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Review Article

REVIEW: Evidence supporting the 'preparation for oxidative stress' (POS) strategy in animals in their natural environment

Maximiliano Giraud-Billoud [a,b,c,*], Daniel C. Moreira [d,e], Marina Minari [d], Aleksandra Andreyeva [f,g], Élida G. Campos [d], Juan M. Carvajalino-Fernández [h], Aleksandra Istomina [i], Basile Michaelidis [j], Cuijuan Niu [k], Yonggang Niu [l], Luciana Ondei [m], Marko Prokić [n], Georgina A. Rivera-Ingraham [o,p], Debadas Sahoo [q], Alexandra Staikou [r], Janet M. Storey [s], Kenneth B. Storey [s], Israel A. Vega [a,b,t], Marcelo Hermes-Lima [d,*]

[a] Instituto de Histología y Embriología de Mendoza (IHEM), Universidad Nacional de Cuyo–CONICET, Mendoza 5500, Argentina
[b] Instituto de Fisiología, Facultad de Ciencias Médicas, Universidad Nacional de Cuyo, Mendoza 5500, Argentina
[c] Departamento de Ciencias Básicas, Escuela de Ciencias de la Salud-Medicina, Universidad Nacional de Villa Mercedes, San Luis 5730, Argentina
[d] Department of Cell Biology, Institute of Biological Sciences, University of Brasilia, Brasilia, Brazil
[e] Research Center in Morphology and Applied Immunology, Faculty of Medicine, University of Brasilia, Brasilia, Brazil
[f] A.O. Kovalevsky Institute of Biology of the Southern Seas of RAS, Moscow 119991, Russia
[g] Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, St-Petersburg 194223, Russia
[h] Laboratory of Adaptations to Extreme Environments and Global Change Biology, Universidad Nacional de Colombia, Bogotá, Colombia
[i] V.I. Il'ichev Pacific Oceanological Institute, Far Eastern Branch, Russian Academy of Sciences, 690041 Vladivostok, Russia
[j] Laboratory of Animal Physiology, Department of Zoology, School of Biology, University of Thessaloniki, GR-54006 Thessaloniki, Greece
[k] Ministry of Education Key Laboratory for Biodiversity Science and Ecological Engineering, College of Life Sciences, Beijing Normal University, Beijing 100875, China
[l] Department of Life Sciences, Dezhou University, Dezhou, China
[m] Universidade Estadual de Goiás, Câmpus Central, 75132-903 Anápolis, GO, Brazil
[n] Department of Physiology, Institute for Biological Research "Siniša Stanković", National Institute of the Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia
[o] Australian Rivers Institute, Griffith University, Southport 4215, Gold Coast, Queensland. Australia
[p] UMR9190-MARBEC, Centre National de la Recherche Scientifique (CNRS), Montpellier, 34090, France
[q] Post Graduate Department of Zoology, S.C.S. Autonomous College, Puri, Odis ha-752001, India
[r] Laboratory of Marine and Terrestrial Animal Diversity, Department of Zoology, School of Biology, University of Thessaloniki, GR-54006 Thessaloniki, Greece
[s] Department of Biology and Institute of Biochemistry, Carleton University, Ottawa, ON, Canada
[t] Departamento de Biología, Facultad de Ciencias Exactas y Naturales, Universidad Nacional de Cuyo, Mendoza 5500, Argentina

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ABSTRACT

Hypometabolism is a common strategy employed by resilient species to withstand environmental stressors that would be life-threatening for other organisms. Under conditions such as hypoxia/anoxia, temperature and salinity stress, or seasonal changes (e.g. hibernation, estivation), stress-tolerant species down-regulate pathways to decrease energy expenditures until the return of less challenging conditions. However, it is with the return of these more favorable conditions and the reactivation of basal metabolic rates that a strong increase of reactive oxygen and nitrogen species (RONS) occurs, leading to oxidative stress. Over the last few decades, cases of species capable of enhancing antioxidant defenses during hypometabolic states have been reported across taxa and in response to a variety of stressors. Interpreted as an adaptive mechanism to counteract RONS formation during tissue hypometabolism and reactivation, this strategy was coined "Preparation for Oxidative Stress"

Abbreviations: CAT, Catalase; CS, Citrate synthase; cMnSOD, Cytosolic manganese superoxide dismutase; G6PDH, Glucose-6-phosphate dehydrogenase; GPX, Glutathione peroxidase; GR, Glutathione reductase; GSR, Global solar radiation; GSH, Reduced glutathione; GST, Glutathione S-transferase; HSP/Hsp, Heat shock proteins; ICDH, Isocitrate dehydrogenase; LPO, Lipid peroxidation; MAPK, Mitogen-activated protein kinase; MDA, Malondialdehyde; miRNA, Micro ribonucleic acid; mMnSOD, Mitochondrial manganese superoxide dismutase; Nrf2, Nuclear factor-erythroid 2-related factor 2; PCO, Protein carbonylation; POS, Preparation for oxidative stress; PTMs, Post-translational modifications; RONS, Reactive oxygen and nitrogen species; SOD, Superoxide dismutase; T-AOC, Total antioxidant capacity; UV, Ultraviolet.

* Corresponding authors.
E-mail addresses: mgiraudbilloud@gmail.com, giraud.maximiliano@fcm.uncu.edu.ar (M. Giraud-Billoud), hermes@unb.br (M. Hermes-Lima).

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(POS). Laboratory experiments have confirmed that over 100 species, spanning 9 animal phyla, apply this strategy to endure harsh environments. However, the challenge remains to confirm its occurrence in the natural environment and its wide applicability as a key survival element, through controlled experimentation in field and in natural conditions. Under such conditions, numerous confounding factors may complicate data interpretation, but this remains the only approach to provide an integrative look at the evolutionary aspects of ecophysiological adaptations. In this review, we provide an overview of representative cases where the POS strategy has been demonstrated among diverse species in natural environmental conditions, discussing the strengths and weaknesses of these results and conclusions.

1. Introduction to the preparation for oxidative stress (POS) theory

Since the 1990s there has been growing evidence that animal tolerance to hypoxia is associated with an improved capacity to deal with reactive oxygen and nitrogen species (RONS) or their downstream cellular effects. Determination of endogenous antioxidants and gene expression levels are the most frequent methods used to study RONS metabolism. This has been addressed in tissues of hypoxia-tolerant species, animals that withstand freezing, severe dehydration (including anhydrobiosis), salinity stress, aerial exposure of gill-bearing species, and estivation/hibernation in non-mammals (see Freire et al., 2011a; Giannetto et al., 2017; Giraud-Billoud et al., 2022; Giraud-Billoud et al., 2018; Hermes-Lima and Zenteno-Savin, 2002; Rivera-Ingraham et al., 2016; Rivera-Ingraham et al., 2013; Rodriguez et al., 2023; Storey and Storey, 2017; Welker et al., 2013). Most of the studies show that during the period of environmental stress (namely when it entails a reduction in metabolic function), the activity/expression of antioxidant enzymes and small non-protein antioxidants increases. For example, catalase (CAT) and glutathione peroxidase (GPX) activities increase during anoxia exposure in leopard frogs, but return to baseline levels upon recovery to normoxic aerobic metabolism (Hermes-Lima and Storey, 1996). In the past 30 years, similar increases in antioxidant capacity have been reported in over a hundred animal species exposed to different stressors. In the late 1990s, this was interpreted as an adaptive mechanism to counteract the negative effects of the increase in RONS formation that occurs with the reactivation of metabolism and it was coined as Preparation for Oxidative Stress (POS) (Giraud-Billoud et al., 2019; Hermes-Lima et al., 1998).

Moreover, about two-thirds of the animal species investigated for this ability to redox-control have been considered as POS-positive (i.e., they showed hallmarks of POS; Moreira et al., 2017; Moreira et al., 2016). The POS strategy has been documented across 9 invertebrate and vertebrate phyla (Moreira et al., 2023a). The environmental conditions in which typical POS responses have been observed are listed in **Table 1**. Six of the stress/adaptive conditions shown in Table 1 are well characterized in the literature (Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015). Ultraviolet (UV) radiation exposure was recently included as the seventh natural environmental stress associated with POS (Geihs et al., 2020).

Mechanisms other than POS may work to maintain RONS homeostasis in at least 1/3 of the investigated species. Examples include **(i)** constitutively high levels of endogenous antioxidants (Hermes-Lima and Zenteno-Savin, 2002), **(ii)** tolerance to oxidative damage (the use of various mechanisms to repair damaged proteins and DNA; Lung and Storey, 2022), and **(iii)** the ability to minimize mitochondrial RONS formation under environmental stress (Eaton and Pamenter, 2022).

2. POS: Past and current challenges

The molecular mechanism underlying POS remained a mystery until approximately a decade ago. This was primarily because there was no clear explanation of how an increase in antioxidant defenses could occur when animals were exposed to hypoxia, as during this phenomenon, the metabolic rate is markedly decreased. The prevailing hypothesis, at that time, suggested that hypoxic cells produce fewer RONS, a hypothetical mechanism derived from the logical direct correlation between the formation of oxy-radicals and the availability of oxygen. Therefore, the precise molecular mechanism of the POS response remained unresolved (Hermes-Lima et al., 1998). This began to change when it was demonstrated that many cell types increase RONS formation under low oxygen conditions, as indicated by direct measurements of oxy-radicals (Clanton, 2007; Giraud-Billoud et al., 2019; Hermes-Lima et al., 2015; Hernansanz-Agustín et al., 2014). Furthermore, increases in lipid peroxidation (LPO) and protein carbonylation (PCO) were reported in tissues of many animal species exposed to severe hypoxia, indicating enhanced RONS production under these conditions. With this new perspective in mind, a plausible mechanism for the POS phenomenon was postulated in 2013, in agreement with the current state-of-art of cellular and comparative physiology (Hermes-Lima et al., 2015; Moreira et al., 2023a; Welker et al., 2013). We proposed that the POS response is initiated by an increase in RONS formation, which would be responsible for triggering several signaling pathways (e.g., kinase/phosphatase systems) including the activation of redox-sensitive transcription factors (such as Nrf2; Breedon et al., 2021). The promotion of the expression of antioxidant proteins and other intracellular changes would ultimately result in the enhancement of antioxidant defenses (Fig. 1).

Although the proposed molecular mechanism for the POS phenomenon contradicts traditional beliefs regarding the proportionality between RONS formation and oxygen availability, the increase in RONS formation under hypoxic conditions is supported by both direct and indirect evidence. Direct evidence comes from cell culture experiments (Smith et al., 2017), while indirect evidence is demonstrated by increases in oxidative damage to molecules found in both hypoxia-tolerant

Table 1
Examples of stress/adaptive conditions resulting in the POS response*.

