**Introduction**

Over the past 30 years, it has become increasingly conspicuous that changes in environmental conditions, both natural and anthropogenic, have negatively impacted amphibian populations and amphibians are now classed as the most threatened vertebrate group globally (Hayes et al. 2010). This global decline is driven by multiple interacting stressors; habitat modification, invasive species, environmental pollution, climate and invasive species (Grant, Miller, & Muths, 2020; Green et al. 2020). Amphibians are extremely sensitive to environmental stressors and human activities are altering environmental conditions and introducing new challenges (pollution and invasive species) at an unprecedented rate and these environmental changes activate physiological, behavioural and morphological plastic responses in amphibians to avoid or reduce damage (Burraco et al. 2020; Strong et al., 2017). For example, stressors including extreme increases in temperature and pond drying induce morphological changes in amphibians by accelerating development and decreasing growth to escape the damaging environment (Burraco, Díaz-Paniagua, & Gomez-Mestre, 2017; Burraco et al. 2020; Richter‐Boix, Tejedo, & Rezende, 2011). Additionally, biotic factors such as predators induce morphological changes, altering their tail length and pigmentation (Burraco, Duarte, & Gomez-Mestre, 2013). However, environmentally induced phenotypic modifications are metabolically demanding and can result 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 & Vonesh 2015; Lee, Monaghan, & Metcalfe, 2013; Metcalfe & Alonso-Alvarez 2010; Smith, Nager & Costantini, 2016). Therefore, understanding the impacts of environmental stressors, particularly those driven by anthropogenic activities, on the antioxidant status and oxidative stress in amphibians is critical for conservation biology.

Oxidative Stress

During normal physiological activities, animals produce excess reactive oxygen species (ROS) including highly reactive and unstable free-radicals, superoxide anion (O2.-) and hydroxyl radicals (OH.), and the less reactive non-radicals, hydrogen peroxide (H2O2) (Finkel & Holbrook 2000). ROS are a toxic metabolic by-product of oxygen-reduction (REDOX) reactions, and to prevent damaging effects the antioxidant system (AOS) releases enzymatic and non-enzymatic antioxidant enzymes to regulate the redox status (Balaban, Nemoto & 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 & Finkel, 2005).). In addition, low ROS molecule concentrations are critical for vital biological processes and 10% 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 & Finkel, 2005; Ježek & Hlavatá 2005).

Antioxidant System

The antioxidant system (AOS) 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 H2O2 and oxygen (Figure 1). There are two different types of SOD enzyme found in amphibians, one containing Copper and Zinc and another containing Manganese on the active site (Pamplona & Costantini 2011). CuZnSOD is found in the cytosol and MnSOD is located within the mitochondrial matrix. The H2O2 produced by SOD is then detoxified by CAT and GPx (Figure 1) by converting the reactive species to water (H2O) (Matés & Sánchez-Jiménez 1999). As demonstrated in Figure 1, GPx detoxifies H2O2 by removing hydrogen from 2 Glutathione (GSH) molecules, producing H2O and Glutathione disulfide (GSSG) and then Glutathione reductase (GR) catalyses the reduction of GSSG to GSH. CAT rapidly converts H2O2 to water despite having a low affinity for the reactive species, whereas GPx has a high affinity and converts H2O2 at a slower rate (Pamplona & Costantini 2011). Therefore, CAT is suitable for controlling high influxes of H2O2 production whereas GPx can be used to control H2O2 that is produced constantly at low levels.

Diagram

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**Figure 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 millimolars (mM) (Smith et al., 1996). Cysteine residue contains an extremely reactive sulfhydryl group (SH)- an exposed group that can easily adopt a number of reversible oxidative states-which in an oxidised state it exists as GSH or GSSG in the reduced form (McBean, 2017). GSH can directly scavenge ROS molecules or, as mentioned previously, GSH works 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). Some antioxidants are obtained exogenously through the diet from fruit and vegetable consumption, including Vitamin E and carotenoids which work collectively to eradicate ROS molecules. Vitamin E is a major lipid-soluble antioxidant located in cell membranes and by reducing lipid peroxyls to hydroperoxides it inhibits the lipid peroxidation chain reaction (Esterbauer, Schaur, & Zollner, 1991). Carotenoids play a crucial role in protecting lipoproteins and cell membranes from peroxidation and it can also recycles Vitamin E from its oxidised form so that it can still protect the body from ROS (Mueller & Boehm 2011; Surai, Speake, & Sparks, 2001).

Measuring Oxidative Stress

To detect the impact of environmental stress on amphibians 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, 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).

Environmental stressors

Amphibians have complex lifecycles with both terrestrial and marine phases, therefore, they are exposed to both natural and anthropogenic stressors on land and in water and can be used as bioindicators (Blaustein & Belden, 2003; Strong et al., 2017). They are extremely vulnerable to stressors 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 & Weber, 2011). In addition, amphibians 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, & Pflugmacher, 2003; Hopkins et al., 2014; Wagner et al., 2017). The effects of exposure to pollution on oxidative stress in amphibians has been largely reviewed in recent literature, exploring the impact of fungicides, pesticides, insecticides 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 disrupt the antioxidant status and cause oxidative damage, resulting in long-term effects such as malformations or damage to tissues. For example, *Xenopus tropicalis* embryos exposed to the pesticide, lambda-cyhalothrin, experience oxidative stress which disrupts their development causing long-term malformations including bent notochord and hypopigmentation (Jiang et al. 2019). The disruption of the ozone layer and reduced vegetation cover due to deforestation is increasing the exposure of UV radiation, causing oxidative stress, and this is considered to have a major role on the global decline of amphibians (Blaustein & Belden, 2003; Londero, Santos & Schuch, 2019). Furthermore, the abiotic stressors, temperature, pH and salinity also have been reviewed and research suggests that extreme changes can induce oxidative stress in amphibians (Burraco & Gomez-Mestre, 2016; Wang et al. 2019). Studies investigating the effects of biotic stressors, predators and food availability, are less reviewed in comparison to abiotic factors. Although, it is important to understand the effects of these two factors as two of the major drivers of the decline in amphibians are invasive species and habitat loss. A recent study demonstrated that tadpoles exposed to the predator, dytiscid water beetle, experienced oxidative stress which resulted in faster growth and development and will lead to a shortened life-span (Burraco, Díaz-Paniagua & Gomez-Mestre, 2017). In addition, low food availability has also been linked to an increase in oxidative stress (Prokić et al., 2020).

Aims and Hypothesis

This study performed a meta-analysis to determine the effects of both abiotic and biotic environmental stressors on oxidative stress levels in amphibians while taking into account confounding variables, species and biological matrix the sample was taken from. The aims of this study are to summarise recent literature investigating the effects of environmental stressors on amphibians to, (1) determine whether changes in environmental conditions affect antioxidant responses (SOD, CAT, GPx and GSH) or cause oxidative stress (MDA, TBARS and GSH:GSSG) to amphibians (2) test if biotic or abiotic stressors impact more on the redox status of amphibians and (3) determine if the antioxidant machinery of amphibians more sensitive to environmental changes at 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, thus negatively impacting the fitness and future survival of the amphibians. Furthermore, considering the large amount of research that has been undertaken to assess the responses of a variety of abiotic stressors on oxidative stress in amphibians, it was hypothesised that abiotic factors would have the most significant impact on the redox status. It was also hypothesised that amphibians are more sensitive to environmental changes during early development.

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