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Impacts of rising temperatures and water acidification on the oxidative status and immune system of aquatic ectothermic vertebrates: a meta-analysis

Running title: CO_2 emissions affect ectotherms physiology

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Abstract

Species persistence in the Anthropocene is dramatically threatened by global climate change. Large emissions of carbon dioxide (CO_2) from human activities are driving increase in mean temperature, intensity of heatwaves, and acidification of oceans and freshwater bodies. Ectotherms are particularly sensitive to CO_2-induced stressors, because the rate of their metabolic reactions, as well as their immunological performance, are affected by environmental temperatures and water pH. We reviewed and performed a meta-analysis of 56 studies, involving 1259 effect sizes, that compared oxidative status or immune function metrics between 42 species of ectothermic vertebrates exposed to long-term increased temperatures or water acidification ($\geq$ 48h), and those exposed to control parameters resembling natural conditions. We found that CO_2-induced stressors enhance levels of molecular oxidative damages in ectotherms, while the activity of antioxidant enzymes was upregulated only at higher temperatures, possibly due to an increased rate of biochemical reactions dependent on the higher ambient temperature. Differently, both temperature and water acidification showed weak impacts on immune function, indicating different direction (increase or decrease) of responses among immune traits. Further, we found that the intensity of temperature treatments (Δ°C) and their duration, enhance the physiological response of ectotherms, pointing to stronger effects of prolonged extreme warming events (i.e., heatwaves) on the oxidative status. Finally, adult individuals showed weaker antioxidant enzymatic responses to an increase in water temperature compared to early life stages, suggesting lower acclimation capacity. Polar species showed weaker antioxidant response compared to temperate and tropical species, but level of uncertainty in the antioxidant enzymatic response of polar species was high, thus pairwise comparisons were statistically non-significant. Overall, the results of this meta-analysis indicate that the regulation of oxidative status might be one key mechanism underlying thermal plasticity in aquatic ectothermic vertebrates.

Keywords: heatwave, water pH, development, oxidative stress, plasticity, acclimation

1. Introduction

Global climatic conditions are changing rapidly, posing new threats to the persistence of species (Pacifici *et al.*, 2015). Emissions of carbon dioxide (CO_2) from anthropogenic activities are inducing a global increase in mean temperatures (IPCC, 2013), and a higher frequency, intensity, and duration of excessive heat periods (i.e., heatwaves; Perkins-Kirkpatrick & Lewis, 2020). Another consequence of this rising concentration of CO_2 in the atmosphere is an increase in its absorption from oceans and freshwater bodies, which causes water acidification (Doney *et al.*, 2009; Hasler *et al.*, 2016). Many species or populations have already shifted their range of distribution in response to climate change, but those with limited possibilities to disperse are challenged to physiologically cope with the new environmental conditions to avoid local extinction (Spence & Tingley, 2020; Donelson *et al.*, 2019).

Ectotherms are particularly sensitive to rising temperatures because of their limited thermoregulatory abilities (Kingsolver *et al.*, 2013). Cellular metabolic activity of ectotherms increases with temperatures until a given threshold value, after which the metabolic rate declines (Schulte, 2015). Similar patterns occur for basic vital functions, such as locomotion, growth, and reproduction (Angilletta, 2009). Thus, by influencing the rate of metabolic processes and biochemical reactions, environmental temperature has the potential to affect organismal fitness (Jørgensen *et al.*, 2022), and to shape the distribution and the abundance of ectothermic species (Huey & Berrigan, 2001; Alfonso *et al.*, 2021).

Water acidification is also an important source of stress for those ectotherms that are dependent on aquatic environments (Heuer & Grosell, 2014). For example, water acidification induces acid-base alteration in blood and extracellular fluids (Nilsson & Lefevre, 2016). Prior work on developing pink salmon (*Oncorhynchus gorbuscha*) and flatfish larvae (*Solea senegalensis*) also found reduced growth and metabolic rate in organisms exposed to water acidification (Ou *et al.*, 2015; Pimentel *et al.*, 2015). In contrast, a recent meta-analysis on the effects of ocean acidification on fish life-history traits found higher resting metabolism in adults (Cattano *et al.*, 2018). Generally, it has been found large interspecific variation in the physiological responses to water acidification, suggesting that species vary significantly in adaptability (Rosa *et al.*, 2017; Hasler *et al.*, 2017; Thomas *et al.*, 2022).

One important consequence of changes in aerobic metabolism could be an increasing mitochondrial production of reactive oxygen species (ROS; Koch *et al.*, 2021). ROS are highly reactive molecules capable of inducing oxidative modifications (i.e., oxidative damages) to vital biomolecules like proteins, lipids, and nucleic acids (Halliwell & Gutteridge, 2015). Generation of oxidative damages can be prevented (or repaired in some cases) by antioxidant molecules, which include endogenously produced enzymes (e.g., superoxide dismutase, catalase, glutathione peroxidase), endogenous compounds (e.g., thiols, uric acid), and diet-derived chemicals (e.g., vitamins, carotenoids; Costantini *et al.*, 2010). It is increasingly recognised that the regulation of cellular oxidative status in changing environments is one major challenge for maintaining physiological homeostasis (Costantini, 2019).