Hypoxia/anoxia (including high altitude stress**)
Freezing exposure
Severe dehydration
Aerial exposure of aquatic animals
Salinity stress of aquatic animals
Estivation/hibernation of non-mammalian species
UV radiation exposure (in small ectotherms/transparent animals)

[*] The POS strategy has not been directly related to high temperature stress nor mammalian hibernation, due to the complexity of these phenomena. However, temperature is designated as a POS-response inducer in the studies described in sections 4.5 (on land snails from Greece) and 5.0 (see Table 2).

[**] High altitude stress includes hypoxia, cold temperatures and UV radiation exposure.

and hypoxia-sensitive animals (Moreira et al., 2017). On the other hand, there is also evidence of mechanisms contrary to the one described above, such as experiments conducted with turtle hearts *in vivo* and *in vitro*, which have not shown an increase in the production of RONS during post-anoxic reoxygenation (Bundgaard et al., 2023; Bundgaard et al., 2018). This indicates that further research is needed on the role of RONS formation in the initiation of the POS response.

Thus, one of the current challenges in POS research is (1) to elucidate the specific mechanisms involved, as the underlying molecular processes remain unresolved. This is the point where researchers' efforts should concentrate in the future, since, as we will see later in the text, the apparent complexity of elucidating molecular mechanisms involving so many animal species is further compounded by the possibility that these mechanisms may depend on the type of stressor, the convergence of more than one of them in the natural environment where the animal inhabits, and, in addition, the possibility of individual variations dependent on biotic and abiotic factors. Another significant challenge for the POS theory is (2) understanding the conditions that trigger this adaptive response, i.e., under laboratory controlled conditions and in the natural environment (Moreira et al., 2017). To date, the POS strategy has been documented in more than 100 animal species, predominantly under experimental laboratory conditions (Giraud-Billoud et al., 2019; Moreira et al., 2016). The next challenge to strengthen the POS theory involves gathering more evidence directly from natural environments. This review, therefore, aims to consolidate existing information on this topic and encourages ecologists and eco-physiologists to consider the possibility that this strategy may contribute to various animal species tolerating diverse environmental conditions.

3. POS in nature

As mentioned above, the evidence regarding the use of POS as a cellular stress response by animals under wild conditions is still very limited, especially when contrasted with the number of studies conducted under laboratory-controlled conditions. Some examples of animals that have been studied under field or natural conditions, demonstrating their use of the POS strategy to cope with the deleterious effects of environmental changes, include a hibernating toads from the cold Indian mountains (Patnaik and Sahoo, 2021), two estivating frog species from semi-desert environments in Brazil (Moreira et al., 2021a; Moreira et al., 2020), and a frog from the Qinghai-Tibet Plateau, experiencing the stress of extreme altitude, characterized by a cold and hypoxic environment (Niu et al., 2023). Moreover, POS in the wild was reported in a fish species exposed to hypoxia in a small stream in the Brazilian savanna during the dry season (Ondei et al., 2020). Among invertebrates, the POS phenomena has also been observed in a small South American mussel species under stress from the tidal cycle (i.e. hypoxia and UV stress) (Moreira et al., 2021b; Moreira et al., 2023b), as well as in a marine gastropod species from the Japanese Sea undergoing the tidal cycle (Istomina et al., 2013). Other animal-examples, either

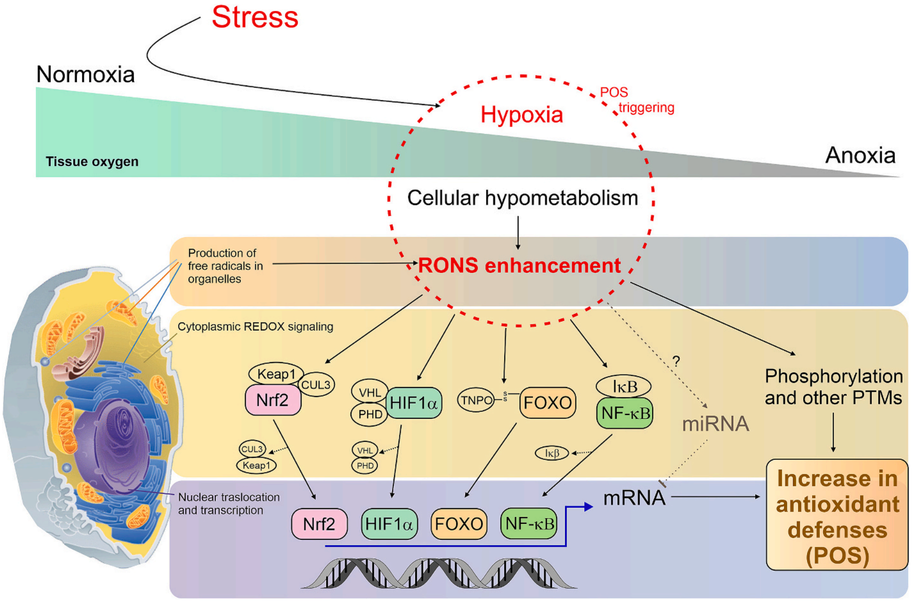


Fig. 1. Proposed Mechanism of Preparation for Oxidative Stress in Response to Changes in Environmental stressors. Hypoxia, for example, can induce a hypometabolic cellular state and trigger the overproduction of reactive oxygen and nitrogen species (RONS) from various organelles, including mitochondria, endoplasmic reticulum, and peroxisomes. This phenomenon can result in oxidative damage to biomolecules but also serves as signaling molecules. The production of RONS can modify the levels of redox-sensitive transcription factors, such as Nrf2, HIF, FOXO, or NF-κB. Under oxidative stress conditions, the KEAP1-CUL3 complex undergoes a conformational change in KEAP1, leading to the liberation of NRF2, disrupting ubiquitination and degradation of NRF2. This allows NRF2 to translocate to the nucleus, where it interacts with other transcription factors and cofactors to regulate the transcription of genes. HIFs are heterodimeric proteins with an O_2-regulated HIF-subunit (1α, 2α, or 3α) and a constitutively expressed HIF-β subunit. The protein Von Hippel-Lindau (VHL) interacts with HIF-1α through prolyl hydroxylase domain (PHD) proteins. In the absence of oxygen or the presence of RONS, oxidative modifications lead to HIF-1α accumulation and translocation to the nucleus. Under oxidative stress, FOXO forms an intermolecular disulfide with transportin (TNPO), promoting nuclear translocation. FOXO proteins regulate downstream target genes encoding antioxidant enzymes, including superoxide dismutase (SOD) and catalase (CAT). RONS can either activate or suppress NF-κB signaling with both anti-oxidant and pro-oxidant roles. REDOX-mediated activation results in the phosphorylation and proteasomal degradation of IκB, releasing NF-κB. In the nucleus, NF-κB must be reduced by nuclear thioredoxin (Trx) or peroxiredoxin (Prx) to enable its DNA-binding capacity. These transcription factors play a crucial role in upregulating antioxidant defenses, including enzymes such as SOD, CAT, glutathione transferase, glutathione peroxidase, Trx, and Prx. Furthermore, other potential mechanism that could be related to the POS response may involve changes in the expression of microRNAs (miRNAs), which could also regulate the expression of redox sensors and modify key components of cellular antioxidant machinery, a hypothesis that deserves to be explored in future research. Finally, oxidative stress could lead to post-translational modifications (PTMs) of antioxidant enzymes and other proteins involved in redox signaling, modifying the antioxidant response. NRF2, Nuclear factor erythroid 2-related factor 2; KEAP1, Kelch-like-ECH-associated protein 1; CUL3, Cullin3; FOXO, Forkhead box O; HIF, Hipoxia inducible factor; NF-κB; (for further information related to pathways regulating the antioxidant response, please refer to Averill-Bates, 2023; Bartel, 2018; de Almeida et al., 2022; Lennicke and Cochemé, 2021; Wang et al., 2023).

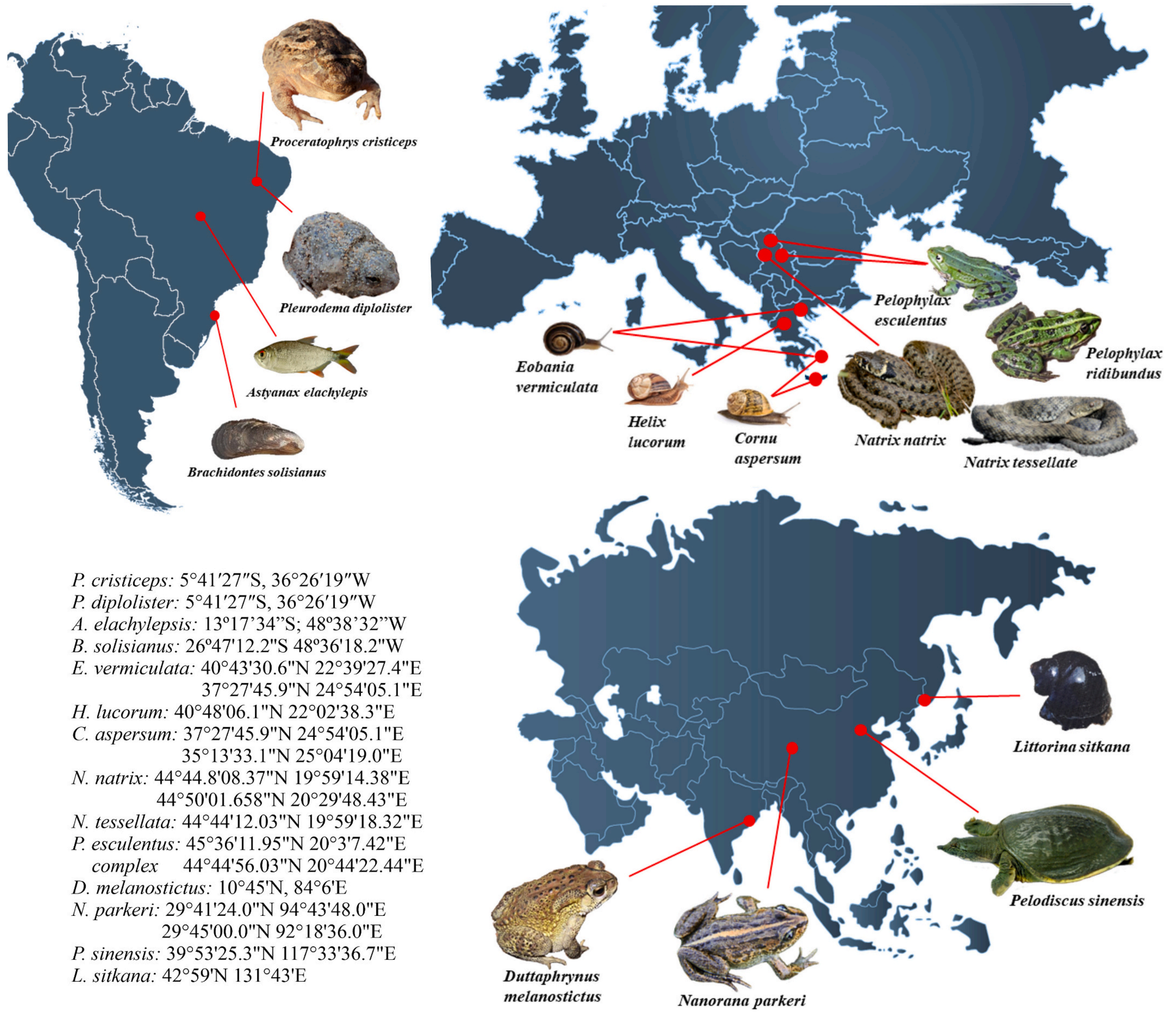
vertebrates or invertebrates, will be provided in the following sections of this article.

