina	chlorpyrifos	10 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Xie et al 2019	China	nitrate	5 mg/L	1.3 to 35.7 mg/L	Low	Xue et al 2016
Xie et al 2019	China	nitrate	50 mg/L	1.3 to 35.7 mg/L	Medium	Xue et al 2016
Xie et al 2019	China	nitrate	200 mg/L	1.3 to 35.7 mg/L	High	Xue et al 2016
Martins et al 2017	Portugal	4-MBC	0.00013 mg/L	0.00114 mg/L-0.0065 mg/L	Low	Martins et al 2017
Martins et al 2017	Portugal	4-MBC	0.013 mg/L	0.00114 mg/L-0.0065 mg/L	Medium	Martins et al 2017
Martins et al 2017	Portugal	4-MBC	1.3 mg/L	0.00114 mg/L-0.0065 mg/L	High	Martins et al 2017

Martins et al 2017	Portugal	triclosan	0.00013 mg/L	0.000048 mg/L-0.0027mg/L	Low	Martins et al 2017
Martins et al 2017	Portugal	triclosan	0.013 mg/L	0.000048 mg/L-0.0027mg/L	Medium	Martins et al 2017
Martins et al 2017	Portugal	triclosan	1.3 mg/L	0.000048 mg/L-0.0027mg/L	High	Martins et al 2017
Svartz et al 2020	Argentina	Ni-Al nanoceramics	5 mg/L	2x10^-7 - 0.00001 mg/L	High	Tiede et al 2009
Svartz et al 2020	Argentina	Ni-Al nanoceramics	25 mg/L	2x10^-7 - 0.00001 mg/L	High	Tiede et al 2009
Freitas et al 2017	Brazil	sulfentrazone	0.01 mg/L	0.000056- 0.0253 mg/L	Low	Thorngren et al 2017
Freitas et al 2017	Brazil	sulfentrazone	0.05 mg/L	0.000056- 0.0253 mg/L	Medium	Thorngren et al 2017
Freitas et al 2017	Brazil	sulfentrazone	0.1 mg/L	0.000056- 0.0253 mg/L	High	Thorngren et al 2017
Lajmanovich et al 2015	Argentina	bacillus thuringiensis var. israelensis	5 mg/L	8 to 40 mg/L	Low	Farajollahi et al 2013
Lajmanovich et al 2015	Argentina	bacillus thuringiensis var. israelensis	10 mg/L	8 to 40 mg/L	Low	Farajollahi et al 2013
Lajmanovich et al 2015	Argentina	bacillus thuringiensis var. israelensis	20 mg/L	8 to 40 mg/L	Medium	Farajollahi et al 2013
Sotomayor et al 2015	Argentina	chlorpyrifos	2 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Sotomayor et al 2015	Argentina	chlorpyrifos	8 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Sotomayor et al 2015	Argentina	chlorpyrifos	14 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Rutkoski et al 2020	Brazil	chlorpyrifos	11 µg/L	0.2 to 10.8 µg/L	Medium	Marino & Ronco 2005
Rutkoski et al 2020	Brazil	chlorpyrifos	90 µg/L	0.2 to 10.8 µg/L	High	Marino & Ronco 2005
Rutkoski et al 2020	Brazil	chlorpyrifos	500 µg/L	0.2 to 10.8 µg/L	High	Marino & Ronco 2005
Coltro et al 2017	Brazil	quinclorac	0.05 µg/L	0.48-6.60 µg/L	Low	Marchesan et al 2007
Coltro et al 2017	Brazil	quinclorac	0.2 µg/L	0.48-6.60 µg/L	Low	Marchesan et al 2007
Coltro et al 2017	Brazil	quinclorac	0.4 µg/L	0.48-6.60 µg/L	Low	Marchesan et al 2007
Barreto et al 2020	Argentina	chlorpyrifos	0.05 mg/L	0.0002-0.0108 mg/L	Medium	Marino & Ronco 2005
Barreto et al 2020	Argentina	chlorpyrifos	0.1 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Barreto et al 2020	Argentina	chlorpyrifos	0.5 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Liendro et al 2015	Argentina	chlorpyrifos	0.1 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Liendro et al 2015	Argentina	chlorpyrifos	0.5 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Carvalhoa et al 2020	Brazil	zinc	1 µg/L	44-122 µg/L	Low	Chiba et al 2011
Carvalhoa et al 2020	Brazil	copper	1 µg/L	4-85 µg/L	Low	Chiba et al 2011
Carvalhoa et al 2020	Brazil	cadmium	1 µg/L	1-5 µg/L	Low	Chiba et al 2011
Pal et al 2018	India	sodium fluoride	64.7 mg/L	96.8 mg/L	Medium	Pal et al 2018
Pal et al 2018	India	sodium fluoride	259 mg/L	96.8 mg/L	High	Pal et al 2018
Pal et al 2018	India	sodium fluoride	518 mg/L	96.8 mg/L	High	Pal et al 2018
Borković-Mitić et al 2016	Serbia	heavy metals	N/A	N/A	Low	Borković-Mitić et al 2016

