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Title: Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later

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Keywords: anoxia; dehydration; estivation; freeze tolerance; hypoxia tolerance; ischemia.

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Abstract: Organisms that tolerate wide variations in oxygen availability, especially to hypoxia, usually face harsh environmental conditions during their lives. Such conditions include, for example, lack of food and/or water, low or high temperatures and reduced oxygen availability. In contrast to an expected strong suppression of protein synthesis, a great number of these animals present increased levels of antioxidant defenses during oxygen deprivation. These observations have puzzled researchers for more than twenty years. Initially, two predominant ideas seemed to be irreconcilable: on one hand, hypoxia would decrease reactive oxygen species (ROS) production, while on the other the induction of antioxidant enzymes would require the overproduction of ROS. This induction of antioxidant enzymes during hypoxia was viewed as a way to prepare animals for oxidative damage that may happen ultimately during reoxygenation. The term "preparation for oxidative stress" (POS) was coined in 1998 based on such premise. However, there are many cases of increased oxidative damage in several hypoxia tolerant organisms under hypoxia. In addition, over the years, the idea of an assured decrease in ROS formation under hypoxia was challenged. Instead, several findings indicate that the production of ROS actually increases in response to hypoxia. Recently, it became possible to provide a comprehensive explanation for the induction of antioxidant enzymes under hypoxia. The supporting evidence as well as the limitations of the POS idea are extensively explored in this review as we discuss results from research on estivation and situations of low oxygen stress, such as hypoxia, freezing exposure, severe dehydration, and air exposure of water-breathing animals. We propose that, under some level of oxygen deprivation, ROS are overproduced and induce changes leading to hypoxic biochemical responses. These responses would occur mainly through the activation of specific transcription factors (FoxO, Nrf2, HIF-1, NF-κB and p53) and post-translational mechanisms; both mechanisms leading to enhanced antioxidant defenses. Moreover, reactive nitrogen species are candidate modulators of ROS generation in this scenario. We conclude by drawing out the future perspectives in this field of research, and how advances in the knowledge of the mechanisms involved in the POS strategy will offer new and innovative study scenarios of biological and physiological cellular responses to stress.

Universidade de Brasília

Brasília, July 7th

Dear Editor,

We are sending the revised version of our manuscript “Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later” accompanied by five figures and five tables. We appreciate the insightful comments made by the referees and the editor. We made substantial changes in the original manuscript (OM) and now present a much improved version. In addition, we were glad to have attracted one more author to contribute to this work, Dr. Thiago C. Genaro-Mattos, a lipid biochemist, who must be officially included as author.

Finally, we are aware that Free Radical Biology and Medicine requires that papers sections should be unnumbered. However, we made the choice to keep the original (submitted) numbered sections format to facilitate the analysis of the revision by the editor and the reviewers. These numbers will be removed in the future, if the paper is accepted. Moreover, we see no problem, whatsoever, to address any additional issues that may appear in this revised version and, thus, make another round of revision.

Prof. Dr. Marcelo Hermes Lima
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*Detailed Response to Reviewers

Manuscript No.: FRBM-D-15-00162

Reviews/Associate Editor's Comments:

1. Your proposed Review manuscript has been reviewed by 4 expert reviewers and, based on their combined comments summarized below, has not been accepted for publication in FRBM. We hope that you will find the time to significantly improve your manuscript by addressing each of the 4 Reviewers' comments. Please note that large sections of this review will need to be written and refocused, with each section ending in a succinct summary. Appropriate alterations and a cover letter with a point by point description of how the manuscript was changed in response to the suggested revisions will be necessary to have this review manuscript re-evaluated. A revised manuscript will be returned to these same reviewers for their reconsideration. Please understand that there is no guarantee that your revision will be sufficient. Nonetheless, I will work with you and the reviewers to hopefully reach a more satisfying outcome.

We are sending the revised version of our manuscript "Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later" accompanied by five figures and five tables. We appreciate the insightful comments made by the referees and the editor. We made substantial changes in the original manuscript (OM) and now present a much improved version. In addition, we were glad to have attracted one more author to contribute to this work, Dr. Thiago C. Genaro-Mattos, a lipid biochemist, who must be officially included as author.

Because the manuscript was thoroughly re-organized, we are not providing the exact page, paragraph and line numbers of each modification. However the major changes in the RM are highlighted in yellow. All issues "provoked" by reviewers are discussed below.

The most important modifications were:

(i) The excessive biology-related information on sections 1 to 3 of the OM was reduced as follows: sections 1 and 2 were reduced by 22 and 10%, respectively, and section 3 was split into new sections 3 and 4 in the RM (which are 30% reduced in relation to section 3 of the OM). Each of these sections (1 to 4) received concluding remarks.

(ii) We made extensive alterations in the ROS formation section (section 5 on the RM), to explain more precisely how mitochondria generates ROS under hypoxia. The section was also reduced by 14%.

(iii) The sections on transcription factors and post translational modifications (sections 6 and 7, respectively, on the RM) were reduced by 13% in total. Both sections received concluding remarks.

(iv) We included two new sections in the RM. One on reactive nitrogen species (RNS) and other on lipid peroxidation issues in hypoxia-exposure studies (sections 8 and 9, respectively). In the case of RNS, the key point was their role in the regulation of mitochondrial ROS formation. In the case of peroxidation, we discussed (a) the apparent paradox of having this oxygen-dependent process going on under hypoxia and (b) the role of aldehydes formed by peroxidation in the regulation of Nrf2.

(v) In the final section of the RM, we discussed some limitations of POS theory and the spread of the POS process in the animal kingdom (a new figure regarding this aspect was added – **Fig 5**). We also presented - on sub-section 10.1 - a new point-by-point summary of our proposal, in a way to unify the POS theory in all situations of low oxygen stress (this text is now much more clear than the old section 7 in the

OM - it is also 24% smaller). We also included a new figure (**Fig 4**) on the overall "take home message" of our article.

(vi) The topic on historical perspectives (section 8 on the OM, now sub-section 10.3) was reduced by 40% in the RM.

Finally, we are aware that Free Radical Biology and Medicine requires that papers sections should be unnumbered. However, we made the choice to keep the original (submitted) numbered sections format to facilitate the analysis of the revision by the editor and the reviewers. These numbers will be removed in the future, if the paper is accepted. Moreover, we see no problem, whatsoever, to address any additional issues that may appear in this revised version and, thus, make another round of revision.