4. Field and natural experiments

Evolutionary and comparative physiology allows us to better understand how modern animals work and identify conservative adaptive physiological and biochemical mechanisms that respond to environmental challenges in specific ecosystems. A few years ago, we postulated the importance of POS studies that compare laboratory results with the adaptive response of animals in field or natural conditions (Giraud-Billoud et al., 2019; Moreira et al., 2017). Now, with a richer literature on this topic in hand, this review aims to consolidate current knowledge on POS under these conditions, highlighting key findings and identifying new areas for future research. We explore a range of studies (Fig. 2), focusing on the phylogenetic and geographic diversity of animal species whose antioxidant systems are up-regulated when challenged by the environment. We will review contributions from researchers around the world that explore whether the POS strategy is used by animals in their own habitats (Fig. 2).

4.1. *Frogs from Tibet, China*

The Qinghai-Tibet Plateau is the highest (with an average elevation of over 4,000 m a.s.l.) and largest (with a total area of about 2.5 million square kilometers) plateau in the world (Tapponnier et al., 2001). As a unique geographic unit on Earth, it is known by many names, such as the Roof of the World, the Third Pole, and the Asian Water Tower (Mao et al., 2021). Over the past few decades, scientists have taken a keen interest in the study of this region and the particularities of the organisms inhabiting an area where extreme conditions occur (Cui et al., 2018; Liu et al., 2021; Yang et al., 2021). As altitude increases, climatic conditions become harsher, with lower atmospheric pressure, oxygen levels and temperature, but with higher UV radiation, making this environment an interesting place to learn how different animal species respond to the challenges of nature. The Xizang plateau frog, *Nanorana parkeri* (Anura: Dicroglossidae), is a representative endemic species of the plateau, living at altitudes ranging from 2,850 to 5,100 m a.s.l. on



P. cristiceps:	5°41′27″S, 36°26′19″W
P. diplolister:	5°41′27″S, 36°26′19″W
A. elachylepsis:	13º17’34”S; 48º38’32”W
B. solisianus:	26º47'12.2"S 48º36'18.2"W
E. vermiculata:	40°43'30.6"N 22°39'27.4"E 37°27'45.9"N 24°54'05.1"E
H. lucorum:	40°48'06.1"N 22°02'38.3"E
C. aspersum:	37°27'45.9"N 24°54'05.1"E 35°13'33.1"N 25°04'19.0"E
N. natrix:	44°44.8'08.37"N 19°59'14.38"E 44°50'01.658"N 20°29'48.43"E
N. tessellata:	44°44'12.03"N 19°59'18.32"E
P. esculentus: *complex*	45°36'11.95"N 20°3'7.42"E 44°44'56.03"N 20°44'22.44"E
D. melanostictus:	10°45'N, 84°6'E
N. parkeri:	29°41'24.0"N 94°43'48.0"E 29°45'00.0"N 92°18'36.0"E
P. sinensis:	39°53'25.3"N 117°33'36.7"E
L. sitkana:	42°59'N 131°43'E

Fig. 2. Examples of animal species that utilize the POS strategy to cope with harsh environmental stresses as confirmed through studies of each under natural living conditions.

the Qinghai-Tibet Plateau (Zhang et al., 2012). Adult individuals from high-altitude (4,600 m a.s.l.), compared to frog populations at lower-altitude (3,400 m a.s.l.), show higher activities of superoxide dismutase (SOD), CAT, and glutathione S-transferase (GST), as well as higher total antioxidant capacity (T-AOC) in the liver (Niu et al., 2022). Similar results are shown by high-altitude juveniles, that have higher antioxidant enzyme activities: CAT activity in liver and muscle, GPX activity in brain, liver, and muscle, and glutathione reductase (GR) activity in muscle, as well as higher T-AOC in the liver (Niu et al., 2022). Maintaining high levels of antioxidant defenses is an adaptation to the stressful environment of high-altitude frogs, as they must endure lower oxygen partial pressure (11.9 kPa) and lower ambient temperature (mean value: 13.7 °C during sampling), as compared with lower-altitude frogs (14.0 kPa, 19.5 °C) (Niu et al., 2022). Although the intensity of UV radiation was not directly measured in the aforementioned study, it is generally accepted that UV levels increase from approximately 9% (UVA irradiance) to 24% (UVB irradiance) with every 1,000 m in altitude (Blumthaler et al., 1997). This means that high-altitude frogs are exposed to an average of 20% higher UV radiation than lower-altitude frogs. All these abiotic factors, alone or in combination, can induce oxidative stress and the enhanced antioxidant defenses seen at high altitude align with the POS phenomenon. Other studies conducted under laboratory-controlled conditions have shown that hibernating frogs exposed to acute freezing (−2 °C for 12 h) or elevated temperatures (10 °C for 12 h) showed tissue-specific increases in antioxidant defenses (Niu et al., 2021; Zhang et al., 2022), further supporting the POS phenomenon among these frogs. On the other hand, winter hibernation induced a significant decrease in antioxidant defenses, as evidenced through enzyme activity and transcription regulation (Niu et al., 2018; Niu et al., 2023). This finding suggests that the POS phenomenon does not hold true in hibernating *N. parkeri*. However, we cannot rule out the possibility that antioxidant defenses are specifically elevated near the end of hibernation, since sampling was conducted in the mid-hibernation period, about 3 months before the end of hibernation. This frog species appears to prefer suppression of their antioxidant defense system during hibernation but restores antioxidant defenses just before the end of hibernation. This may be related to ensuring long-term survival by reducing energy consumption during the winter months. Altogether, these results lead to the hypothesis that the initiation of POS may not be triggered only by environmental factors but other intrinsic factors could also play roles and these deserve to be studied in the future (e.g. circannual rhythmicity). Overall, the unique and complex climatic conditions of the Qinghai-Tibet Plateau make it an ideal site for studying POS in the natural conditions.

4.2. *Turtles from Hebei, China*

The Chinese soft-shelled turtle, *Pelodiscus sinensis*, is a widespread freshwater species in China and is valued in aquaculture for its economic importance. However, natural populations have declined significantly due to the unsustainable harvesting of wild animals, to the extent that the species is now listed as “vulnerable” on the IUCN Red List. This subtropical species has been very well studied and is an interesting case study due to its distinctive traits; e.g. as a lung-breathing, bottom-dwelling species, these turtles also have a preference for basking, thriving at temperatures around 31 °C (Niu et al., 1999). During the winter months, these turtles typically undergo hibernation beneath ice-sealed bodies of water, burying themselves in the anoxic bottom mud with the surrounding temperature dropping to approximately 5 °C in the Beijing area (Jackson and Ultsch, 2010). In the spring, when water temperature rises above 18 °C, *P. sinensis* re-emerges, returning to the water surface to breathe and feed. This transition is accompanied by a notable increase in oxygen consumption and energy metabolism (Costanzo et al., 2008). The strategies employed by these turtles to endure low tissue oxygenation during hibernation and the ischemia-reperfusion-like arousal phase have long captivated the attention of ecophysiologists (e.g. Storey and Storey, 1988; Tang et al., 2021; Zhang et al., 2017a, 2017b).

It has been documented that other anoxia tolerant turtles, especially those such as *P. sinensis* that experience freezing in their winter environment, use the POS strategy to protect their tissues from oxidative damage induced during the transition from low oxygenation to reoxygenation (Giraud-Billoud et al., 2019; Hawkins and Storey, 2020). However, there are few eco-physiological studies on how wild soft-shelled turtles adapt to endure the challenges of hibernation beneath ice-sealed waters and the subsequent reoxygenation arousal phase. Zhang et al. (2017a, 2017b) studied gene expression and enzyme activities of the antioxidant system of *P. sinensis* hatchlings farmed in a natural pond during hibernation and arousal periods. This research highlighted the role of Nrf2 in regulating antioxidant enzyme transcript levels and enzyme activities in brain, liver, and heart. Notably, high levels of hepatic both Cu/Zn-SOD and Mn-SOD transcripts and cardiac GPX (GPX4) mRNA were found during hibernation (Zhang et al., 2017a). On the other hand, out of the 30 measured antioxidant defense parameters in the glutathione system, only the expression levels of four genes in the liver and one in the brain exhibited an increases during the hibernation of the hatchlings (Zhang et al., 2017b). However, interestingly, hepatic GR expression displayed a 2.3-fold increase. This field study provided inconclusive evidence regarding the presence of the POS strategy in the hibernating soft-shelled turtle hatchlings.

By contrast, for *P. sinensis* juveniles examined during hibernation and arousal stages, among the 63 antioxidant parameters examined (including both the Nrf2-regulated antioxidant enzymes and the glutathione system) in brain, liver, and kidney, the transcript levels of only three genes (cerebral GPX1, hepatic GPX4 and GR) showed significant increases during hibernation. By contrast, the majority of gene transcripts in these tissues experienced a decline (Tang et al., 2021). During hibernation of juvenile turtles and hatchlings, an intriguing observation arose when comparing gene expression. While the majority of genes were down-regulated, there was a consistent increase in hepatic GR gene expression. Furthermore, genes that exhibit upregulation during hibernation are primarily associated with the glutathione redox system. Overall, field studies suggest that only a limited number of molecular markers potentially linked to the POS strategy have been identified in the liver of hibernating *P. sinensis* hatchlings. However, this weak trend appears to diminish as the turtles mature. Still, the glutathione redox system appears to play a pivotal role in this turtle's antioxidant defense system.