Energy required for maintaining oxidative status homeostasis can come at a cost for other energy-demanding physiological functions. For example, due to elevated energetic and physiological demands, vertebrates may trade off investment in baseline innate immune function against investment in antioxidant defence (Eikenaar *et al.*, 2018; Cram *et al.*, 2015). Immune function is one major player in the maintenance of physiological homeostasis, but its large metabolic costs also make it particularly vulnerable to environmental changes (Sorci & Faivre, 2022; Ohmer *et al.*, 2021). Studies on ectotherms found that immune function can be suppressed, reconfigured, or stimulated by changes in ambient temperature and/or water acidification (Moretti *et al.*, 2019; Bresolin de Souza *et al.*, 2016; Stahlschmidt *et al.*, 2017).

Stressor intensity and duration of exposure to a given stressor are key factors that affect the physiological response of organisms (Moyano *et al.*, 2017; Isotalo *et al.*, 2022). Gradual and prolonged exposure to changing environmental conditions may favour acclimation, which is reversible adjustments of physiological traits to the prevailing environmental conditions (Kingsolver & Huey, 1998; Fox *et al.*, 2019). Acclimation is enhanced by changes in cellular biochemistry that aim to maximize organismal fitness in the new environment (Ohlberger & Fox, 2013). A recent study on mosquitofish (*Gambusia holbrooki*) found that experimentally increasing antioxidant capacities buffered any increase in oxidative damage due to thermal acclimation, suggesting that oxidative stress may be a constraint for acclimation capacity (Loughland & Seebacher, 2020).

Sensitivity to the effects of climate change is also affected by developmental stage and environmental stability (Pörtner & Farrell, 2008; Somero, 2010). A recent study on physiological sensitivity to heatwaves in Atlantic sea-bream (*Sparus aurata*) found higher acclimation capacity in juveniles, as compared to adults (Madeira *et al.*, 2020). In addition, higher ability to physiologically cope with increasing temperature is expected in temperate species, having evolved in more variable environments as compared to tropical and polar species (Birnie-Gauvin *et al.*, 2017; Tewksbury *et al.*, 2008). If oxidative stress is a limit to acclimation (Loughland & Seebacher, 2020), we expect that adults, tropical and polar species will show lower antioxidant defences in response to increased levels of oxidative damage, compared to, respectively, early life stages, and temperate species.

In this article, we (i) reviewed, for the first time, studies that compared markers of oxidative status and immune function between aquatic ectothermic vertebrates exposed to increased temperatures or water acidification and to control parameters resembling natural conditions, and (ii) conducted meta-analyses to estimate the effect sizes and relative impacts on different markers of oxidative status and of immune response. We also assessed the impacts of stressor intensity and duration, providing insights for potential effects of heatwaves. Finally, we tested for the effects of developmental stage and climatic zone of origin on the response of oxidative status markers of ectotherms to rising temperature.

2. Methods

2.1 Literature search and inclusion/exclusion criteria

We conducted a systematic literature review of studies comparing markers of oxidative status or immune function in ectothermic vertebrates exposed to warmer temperature or decreased pH levels (i.e., water acidification). We focused on studies comparing one or more treatment groups with a control group resembling normal habitat temperature and pH conditions. We included studies measuring oxidative status markers in different tissues or organs, since they were the vast majority found in literature. Differently, for immune markers we selected studies focusing on the immune response at the organismal level, avoiding tissue specific responses. Thus, we included studies measuring immune markers in whole body, body fluids, and the head kidney which is the major haematopoietic-lymphoid organ in teleost fish (Geven & Klaren, 2017). In addition, we included one study that measured the swelling response to the injection of the antigen phytohaemagglutinin, a marker of integrated immune response (Brown *et al.*, 2011), in organisms exposed to elevated and control temperature.

Any study explicitly testing for thermal or pH shock was excluded from our meta-analysis. We considered studies that exposed animals to a gradual increase of temperatures or of water acidification, avoiding sudden thermal or pH challenges. In addition, to focus our meta-analysis on the medium-to-long term physiological effects of CO_2-induced environmental changes, we included only studies that measured the physiological response at least 48h after the start of treatment. Furthermore, we included studies that measured the effects of treatments i*n vivo*, and reported data on the effects of temperature or acidification separated from those of other study variables (e.g., pathogens and diet). Studies that exposed organisms to more than one different temperature or pH treatment were also included. We considered the temperature and pH of acclimation in captivity, that resembled normal habitat conditions, as control references.

In order to control for effects of stressor intensity and duration in our analyses, we included in the database only studies that reported the exact temperature or pH variation, and the number of days of exposure. Thus, we excluded studies that exposed animals to sources of heat (unknown exact temperature variation), and studies that reported only developmental stages as measure of exposure length.

Literature screening was performed on the Web of Science and on Scopus, using a combination of the keywords “high temperature”, “water ph”, “water acidification” with “immun*”, “oxidat* stress” and with “ectotherm”, “fish”, “amphibian”, “tadpole”, “reptile”. The last search was conducted on 19 October 2022, and resulted in the screening of 4783 studies. Paper selection was conducted according to PRISMA guidelines (Fig. A.1; O'Dea *et al.*, 2021). We retained 56 studies that matched our selection criteria. All studies were conducted in captivity.

2.2 Effect sizes calculation

We calculated Hedges’ *g* effect sizes (i.e. bias-corrected standardized mean difference; Nakagawa & Cuthill, 2007) and their variances (Var-*g*) using the *compute.es* package in R (R Core Team, 2021). Effect sizes were calculated from descriptive statistics (means and standard deviations) and sample sizes, referred to markers of interest, of the study control and experimental groups. If data were not reported in the text or tables, we extracted them from graphs using the software GetData Graph Digitizer (Fedorov, 2014). In case of missing data or information, the authors were contacted. We contacted 16 authors, of which 7 responded providing requested data or information.