Reviewer #1:

2. This is a review/hypothesis paper that summarizes a great deal of literature on the controversial topic of preparation for oxidative stress under hypoxia and metabolic depression. The authors discuss various research findings from situations of low oxygen stress, such as hypoxia and compare them to freezing exposure, severe dehydration, estivation and air exposure of water-breathing animals. They make the proposal that under some level of low oxygen stress, ROS are overproduced and may induce changes in the activity of antioxidant enzymes most likely by regulating specific transcription factors. In general, the paper is interesting and thought-provoking, but has over-simplified the issue by centering on the production of oxidative stress and ROS from molecular O_2 entering the organism from the environment, for example, for use by respiration. This argument sets up the famous false dichotomy that as the PO_2 falls to very low levels, the rate of ROS production should fall, but does not always, resulting in an apparent paradox, especially when the anti-oxidant enzyme content or activity of the tissue increase.

We agree that we had exclusively focused on O_2 and ROS production in the OM. As recommend by the reviewers, we have considered the role of other pathways/molecules in the RM. Specific alterations in the RM are described below for each specific point raised by this reviewer. We anticipate that the role of reactive nitrogen species and compounds associated with lipid peroxidation are discussed in the RM.

3. The paper has glossed over some key free radical biology, for example, related to organic hydroperoxides, which may exist for minutes or hours in cells and tissues even under hypoxia and exhibit non-PO_2 dependent behaviors leading to oxidative stress. For example, fatty acid hydroperoxides, protein hydroperoxides, phospholipid hydroperoxides all exist in modest abundance and along with the related chemistry of alkoxyl radicals, enzymatic alkoxy formation, singlet oxygen, and peroxynitrite and other organic nitro- compounds, produce a rich biological chemistry for the generation of oxidative stress that has been primarily the purview of chemists and biochemists and too often ignored by the biologist. These compounds and their reactions are sufficiently plentiful in cell membranes, etc., to explain many apparently puzzling or oxidative effects when free molecular O_2 is reduced to trivial levels.

We created a new topic (Section 9, begins at line 806) in the manuscript to address the issue of lipid peroxidation raised by the reviewer. In this new section we briefly discussed the chemical nature of O_2 and membranes, and the solubility of O_2 in the membrane core. The fact that O_2 is soluble in the membrane core implies that it could be available for lipid peroxidation even after long periods of hypoxia. We also introduced a brief explanation (and a scheme of reactions, line 854, on Section 9) on the biological chemistry of alkoxyl, peroxyl and other oxidizing species (such as singlet oxygen), which are key players in membrane peroxidation. Therefore, the new section 9 provides a biochemical explanation at the molecular

level for the oxidative stress results discussed in our manuscript. We thank the reviewer for pointing out these issues and believe that this new section on peroxidation should enrich our manuscript.

4. The investigators also seem to place excessive emphasis on the work of Chandel and Schumacher, which has rarely resulted in the actual demonstration of excessive ROS production by mitochondria under hypoxia—in the few situations where this has been done, the increment is no the order of 20-25 % for brief periods of time. In this regard, figure 2 is misleading as it displays a doubling (or more) of the ROS production rate during hypoxia and about 2/3 of the apparent ROS generation during reperfusion. This hypoxic course for mitochondria flies in the face of the Km for O_2 of cytochrome oxidase, which is below 1 torr (See Chance et al) and thus gives the oxidase a favored position with respect to O_2 reduction.

We revised section 4 (on ROS formation) of the OM (now section 5 of the RM) according to the reviewer's suggestions. In addition, Figure 2 (we added a comment in the caption, line 1744)was revised to match a more realistic ROS generation under hypoxia (even though it is quite speculative any kind of "prediction", since our theory covers animals from many different groups, from corals to reptiles, and so each one should respond differently in terms of rates of ROS formation).

5. The literature is replete with post mortem "oxidative stress", especially of lipid peroxidation, which is notorious issue when the tissues are not handled anaerobically (i.e. artificially reperfused).

We recognize that many sources of errors might occur when measuring, for instance, levels of lipid peroxidation. For example, if the tissues are not handled properly, the possibility of post-mortem lipid peroxidation becomes relevant (as pointed out by the reviewer). We cannot account for every study discussed in this review. However, all of such studies used proper controls and compared levels of lipid peroxidation between the control animals (normoxia) and animals submitted to low oxygen stress. We can infer that samples from both conditions were submitted to the same procedure (handling), which ensures that the levels of post-mortem peroxidation are the same in both conditions. Therefore, any difference observed between these two groups must have happened before the handling. In other words, we believe that these differences are due to biological/biochemical effects. Finally, we mentioned the issue of post mortem oxidative stress in the new section 9 (on peroxidation) of the RM (line 917).

6. Although at normal conditions most LOOH are reduced to the less reactive hydroxides, there are conditions where LOOH are not efficiently detoxified and thus can participate in reactions leading to increased toxicity. LOOH causes changes in the structural organization and packing of membrane lipid components, leading to alteration in membrane fluidity and properties. LOOH can affect many cellular processes, leading to increased cellular protection or promoting cell death. Hydroperoxides may accumulate in several pathological conditions and some are stable at room temperature, but can be decomposed by heating, exposure to UV or by addition of transition metals. Metal-induced decomposition of LOOH yields lipid alkoxyl (LO.) and/or lipid peroxyl radicals (LOO.). These oxyl radicals propagate, as well generate reactive products capable of modifications in proteins. These products are electrophilic aldehydes, epoxides, ketones, and excited species, such as singlet and and excited cabonyl species. In fact fatty acid and phospholipid hydroperoxides formed in membranes can generate singlet O_2.

A new section was added to the RM discussing the molecular basis of lipid peroxidation and its biological effects, identified as Section 9 (line 806). In this section we discuss the implications of lipid peroxidation products and we provided the explanation for the intriguing peroxidation after hours/days of

hypoxia. In addition, we discussed how lipid peroxidation products – in particular electrophilic aldehydes - might affect signaling events in the cell. For instance, we discuss that since lipid peroxidation can occur independently from the pO2 in the cell, it can produce aldehydes long after the beginning of hypoxia. Once produced, these species can activate transcription factors (such as Nrf2), which ends up activating antioxidant response systems (line 887). This effect of lipid peroxidation products on Nrf2 regulation and the interplay with hypoxia exposure time is illustrated in the new Figure 4 added to the RM.

7. the tables do not include a column for whether the work actually measured ROS production or specific footprints of the free radicals that might be involved, and which at the least would be a helpful addition.