4.3. *Toads from Paralakhemundi, India*

The common Asian toad, *Duttaphrynus melanostictus,* is found mainly in close proximity to human settlements in South and Southeast Asian countries (Van Dijk et al., 2004). This species is well-known for its docile and nocturnal behavior. With the objective of studying physiological responses under natural conditions, a research team conducted an evaluation of several specimens during the early rainy season (June–July), in a well-protected area of nearly 604 km^2 in Paralakhemundi, India. By early winter (October–November), the toads gradually enter a hibernation phase and, by mid-winter (December–January), getting buried in loose and moist soil at a depth of 35–40 cm. During this immobile state, the hibernating toads exhibits a thick grey-colored covering on the dorsal surface (Lin et al., 2011; Patnaik and Sahoo, 2021; Pratihar and Kumar, 2010; Sahoo and Patnaik, 2020). Throughout hibernation, animals remain inactive, resulting in observable metabolic depression (Patnaik and Sahoo, 2021; Pratihar and Kumar, 2010; Sahoo and Patnaik, 2020). Unlike other anuran species that endure hibernation in a hypoxic microhabitat, the common Asian toads utilize an aerobic metabolic depression while residing within a normoxic microhabitat (Patnaik and Sahoo, 2021). This is similar to the behavior of other toad and frog species such as *Scaphiopus couchi* (Seymour, 1973), *Pleuroderma diplolistris* (Moreira et al., 2021a) and

Proceratophrys cristicep (Moreira et al., 2020). These particularities have allowed researchers to differentiate between hypometabolic situations and evaluate whether the common Asian toad implements the POS strategy. During hibernation, toads decrease oxygen intake and depress their metabolic rate, generating higher RONS levels as indicated by elevated levels of LPO, PCO and an increase of the GSSG/GSH ratio in liver and brain (Patnaik and Sahoo, 2021). Furthermore, there is a simultaneous rise in the activity of antioxidant enzymes (SOD and CAT) and the non-enzymatic antioxidant, ascorbic acid in liver and brain (Patnaik and Sahoo, 2021; Sahoo and Patnaik, 2020). Likewise, Sahoo and Patnaik (2020) reported an increase in the levels of different antioxidants (CAT, SOD, GSH, ascorbic acid and uric acid) upon arousal from hibernation, when the toads restore their oxygen consumption and metabolism. Altogether, these antioxidant responses against oxidative stress in the common Asian toad during hibernation and arousal suggest the use of the POS strategy in nature by this species.

4.4. *Marine mollusks from Russia and Brazil*

Intertidal sites or zones represent distinctive coastal habitats characterized by daily fluctuations in environmental conditions that are driven by the regular ebb and flow of tides (i.e., the tidal cycle). Therefore, organisms residing in intertidal coastal regions confront multiple stressors, including temperature variations, physical stress induced by wave action and winds, salinity fluctuations, intense exposure to UV radiation, and deficiency of oxygen availability due to closing or retreat into shells (Cubillos et al., 2023; Freire et al., 2011b; Gostyukhina et al., 2023; Nie et al., 2020). Indeed, intertidal zones represent one of the most challenging habitats for organisms.

Natural experiments in these environments can reveal ecological and evolutionary changes that occur over long periods of time and influence the distribution of biota along the coast (Petraitis et al., 2008). Air exposure is an interesting stressor to study in the intertidal zone, since when the tide is low, gill-breathing organism such as gastropods, struggle to maintain aerobic metabolism due to their limited ability to efficiently acquire oxygen from the atmosphere (Larade and Storey, 2002). An interesting example is provided by *Brachidontes solisianus*, commonly known as the striped mussel or ribbed mussel of the *Mytilidae* family. Predominantly found along the Atlantic coasts of South America, specifically in regions such as Brazil, Argentina, and Uruguay, striped mussels typically inhabit intertidal and shallow subtidal zones. Like other bivalves, *B. solisianus* plays a crucial role as an important filter feeder, and is recognized for its remarkable ability to thrive under a wide range of environmental conditions, including temperature fluctuations, salinity variations, and different levels of water quality. This adaptability contributes to their successful colonization of a diverse range of coastal habitats. Recent studies conducted by Moreira and colleagues have shown the effect of tidal cycles on the redox metabolism of *B. solisianus* (Moreira et al., 2021b; Moreira et al., 2023b). These studies involved a comparative analysis of redox biochemical responses in mussels exposed to air or submerged under natural conditions over two consecutive days. Throughout the experiment, air and water temperature remained stable, ranging from 22.5 °C to 26 °C, and followed the same patterns during both days. The most significant environmental distinction between day 1 and day 2 was related to global solar radiation (GSR). Day 1 exhibited a cumulative GSR of 15,381 kJ/m^2, whereas day 2 had a cumulative GSR of only 5,489 kJ/m^2. Mussels were collected following a sequence of exposure to air in the early morning (7:30 AM), immersion during late morning and afternoon (8:45 AM-3:30 PM), and subsequent exposure to air in the late afternoon/evening (5:45 PM-9:25 PM) on both days. When comparing the mussels exposed to air with those underwater, no significant alterations in redox biomarkers were observed during the early morning emersion on both days. However, exposure to air for 4 h in the late afternoon/evening induced oxidative damage to proteins and lipids, as measured by the levels of TBARS (LPO marker) and PCO on Day 1. Moreover, this air exposure led to the elevation of reduced glutathione (GSH) resulting in a significant increase of ~60% in GSH levels of mussels that had previously experienced high GSR levels earlier in the day. Interestingly, on the subsequent day with significantly lower GSR, exposure to air under identical conditions (duration, time, and temperature) had no effect on any of the redox biomarkers. These findings suggest that air exposure under low-intensity solar radiation is insufficient to prompt a redox response in *B. solisianus* within its natural habitat (Moreira et al., 2023b). Instead, it appears that natural UV radiation, combined with air exposure, plays a pivotal role in triggering the POS response to the challenging event of tidal variation in this coastal species.

Another study regarding air exposure of littoral species was undertaken on periwinkles (*Littorina* sp.*)*. These gastropods inhabit rocky shores, and are typically found between the tidal low and high water environments and the littoral fringe (Johannesson, 2003). Various species of *Littorina* display adaptive plasticity in response to variation in oxygen availability and resistance to thermal stress in laboratory experiments (Davenport and Davenport, 2007; Lama et al., 2013; Melatunan et al., 2011). Istomina et al. (2013), provided an interesting illustration of how the tidal cycle affects redox balance in the Sitka periwinkle *Littorina sitkana* (formerly *L. kurila)*. This species is commonly found along the high intertidal shorelines of the Northern Pacific, including the Bering Sea, the Sea of Okhotsk, and the west coast of Canada and the U.S.A. (Yamazaki and Goshima, 2012). For the study, adult *L. sitkana* (2–3 years old) were collected in the intertidal zone of Alekseeva Bay (Peter the Great Bay, Russia). Environmental conditions were stable, with air and water temperatures of 22 °C and 19 °C, respectively. During low tide, mollusks were collected in the evening (at 8:00 PM) after being exposed to air for 2 h and then again at midnight after 6 h of hypoxia. When high tide occurred, the periwinkles were submerged in water, and sampling took place after 12 h submergence. The authors evaluated the activity of key antioxidant enzymes (SOD, CAT, GR), as well as low molecular weight antioxidants, like GSH. They also measured the levels of ROS production and malondialdehyde (MDA) in the hepatopancreas during low tide (representing functional hypoxia) and high tide (representing “reoxygenation animals”). After 2 h of air exposure, Istomina et al. (2013) observed no significant changes in the activity of antioxidant enzymes and GSH levels. However, they did note an increase in MDA levels that indicated LPO in the hepatopancreas (Istomina et al., 2013). Further air exposure for 6 h led to a notable 60% increase in CAT activity as compared to high tide animals, whereas MDA levels returned to baseline levels. Notably, 6 h exposure at low tide resulted in lower RONS levels in the hepatopancreas as compared to the group of animals that remained underwater for 12 h during high tide. These findings support the hypothesis that intertidal marine gastropods possess a reliable and responsive antioxidant system to combat oxidative stress caused by periodic exposure to air, that is reversed when snails are again submerged in water. Furthermore, the hepatopancreas of intertidal *L. sitkana* when submerged, exhibited higher levels of antioxidants and lower MDA levels as compared to the subtidal gastropod *Littorina squalida* (Istomina et al., 2013). These data correlate well with previous studies that support the conclusion that intertidal species (i.e. *Littorina mandschurica*) tend to have a more stress-resistant antioxidant system than their subtidal counterparts (i.e. *Tegula rustica*) (Istomina et al., 2011).

4.5. *Land snails from mainland regions and islands of Greece*

It is well known that under stressful conditions, snails enter a quiescent stage characterized by a marked reduction in metabolic rate and ATP turnover (Storey and Storey, 2004; Storey and Storey, 2010). Using the POS strategy, some gastropod species protect themselves against RONS production and oxidative stress when they resume full oxidative metabolism from a state of metabolic depression, such as estivation or hibernation (Giraud-Billoud et al., 2011; Giraud-Billoud et al., 2018; Hermes-Lima et al., 2015; Jiang et al., 2023; Nowakowska et al., 2014; Ramos-Vasconcelos and Hermes-Lima, 2003). This defense strategy involves, among others, modification of the expression of heat shock proteins (HSPs) to protect the proteome (Giraud-Billoud et al., 2013; Storey and Storey, 2023; Storey and Storey, 2011).

Land snails occupy a variety of habitats with differing thermal regimes, either along altitudinal or latitudinal gradients, and exhibit different physiological adaptations to their environment (Arad et al., 2010; Gaitán-Espitia et al., 2013; Köhler et al., 2009; Mizrahi et al., 2010; Staikou et al., 2017; Staikou, 1999). A recent study of two populations of *Helix lucorum* from different altitudes revealed different profiles of HSPs expression and activation of the MAPK cascade during winter hibernation and arousal. This strategy appears to be closely linked to the climatic conditions in their habitats and their biological cycle (Staikou et al., 2021). Specifically, individuals from the coastal population at low altitude seem to maintain high levels of Hsp70 throughout the entire year (Staikou et al., 2021), whereas, high-altitude snails showed lower constant levels of Hsp70 throughout the winter months, compared with summer. It is well known that protein synthesis is a highly energy-demanding process. Consequently, maintaining high levels of HSPs may serve as an energy-saving protective strategy during hibernation and winter hypometabolism. On the other hand, the early activation of ATP turnover before snails enter hypometabolism may fulfill the energy requirements for HSPs synthesis (Staikou et al., 2024). Additionally, both populations differed in their preparation for arousal. The mountain population initiated this process in February (<0 °C), whereas the coastal population began the process two months earlier. Similarly, snails from the coastal population resumed activity in early March, whereas those from the mountain population did so in April. In both cases, snails faced the challenges posed by the higher production of RONS, resulting from intense reoxygenation-like conditions (Hermes-Lima et al., 2015). Likewise, studies on reciprocally transplanted populations of land snails along an altitudinal gradient have shown different seasonal role for Hsp70, suggesting a reduced demand for molecular chaperones in individuals at lower altitude (Staikou et al., 2021). These data are in line with the hypothesis that ectotherms that occupy extreme and/or unpredictable environments maintain constitutively high levels of HSPs, thus allowing for an immediate response to potentially harmful effects of heat stress.