Following the classification of effect sizes proposed by Cohen (1988), we considered effect sizes to be small (g = 0.2, explaining 1% of the variance), intermediate (g = 0.5, explaining 9% of the variance) or large (g = 0.8, explaining 25% of the variance). Positive values of effect size indicate that the stressor increases the concentration of a given marker in the treatment group as compared to the control group, whereas negative values indicate the opposite pattern.

2.3 Meta-analytic technique

We used the *rma.mv* function of package *metafor* (Viechtbauer, 2010) to perform meta-analytic multi-level mixed effects models in the R environment. We implemented phylogenetic random effects meta-regression models testing for oxidative status and immune system response of ectothermic vertebrates to CO_2-induced stressors (i.e., rising temperatures and water acidification). Models were run separately for rising temperatures and water acidification. We set the minimum number of studies and effect sizes to include a given physiological parameter in the model at 6 and 25, respectively.

Given the large variation among and within studies in temperature and pH treatments, our models included the covariates *Intensity* (continuous), measured as the difference between treatment and control parameters, and *Days of Exposure* (continuous), measured as the number of days in which the animals were exposed to treatments. Both covariates can affect physiological responses by favouring acclimation or chronic stress, thus we included in our models the interaction between *Intensity* and *Days of Exposure*. Covariates were Z-transformed to place them on the same scale for comparison (Noble *et al.*, 2022).

Our models included the random factors *Study ID* and *Observation ID* to control, respectively, for non-independence of effect sizes extracted from the same study, and for within-study effect size variance additional to sampling error. Initially, we also included the random factors *Tissue* and *Species*. However, the random factors *Tissue* and *Species* showed variance closed to zero and did not improve the AIC of the models, thus we removed them from the analyses and present only results of best models. To control for shared evolutionary history among species, we included the random factor *Phylogeny*. To this aim, we used the package *rotl* (Michonneau *et al.*, 2016) to build phylogenies by retrieving the phylogenetic relationships from the Open Tree of Life (Fig. A.2; Hinchliff *et al.*, 2015). The branch lengths of phylogenetic trees were estimated using the *compute.brlen* function from the *ape* package (Paradis & Schliep, 2018), and the phylogenetic relatedness was modelled as a variance-covariance matrix.

Our dataset contained numbers of effect sizes calculated from shared controls (i.e., studies with one treatment control and more than one temperature of pH treatments). To account for such form of data non-independence, the effect sizes of our models were weighted on the variance/co-variance matrix of Var-*g* (Noble *et al.*, 2017). Furthermore, we obtained estimates of the overall mean degree of heterogeneity $I^2 Total$, and relative to each random factor, implementing the function *i2_ml* (Nakagawa *et al.*, 2020). The degree of heterogeneity is the proportion of variance between effect sizes not attributed to sampling variance (Senior *et al.*, 2016). Estimates of I^2 over 75% are considered high, 50% medium, and 20% small (Higgins *et al.*, 2003).

2.4 Meta-regression models

First, we first implemented two models to assess the relative impact of rising temperatures and water acidification on different components of the physiological and immunological responses, respectively. Such models included the factor ‘Response Parameter’ as moderator, that was categorized as follow: ‘Non-enzymatic antioxidants’, ‘Antioxidant enzymes’, ‘Oxidative damage’, ‘Immune response’. We could not discern between humoral and innate immunity since the vast majority of studies focused on the latter. Moreover, the Acidification model included only the category ‘Antioxidant enzymes’ as measure of antioxidant response, due to a low number of studies and effect sizes for the category ‘Non-enzymatic antioxidants’. Models also included the covariates ‘Intensity’, ‘Days of Exposure’ and their interaction, and the random effects *study ID*, *Observation ID*, and *Phylogeny*.

Then, we implemented two models testing for the effects of rising temperature on the oxidative status markers at different life stages and climate zones, respectively. Life-history traits can affect species sensitivity to thermal stress, influencing their physiological response. Thus, we ran a model including the interaction between the moderator ‘Response Parameter’ and ‘Developmental Stage’ (Adult *vs* Early Life; description of moderators in Table A.1). Also, the environment in which species are living can determine their capacity to physiologically respond to environmental perturbations. Thus, we tested for the interaction between ‘Response Parameter’ and ‘Climate Zone’ to assess differences in the strength of physiological response between ‘Tropical’, ‘Temperate’ or ‘Polar’ species. Models also included the covariates ‘Intensity’, ‘Days of Exposure’ and their interaction, and the random effects *study ID*, *Observation ID*, and *Phylogeny*. Due to a low number of studies and effect sizes, we could not test for the effects of acidification on those interactions.

Data on fish and amphibian distribution were found on the websites fishbase.se and amphibiaweb.org. Species distributed between the parallels 23°27’N and 23°27’S for most of their range were classified as ‘Tropical’. The subcategory ‘Temperate’, including also species with subtropical distribution, included species ranging between parallels 23°27’ and, either, 66°33’N, or 60° S, for most of their range. Lastly, species classified as ‘Polar’ have a distribution exceeding either the parallel 66°33’ N, or the parallel 60° S.

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To assess the significance of interactions based on categorical variables (i.e., ‘Response Parameter * Developmental Stage’ and ‘Response Parameter * Climate Zone’), we performed

Wald-type tests trough the *anova* function of package *metafor*, involving all the interactions coefficients. Then, the Wald-type tests were implemented to explore significant contrasts between coefficients of an interaction in pairwise comparisons (Viechtbauer, 2010).