Honestly, we did not understand what the reviewer meant in this point. What we can say about the tables is: Table 4 shows oxidative stress markers (footprints of free radicals) in animals exposed to low oxygen stress; and Table 5 shows studies on ROS production in mammalian systems exposed to hypoxia, describing the methods, the probes and the molecule(s) expected to react with each probe.

Reviewer #2:

8. The review by Hermes-Lima et al. summarizes the biological and physiological cellular responses to hypoxic and metabolic stress across many unique species. The MS is dealing with rarely reviewed topic by discussing how organisms facing harsh environmental conditions deal with oxidative stress. To further enhance the potential impact of this Review, I would suggest the authors to amend their MS with attractive figures. An additional figure may show e.g. the evolutionary development of ROS defense - ROS production.

We followed the recommendation of Reviewer 2. We reviewed Figure 2 (incorporating referee #1 comments) and created a new figure (Figure 5) showing the animal groups with enhanced antioxidant defenses under low oxygen stress and estivation. Another figure (Figure 4) that summarizes the "take home message" of the manuscript was also created.

9. Authors should also consider mentioning, even if not going to details, that a similar scenario to their proposal with ROS may also exist in case of reactive nitrogen species such as peroxynitrite, which is in part the consequence of increased superoxide generation. There is substantial evidence that it can be formed during both hypoxia, as well as reoxygenation/reperfusion, and it may exert important signaling roles (both protective and detrimental) modulating the expression/function of all major components involved in ROS generation/defense, as well as in metabolic regulation (PMID: 17237348)

As correctly pointed out by the referee, there is an important amount of literature that points to reactive nitrogen species (RNS) being involved in the hypoxic response. Following the suggestions of reviewer 2 , we added a new section (Section 8, line 759) in the RM, where we briefly discuss the potential role of RNS on effecting changes and signaling under hypoxia. We are aware of all the evidence pointing to the important role of RNS in redox metabolism and the regulation of metabolic rates in hypoxia-tolerant animals. However, we decided to concentrate on the consequences of nitric oxide (NO) and its derivatives interacting with the electron transport chain. This is, in our opinion, the most important piece of information

in the context of the subject of our article: through this RNS-electron transport chain interaction, regulation of electron flow is possible and thus, also the modulation of mitochondrial ROS production.

Reviewer #3:

10. A comprehensive review of oxidative stress under conditions of hypoxia and metabolic depression with a focus on comparative biology is presented. This reviewer finds the paper a bit tedious to read, yet informative with an excellent review of material not often covered in FRBM. It is unfortunate that so many of the comparative studies still focused on tissue TBARS!!!

We thank the reviewer for his comment about the way the manuscript was written and we re-wrote and re-organized almost the whole manuscript to make it "easier" to read. We also agree with the reviewer that TBARS is a poor method to address lipid peroxidation in samples with complex animal tissues, which is the case of all studies discussed in our manuscript. Unfortunately, the field of comparative biology uses this method to measure lipid peroxidation, as already pointed out by the reviewer. We included a section discussing the negative points of TBARS referring to a recent editorial of FRBM (Forman et al. 2015) criticizing the method (line 906). We also make a strong suggestion to all researchers in the field to abandon TBARS and move towards more precise and specific methods, such as measurements of F2-isoprostanes by GC-MS.

Reviewer #4:

11. For this reviewer, I have long yearned for a FRBM-centric review that covers the general biology of how animals cope with environments where oxygenation cycles. I recall requesting a consideration of oxygenation in tidal cycles while reviewing a recent FRBM submission on ROS catabolizing enzymes in the gills of mollusks that inhabit this zone, and was wholly unsatisfied with the usual dodged response. So I applaud these authors in their comprehensive consideration of this topic in this forum. I find great value in the work done here to chronical these comparative biology studies of how animals are redox adapted to niches that experience oxygenation cycles. That said, the descriptive portion of the review is entirely too long for the casual FRBM reader, and each subsection and the work overall concludes with little to none of the synthesis viewpoint by these experts that I was expecting. Several review works including these authors (2002) contain much of this information (Guppy M and Withers PC. 1999; Hochachka PW and Somero GN 2002; Bickler PE and Buck LT, 2007; etc.).

We have reduced significantly the amount of information regarding basic comparative biology stuff. The sections where most of these information were present in the OM where 1, 2 and 3 (sections 1 to 4 on the RM; we decide to make "estivation" as a separated section). Sections 1 to 4 of the RM are **20% smaller** (in terms of word-count) than sections 1 to 3 from the OM. Moreover, the basic biology and physiology of metabolic depression and hypoxia tolerance was also much reduced. We hope this new version of the manuscript is more pleasant to the FRBM reader.

12. In summary, I will not quibble on the small points, but go 10K foot: my recommendation is for the authors to consider honing the current work to concentrate on specific examples that support common themes in animal strategies to cope with acutely or to thrive in scenarios or niches of varied oxygenation, and lay out those themes in a clear summary section. I see your historical approach, but it suffers from a forest through the trees problem. I am just left with a sense of after wading through 45 pages of interesting examples; a clear explanation to reconcile the points raised seems to be lacking.

We added a clear and summarized explanation in section 10.1 (former section 7 of OM) and included Figure 4. Moreover, we added closing statements to each section (as also requested by the editor). The "historical section" from the OM was reduced in the RM (by 40% - it is now section 10.3).

13. how does biochemistry under varied oxygenation interface with Darwinian selection (or is it Lamarkian)?; convergent evolution versus unique strategies

The heterogeneity in experimental designs and in the methods used to determine oxidative damage and activity of enzymatic and non-enzymatic antioxidants only let us hypothesize the clades that could develop the POS strategy. Therefore,, we added a new figure (Fig. 5 of the RM) to schematically represent the groups of animals that could use the POS strategy and a related commentary in Section 10 (line 983).

An interesting and extensive review could be made in the future about whether there is any particular antioxidant mechanism shared between species of different groups, and if comparable, maybe mechanisms of convergent evolution could be established. Such review could instead indicate if unique strategies are used by particular species to cope with oxidative damage occurred during hypometabolic situation.