Research conducted on three land snail species, *Cornu aspersum, Eobania vermiculata* and *Helix lucorum*, along a latitudinal gradient, from northern Greece to the island of Crete (in the southernmost part of the country), has unveiled adaptive antioxidant defense strategies and different heat shock responses during aestivation (Staikou et al., 2024). Species from all populations exhibited increased antioxidant enzyme activities (SOD, CAT and GR) and a significant increase in Hsp70 and Hsp90 expression within the hepatopancreas and foot muscle during the first fifteen days of exposure to elevated temperatures. After that, levels dropped to the values observed during active periods. Furthermore, the activity of SOD and the levels of HSPs were higher in snails from two sample sites (the northern site Axios and the island of Syros, with lower summer temperatures and higher humidity), whereas CAT and GR activities were higher in snails from the Crete populations (naturally exposed to higher temperatures and lower rain intensity). Statistical analyses revealed differences among species in the degree to which antioxidant enzymes and HSPs were induced. These changes may be associated with the ability of these snails to endure harsh environmental conditions and thrive in stressful habitats. Moreover, the results indicated that the induction of HSPs and antioxidant enzyme activities was tissue specific (Staikou et al., 2024). These results also suggest that, in land snails, both antioxidants and HSPs work in concert in the mechanism of the POS strategy.

Taken together with previously published data (Michaelidis and Pardalidis, 1994; Staikou et al., 2021; Staikou et al., 2017), these findings depict a dynamic interplay between metabolic reorganization and antioxidant defense. This interplay serves not only to maintain protein homeostasis but also to regulate water balance during long term estivation of terrestrial snails.

4.6. Snakes and frogs from Vojvodina, Serbia

In Europe's temperate zone the herpetofauna, that include amphibians and reptiles, face harsh winter conditions characterized by low temperatures and lack of food. In the wild, many of these animals survive by hibernating in secure locations where they are (Ultsch, 1989). Despite hibernation being an integral part of their life cycle, there is a notable lack of studies on this phenomenon in these groups, especially within natural populations (Giraud-Billoud et al., 2019). Some investigations have been conducted on semi-aquatic snake species (*Natrix natrix* and *N. tessellata*) and frog species (*Pelophylax ridibundus* and *P. esculentus*). These studies have focused on the biochemical and physiological changes induced during the preparation for hibernation and the arousal after hibernation (Gavrić et al., 2017; Gavric et al., 2015; Prokić et al., 2017).

Snakes belonging to the genus *Natrix* are wide-ranging, long-lived predators that occupy a place at the top of the food chain and play crucial roles in both terrestrial and aquatic ecosystems (Guicking et al., 2006). During winter months these animals hibernate in underground holes (Hermes-Lima and Zenteno-Savin, 2002; Ultsch, 1989). Some studies have been conducted on *Natrix natrix* and *N. tessellata* females from the Nature Reserve Obedska Bara, the oldest protected area in Serbia (Gavrić et al., 2017; Gavric et al., 2015). Individuals were captured during the pre-hibernation period (late October) and after arousal during the post-hibernation period (early April). In comparison to pre-hibernation, *N. natrix* snakes showed reduced blood LPO levels during arousal, indicating their capacity to maintain balanced oxidative stress levels in the post-hibernation period (Gavric et al., 2015). However, the findings regarding antioxidant defenses in this species are inconclusive, as enzymatic activity may have been influenced by the presence of metals in the environment. Only CAT and GR demonstrated increased activity in red blood cells during pre-hibernation (Gavric et al., 2015). In contrast, the liver of *N. tessellata* exhibited the opposite pattern with a significant increase in LPO levels in post-hibernation animals, despite significantly higher levels of SOD, GST activity, and GSH concentration in pre-hibernating snakes (Gavrić et al., 2017). Conversely, augmented GPX and GR activities during post-hibernation could have prevented a higher increase in LPO in *N. tessellata*.

The same authors also explored the POS hypothesis in two European frog species within the *Pelophylax esculentus* complex, *P. ridibundus* and *P. esculentus* (Prokić et al., 2017). Both species are semiaquatic, hibernate in winter and are distributed in freshwater habitats throughout Europe (Bagnyukova et al., 2003; Falfushinska et al., 2008). However, variations in life history traits, such as metabolic rates, food preferences and overwintering modifications, may influence their response to arousal-induced stress. *Pelophylax ridibundus* hibernates underground whereas *P. esculentus* hibernates both in the water or underground (Holenweg and Reyer, 2000). Adult individuals were collected during the pre- and post-hibernation periods from two localities with different habitat characteristics; the Danube-Tisza-Danube canal (affected by intense anthropogenic activity in the area) and the River Ponjavica Nature Park, a pristine site (Prokić et al., 2016). Regardless of water quality, both frog species exhibited similar responses to arousal. The highest antioxidant response was reported in skin and muscle during post-hibernation. The glutathione system (GSH, GR and GST) played a central role in the antioxidant defense system in response to arousal-induced RONS production in the skin whereas SOD and CAT were highest in muscles. These differences can be attributed to the distinct oxygen requirements, functions, vascularity, and metabolism of the different tissues (Prokić et al., 2017).

Studying the biological and physiological cellular responses to arousal stress by snakes and frogs from natural populations, the phenomenon of POS can provide new insights into the hibernation process in the wild. Additionally, from a physiological perspective, understanding the hibernation process in European reptile and amphibian species has great importance for ecology and conservation studies, since these groups are among the most globally threatened vertebrates.

4.7. Frogs of the Caatinga, Brazil

Different physiological strategies are commonly observed among frogs inhabiting extreme environments in the northeastern region of Brazil, especially in the Caatinga biome. This region is characterized by unpredictable rainfall, in which a diversity of frog species have adapted their behavior and physiological strategies to thrive in the harsh and arid conditions. Based on their activity patterns, the frogs in this area can be divided into two groups: continuous or seasonal. The first group, known as “persistent species”, includes notable examples like *Rhinella granulosa* and *R. jimi*. These species are active throughout the year and exhibit behaviors such as strict nocturnal foraging. They also possess thermal tolerance plasticity, including variations in Krebs cycle enzymes, such as citrate synthase (CS) (Navas et al., 2007). These adaptations enable persistent species to remain active and survive in the challenging environmental conditions in the Caatinga biome (Madelaire et al., 2017; Navas et al., 2007). By contrast, the second group, referred to as “nap and wait species”, displays extreme season-dependent activity (Carvalho et al., 2010; Madelaire et al., 2020; Navas et al., 2004). Frogs like *Pleurodema diplolister* and *Proceratophrys cristiceps* have evolved a strategy for the unpredictable and arid dry season. They engage in estivation by burying themselves in drying riverbeds and remain there until the onset of the rainy season (Carvalho et al., 2010; Jared et al., 2020). This seasonal behavior allows them to conserve energy and survive adverse conditions until more favorable environmental conditions return. Whereas both species undergo an estivation period, there are notable differences in their physiological strategies that are set to counteract deleterious effects. For example, despite estivating, *P. diplolister* maintains a higher resting aerobic metabolic rate compared to *P. cristiceps* (Pereira, 2016). Moreover, their responses to external stimuli during the dry season also differ. When disturbed, estivating *P. diplolister* promptly resumes locomotor activity as an escape response (Jared et al., 2020; Pereira, 2016), whereas *P. cristiceps* remains relatively inactive and takes a much longer time to exhibit its initial response (Pereira, 2016). These disparities in the locomotion response contribute to the distribution of resources in the anuran community during the transition between the dry and wet season. In this context, species like *P. diplolister* and *P. cristiceps* take advantage of resource availability during the wet season in a scaled manner in time, owing to interspecific differences in morphological, metabolic, and physiological, adaptations associated with locomotion, as they emerge from their underground shelters.

Oxidative damage, particularly during reactivation of metabolic processes following a period of estivation, poses a significant risk for “nap and wait species” at the end of the dry season. It can impair various physiological functions, including the ability of frogs to emerge from their shelter in the dry riverbeds. Therefore, both *P. cristiceps* and *P. diplolister* have been utilized as models to study the modulation of redox metabolism in skeletal muscle of estivating anurans under natural conditions in the wild. Researchers aimed to gain insights into how these species cope with oxidative stress and maintain cellular balance as they transition from estivation to activity in their challenging environment (Moreira et al., 2021a; Moreira et al., 2020). To study this phenomenon, adult specimens of *P. diplolister* and *P. cristiceps* were collected during four expeditions spanning 2005 to 2012. During the rainy season, active animals were collected on the surface of sandy soil. Estivating animals, on the other hand, were collected during the dry season by excavating dry riverbeds and locating animals buried in the soil. In this region, surface temperatures can exceed 53 °C during the hottest hours, whereas soil temperature at 30 cm below the surface drops to ∼30 °C and remains stable down to a depth of 100 cm. Male specimens of *P. cristiceps* were collected during two specific periods: (i) in April 2007, when active frogs were found in water ponds and were collected after a cumulative total precipitation of 478 mm in the previous three months, and (ii) in August 2012, when six estivating frogs were located by digging dry riverbeds and collected after a dry period of 5 months with a total precipitation of only 14 mm. Similarly, male specimens of *P. diplolister* were collected during two distinct periods: (i) in December 2005, when active *P. diplolister* frogs were located in water ponds and were collected in an atypical season when the cumulative total precipitation of the previous 5 months was only 6.7 mm, and (ii) in November 2012, when estivating frogs were found by excavating dry riverbeds and were collected after a dry period of 8 months with a total precipitation of 14.4 mm.

The studies revealed that metabolic state (active versus estivating) had a significant impact on key metabolic enzymes, namely CS and isocitrate dehydrogenase (ICDH), in both species. The activities of these enzymes were found to be 36% (CS for both species), 25% (ICDH in *P. cristiceps*), and 48% (ICDH in *P. diplolister*) lower during estivation as compared to the active period. Furthermore, activities of the peroxide-detoxifying enzymes GPX and CAT were also significantly influenced by the metabolic state, but in the opposite direction. During estivation, their activities were 48%–78% higher compared to during the rainy season (Fig. 3). Activities of SOD and GST were similar between estivating and active animals for both species (Moreira et al., 2021a; Moreira et al., 2020). In the case of *P. cristiceps*, levels of GSH were higher in the skeletal muscle of estivating frogs than in active frogs (Moreira et al., 2020). Although endogenous antioxidants were activated, there were no significant differences in the levels of oxidative damage to proteins between animals collected during the dry season and those collected in the rainy season for either species (Fig. 3).