2.5 Heatwaves effect

Frich *et al.* (2002) defined heatwaves as periods of five consecutive days, or more, in which the daily maximum temperature exceeds the average maximum temperature of the previous 30 years by 5°C. Ecological studies exposing animals to heatwaves, however, generally do not follow a strict climatic definition of the phenomenon, rather they discretionally consider heatwaves as prolonged extreme warming events (e.g., Smale *et al.*, 2019; Ujszegi *et al.*, 2022; Isotalo *et al.*, 2022). Therefore, we consider the effects of the covariates 'Intensity', 'Days of Exposure' and their interaction, in temperature models, as reliable proxies for the effects of heatwaves on the oxidative status and immune function of aquatic ectothermic vertebrates.

2.6 Publication bias

We performed funnel plot analysis to test for absence of publication bias. To this aim, we plotted effect sizes on the *y*-axis and the inverse of the sample standard error, that is a measure of precision, on the *x*-axis (Nakagawa *et al.*, 2022). We calculated precision from the variance of Hedges' *g*. Additionally, we tested for the presence of bias in reporting effect sizes among years, by running full factorial meta-regression models, separated between databases on temperature and acidification, including: (a) covariates Year, Intensity, Days of Exposure, interaction between Intensity and Days of Exposure; (b) fixed factors Response Parameters; (c) interactions between Response Parameters and, respectively, Developmental Stage and Climatic Zone (both interactions included only in the model on temperature); and (c) random factors *Study ID*, *Observation ID*, and *Phylogeny*.

3. Results

3.1 Descriptive information

Overall, our work included 1259 effect sizes from 56 studies and 42 species (Table 1). Fish (including one species of Chondrichthyes) were the most represented taxon (1097 effect sizes, 34 species, of which 19 temperate, 8 tropical and 8 polar), followed by amphibians (148 effect sizes, 6 species of which 3 temperate and 3 tropical) and reptiles (14 effect sizes, 2 species of which 1 temperate and 1 tropical). The dataset on Temperature included 1042 effect sizes, 45 studies and 36 species. There were 100, 652, 189 and 101 effect sizes from non-enzymatic antioxidants, antioxidant enzymes, oxidative damage, and immunity, respectively. Moreover, the dataset on Temperature included 499 and 442 effect sizes from adult and early life stages, and 259, 452 and 130 effect sizes from polar, temperate and tropical species, respectively. The dataset on Acidification included 213 effect sizes, 22 studies and 18 species. The Acidification dataset involved 129, 36, and 48 effect sizes from antioxidant enzymes, oxidative damage, and immunity, respectively.

3.2 Meta-regression models

The moderator ‘Response Parameter’ was significant both for the model on Temperature ($Q_M = 55.609$, df = 6, $P < 0.001$) and Acidification ($Q_M = 16.684$, df = 5, $P = 0.005$). Both CO_2-induced-stressors increased oxidative damage in ectotherms. This effect was large and significant either for increasing temperatures (estimate ± SE: 0.759 ± 0.195; CI: 0.378, 1.141), and for decreasing pH (estimate ± SE: 0.641 ± 0.302; CI: 0.050, 1.232). In addition, higher temperatures significantly increased the activity of antioxidant enzymes (estimate ± SE: 0.430 ± 0.180; CI: 0.076, 0.783). We found no significant effects of temperature on the non-enzymatic antioxidant response, and both temperature and acidification on the immune system of ectothermic vertebrates (Fig. 1). Covariates *Intensity*, *Days of Exposure*, and their interaction, were important predictors of how strongly the temperature affected the physiological response (Table A.2, Fig. A.3). Specifically, effect sizes of physiological response to temperature significantly increased with intensity (estimate ± SE: 0.638 ± 0.187; CI: 0.272, 1.004), exposure length (estimate ± SE: 0.444 ± 0.192; CI: 0.067, 0.821) and their interaction (estimate ± SE: 0.499 ± 0.193; CI: 0.121, 0.876), indicating stronger effects of prolonged conditions of exceptionally high temperatures (e.g., heatwaves) on ectotherms' physiology. Such effects of covariates were not found for acidification (Table A.2, Fig. A.4).

The interaction between 'Response Parameter' and 'Developmental Stage' was significant in our model on Temperature (Q_M = 48.305, df = 5, $P < 0.001$). Pairwise comparisons showed that higher temperatures induce a significantly higher activity of antioxidant enzymes in early life stage compared to adult, while no significant differences between life stages occurred for non-enzymatic antioxidants and oxidative damage (Table 2; Fig. 2). Even the interaction between 'Response Parameter' and 'Climate Zone' was significant (Q_M = 27.802, df = 5, $P < 0.001$), however, pairwise comparisons showed no significant differences in levels of antioxidant enzymes and oxidative damage between climate zones (Table 2). Polar species showed weaker antioxidant enzymatic response to increasing temperatures, than temperate and tropical species, but the mean effect sizes was associated to high levels of uncertainty (Fig. 3).

3.3 Publication bias

We found no evidence of publication bias from funnel plot visualization (Fig. A.5). We also found no bias driven by the year of publication, neither for data on temperature (estimate ± SE: -0.05 ± 0.03; $P = 0.18$) nor for acidification (estimate ± SE: 0.04 ± 0.06; $P = 0.49$).