Borković-Mitić et al 2016	Serbia	heavy metals	N/A	N/A	High	Borković-Mitić et al 2016
Lajmanovich et al 2015	Argentina	chlorpyrifos	10 mg/L	0.0002-0.0108 mg/L	High	Marino & Ronco 2005
Lajmanovich et al 2015	Argentina	2,4-D	20 mg/L	0- 0.00153 mg/L	High	Suárez et al 2021
Lajmanovich et al 2015	Argentina	glyphosate	20 mg/L	0.0001-0.165 mg/L	High	Bruggen et al. 2018
Falfushynskaa et al 2017	Ukraine	zinc oxide	252.276 µg/L	0.1-0.5 µg/L	High	Dumont et al 2015
Falfushynskaa et al 2017	Ukraine	nifedipine	3463 µg/L	1004.27 µg/L	High	Benotti & Brownawell 2007
Silva et al 2020	Brazil	sulfentrazone	130 µg/L	0.056–25.3 µg/L	High	Thorngren et al 2017
Silva et al 2020	Brazil	sulfentrazone	980 µg/L	0.056–25.3 µg/L	High	Thorngren et al 2017
Silva et al 2020	Brazil	glyphosate	234 µg/L	0.0001-0.165 mg/L	Medium	Bruggen et al. 2018
Silva et al 2020	Brazil	glyphosate	2340 µg/L	0.0001-0.165 mg/L	High	Bruggen et al. 2018
Zhang et al 2019	China	ciproconazole	1 mg/L	0.049054 mg/L	High	Wightwick et al 2012
Zhang et al 2019	China	ciproconazole	10 mg/L	0.049054 mg/L	High	Wightwick et al 2012
Zhang et al 2018	China	triadimefon	20 mg/L	0.00000152-0.00522 mg/L	High	Liu et al 2018
Zhang et al 2018	China	triadimefon	23 mg/L	0.00000152-0.00522 mg/L	High	Liu et al 2018
Zhang et al 2018	China	triadimefon	25 mg/L	0.00000152-0.00522 mg/L	High	Liu et al 2018
Zhang et al 2018	China	triadimenol	20 mg/L	0.000342-0.00048 mg/L	High	Xie et al 2019
Zhang et al 2018	China	triadimenol	30 mg/L	0.000342-0.00048 mg/L	High	Xie et al 2019
Zhang et al 2018	China	triadimenol	38 mg/L	0.000342-0.00048 mg/L	High	Xie et al 2019
Yologlu & Ozmen 2015	Turkey	cadmium	0.005 mg/L	0.00023-0.001368 mg/L	Medium	Varol & Şen 2012
Yologlu & Ozmen 2015	Turkey	cadmium	0.518 mg/L	0.00023-0.001368 mg/L	High	Varol & Şen 2012
Yologlu & Ozmen 2015	Turkey	cadmium	2.59 mg/L	0.00023-0.001368 mg/L	High	Varol & Şen 2012
Yologlu & Ozmen 2015	Turkey	lead	0.01 mg/L	0.000342-0.00048 mg/L	High	Varol & Şen 2012
Yologlu & Ozmen 2015	Turkey	lead	12.3 mg/L	0.000342-0.00048 mg/L	High	Varol & Şen 2012
Yologlu & Ozmen 2015	Turkey	lead	61.53 mg/L	0.000342-0.00048 mg/L	High	Varol & Şen 2012
Yologlu & Ozmen 2015	Turkey	copper	0.01 mg/L	0.00092-0.165 mg/L	Medium	Cengiz et al 2017
Yologlu & Ozmen 2015	Turkey	copper	0.085 mg/L	0.00092-0.165 mg/L	Medium	Cengiz et al 2017
Yologlu & Ozmen 2015	Turkey	copper	0.425 mg/L	0.00092-0.165 mg/L	High	Cengiz et al 2017
Wilkens et al 2018	Brazil	sulfentrazone	130 µg/L	0.056–25.3 µg/L	High	Thorngren et al 2017
Wilkens et al 2018	Brazil	glyphosate	234 µg/L	0.1-165 µg/L	High	Bruggen et al. 2018
Cheng et al 2017	China	(-)myclobutanil	7 mg/L	0.0000035-0.000148 mg/L	High	Zhao et al 2018
Cheng et al 2017	China	(-)myclobutanil	8 mg/L	0.0000035-0.000148 mg/L	High	Zhao et al 2018
Cheng et al 2017	China	(-)myclobutanil	9 mg/L	0.0000035-0.000148 mg/L	High	Zhao et al 2018
Cheng et al 2017	China	(+)myclobutanil	7 mg/L	0.0000033-0.000152 mg/L	High	Zhao et al 2018