In April 2018 another group of eight active *P. cristiceps* was collected in a transitional belt region between the Caatinga and Atlantic rainforest ecosystems (known as “Escola Agrícola de Jundiaí”). This area is characterized by a high presence of water bodies and streams, particularly during the rainy season. Notably, there were no discernible disparities in

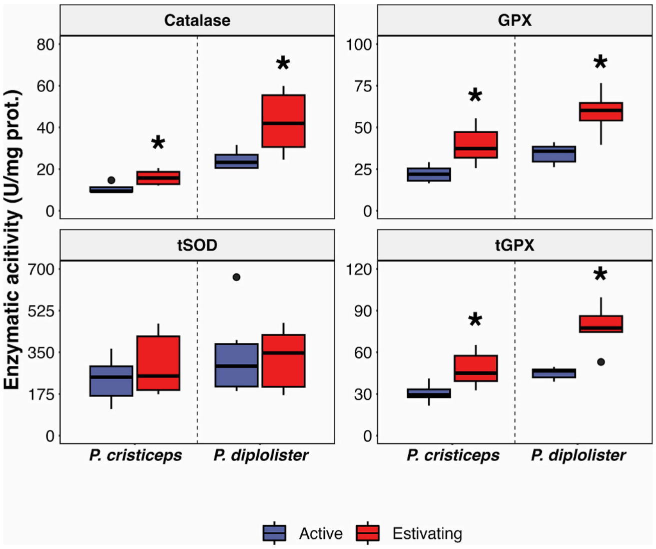


Fig. 3. Antioxidant enzymes activities of the skeletal muscle of *Pleurodema diplolister* and *Proceratophrys cristiceps* living in the Caatinga, collected during the rainy (active) and dry (estivating) seasons. The data are shown as boxplots, with the central box representing the interquartile range. Whiskers extend from the box to show the range of the data, excluding outliers. Outliers are depicted as individual dots beyond the whiskers, indicating data points that differ significantly from the rest of the sample. Asterisk indicates significant difference between active and estivating groups. Data obtained from Moreira et al., 2020, 2021a. The activities of GPX and tGPX are reported in milli-units per milligram of protein.

the measured biochemical parameters of the skeletal muscle between frogs collected from the arid Caatinga region and those collected from the Caatinga-Atlantic rainforest transition zone (Moreira et al., 2020).

Our results indicate that both, *P. diplolister* and *P. cristiceps* exhibit characteristics of the POS phenotype under conditions of aerobic metabolic depression. This adaptation might offer an advantage for coping with the seasonal conditions of the Caatinga and could be crucial throughout multiple seasons. Notably, there were species-specific differences, with *P. diplolister*, being more metabolically active and responsive, showing higher activity of both metabolic and antioxidant enzymes (Fig. 3) (Moreira et al., 2021a; Moreira et al., 2020).

4.8. *Fish from Araguaia-Tocantins, Brazil*

Seasons in the Brazilian Cerrado, a savannah-like biome, are well defined but do not follow the prototypic four seasons. Instead, this region experiences six months of rain (from October to March), and six months of drought (from April to September). Notably, the dry season is characterized by severe water scarcity (Ferreira et al., 2007). During this period, water flow may decrease or cease entirely, leading to the formation of isolated pools. These pools become ecological traps for aquatic life, including fish species (Chapman and Kramer, 1991; Matthews and Marsh-Matthews, 2003; Silva and Teresa, 2017). As water levels in these isolated pools of the Brazilian Cerrado diminish, critical changes in the environmental factors occur. Firstly, there is a reduction in oxygen availability, leading to hypoxic conditions. Concurrently, water temperatures rise, and there is an accumulation of toxic nitrogen compounds (Matthews and Marsh-Matthews, 2003). These altered conditions can adversely impact the normal metabolic processes and survival of aquatic animals. Aquatic organisms in these pools are likely to undergo increased oxidative stress compared to their counterparts in areas of the Cerrado where water flow is maintained. However, the precise biological implications of the challenges imposed by the droughts remain poorly understood.

To better understand the antioxidant responses of fish to oxidative stress under varying environmental conditions, Ondei et al. (2020) used, as a study model, *Astyanax elachylepis*, an indigenous fish of the Tocantins-Araguaia river basin (da Silva Coelho et al., 2020). The research aimed to investigate the impact of spatial and temporal environmental variations on fish physiology. Fish were collected from two distinct locations, one with intermittent water flow and the other with perennial flow, during both the dry (September) and rainy (February) seasons. This approach resulted in four distinct groups for comparative analysis: intermittent-dry, intermittent-rainy, perennial-dry, and perennial-rainy. For the study, liver and gill tissues were excised immediately upon collection, promptly frozen in liquid nitrogen, and preserved at −80 °C until further analysis. The findings revealed differences in MDA levels in the gills and liver tissues across the groups. In gills, MDA levels were significantly higher in the intermittent-dry group than those in the intermittent-rainy group. Malondialdehyde levels in the intermittent-dry animals were also higher when compared to levels in perennial-dry animals. However, MDA levels in perennial-dry and perennial-rainy animals remained unchanged. In the liver, MDA levels varied according to the season, being higher during the dry season compared to the rainy season, regardless of the location where the fish were collected (Ondei et al., 2020).

The study further investigated the enzymatic activities of several antioxidants in *A. elachylepis* under the four environmental conditions. The enzymes examined were CAT, glucose-6-phosphate dehydrogenase (G6PDH), GR, and GPX. The enzymatic activities of CAT, G6PDH and GR in gills, as well as GPX in liver, were higher during the dry season than those recorded during the rainy season, irrespective of the water flow conditions at the collection sites. However, the collection site specifically influenced the activity of GPX and GST in the gills, as well as CAT and GR in the liver (Ondei et al., 2020). In the gills, both the intermittent-dry and perennial-dry groups exhibited higher GPX activity than their respective rainy season counterparts. Notably, GPX activity was higher in the gills of intermittent-dry fish compared to perennial-dry fish. In contrast, GST enzyme activity showed a significant change only in perennial-dry animals, where it was higher than in perennial-rainy animals. In the liver, a distinct pattern emerged. CAT activity was elevated in perennial-dry animals compared to perennial-rainy animals, but there was no significant change between the intermittent-dry and intermittent-rainy groups. GR activity, on the other hand, was higher in animals collected during the dry season, with intermittent-dry animals showing higher levels than perennial-dry animals. These results collectively suggest that *A. elachylepis* experiences increased oxidative stress during the dry season, particularly in areas with disrupted water flow (Ondei et al., 2020).

The study's findings indicate that redox metabolism in *A. elachylepis* primarily exhibit a seasonal response, largely independent of water flow conditions. This pattern is characterized by an increase in antioxidant enzyme activities during the dry season compared to the rainy season, aligning with elevated MDA levels observed during the dry season. The authors hypothesized that the fish's response would be similar in both stream types during the rainy season due to comparable water flow conditions. However, they anticipated distinct responses in the dry season, particularly in intermittent streams where water flow is interrupted, resulting in more challenging conditions for fish in isolated pools. This hypothesis was corroborated by the observed increase in GPX enzyme activity in fish from intermittent streams during the dry season, compared to those in perennial streams. Increased GPX activity was associated with low oxygen availability. Dissolved O_2 was below 2 mg/mL in pools of intermittent streams during the dry season, while O_2 levels remained normoxic in the other three sampling conditions. This suggests that the antioxidant response in fish during extreme drought conditions might be influenced by oxygen availability in the pools, which aligns with the predictions of the POS theory. Considering that changes in stream water flow in the Cerrado predictably occur during each dry season, could antioxidant responses start occurring before water flow interruption as a preparation for the harsh environmental conditions resulting from disrupted water flow? This is an intriguing question that remains open, inviting researchers to return to the field and uncover the nuances of these physiological adaptations.

5. Other studies in natural environments that may evidence the POS strategy

Laboratory experimentation in animals is intrinsically limited by different factors, and any observation can hardly be generalized without taking into account what occurs under natural conditions. In the pursuit to understand complex ecophysiological processes, especially those involving an evolutionary component, we require working across broad scales of space and time. Such a broad approach is not straightforward, as the interpretation of results becomes challenging when experimental conditions are not controlled. We may encounter intrinsic sources of variation (e.g. life cycle of the animal), as well as environmental variation that may include seasonal modifications in water or air temperature, presence of pollutants (organic or metallic) and eutrophication of water bodies (Le Moal et al., 2019). However, working in nature presents the challenge of accessing a complex combination of stressors in the field that truly reveals, from an organismic perspective, how animals adapt to their natural environment.

The examples detailed above illustrate the difficulty that is often encountered when conducting experiments with animals exposed to real environmental conditions. This is clearly supported by the fact that in the over 100 animal species in which the POS phenomenon has been described, the majority of studies have been conducted under controlled laboratory conditions. In contrast, studies under field or natural conditions have only been carried out in 32 animal species (excluding mammals), as detailed in Table 2, and the number of publications on this matter is much lower. Even though temperature has not been included

Table 2
Record of species that use the preparation for oxidative stress strategy in studies carried out in field and natural conditions (mammals have been excluded from this analysis, given the complexities of these organisms).