4. Discussion

Mean higher temperatures and water acidification are ongoing effects of anthropogenic emissions of CO_2 in atmosphere. Many species are forced to cope with such environmental changes, but how ectothermic vertebrates respond physiologically to rising temperatures and acidification remains an open question. Environmental temperature and water pH levels are mediators of cellular oxidative status and immune function in ectotherms (e.g., Butler *et al.*, 2013; Thomas *et al.*, 2022; Ritchie & Friesen, 2022). Using a meta-analytical approach, we found that elevated temperatures increase oxidative damage and antioxidant enzymes in ectothermic vertebrates, while the impact on immune function is generally weak (Fig. 1). Such effects were positively associated with temperature intensity and duration, suggesting stronger physiological impacts of heatwaves. Even water acidification increased levels of oxidative damage, but we found no effects on antioxidant defences and immune function. Furthermore, warming temperatures induce stronger increase in the antioxidant enzymatic activity of early life stage, compared to adult (Fig. 2), and of temperate and tropical species, compared to polar (Fig. 3; although this result is non-significant).

4.1 Impacts of temperatures and acidification on oxidative status

Increasing temperature and water acidification can induce higher metabolic rate and oxygen consumption in ectotherms (Sokolova, 2021). As a consequence, an increased flux of protons at level of mitochondrial electron transport chain can lead to greater production of ROS, and oxidative damage to biomolecules (Hou *et al.*, 2021). Since oxidative stress can impair fitness of organisms (Costantini *et al.*, 2010), mechanisms of oxidative status regulation might underlie populations response of ectotherms to CO_2-induced environmental changes.

Elevated oxidative damages can reduce fitness of organisms through negative impacts on reproduction and survival (Dupoué *et al.*, 2020; Castro *et al.*, 2020), which might translate into detrimental consequences for population stability and local abundance. Another way through which oxidative stress can reduce organismal fitness is by accelerating the rate of telomere erosion, which is an important driver of the senescence rate (Metcalfe & Olsson, 2022; Burraco *et al.*, 2022a). In accordance, a long-term study on wild populations of common lizard (*Zootoca vivipara*) found that shorter telomeres were positively correlated with the risk of extinction caused by increased local temperatures (Dupoué *et al.*, 2017).

Results of our meta-analysis showed upregulated activity of antioxidant enzymes in response to warmer environmental conditions. Antioxidant enzymes play a major role in cellular detoxification of ROS. In particular, antioxidant enzymes are involved in scavenging of superoxide anion and its damaging derivates, by-products of cellular respiration (Halliwell & Gutteridge, 2015). Experimental studies found upregulated activity of antioxidant enzymes in fish exposed to thermal stress (Madeira *et al.*, 2013), and in response to thermal acclimation (Loughland & Seebacher, 2020), indicating that antioxidant enzymes play a major role in the physiological response of ectotherms to temperature increase.

One explanation for the higher enzymatic antioxidant activity lies with the higher ambient temperature. Increased kinetic energy and collisions among molecules, trigger more reactions between enzymes and their substrates, that occur as a consequence of the increased temperature (Tattersall *et al.*, 2012). Another possible explanation is that higher temperature induces a direct upregulation of genes expression for antioxidant enzymes (Cheng *et al.*, 2015; Cheng *et al.*, 2018). Differently, we found no increase in activities of antioxidant enzymes in response to water acidification. It is possible that other antioxidant compounds than enzymes are preferentially involved to contrast increasing ROS production induced by water acidification (e.g., thiols; Carneiro *et al.*, 2021). Further experimental studies are needed to understand mechanisms of oxidative status regulation in response to water acidification.

4.2 Heatwaves and oxidative status

Our results show that thermal effects on ectotherms physiology are affected by intensity and exposure length of the treatment, indicating stronger effects of prolonged extreme warming events (i.e., heatwaves) on the oxidative status. Given the ongoing increase of frequency and intensity of heatwaves (Perkins-Kirkpatrick & Lewis, 2020), the study of physiological and fitness consequences of exceptionally high temperatures is pivotal to understand long-term effects on viability of ectotherms populations (Burraco *et al.*, 2020; Morley *et al.*, 2019). In ectotherms, physiological dysfunction at (tolerated) temperatures higher and lower than the ‘thermal optimum’ is described by thermal performance curves commonly shaped as an inverted ‘U’ (Martin & Huey, 2008; Jørgensen *et al.*, 2022). The further the environmental temperature is from the thermal optimum of the species, the more oxidative stress is predicted (Ritchie & Friesen, 2022). Thus, heatwaves may accelerate senescence rate in ectotherms by increasing oxidative lesions to the guanine of telomeric DNA (Metcalfe & Olsson, 2022; Burraco *et al.*, 2020). Accordingly, a review on thermal effects on telomere length in ectothermic animals found faster rate of telomeres shortening at temperatures close to the species’ thermal limits (Friesen *et al.*, 2022).

4.3 Impacts of temperatures and water acidification on immune function

One way through which rising temperatures and acidification may threaten the persistence of ectotherms populations is by impairing the immune system, thus exposing individuals to higher risk of disease (Rohr & Raffel, 2010; Mahmud *et al.*, 2017). However, general trends of immune response may stay hidden due to different direction of responses among immune traits (Ohmer *et al.*, 2021; Fig. A.6). Across vertebrates, altered environmental conditions are known to induce upregulated production of heterophils (neutrophils in mammals), the primary phagocytic leucocyte (Davis *et al.*, 2008). Studies on ectothermic vertebrates exposed to increasing environmental temperatures or water acidification found upregulated activities of phagocytes and complement system (both markers of innate immunity; Bresolin de Souza *et al.*, 2016; Butler *et al.*, 2013; Hudson *et al.*, 2020). While it is important to point out that an upregulated innate immunity may not translate into better fitness (Graham *et al.*, 2011), it is possible that it is part of acclimatization mechanisms.