14. a list of common strategies to deal with changes in oxygenation (ROS formed under hypoxia) and their use dependence on time (minutes, hours, months//acute v chronic)

The only common strategy we can describe is that many animals - from corals to reptiles – show oxidative stress and enhanced antioxidant defenses when oxygen availability is reduced. As we mentioned in point 13, the studies are highly heterogeneous. Let us consider a few examples (looking exclusively at the cases of increased antioxidants under low oxygen stress): (i) among the studies on hypoxia, the concentration of oxygen regarded as hypoxic ranges from 0.5 to 3.9 mg O_2 L^{-1}; (ii) the complexity and, thus, the respiratory physiology, ranges from cnidarians to reptiles; (iii) also, we are not considering other adaptations, such as behavioral and physiological adaptations, used to counteract the fall in internal pO2; (iv) the responses seem to be tissue specific, and in a given animal one organ may increase antioxidants while in other it may not (e.g. catalase activity increases in liver, decreases in kidney, and does not change in muscle and brain of anoxic goldfish; Lushchak et al., 2001); (v) as we discuss in section 10.2, the exposure time varies largely from minutes to days. For these reasons, we are not able to further elaborate (with the level of detail this referee asked) on biochemical mechanisms/strategies of POS for the animals.

Responding to another "POS problem", the vast majority of studies discussed in the manuscript are those using an acute exposure protocol. There are fewer works examining oxidative stress markers the role of antioxidants in animals chronically exposed to hypoxia. Fingernail clams *Sphaerium* sp. (Joyner-Matos and Chapman, 2013) and mole rats (Schülke et al., 2012) are examples of animals chronically exposed to hypoxia. Even though we expect that animals chronically exposed to hypoxia should behave differently in the long term, we do not have enough data to make conclusions about the similarities and discrepancies of acute versus chronic exposures regarding the redox metabolism. We kept in the manuscript a footnote on what happens in clams under chronic exposure to hypoxia (on section 10 of RM).

Lushchak, V. I.; Lushchak, L. P.; Mota, A. A.; Hermes-Lima, M. Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. *Am. J. Physiol.* **280**: R100–R107; 2001.

Joyner-Matos, J.; Chapman, L. J. Persisting in papyrus: size, oxidative stress, and fitness in freshwater organisms adapted to sustained hypoxia. *Comp. Biochem. Physiol. A* **165**: 405–416; 2013.

Schülke, S.; Dreidax, D.; Malik, A.; Burmester, T.; Nevo, E.; Band, M.; Avivi, A.; Hankeln, T. Living with stress: regulation of antioxidant defense genes in the subterranean, hypoxia-tolerant mole rat, *Spalax*. *Gene* **500**: 199–206; 2012.

15. a clear explanation of how ROS are formed as pO2 transits a crucial saddle point, quantitatively what is that point, all ROS or specific ROS? does it mechanistically differ in one directions versus another (going down v going up in pO2) and if so how?, and does that vary for different animals…

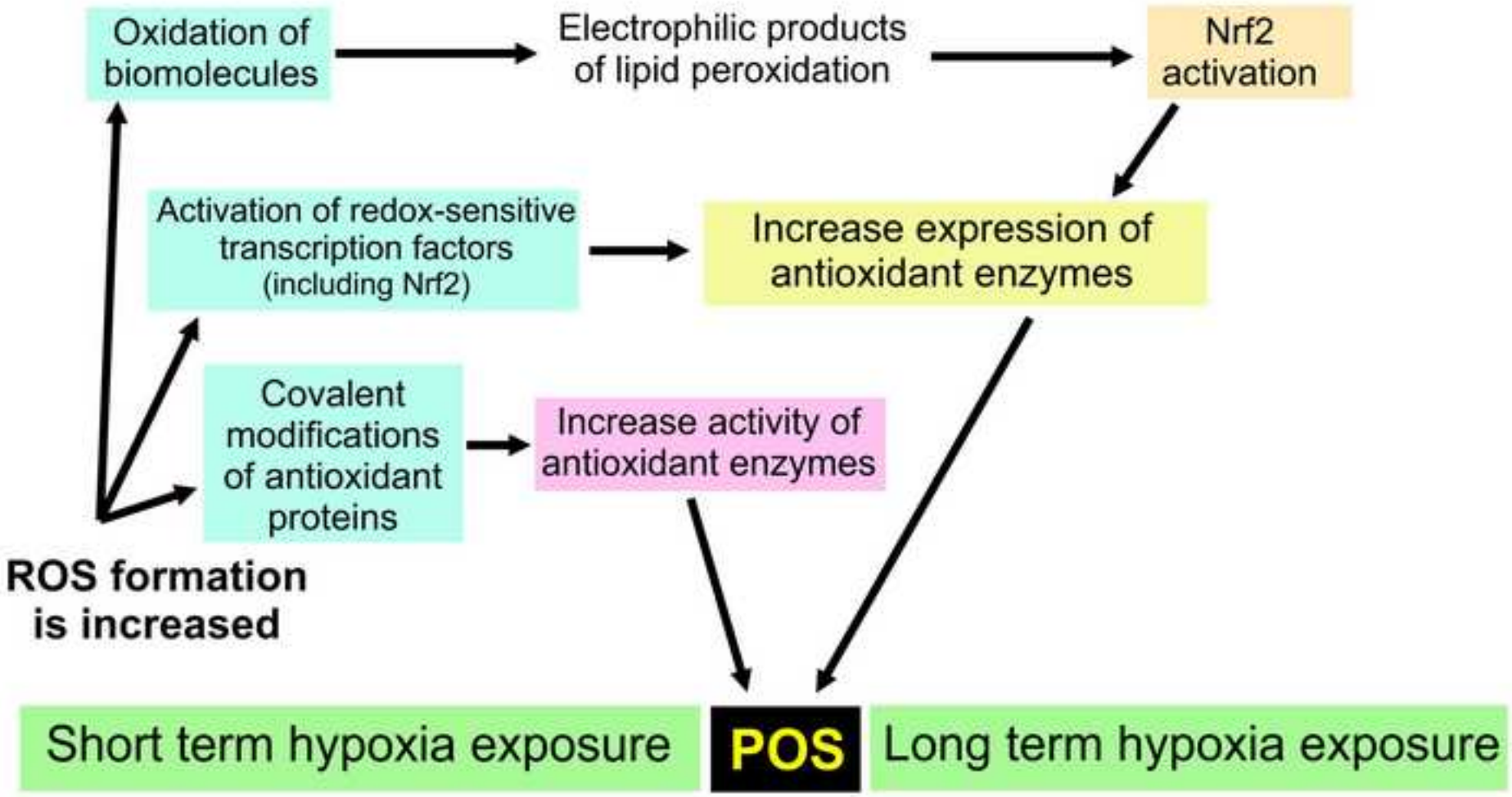
We can not make the predictions requested by the reviewer. The reason is that so far there is no study showing an increase in ROS generation in hypoxia tolerant animals. We are making an assumption based on data about antioxidant expression and oxidative stress markers. Once again, the time in which each one of the animals responded should greatly vary; thus, we can not even estimate when this hypothetical phenomenon shall occur. We hypothesized that, as pO_2 decreases, there should be a lower level of reduction of O_2 to water in mitochondrial complex IV. The degree of reduction of the electron carriers preceding complex IV would be increased with consequent increase in O_2 reduction to superoxide. This "excess" electron should favor superoxide formation up to certain point, because, when oxygen is too low, ROS formation should decrease. On the other hand, when oxygen concentration gets higher than a certain point (a pO_2 that we cannot predict!), ROS formation should decrease again, because there would be no more electron "excess". Thus, we are talking about a bell-shaped curve of oxygen concentration versus ROS formation (as depicted in figure 2). And this should vary considerably in hypoxic-tolerant organisms. We only imagine that superoxide is the "primary chemical species" formed by mitochondria, in all hypoxic-tolerant animals, which will activate the POS response (other reactive species should be formed after that: hydrogen peroxide, hydroxyl radical and even peroxynitrite, when superoxide reacts with NO).