Cheng et al 2017	China	(+)-myclobutanil	8 mg/L	0.0000033-0.0000152 mg/L	High	Zhao et al 2018
Cheng et al 2017	China	(+)-myclobutanil	9 mg/L	0.0000033-0.0000152 mg/L	High	Zhao et al 2018
Freitas et al 2017	Brazil	clomazone	0.01 mg/L	0.2-0.4 mg/L	Low	Zanella et al 2002
Freitas et al 2017	Brazil	clomazone	0.05 mg/L	0.2-0.4 mg/L	Low	Zanella et al 2002
Freitas et al 2017	Brazil	clomazone	0.1 mg/L	0.2-0.4 mg/L	Low	Zanella et al 2002
Ejilibe et al 2018	Nigeria	butaforce	7 µg/L	0.1-1.4 µg/L	High	Zhu et al 2014
Ejilibe et al 2018	Nigeria	butaforce	9 µg/L	0.1-1.4 µg/L	High	Zhu et al 2014
Ejilibe et al 2018	Nigeria	butaforce	11 µg/L	0.1-1.4 µg/L	High	Zhu et al 2014

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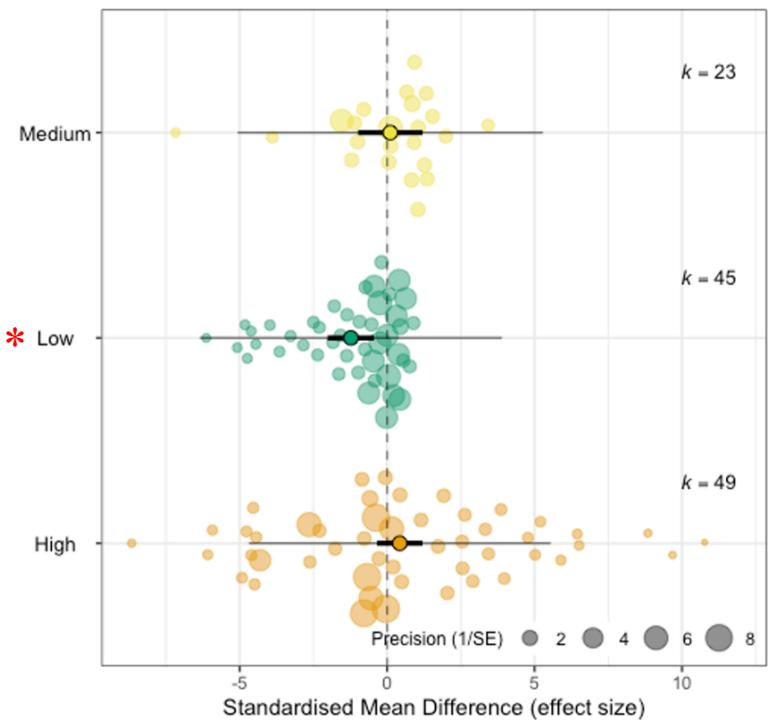
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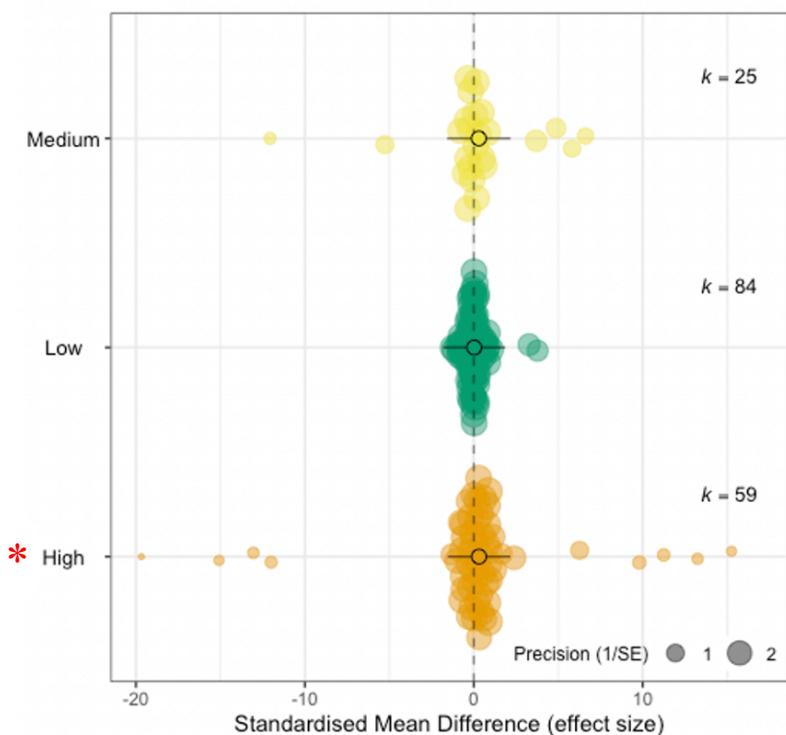
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Appendix C: Toxicity Level data