Phylum	Class	Species	Stressor*	Experimental condition	Antioxidant response to stress	Reference
Arthropoda	Copepoda	***Acartia bifilosa***	↑ temperature	Natural condition	↑ GST activity (whole soft tissue)	Glippa et al., 2018
	Malacostraca	***Euphausia eximia***	Hypoxia, temperature (water depth)	Natural condition	↑ GPX activity (whole soft tissue)	Tremblay et al., 2010
		Litopenaeus vannamei	Hypoxia	Field study	↑ cMnSOD expression (hepatopancreas), ↑ cMnSOD-mMnSOD-CAT expression (gill)	González-Ruiz et al., 2023a
		Nematoscelis difficilis	Hypoxia, temperature (water depth)	Natural condition	↑ GST activity (whole soft tissue)	Tremblay et al., 2010
		Nyctiphanes simplex	Hypoxia, temperature (water depth)	Natural condition	↑ CAT-SOD-GST-GR-GPX activity (whole soft tissue)	Tremblay et al., 2010
Mollusca	Gastropoda	***Cornu aspersum***	↑ temperature (estivation)	Natural condition	↑ SOD-CAT-GR activity (hepatopancreas and foot)	Staikou et al., 2024
		Eobania vermiculata	↑ temperature (estivation)	Natural condition	↑ SOD-CAT-GR activity (hepatopancreas and foot)	Staikou et al., 2024
		Helix lucorum	↑ temperature (estivation)	Natural condition	↑ SOD-CAT-GR activity (hepatopancreas and foot)	Staikou et al., 2024
		Helix pomatia	↓ temperature (winter torpor)	Field study	↑ CAT activity (kidney and hepatopancreas), ↑ GST activity (hepatopancreas)	Nowakowska et al., 2009
			Hypoxia (estivation)	Natural condition	↑ GST activity (hepatopancreas)	Nowakowska et al., 2010
		Littorina kurila	Aerial exposure (intertidal)	Natural condition	↑ CAT activity (hepatopancreas)	Istomina et al., 2013
		Nerita oryzarum	↑ Temperature (water)	Field study**	↑ SOD-CAT-GPX-GST activity (whole soft tissue)	Ambekar et al., 2023
		Pila globosa	↑ Temperature, pH and salinity (water depletion)	Natural condition	↑ SOD-CAT-GR-GPX-GST activity, ↑Ascorbic acid concentration (foot muscle)	Pati et al., 2023; Panda et al., 2022
	Bivalvia	***Brachidontes solisianus***	Aerial exposure (intertidal)	Natural condition	↑ GSH concentration (whole soft tissue)	Moreira et al., 2021b
		Mytilus edulis	↑ temperature, aerial exposure (intertidal)	Natural condition	↑ SOD-CAT-GPX-GST activity (gill and digestive gland)	Letendre et al., 2009
		Ruditapes decusatus	↑ temperature, ↑ salinity, hypoxia (water)	Natural condition	↑ SOD-CAT activity, ↓GSH concentration (gill)	Maisano et al., 2016
		Ruditapes laeta	↑ temperature, ↑ salinity, hypoxia (water)	Natural condition	↑ SOD-CAT activity, ↓GSH concentration (gill)	Maisano et al., 2016
Echinodermata	Holothuroidea	***Apostichopus japonicas***	↑ temperature (estivation)	Field study	↑ SOD-CAT activity (coelomic fluid)	Fangyu et al., 2011
	Echinoidea	***Tripneustes gratilla***	UV-B exposure	Field study	↑ SOD-CAT-GPX-GR activity, ↓GSH concentration (larvae extract)	Lister et al., 2010
Chordata	Actinopterygii	***Astyanax elachylepis***	Hypoxia (drought)	Natural condition	↑ GST-GPX activity (gill), ↑ CAT-GR activity (liver)	Ondei et al., 2020
	Teleostei	***Benthosema panamense***	Hypoxia, temperature (water depth)	Natural condition	↑ GST activity (whole soft tissue)	Lopes et al., 2013
		Gymnocephalus cernua	Hypoxia and temperature (water)	Natural condition	↑ SOD-2 expression (heart)	Tiedke et al., 2014
		Triphoturus mexicanus	Hypoxia, temperature (water depth)	Natural condition	↑ CAT-GST activity (whole soft tissue)	Lopes et al., 2013
	Amphibia	***Bufo melanostictus***	↓ temperature (season)	Natural condition	↑ CAT activity (gonad and liver), ↑ GSH concentration (liver)	Samanta and Paital, 2016
		Duttaphrynus melanostictus	↓ temperature (hibernation)	Natural condition	↑ CAT activity and Asc.Acid concentration (liver-brain)	Sahoo and Patnaik, 2020
		Nanorana parkeri	Hypoxia, ↓ temperature, UV (high altitude)	Natural condition	↑ SOD-CAT-GPX-GST activity and T-AOC capacity (liver)	Niu et al., 2022
		Pelophylax esculentus	↓ temperature (hibernation)	Natural condition	↑ SOD activity (skin), ↑ GSH activity (muscle-skin)	Prokić et al., 2017
		Pleurodema diplolister	Hypoxia (estivation)	Natural condition	↑ CAT-GPX activity (skeletal muscle)	Moreira et al., 2021a
		Proceratophrys cristiceps	Hypoxia (estivation)	Natural condition	↑ CAT-GPX activity and GSH concentration (muscle)	Moreira et al., 2020
	Sauropsida	***Natrix tessellata***	↓ temperature (hibernation)	Natural condition	↑ SOD-GST-GSH activity (liver)	Gavrić et al., 2017
		Pelodiscus sinensis	↓ temperature (hibernation)	Field study	↑ SOD-GPX expression (heart)	Zhang et al., 2017a
		Uromastyx philbyi	↓ temperature (hibernation)	Natural condition	↑ GSH concentration and GPX-GR-SOD-CAT activity (liver, brown adipose tissue and serum), ↑ GPX-GR-SOD-CAT activity (brain)	Afifi and Alkaladi, 2014
	Reptilia	***Phrynocephalus vlangalii***	Hypoxia, ↓ temperature, UV (high altitude)	Natural condition	↑ CAT activity (brain)	Zhang et al., 2015b

[*] The stressors on the list are those that the authors of the publications have identified at the sampling site.

[**] The animals were collected around Tarapur nuclear power plant, from water heated by plant effluents (thermal impact).

as a typical POS inducer in animals (see Table 1), we added in Table 2 (as well as in Section 4.5) some references showing the activation of antioxidant defenses prompted by increases in temperature in nature.

A couple of weeks before the submission of the present article, a particularly interesting study was published by Dr. Gloria Yepiz-Plascencia's research group (González-Ruiz et al., 2023a). They have been examining, the response of *Litopenaeus vannamei,* the white shrimp, to environmental changes through controlled laboratory experiments for over a decade (González-Ruiz et al., 2023b; Soñanez-Organis et al., 2012; Trasviña-Arenas et al., 2013). Moreover, they recently investigated the redox response of L. *vannamei* to semi-natural fluctuations in oxygen and temperature in a pond used for shrimp farming, located at the coast of Hermosillo, Sonora, Mexico. The animals were collected at four different time-points, T1 through T4, which corresponded to expected stress conditions: highest temperature (T1, collection at 5:45 PM, 33.5 °C, DO 8.6 mg O_2/L), recovery from high temperature (T2 at 10 PM, 31 °C, DO 5.8 mg O_2/L), lowest dissolved oxygen (T3 at 4:50 AM, 30.3 °C, DO 3.7 mg O_2/L), and reoxygenation (T4 at 7:50 AM, 30 °C, DO 4.8 mg O_2/L). The animals in the T2 group were considered normoxic controls. The researchers followed the philosophy of a natural experiment, as described by Diamond (1986). They measured the gene expression levels of CAT and cytosolic and mitochondrial SOD (cMnSOD and mMnSOD), as well as CAT and total SOD enzyme activities. Higher levels of cMnSOD mRNA in the hepatopancreas and gills, as well as catalase mRNA in gills, were found in shrimps under hypoxia (T3) and reoxygenation (T4). The activities of SOD and CAT in gills remained unchanged in all animal groups, while both enzyme activities decreased in the hepatopancreas in T1 and T4. Even though the actual antioxidants were not increased during hypoxia/reoxygenation time-points, the activation of mRNA expression points to an enhanced response of the organisms towards the management of oxidative stress. Moreover, what is relevant herein are not solely the specific findings, but rather the methodology employed in conducting the experiment: the abiotic variables were left undisturbed, thereby not being manipulated by the researchers (González-Ruiz et al., 2023a).

It should be considered that tackling the challenge of conducting natural experiments allows for interpreting physiological adaptation from a broader perspective. While they indeed add complexity to the understanding of the involved phenomena, the benefits for comparative physiology and the knowledge of adaptive responses to stress are substantial. Following Table 2, which resumes POS responses in animal species under field and natural experiments, in the next section, we discuss views both in favor of and against testing hypotheses in experiments where the researcher has greater or lesser influence on the modification of variables.

6. Laboratory, field and natural experiments: The drawback of pseudo-replicas and appropriate controls

In traditional scientific research, experiments involve the deliberate manipulation of an independent variable to observe its effects on dependent variables. For instance, experiments, as detailed by Bunge (2018), offer researchers a systematic methodology to enhance the knowledge about the interplay between the environment of living organisms and their physiological and behavioral responses. When scientific research is conducted with living organisms as the unit of analysis, they may be chosen randomly or by other methods, which ensures that all individuals of a population have an equal chance of being included in the assays. Random selection of unit of analysis should be made in laboratory experiments, as well in the other forms of experimentation (see below), to avoid sampling bias.

Ecology and ecophysiology distinguish three types of experiments: laboratory experiments, in which the intentional alteration of the independent variable is carried out by the researcher under controlled laboratory conditions to emulate natural settings; field experiments involve the manipulation of the independent variable in outdoor settings, allowing the researcher to approximate the experiment to natural conditions while still controlling and/or isolating the variable; and natural experiments, on the other hand, entail changes in the independent variable that occur spontaneously due to natural processes, without any intentional manipulation by the researcher; in other words, the independent variable is a result of natural occurrences (Diamond, 1986).

Scientific investigation, in any of these methodological approaches, raises numerous questions when observations are generalized. Therefore, it is necessary to primarily identify the relative strengths and limitations of the three possibilities (Fig. 4) (Schoener, 1982; Schoener and Schoener, 1983; Schoener and Toft, 1983). We must consider that laboratory experiments provide the advantage of controlling independent variables (although theoretically), whereas field and natural experiments, reliant on various species and environmental factors, tend to exhibit greater realism compared to their laboratory counterparts. Furthermore, some species can only be studied using one type of experiment, while others can be studied using two or more types of these experiments (Diamond, 1986; Spicer, 2014). The strengths and weakness of the three different experimental approaches are further detailed in Fig. 4, based on the remarks of Spicer (2014). Moreover, "*to understand 'how animals work' in the wild demands that the ecophysiologist draw on the strengths and capabilities of a number of different disciplines encouraging collaboration*" (Spicer, 2014).