We discuss in section 5 (this is a revised form of section 4 from the OM) how ROS are formed in mitochondria under hypoxia. In section 10 we comment that it is not possible (so far) to predict the time course of POS response in hypoxia-tolerant animals. We also comment that we cannot predict which pO_2 would be the peak of ROS formation. This comment was added in the legend to figure 2.

Highlights

- Many hypoxia-tolerant animals increase their antioxidants during oxygen deprivation
- ROS may be overproduced in these conditions causing oxidative damage
- ROS activate transcription factors leading to increase in expression of antioxidants
- Covalent modification in antioxidant proteins may increase their activities
- These are the key molecular basis of the “preparation for oxidative stress” theory

Graphical Abstract (for review)



Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later

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Abstract

Organisms that tolerate wide variations in oxygen availability, especially to hypoxia, usually face harsh environmental conditions during their lives. Such conditions include, for example, lack of food and/or water, low or high temperatures and reduced oxygen availability. In contrast to an expected strong suppression of protein synthesis, a great number of these animals present increased levels of antioxidant defenses during oxygen deprivation. These observations have puzzled researchers for more than twenty years. Initially, two predominant ideas seemed to be irreconcilable: on one hand, hypoxia would decrease reactive oxygen species (ROS) production, while on the other the induction of antioxidant enzymes would require the overproduction of ROS. This induction of antioxidant enzymes during hypoxia was viewed as a way to prepare animals for oxidative damage that may happen ultimately during reoxygenation. The term "preparation for oxidative stress" (POS) was coined in 1998 based on such premise. However, there are many cases of increased oxidative damage in several hypoxia tolerant organisms under hypoxia. In addition, over the years, the idea of an assured decrease in ROS formation under hypoxia was challenged. Instead, several findings indicate that the production of ROS actually increases in response to hypoxia. Recently, it became possible to provide a comprehensive explanation for the induction of antioxidant enzymes under hypoxia. The supporting evidence as well as the limitations of the POS idea are extensively explored in this review as we discuss results from research on estivation and situations of low oxygen stress, such as hypoxia, freezing exposure, severe dehydration, and air exposure of water-breathing animals. We propose that, under some level of oxygen deprivation, ROS are overproduced and induce changes leading to hypoxic biochemical responses. These responses would occur mainly through the activation of specific transcription factors (FoxO, Nrf2, HIF-1, NF-κB and p53) and post-translational mechanisms; both mechanisms leading to enhanced antioxidant defenses. Moreover, reactive nitrogen species are candidate modulators of ROS generation in this scenario. We conclude by drawing out the future perspectives in this field of research, and how advances in the knowledge of the mechanisms involved in the POS strategy will offer new and innovative study scenarios of biological and physiological cellular responses to stress.

Keywords: anoxia, dehydration, estivation, freeze tolerance, hypoxia tolerance, ischemia.

1. Introduction

Oxygen is essential for the majority of organisms on Earth. For most of them, oxygen restriction is highly deleterious. However, many animal species are able to survive long periods of oxygen deprivation, including a variety of invertebrates and vertebrates. In nature, oxygen deprivation ranges from mild hypoxia to complete anoxia (Welker et al., 2013). Information on the physiological processes and biochemical mechanisms to cope with hypoxia, in special metabolic depression, has been amassed for the past 30-40 years, and several mechanisms have been unveiled (Storey and Wu, 2013). One of the issues that have interested researchers since the 1990s is the role of free radicals in the process of natural hypoxia tolerance, and our aim is to discuss the interplay between reactive oxygen species and this process. Additional natural conditions that involve reduced oxygen delivery to organs, such as freezing stress and severe dehydration will also be covered in this article.

One well-known mechanism used to survive low oxygen levels is the severe depression of the metabolic rate during oxygen deprivation in association with lower rates of ATP production via fermentative pathways. Such ability to severely slow down many energy-consuming pathways is a key strategy for survival. It includes reduction of key metabolic enzymes activities via post translational modifications, or via decreased transcription and/or translation. Changes in expression/activity of several kinases and phosphatases, transcription factors and microRNAs participate in such responses (Biggar and Storey, 2015; Staples and Buck, 2009; Storey and Storey, 2007; Storey and Storey, 2012). Next, the modulation of free radical metabolism during oxygen deprivation in hypoxia tolerant animals is presented.

In the early 1990s it was well established that ischemia and reperfusion episodes in mammalian organs elicited intense formation of reactive oxygen species (ROS) during the recirculation of oxygenated blood (Zhu et al., 2007; Zweier et al., 1987). There were unequivocal evidences that increased formation of ROS during reperfusion was one of the

key factors for cell damage under these conditions. Mitochondria were already recognized as the major source of excess ROS formation during reoxygenation. Based on the knowledge about ischemia/reperfusion injury it was reasonable to argue that hypoxia tolerant animals would be adapted to endure the potential dangers of reoxygenation. At that time, it was hypothesized by one of us that these animals would rely on high levels of antioxidant defenses to control the effects of a putative overproduction of ROS during reoxygenation.