The other side of immune response, adaptive immunity, is inhibited during stressful conditions by increased levels of glucocorticoid hormones (Cain & Cidlowski, 2017; Billig *et al.*, 2020). Accordingly, the number of lymphocytes, i.e. leucocytes responsible for adaptive immune response, decreases in response to stress in vertebrates (Davis *et al.*, 2008). Additionally, upregulated phagocytic activity enhances levels of oxidative stress (Schulenburg *et al.*, 2009; Costantini, 2022), potentially reducing the amount of energy available to invest into adaptive immune response. Given the low number of studies investigating adaptive immune response to CO_2-induced stressors (matching our criteria of inclusion for temperature: num. study = 3, num. effect sizes = 17; matching our criteria of inclusion for acidification: num. study = 2, num. effect sizes = 9), we encourage future experimental studies to assess the impact of higher temperature and water acidification on baseline and pathogen-induced adaptive immunity.

4.4 High temperature, developmental stage and climate zone

In accordance with our expectation, we found that individuals at early life stage show higher activity of antioxidant enzymes than adults, in response to higher temperature. Metabolic requirements and responses to environmental stimuli vary among developmental stages (Pörtner *et al.*, 2010; Kingsolver *et al.*, 2011). For example, early life stages may prioritize investments in enzymatic antioxidant machinery to fight oxidative stress, which is an important physiological constrain of growth (Janssens & Stoks, 2020; Smith *et al.*, 2016). In turn, adults may need to preserve energies when facing environmental challenges to prioritize investment into reproduction (Harshman & Zera, 2007; Marasco *et al.*, 2018). Such intrinsic physiological differences may concur in explaining the higher levels of antioxidant enzymes in early life stage, compared to adult, in response to increased temperature.

A balanced upregulation of both oxidative damage and antioxidant defences favours acclimation response in ectotherms. Accordingly, a recent study on spadefoot toad (*Pelobates cultripes*) larvae found positive associations between the degree of individuals phenotypic plasticity (measured as developmental, growth, and morphological responses to a stressor) and levels of antioxidant enzymatic activity (Burraco *et al.*, 2022b). Thus, regulation of oxidative status may be one key mechanism underlying the phenotypic responses to environmental changes in ectotherms. The potential for phenotypic plasticity may be either enhanced or constrained depending on the ability of organisms to counterbalance increasing levels of oxidative damage with upregulated antioxidant defences.

Organisms living at relatively stable environmental conditions, such as polar and tropical species, are more susceptible to climate change because they evolved physiological specializations at expenses of reduced acclimation capacity (Peck *et al.*, 2014; Somero, 2010; Morgan *et al.*, 2022). Partially in accordance, we found that polar species show lower antioxidant enzymatic activity in response to increased temperatures, compared to temperate and tropical species. This result, however, is statistically non-significant because of the high level of uncertainty in antioxidant enzymatic response to temperature in polar species, as shown by the wide confidence interval. Further experimental studies are needed to unravel mechanisms of oxidative status regulation and evolutionary constraints in Arctic and Antarctic species.

5. Conclusions

In conclusion, our meta-analysis provides mechanistic evidence underlying the effects of increasing temperature and water acidification on vertebrate ectotherms. We found that both stressors induce a higher level of oxidative damage in ectothermic vertebrates, and that increasing temperatures also caused upregulation of antioxidant enzymes. The magnitude of physiological response increased with higher temperatures and exposure, suggesting stronger negative impacts of heatwaves on the oxidative status and immunity of ectotherms. Overall, these results indicate that the regulation of oxidative status might be one key mechanism underlying thermal plasticity, while immune function might be less affected.

A better capacity to upregulate antioxidant enzymatic activity in concert with increased oxidative damage, may indicate higher acclimation capacity to thermal stress in individuals at early life stages, compared to adults. Furthermore, higher temperature has minor effects on the antioxidant enzymatic activity of polar species, compared to temperate and tropical, possibly due to physiological and evolutionary constraints (Birnie-Gauvin *et al.*, 2017). Our results on the impact of climate zone on the oxidative status response to increased temperature suggest that Arctic and Antarctic species may be particularly susceptible to the effects of climate change, but further studies are needed to assess this pattern. Furthermore, we recognize that our sample included an unbalanced representation of climate zones in favour of temperate species, indicating that more work is needed on both polar and tropical species.

We also found high levels of heterogeneity in our models, indicating a large proportion of unexplained variance among effect sizes. Since thermal biology of ectothermic animals is widely affected by ontogenetic environmental conditions, populations of origin, and intrinsic characteristics of individuals (Seebacher & Little, 2021; Hossack *et al.*, 2013), we hypothesise that these factors might substantially contribute to the unexplained levels of variance in our models. Finally, we highlight the importance of assessing how metamorphosis would affect the consequences of environmental changes on amphibians (Stoks *et al.*, 2022). Given the strong tissue remodelling, metamorphosis might limit carry-over effects of accumulated damage very early in life.

Finally, we call for more studies investigating co-occurrent effects of higher temperature and water acidification on the physiology of ectothermic vertebrates. As today, there are data for only a few numbers of species (reviewed in Baag & Mandal, 2022). Some studies found that the combined effects of these two stressors have stronger impacts on the oxidative status and immune system of fish, than those of single stressors (Feidantsis *et al.*, 2015; Bresolin de Souza *et al.*, 2016; Carney Almroth *et al.*, 2019). However, those effects might be species-specific as suggested by the heterogeneity of results found in literature (Lee *et al.*, 2022b; Enzor & Place, 2014).