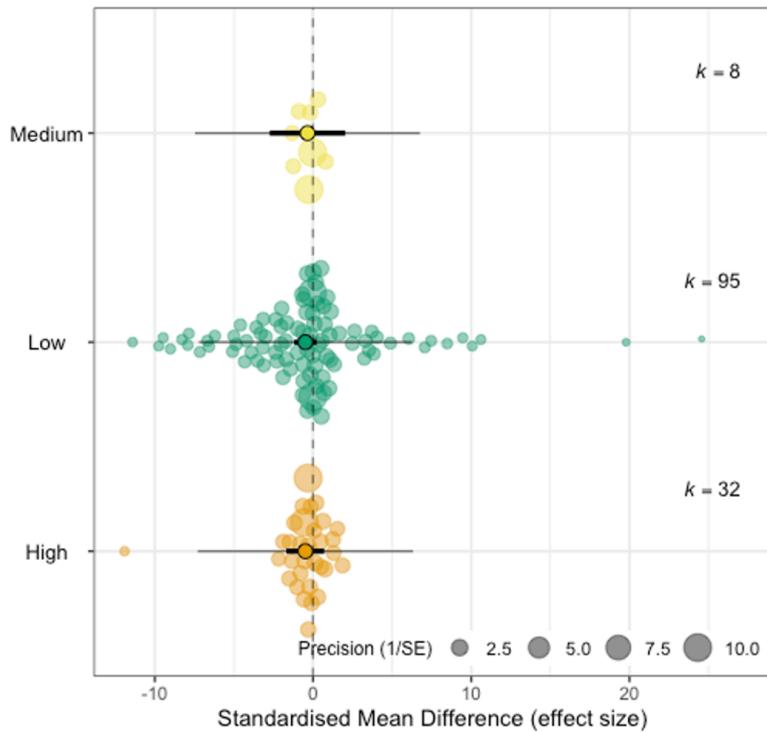
A. Pesticide



B. Herbicide



C. Heavy Metal



D. Wastewater Contaminants

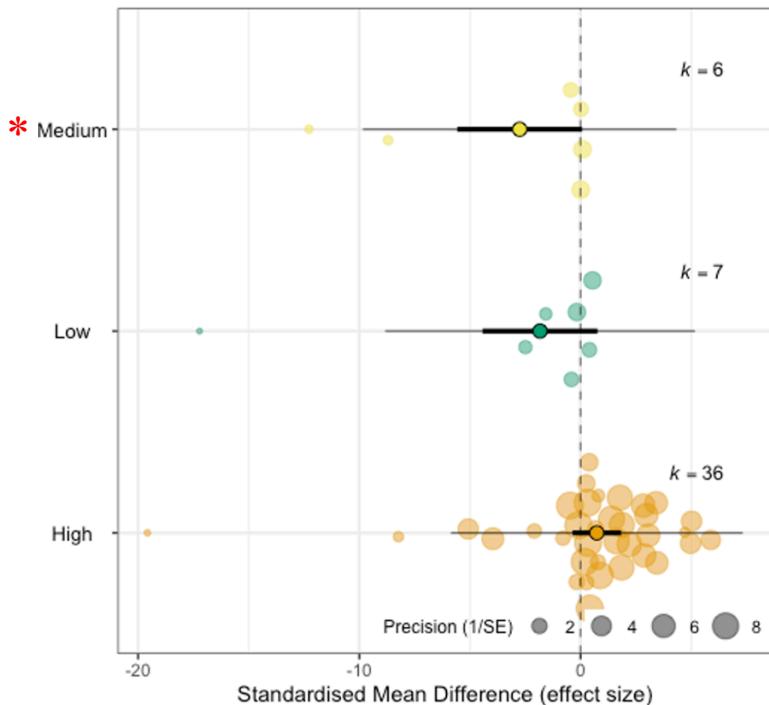


Figure 1: Orchard plot of the effect of different levels of pollutant toxicity on the oxidative stress biomarker levels.

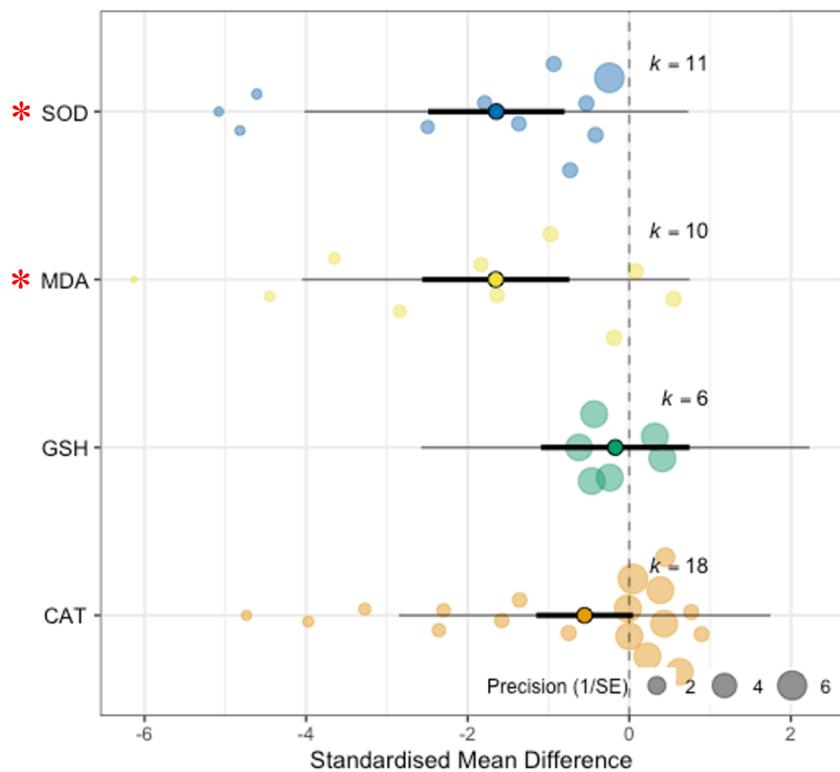
These orchard plots show the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p < 0.05$). Significant effects on biomarkers is

indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage. A-Overall dataset; B- Pesticide; C-Herbicide; D- Heavy Metals.

Table 3: Meta-regression estimates for pollutant toxicity range (low, medium and high). P-values significant at (<0.05).

Toxicity Levels	No. of effect sizes	Main Effect	
		Hedges g' (95% CI)	P-value
Pesticides	117		
Low	45	-1.22 (-2.016-(-0.43))	0.0023
Medium	23	0.10 (-0.98-1.19)	0.84
High	49	0.42 (-0.34-1.19)	0.27
Herbicides	168		
Low	84	0.025 (-0.19-0.24)	0.82
Medium	25	0.29 (-0.13-0.72)	0.17
High	59	0.3 (0.026-0.59)	0.032
Heavy Metal	135		
Low	95	-0.48 (-1.19-0.23)	0.18
Medium	8	-0.34 (-2.73-2.04)	0.77
High	32	-0.48 (-1.68-0.71)	0.42
Wastewater contamination	49		
Low	7	-1.83 (-4.43-0.76)	0.16
Medium	6	-2.75 (-5.57-0.07)	0.056
High	36	0.73 (-0.36-1.83)	0.19