Although the POS theory is firmly established in animals that have been studied under controlled laboratory conditions, its applicability to wild animals is still in its early stages, with studies confined to specific taxonomic groups. To evidence the use of the POS strategy in animals in their habitats, a synergistic approach is ideal, which involves both laboratory studies (with a biochemical and molecular approach), and the use of field and natural experiments, a common practice in ecology. Unlike laboratory experiments, studying animals in the wild can reveal ecological and evolutionary processes that operate over long periods of time and large areas. In these cases, researchers are obliged to clearly identify potential elements that may constitute a confounding factor, such as unidentified environmental factors (e.g. predation, parasitism or an unidentified nutrient), or another conditioning aspect (e.g. presence

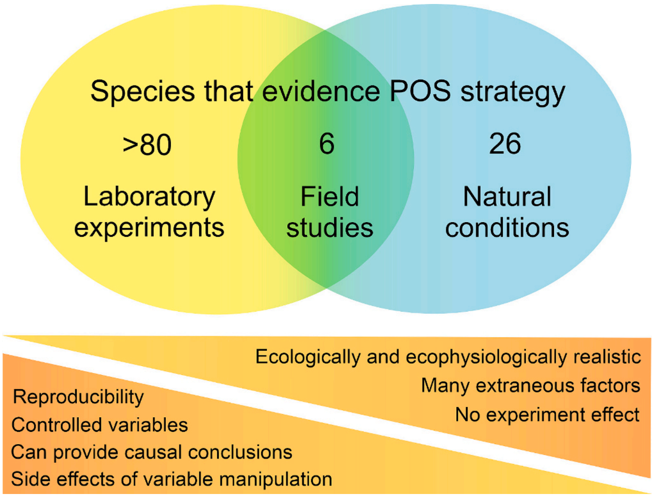


Fig. 4. Number of animal species currently described employing the preparation for oxidative stress (POS) strategy to tolerate hypometabolic conditions, studied under different experimental settings. Strengths and weaknesses of different types of experiments employed in the field of ecophysiology (Adapted from Spicer, 2014).

of contaminants or another environmental stressor) which could affect the experiment. In natural settings, multiple environmental factors may influence physiological responses, necessitating an analysis of potential interferences and cross tolerances by researchers (Rodgers and Gomez Isaza, 2023). Moreover, it is even possible that the results of laboratory experiments differ from the results obtained in the wild, particularly in experiments involving endocrinology and behavior (Calisi and Bentley, 2009)

Most biochemists and molecular biologists primarily focus on laboratory-based bench work, prioritizing it over natural experiments, which sacrifice all manipulative control of variables. Several reasons account for this preference. Experiments in these disciplines require rigorous control by researchers to ensure the precision of the nominal treatment. To understand the mechanisms governing life and cellular processes, researchers must initially identify independent variables that can elucidate theoretical proposals or concepts before applying a nominal treatment. Once the treatment is applied, unintended effects that may arise can potentially impact the data. To address this problem, researchers may introduce multiple appropriate controls that reduce the error of the treatment's effect. An empirical investigation using controlled experiments could help maintain the independence of the variable.

The importance of experimental design in field studies and animal behavior has been intensely discussed, due to its impact on the reproducibility and the construction of knowledge (Hurlbert, 1984, 2004). The use of observational investigations through field experiments may pose certain statistical and/or methodological challenges due to the difficulty of establishing a clear cause-and-effect relationship (Schank and Koehnle, 2009). Maintaining the independence of a variable is crucial for applying inferential statistics. However, in many cases, a population of the studied animal species (the observational unit) may be influenced by various concomitant environmental fluctuations, and the physiological response can depend on this correlation of stressors, leading to a conceptual loss of independence (Rodgers and Gomez Isaza, 2023). Researchers often attempt to address this issue by seeking experimental controls, but this isn't always feasible. It is crucial to detect false or misleading effects when engaging in natural experiments. For example, Moreira et al. (2023b) reported POS in nature in mussels under the simultaneous influence of two stressors: UV radiation and aerial exposure. In this case, the POS response happened under conditions of high solar radiation (while mussels were with open valves underwater) preceding a few hours of aerial exposure (during low tide); daily temperature changes had no effect (Moreira et al., 2023b). Possibly, if this experiment was conducted in laboratory settings using only the aerial exposure factor as an independent variable, the POS-response would have been missed.

Another issue arises when experimentally defining the unit of analysis. Natural and field studies conducted beyond the individual level must grapple with the issue of pseudoreplication (Hurlbert, 1984). The POS theory focuses on exploring cellular/molecular (causal) mechanisms, suggesting that the studied phenomena occur mainly spatiotemporally below the individual level. Consequently, the tissues or cells under study represent the "observational unit", while the individual animal constitutes the "experimental unit" despite the spatial and temporal proximity within which animals exist. It can be argued that selecting the appropriate experimental and observational units depends on the specific question we aim to address. However, the issue of pseudoreplication is always present as data points are often treated as independent biological estimates when in fact, they are technical replicates (Eisner, 2021). Furthermore, it is always advisable to recognize a possible pseudoreplication event at the experimental level (Monteiro et al., 2017). As a final consideration, defining an individual in colonial animals such as sponges and corals becomes challenging. One approach to address this issue is to collect samples at a significant distance from the place that researchers made the laboratory assays. Nevertheless, even in doing so, it remains impossible to fully replicate the natural conditions that occurred during the sampling process.

In the last two decades, the theoretical foundation of the POS theory has been significantly developed through laboratory experiments utilizing numerous control treatments, aiming to ensure (to the extent possible) the independence of variables under investigation. However, field experiments, while helpful in identifying POS-positive animals, often lack controls and encounter challenges in visualizing various interacting factors (such as hypoxia, desiccation, and UV radiation), which may be in turn crucial for activating the POS strategy. The most effective approach to address these issues is to combine two types of experiments. Firstly, conduct a natural experiment to observe all stressors simultaneously. After identifying the stressors most likely to cause the observed biochemical/physiological changes, proceed with laboratory experiments to control each 'suspect' individually. This methodology facilitates a clearer definition of cause-and-effect relationships between each variable and metabolic responses. Consequently, it becomes more feasible to determine cross-tolerances among the stressors.

Another approach that deserves attention is the exploration of possible effect of among-individual variation in the manifestation of POS phenotype. The influence of factors such as sex, age, and physiological condition in POS have been underexplored to some degree. Considering that POS requires resource allocation in antioxidant defenses, its expression might not be uniform across individuals but could vary significantly in response to these factors. Exploring the interplay between these factors and POS might provide valuable insights (Costantini, 2019). Sex-specific differences, for instance, could lead to divergent expression patterns of the POS, potentially due to differing reproductive strategies, hormonal influences and others. Indeed, sex-specific responses to hypoxia are observed in zebrafish embryos whose one of their parents (either male or female) were exposed to hypoxia before fertilization (Heinrichs-Caldas et al., 2023). Moreover, sex-related differences in cellular stress responses have been widely documented (see Tower et al., 2020). Similarly, the general condition or health status of an individual could also significantly impact the expression of the POS. Individuals in prime condition might afford to express the trait more robustly, whereas those in poorer condition might exhibit a subdued response. This variation might be particularly relevant in understanding the adaptive significance of the POS, as it may indicate the trait's role in survival, reproduction, or overall fitness (Costantini, 2019). Lastly, for a long time, aging and life span have been associated with changes in the ability to effectively mount a cellular stress response (Finkel and Holbrook, 2000). From a redox metabolism point of view, there is general tendency of increasing levels of redox imbalance and oxidative stress markers (Balaban et al., 2005; Sohal and Orr, 2012) and decreasing the levels of endogenous antioxidants and oxidative damage repair pathways as animals age increases (Zhang et al., 2015a). Moreover, the ability to respond to oxidative stress through Nrf2-induced expression of antioxidants and repair mechanisms is impaired with age (Zhang et al., 2015a). Therefore, it is expectable that the POS response should be affected by age and differ between young and old individuals. To sum up, exploring how sex, age, and physiological condition influence POS manifestation might provide insights into its ecological and evolutionary implications. Such investigations would not only elucidate the underlying mechanisms governing this variation but also enhance our understanding of the adaptive value and functional significance of the POS in natural populations.

Finally, we value the significance of establishing nationwide collaborative research initiatives that involve studying diverse species across different continents and employing diverse methodological approaches, ranging from observational to experimental studies. Consequently, we believe that beyond the hurdle of replication and its impact on statistical inference, field and natural experiments should be of help for a further understanding of how animals work in the wild (in terms of redox molecular physiology) under natural stress/stresses. Moreover, it is critical the observation of the effects of multi-stressors in nature acting together to influence how animals would respond in terms of redox metabolism and physiology. For example, in studies on low/high tides in mollusks, we should consider, beyond the obvious aerial exposure, the effects of temperature (in air and water), the animal's dehydration, solar radiation, salinity and water turbidity (Moreira et al., 2023b).

7. Conclusion

For enzymologists, the scientific bywords for researchers came to be "don't waste clean thinking on a dirty (unpurified) enzyme"(Kornberg, 2000). This perspective, which involved simplifying and eliminating "side-effects", revolutionized enzyme studies. However, this approach also lost important interactive cellular information, including **(a)** the separation of proteins and enzymes that interacted *in vivo* to achieve metabolic regulation, **(b)** the depletion of key metabolites acting as driving forces for metabolism, and **(c)** the reduction of complexity held back holo-knowledge of cell and organ functionality.

There is a similar current tendency for reductionist measurements during live animal experiments: i.e. control ALL the variables but ONE and then vary that one variable to get the best and most accurate results. Bring animals to the laboratory and control environmental conditions in order to increase reproducibility while reducing variables. This is certainly a valuable first stage, but by not allowing complex interactions much can been lost in our understanding of environmental adaptation.

The arguments herein in this article and in the whole enterprise of Preparation of Oxidative Stress (POS) are a valuable step-forward and stand in opposition to past practices. Allowing experiments to move "outdoors" with many variables uncontrolled and then checking the values of key components (enzymes, metabolites, miRNA, gene expression), allows a new way of looking at natural adaptation. Surprisingly, these new experiments yield reproducible, accurate, and important insights. While our focus here is on oxidative stress, analogous breakthroughs are anticipated in all aspects of cell, organ, and organismal function, as have been happening in field ecology and ecophysiology. This new paradigm will allow a new way of looking at biochemical adaptation and a new way to look at animals in nature.

In the words of our friend and forever-teacher, Dr. Ken Storey: "*This new course/approach may represent a revolution, and could be likened to the paradigm shift that led to the real world, outdoor, interactive painting experiments of the artists that made up French Expressionism. Selected artists broke away from the constraints of their existing painting academies to observe nature-complexity, genuine interactions, and environments. They pursued studies in the raw and coined the new movement "en plein air". Attitudinal shifts and changes in approach are underway, offering exciting possibilities for future discoveries.*"

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Maximiliano Giraud-Billoud: Writing – review & editing, Writing – original draft, Visualization, Methodology, Conceptualization. **Daniel C. Moreira:** Writing – review & editing, Writing – original draft, Visualization. **Marina Minari:** Writing – original draft. **Aleksandra Andreyeva:** Writing – original draft. **Élida G. Campos:** Writing – review & editing, Writing – original draft. **Juan M. Carvajalino-Fernández:** Writing – original draft. **Aleksandra Istomina:** Writing – original draft. **Basile Michaelidis:** Writing – original draft. **Cuijuan Niu:** Writing – original draft. **Yonggang Niu:** Writing – original draft. **Luciana Ondei:** Writing – original draft. **Marko Prokić:** Writing – review & editing, Writing – original draft. **Georgina A. Rivera-Ingraham:** Writing – review & editing, Writing – original draft, Visualization. **Debadas Sahoo:** Writing – original draft. **Alexandra Staikou:** Writing – original draft. **Janet M. Storey:** Writing – review & editing. **Kenneth B. Storey:** Writing – review & editing, Writing – original draft. **Israel A. Vega:** Writing – review & editing, Writing – original draft. **Marcelo Hermes-Lima:** Writing – review & editing, Writing – original draft, Visualization, Supervision, Methodology, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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