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Declaration of competing interest

The authors of this study declare no competing interests.

Figure legends

Fig. 1 – Effect sizes of immune response and oxidative status in aquatic ectothermic vertebrates exposed to warming temperatures (red) and water acidification (blue). nES indicates the number of effect sizes, and nStudy the number of studies included in the meta-analytical model for any given physiological marker.

Fig. 2 – Impacts of warming temperatures on the oxidative status of adult (orange) and early life (violet) stages, in aquatic ectothermic vertebrates. Statistically significant contrasts between same marker of oxidative status in adult and early life stages are indicated by an asterisk. nES indicates the number of effect sizes, and nStudy the number of studies included in the meta-analytical model for any given physiological marker.

Fig. 3 – Impacts of warming temperatures on the oxidative status of aquatic ectothermic vertebrates living at different climatic zones. Light blue = polar species; dark blue = temperate species; dark green = tropical species. nES indicates the number of effect sizes, and nStudy the number of studies included in the meta-analytical model for any given physiological marker.

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Table 1 – List of species included in the meta-analysis, with relative taxonomic class, climate zone, and study citation.

Class	Species	Climate Zone	Study
Amphibia	*Osteopilus septentrionalis*	Tropical	Peck & Wilcoxen (2018)
Amphibia	*Pelobates cultripes*	Temperate	Burraco & Gomez-Mestre (2016); Florencio *et al.* (2020)
Amphibia	*Physalaemus nattereri*	Tropical	Freitas & Almeida (2016); Freitas *et al.* (2017a,b)
Amphibia	*Rana catesbeiana*	Temperate	Albin & Wilcoxen (2020); Krynak *et al.* (2015)
Amphibia	*Rana pipiens*	Temperate	Vatnick *et al.* (2006)
Amphibia	*Rhinella schneideri*	Tropical	Freitas *et al.* (2017a,b)
Fish	*Acipenser baerii*	Temperate	Yang *et al.* (2021)
Fish	*Anguilla anguilla*	Temperate	Lopes *et al.* (2021)
Fish	*Argyrosomus regius*	Temperate	Antonopoulou *et al.* (2020); Sampaio *et al.* (2018)
Fish	*Atherina presbyter*	Temperate	Silva *et al.* (2016)
Fish	*Channa punctata*	Tropical	Singh *et al.* (2021)
Fish	*Chiloscyllium punctatum*	Tropical	Rosa *et al.* (2016)
Fish	*Chionodraco rastrospinosus*	Antarctic	Mueller *et al.* (2014)
Fish	*Colossoma macropomum*	Tropical	Pereira *et al.* (2021)
Fish	*Dicentrarchus labrax*	Temperate	Cecchini & Saroglia (2002); Chang *et al.* (2021); Pereira *et al.* (2017); Vinagre *et al.* (2012)
Fish	*Gasterosteus aculeatus*	Temperate	Dittmar *et al.* (2014)
Fish	*Genypterus chilensis*	Tropical	Dettleff *et al.* (2020)
Fish	*Gobiusculus flavescens*	Temperate	Faria *et al.* (2018)
Fish	*Hippoglossus hippoglossus*	Temperate	Bresolin de Souza *et al.* (2016); Carney Almroth *et al.* (2019)
Fish	*Hoplosternum littorale*	Tropical	Rossi *et al.* (2017)
Fish	*Labeo rohita*	Tropical	Roychowdhury *et al.* (2021)
Fish	*Miichthys miiuy*	Temperate	Liu *et al.* (2019)

Fish	*Notothenia coriiceps*	Antarctic	Klein *et al.* (2017); Mueller *et al.* (2014); O'Brien *et al.* (2022)
Fish	*Notothenia rossii*	Antarctic	Guillen *et al.* (2022)
Fish	*Oncorhynchus mykiss irideus*	Temperate	Defo *et al.* (2019)
Fish	*Pagothenia borchgrevinki*	Antarctic	Carney Almroth *et al.* (2015); Enzor & Place (2014)
Fish	*Paralichthys olivaceus*	Temperate	Cui *et al.* (2022); Lee *et al.* (2022a,b); Kim *et al.* (2019a,b); Kim *et al.* (2021)
Fish	*Piaractus mesopotamicus*	Temperate	Pinto *et al.* (2019)
Fish	*Poecilia reticulata*	Tropical	Breckels & Neff (2013)
Fish	*Pomatoschistus microps*	Temperate	Vieira *et al.* (2021)
Fish	*Salmo marmoratus*	Temperate	Simčič *et al.* (2015)
Fish	*Salmo salar*	Temperate	Zanuzzo *et al.* (2020)
Fish	*Scophthalmus maximus*	Temperate	Huang *et al.* (2011)
Fish	*Solea senegalensis*	Tropical	Machado *et al.* (2020)
Fish	*Sparus aurata*	Temperate	Madeira *et al.* (2020)
Fish	*Squalius carolitertii*	Temperate	Jesus *et al.* (2018)
Fish	*Squalius torgalensis*	Temperate	Jesus *et al.* (2018)
Fish	*Tor putitora*	Temperate	Akhtar *et al.* (2013; 2020)
Fish	*Trematomus bernacchii*	Antarctic	Enzor & Place (2014); Giuliani *et al.* (2021)
Fish	*Trematomus newnesi*	Antarctic	Enzor & Place (2014)
Reptilia	*Caiman latirostris*	Tropical	Moleon *et al.* (2018)
Reptilia	*Mauremys mutica*	Temperate	Li *et al.* (2021)

Table 2 – Pairwise comparisons between predicted effect sizes for levels of the interactions between Response Parameter and, respectively, Developmental Stage and Climate Zone. Significant contrasts are shown in bold.