A. Pesticide dataset- Low Pollutant Concentration



B. Herbicide dataset- High Pollutant Concentration

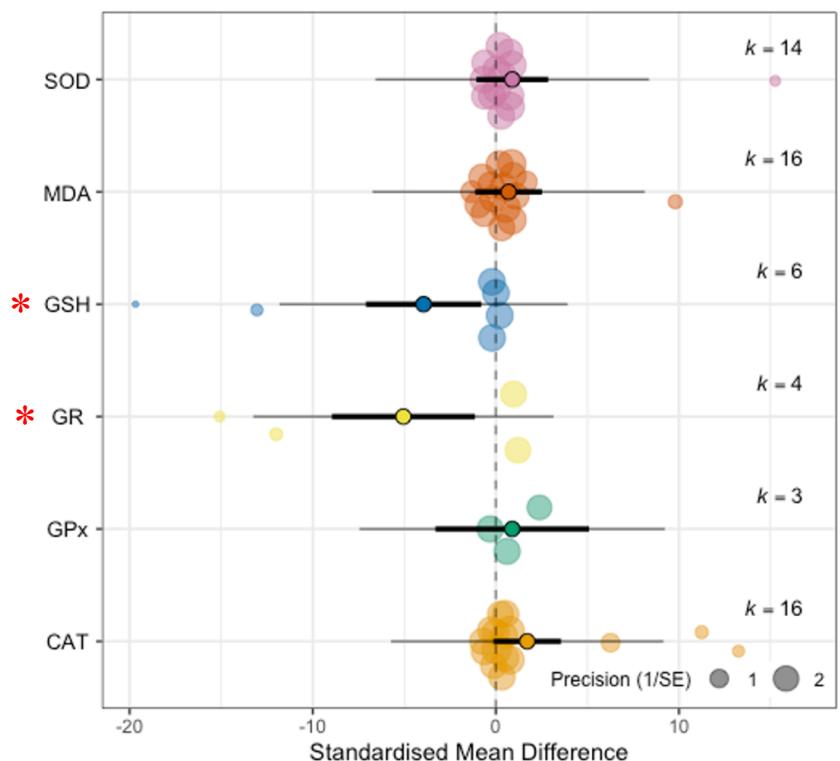


Figure 2: Orchard plot of the effect of different levels of pollutant toxicity on the oxidative stress biomarkers.

These orchard plots show the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the

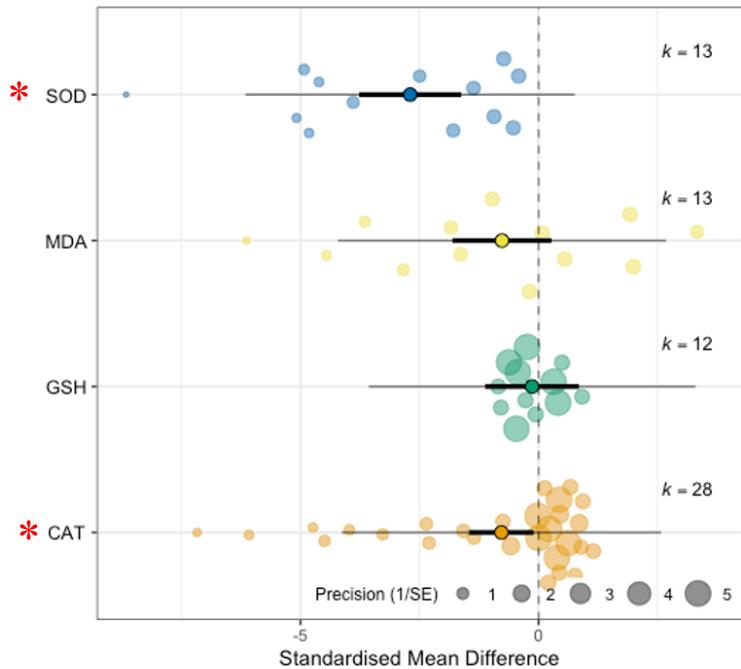
individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p<0.05$). Significant effects on biomarkers is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size indicates an increase in oxidative stress and damage.. A-Overall dataset low pollutant concentrations; B- Overall dataset high pollutant concentrations; C-Pesticide low pollutant concentrations; D- Herbicide dataset high pollutant concentrations.