The first examination of this hypothesis was performed using garter snakes *Thamnophis sirtalis parietalis*, which are naturally tolerant to anoxia for a few days, and freezing for several hours. The activities of garter snake antioxidant enzymes, such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S-transferase (GST) in liver, muscle and lung were determined. Unexpectedly, garter snake enzyme activities (in tissues from control animals) were much lower than those reported, for example, in mice and rats. On the other hand, it was also unexpected that some of the activities increased during exposure to anoxia for 10 h at 5°C or freezing for 5 h (with 40-50% of the total body water frozen at −2.5°C) (Hermes-Lima and Storey, 1993). This increase was observed for SOD in muscle and liver from anoxia-exposed snakes, as well as for catalase in muscle and lung and for GPX in muscle from frozen animals. In both conditions the increase in enzyme activities was higher than 100% (liver SOD and muscle GPX) when compared to controls. Moreover, levels of reduced glutathione (GSH) increased by about 1.6-fold in muscle of anoxia-exposed garter snakes (Hermes-Lima and Storey, 1993).

In the light of these results, it was suggested that garter snakes activated their antioxidant defenses during conditions when ROS production would be low (freezing) or absent (anoxia). The reasoning at that time was that free radical formation would always be dependent on and directly proportional to oxygen availability. Thus, under the hypoxic

condition of freezing oxygen radicals production would be low. This assumption hampered the understanding of how antioxidant defenses were being activated. In those days, it was already known that up-regulation of genes for antioxidant defenses happens under oxidative stress conditions, i.e. rises in ROS formation should stimulate mechanisms that result in increased expression of antioxidant enzymes. Because garter snakes under anoxia or freezing would be under severe oxygen limitation, it was improbable (at 1990s) that ROS formation would increase under these conditions. Therefore, it was proposed that these snakes enhanced their antioxidant capacity in order to prepare them for a putative increase in ROS generation and oxidative stress during reoxygenation (i.e. increasing the defenses before oxidative stress). The conclusion of the study (Hermes-Lima and Storey, 1993) was:

> "*The antioxidant defenses are built when oxyradical formation is not likely to occur (under the frozen or anoxic condition) in anticipation of their need when the perfusion of oxygenated blood is reinitiated. A nonradical messenger must stimulate these enzyme systems at either transcriptional, translational, or posttranslational levels.*"

Since then, many studies reported that when hypoxia-tolerant animals are exposed to low oxygen stress there is an increase in their antioxidant defense capacity (Hermes-Lima and Zenteno-Savín, 2002; Hermes-Lima et al., 2001; Storey, 1996; Welker et al., 2013). Low oxygen stress situations include anoxia, hypoxia, freezing, severe dehydration and aerial exposure of water-breathing animals. Estivation is another situation related to the adaptation to harsh environmental conditions (and, in the case of gastropods, decreased O_2 availability in tissues) in which endogenous antioxidants were found increased. In the late 1990s, this process was coined as "preparation for oxidative stress" (POS) (Hermes-Lima et al., 1998).

Although there were growing evidences of increased antioxidant levels in animals under low oxygen stress (i.e. POS), a biochemical explanation on the molecular basis of this

mechanism was still missing. The first observation of POS dates back to 1993 and only in 2005 the first attempt to explain the molecular mechanisms underlying antioxidant activation appeared in the literature (Almeida et al., 2005). The present work describes how we put together the puzzle about the POS mechanism and discuss a rational for the understanding of this process.

2. Preparation for oxidative stress (POS) under low oxygenation

After the pioneering work on garter snakes (Hermes-Lima and Storey, 1993) many studies reported that anoxia or hypoxia exposure in several other organisms induces increased activity or gene expression of antioxidant enzymes. Leopard frogs (*Rana pipiens*) under 30 h anoxia presented increased activities of catalase in muscle and heart, GPX in heart and brain, as well as GST in brain (Hermes-Lima and Storey, 1996). Goldfish (*Carassius auratus*) under anoxia showed increased GPX activity in brain and catalase activity in liver (Lushchak et al., 2001). Crabs (*Chasmagnathus granulata*) under anoxia showed increased catalase and GST activities in both anterior and posterior gills (de Oliveira et al., 2005). Anoxia exposure for six days of the marine gastropod (*Littorina littorea*) caused an increase in GSH levels, although the activity of antioxidant enzymes were reduced (Pannunzio and Storey, 1998). Furthermore, pupae of the Caribbean fruit fly, *Anastrepha suspensa*, presented higher GPX and MnSOD activities, while the activities of catalase and CuZnSOD remained unchanged in response to 1 h anoxia (Lopez-Martinez and Hahn, 2012).

Similar results have been observed in several animals exposed to hypoxia. Common carps (*Cyprinus carpio*) under hypoxia for 5 h presented increased catalase and GPX activities in brain (Lushchak et al., 2005). Pacific oysters (*Crassostrea gigas*) under hypoxia (from 3 to 24 days) showed increased mRNA expression for GPX in mantle, gill and hepatopancreas (David et al., 2005). Moreover, microarray analysis of these oysters under

hypoxia (for 20 days) also showed increased expression of peroxiredoxin-5 in hepatopancreas (Sussarellu et al., 2010). In the disc abalone, *Haliotis discus discus*, hypoxia for 8 h prompted increased expression (mRNAs) of MnSOD, SeGPX, catalase and thioredoxin (De Zoysa et al., 2009). Exposure of amphipods *Monoporeia affinis* to hypoxia for 5 or 9 days induced a rise in the activities of catalase and SOD (Gorokhova et al., 2010; Gorokhova et al., 2013). In a study with *Balanus amphitrite* barnacles under anoxia, severe hypoxia and mild hypoxia (24 h) resulted in a sharp increase in catalase and SOD activities in larvae and adults (Desai and Prakash, 2009). In another crustacean, the shrimp *Litopenaeus vannamei*, hypoxia exposure for 4 hours elicited an up-regulation of the genes coding for GPX, cytoplasmic MnSOD and peptide-methionine (R)-S-oxide reductase (Kniffin et al., 2014). The later enzyme participates in the repair of oxidatively damaged proteins, specifically methionyl residues. In the case of the subterranean amphipod *Niphargus rhenorhodanensis*, an increase in GPX activity was observed after exposure to either anoxia (24 h) or hypoxia (10 days) (Lawniczak et al., 2013). Other cases of increased antioxidant levels in response to anoxia or hypoxia are presented in Tables 1 and 2.