Model	Marker	Pairwise comparison	Estimate	SE	Z-value	*P* value
Developmental Stage	AO Enzymes	Adult *vs.* Early Life	-0.412	0.144	-2.862	**0.004**
	Non-Enzy. AO	Adult *vs.* Early Life	0.004	0.280	0.015	0.988
	Ox. Damage	Adult *vs.* Early Life	0.209	0.202	1.035	0.301
Climate Zone	AO Enzymes	Polar *vs.* Temperate	-0.553	0.398	-1.383	0.167
		Polar *vs.* Tropical	-0.789	0.486	-1.622	0.104
		Temperate *vs.* Tropical	-0.236	0.355	-0.666	0.506
	Ox. Damage	Polar *vs.* Temperate	0.087	0.428	0.203	0.839
		Polar *vs.* Tropical	0.014	0.513	0.027	0.979

		Temperate *vs.* Tropical	-0.073	0.396	-0.184	0.854

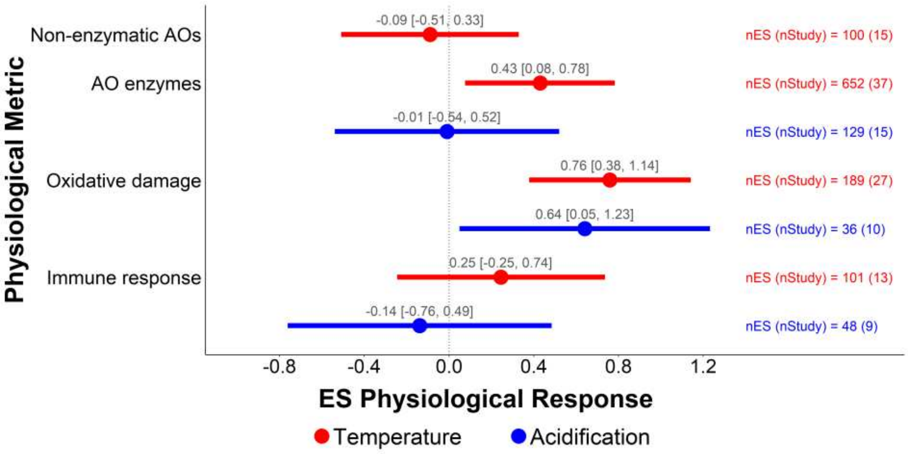

Fig. 1

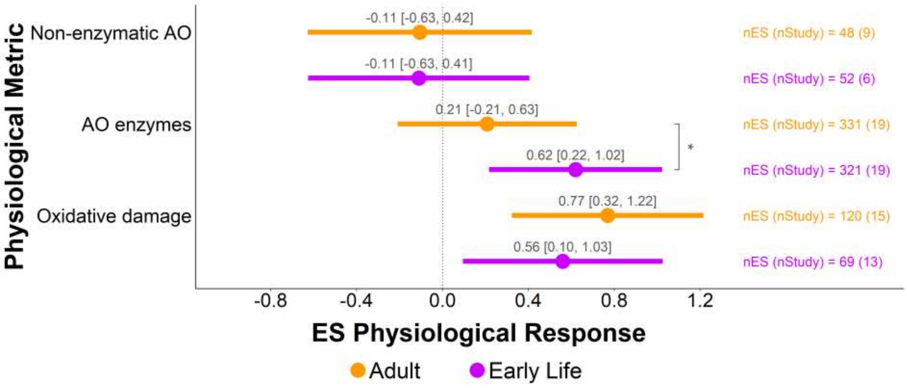

Fig. 2

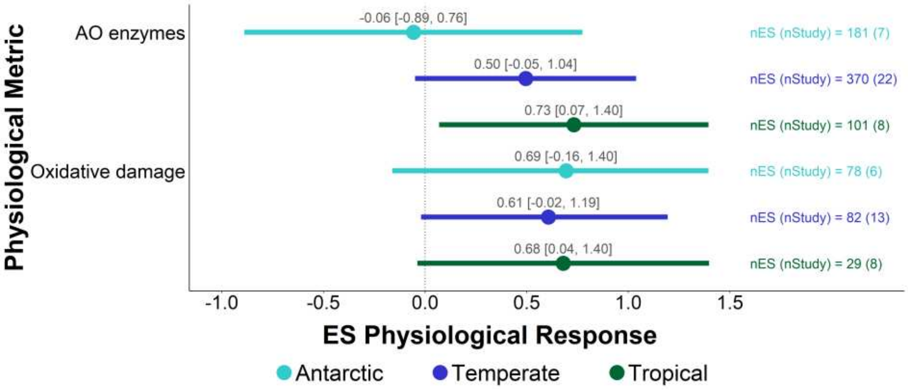
Physiological Metric
AO enzymes
Oxidative damage
-0.06 [-0.89, 0.76]
0.50 [-0.05, 1.04]
0.73 [0.07, 1.40]
0.69 [-0.16, 1.40]
0.61 [-0.02, 1.19]
0.68 [0.04, 1.40]
nES (nStudy) = 181 (7)
nES (nStudy) = 370 (22)
nES (nStudy) = 101 (8)
nES (nStudy) = 78 (6)
nES (nStudy) = 82 (13)
nES (nStudy) = 29 (8)
-1.0
-0.5
0.0
0.5
1.0
1.5
ES Physiological Response
Antarctic
Temperate
Tropical

Fig. 3