Table 4: Meta-regression estimates for each biomarker of oxidative stress and oxidative damage exposed to low concentrations of pesticides and high concentrations of herbicides. P-values significant at (<0.05).

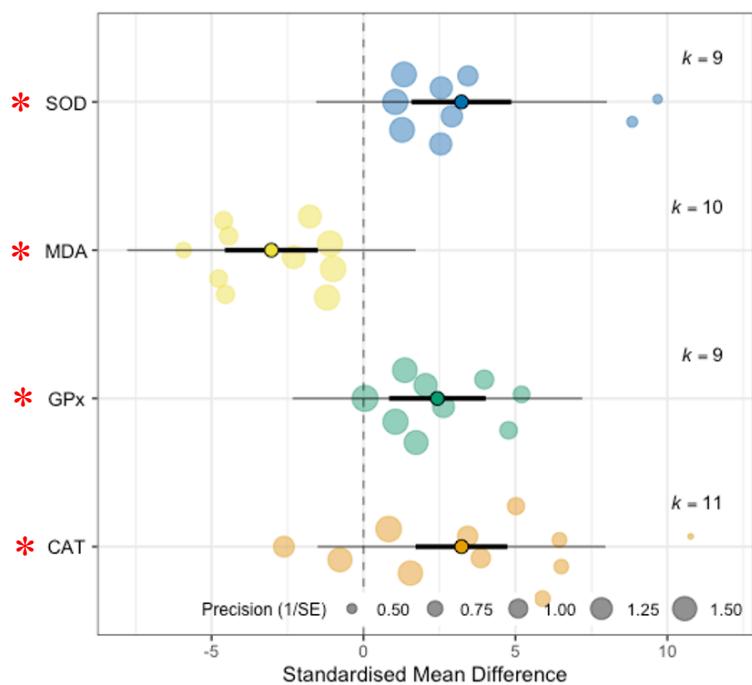
Oxidative stress biomarker levels	No. of effect sizes	Main Effect	
		Hedges g' (95% CI)	P-value
Pesticides- low pollutant concentration	45		
SOD	11	-1.64 (-2.48-(-0.80))	0.0001
MDA	10	-1.65 (-2.56-(-0.74))	0.0004
GSH	6	-0.17 (-1.09-0.74)	0.71
CAT	18	-0.55 (-1.15-0.047)	0.07
Herbicides- high pollutant concentration	59		
SOD	14	0.89 (-1.06-2.86))	0.36
MDA	16	0.69 (-1.13-2.52)	0.45
GSH	6	-3.94 (-7.09-(-0.79))	0.014
GR	4	-5.05 (8.95-(-1.14))	0.011
GPx	3	0.89 (-3.30-5.09)	0.67
CAT	16	1.71 (-0.13-3.55)	0.068