In addition to hypoxia/anoxia, other conditions are also related to functional low oxygenation, such as freezing stress, severe dehydration and aerial exposure of water-breathing animals. We and other authors observed that leopard frogs under dehydration (Hermes-Lima and Storey, 1998), wood frogs, garter snakes and turtle hatchlings under freezing (Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996; Krivoruchko and Storey, 2010b), and crabs and bivalves under aerial exposure (Almeida and Bainy, 2006; Freire et al., 2011a; Romero et al., 2007) presented increased activities of antioxidant enzymes. The endogenous antioxidants that were found to be increased in response to freezing, dehydration or air exposure are presented below (Tables 1 and 2).

In the majority of the studies the enhancement of antioxidant defenses was regarded as an important adaptation to deal with the recovery/reoxygenation phase from oxygen restriction, in which a sharp ROS overproduction is expected to occur. The trigger to such response, however, was unknown and few attempts were made to explain at the molecular level this phenomenon. If one looks closely, there are clues that indicate a putative trigger to the increase in endogenous antioxidant. These clues are the observations of altered redox balance and increased oxidative stress during low oxygen stress discussed in the next section.

Not all animals respond to oxygen restriction by increasing activity/expression of endogenous antioxidants. Examples are salamanders under anoxia exposure (Issartel et al., 2009), three fish species under hypoxia (Leveelahti et al., 2014), golden gall fly larvae exposed to freezing (Joanisse and Storey, 1998), and in the fish *Heteropneustes fossilis* exposed to air exposure (Paital, 2013; Paital, 2014). Thus, as mentioned in previous works (Hermes-Lima and Zenteno-Savín, 2002), the process of "preparation for oxidative stress" (POS) is not a universal adaptive mechanism in animals for dealing with the stress of low oxygenation. However, POS is present in a great number of species that evolved under the pressure of low oxygen stress.

3. Redox imbalance and oxidative stress under low oxygen stress and metabolic depression

Following the proposal that a "non-radical" messenger would trigger the activation of endogenous antioxidant defenses (Hermes-Lima and Storey, 1993) (Figure 1), there were many observations indicating oxidative stress and/or a redox imbalance during low oxygen stress and estivation. The most relevant examples are discussed below for studies on the stresses of anoxia, hypoxia, freezing, severe dehydration, aerial-exposure of aquatic animals and estivation.

3.1. Anoxia

A 1990s study on the free radical metabolism in leopard frogs (*R. pipiens*) under anoxia indicated that levels of disulfide glutathione (GSSG) were increased in muscle and liver after 30 h anoxia exposure (Hermes-Lima and Storey, 1996). Moreover, the ratio GSSG:GSH-eq was also increased in muscle after 30 h anoxia and in liver at 10 and 30 h of anoxia, followed by a decrease in the GSSG:GSH-eq ratio during recovery. The elevation in this ratio was an indicative of redox imbalance, indicating that GSSG accumulation occurred at 10 and 30 h anoxia. These results were interpreted as a consequence of diminished capacity of anoxic frogs to recycle GSSG, possibly by a reduction in the carbon flux via the pentose pathway, that provides NADPH for glutathione reductase-catalyzed reactions. However, such interpretation did not explain why GSH was still being oxidized under anoxia.

Indeed, in the earlier garter snake study (Hermes-Lima and Storey, 1993), it was observed that GSSG levels increased in muscle from animals exposed to anoxia for 10 h, but not in liver and lung. This was explained as a consequence of the elevation in total-glutathione under anoxia. This increase was possibly due to activation of GSH biosynthesis, because the GSSG:GSH-eq ratio was maintained and levels of GSH increased under anoxia in muscle. This interpretation however failed to explain why oxidation of GSH to GSSG increased under anoxia. Oxidation of GSH by GPX-catalyzed reaction requires H_2O_2 or other hydroperoxides. How would hydroperoxides still be available (or formed) in muscle tissue after 10 h under anoxia? Other intriguing observations were made in a study with marine gastropods *Littorina littorea* under anoxia (Pannunzio and Storey, 1998) showing that lipid peroxidation (determined by two different methods) increased in foot muscle after 6 days anoxia, returning to control levels following recovery. No explanation could be given as how lipids would undergo peroxidation under anoxia. Recently, it was suggested that there might be an increase in mitochondrial ROS formation during the hypoxic phase that preceded full

anoxia (Welker et al., 2013). Figure 2 shows a proposed connection between ROS formation under anoxia/hypoxia exposure and the activation of endogenous antioxidant defenses.

In addition, a Brazilian study with crabs (*C. granulata*) under 8 h anoxia showed an increase in lipid peroxidation in hepatopancreas (de Oliveira et al., 2006). Lipid peroxidation was determined by two methods (conjugated dienes and TBARS) and in both methods the levels increased after 8 h anoxia and diminished upon recovery. The authors explained that by the presence of residual O_2 in the internal tissues after exposure to anoxia, which could trigger ROS formation. However, the general view in those days was that under hypoxia there would be less ROS formation. As discussed in more detail in following sections, since the late 1990s there are amounting evidences for increased ROS formation, at least in mammalian cells, under hypoxia.

3.2. Hypoxia

A Ukrainian study with carps exposed to hypoxia reported an increase in liver lipid peroxidation, determined as TBARS, while no changes were observed in brain, kidney and muscle (Lushchak et al., 2005). At that time, it was expected that low oxygenation would cause a decrease or no change in lipid peroxidation, since it was assumed that ROS formation would be lower. In fact, levels of lipid peroxides (measured as cumene hydroperoxide equivalents by the xylenol orange method), initial products of peroxidation, decreased in carp liver and brain under hypoxia (Lushchak et al., 2005). Based on these findings, the authors speculated that non-radical molecules would be responsible for the activation of antioxidants (Lushchak and Bagnyukova, 2006). However, in their next publication, an increase in oxidative stress markers was observed in rotan fish *P. glenii* exposed to hypoxia (Lushchak and Bagnyukova, 2007). Protein oxidation (as carbonyl protein) increased in brain, liver and muscle after 2 to 6 h of hypoxia exposure. Lipid peroxides concentration also increased in

brain and liver after 2 h of hypoxia. Based on such findings, authors suggested that mitochondrial ROS production could be increased under hypoxia (Lushchak and Bagnyukova, 2007). This series of publications by Lushchak et al. illustrates the transition from the idea of a non-radical messenger (Figure 1) to the current view of increased ROS production during oxygen deprivation that the field has gone through (Figure 2).