Appendix D: Development Stage effect on oxidative stress biomarkers data

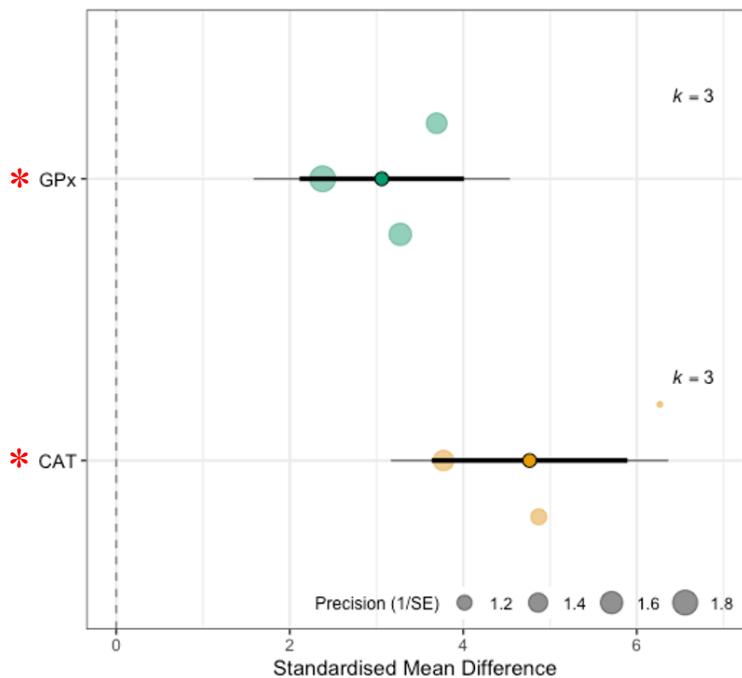
A. Pesticides- Tadpoles



B. Pesticides- Adults



C. Herbicides- Embryos



D. Wastewater contaminants- Adults

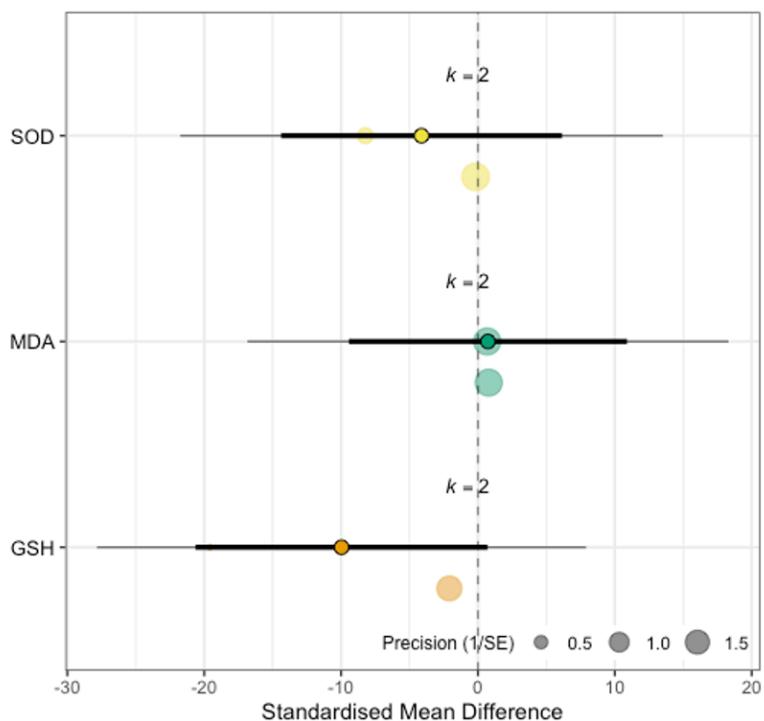


Figure 3: Orchard plot of the effect of different levels developmental stages on the oxidative stress biomarkers.

These orchard plots show the mean effect size (Standard mean difference, SMD), CI (bold line), PI (thin line) and the individual effect sizes and their precision for oxidative stress biomarkers. Where the 95% CIs did not cross over zero, the effect was significant ($p < 0.05$). Significant effects on biomarkers is indicated by a red asterisk on the y-axis. The vertical dashed line indicates no effect and a positive effect size indicates a decrease in oxidative stress and a negative effect size

indicates an increase in oxidative stress and damage. A- Tadpoles exposed to pesticides; B- Adults exposed to pesticides; C- Embryos exposed to herbicides; D- Adults exposed to wastewater contaminants

Table 5: Meta-regression estimates for each biomarker of oxidative stress and oxidative damage exposed to low concentrations of pesticides and high concentrations of herbicides. P-values significant at (<0.05).

Oxidative stress biomarker levels	No. of effect sizes	Main Effect	
		Hedges g' (95% CI)	P-value
Pesticides- tadpole	77		
SOD	13	-2.69 (-3.76-(-1.62))	<0.0001
MDA	13	-0.76 (-1.80-0.27))	0.14
GSH	23	-0.13 (-1.12-0.84)	0.78
CAT	28	-0.77 (-1.45-(-0.10))	0.024
Pesticides- adult	39		
SOD	9	3.22 (1.58-4.86)	0.0001
MDA	10	-3.03 (-4.56-(-1.49))	0.0001
GPx	9	2.43 (0.84-4.02)	0.0028
CAT	11	3.22 (1.71-4.73)	<.0001
Herbicides- embryo	6		
GPx	3	3.06 (2.11-4.0)	<.0001
CAT	3	4.76 (3.63-5.89)	<.0001
Wastewater contaminants- adult	6		
SOD	2	-4.12 (-14.38-6.13)	0.43
MDA	2	0.73 (-9.43-10.89)	0.88
GSH	2	-9.96 (-20.63-0.69)	0.067