An interesting study by UK researchers showed that erythrocytes from carps exposed to hypoxia for 30 days present similar levels of DNA damage when compared to carps exposed to hyperoxia (Mustafa et al., 2011). This result was obtained using three different protocols of the Comet assay. It is well known that hyperoxia induces ROS formation and oxidative stress, therefore the authors concluded that ROS formation also increased under hypoxia, possibly by "electron scape" from the mitochondrial respiratory chain. Therefore, fish oxidative stress would result from either excess or diminished oxygen availability. Since 2007-2008, researchers in general studying hypoxia tolerant animals begun to be aware of the possibility of increased mitochondrial ROS formation under hypoxia (see (Bickler and Buck, 2007)).

A study with two species of subterranean mole rats (*Spalax galili* and *S. judaei*) revealed that these hypoxia tolerant species have increased constitutive expression levels (mRNA) of many genes related to antioxidant protection when compared to laboratory rats (Schülke et al., 2012). This observation was interpreted as a way to counteract the effects of ROS formation under hypoxia: *"The permanent subterranean lifestyle of Spalax most probably requires constant protection against hypoxia-generated ROS injury. If O_2 tension suddenly drops even more, e.g. by flooding of the soil in the rainy season (Shams et al., 2005), immediate prophylactic protection by high antioxidant levels is required."* (Schülke et al., 2012). As far as we know, this is the first study in comparative biology to treat increased ROS formation during hypoxia as an assured phenomenon. Accordingly, the idea that

mitochondrial ROS formation is increased under hypoxia in mammalian cells is presented in presented in the 2008 edition of the Lenhinger text-book.

3.3. Air exposure

Aerial exposure of aquatic animals, that causes functional hypoxia to internal tissues, has been the subject of many studies (Freire et al., 2011b). Many water breathing sessile animals are periodically subjected to air exposure as a consequence of tidal height variations. In this context, bivalves compose a group of extensively studied animals. Mussels *Perna perna* exposed to air for 18 h showed 1.5-fold increase in hepatopancreas GST activity (Almeida et al., 2005). Moreover, when the same species was exposed to air for 24 h increased lipid peroxidation (as TBARS) in gills and hepatopancreas was observed. They also had increased DNA damage in gills measured as levels of 8-oxodGuo (Almeida et al., 2005). The authors originally proposed the idea (quoted below) that increased mitochondrial ROS formation during hypoxia would modulate antioxidant defenses in mussels. This was the first biochemical explanation by which the POS process functions and was also discussed in following publications (Almeida and Di Mascio, 2011; Almeida et al., 2007).

> *"The decrease in cytochrome oxidase V_{max} during hypoxia is responsible for an increase in mitochondrial redox state (Chandel and Schumacker, 2000), which, in turn, accelerates ROS generation during hypoxia, triggering the activation of different transcriptional factors involved in numerous cellular hypoxia responses. Despite its modulator effect, such increase in ROS production would be also accounted for increases in lipid and DNA damage in cells."*

As in mussels, stone crabs *Paralomis granulosa* exposed to air for 3 to 24 h presented increased protein oxidation in gills, measured as carbonyl protein, while no changes happen in muscle or hepatopancreas (Romero et al., 2007). Moreover, lipid peroxidation, determined

as levels of lipid peroxides, increases in muscle and hepatopancreas after 6 to 24 h of aerial exposure. This indicative of increased ROS formation could be a trigger for regulation of several antioxidant enzymes in the tissues of *P. granulosa*.

In cnidarians, the air exposure of corals *Veretillum cynomorium* for 2.5 h resulted in no change in TBARS levels, but a sharp increase (by 10-fold) in TBARS levels occurs after 30 min re-immersion (Teixeira et al., 2013). Catalase and GST activities increased during air exposure, and SOD activity shows no changes. Interesting, catalase and GST activities returned to control levels upon re-immersion, while SOD activity increased upon re-immersion. In spite of a probable ROS overgeneration upon return to water, an increase in ROS of smaller magnitude may have happened under air exposure, inducing catalase and GST without an increase in oxidative damage.

3.4. Freezing and Dehydration

In the mid 1990s, a Canadian study (Joanisse and Storey, 1996) described the alterations in the redox metabolism in a cycle of freeze and thaw in the wood frog *R. sylvatica*. The main observation was the increase in GPX activity in several tissues (muscle, liver, kidney, brain and heart) after 24 h of freezing exposure at −2.5°C. Overall lipid peroxidation levels (determined as TBARS and total lipid peroxides) remained stable in all tested tissues after thawing (30 min, 90 min and 4 h). Moreover, GSSG levels remained stable after 24 h thawing in five tissues. It was concluded that thawing would cause no oxidative stress because tissues had increased antioxidant capacity during freezing, in a way to prepare tissues for potentially deleterious effects of ROS. This was in agreement with previous observations in garter snakes under freezing stress (Hermes-Lima and Storey, 1993). However, a couple of observations indicated a redox imbalance in kidney and brain during freezing in wood frogs: the increase in GSSG levels and GSSG:GSH-eq ratio. These changes

were not observed in muscle, liver and heart. The unexpected increase in GSSG and GSSG:GSH-eq ratio were interpreted by the authors as a consequence of reduced capacity (due to hypometabolism) for glutathione recycling (Joanisse and Storey, 1996), the same explanation presented in the study with leopard frogs under anoxia (Hermes-Lima and Storey, 1996). Alternatively, we can currently consider that an increase in ROS formation during freezing could cause GSH oxidation to GSSG in the two frog organs (kidney and brain), even though this was unable to induce lipid peroxidation.

Severe dehydration is a condition that resembles the effect of freezing on internal organs. Dehydration tolerant anurans may endure up to 60% loss of body water. The consequences of this condition are reduced blood volume and increased blood viscosity, which induces a severe loss in aerobic cardiovascular capacity, including a decline in pulse rate and oxygen consumption. Therefore, internal organs become hypoxic during severe dehydration. Restoration of body fluids in dehydrated anurans is comparable to reoxygenation/reperfusion, in which an increase in ROS formation would be expected (Hermes-Lima and Zenteno-Savín, 2002; Hermes-Lima et al., 2001).

The observed increase in the activity of antioxidant enzymes in liver and muscle (and levels of hepatic GSH) during severe dehydration (50% body water loss) in leopard frogs was considered a preventive process to counteract the effects of ROS during rehydration (Hermes-Lima and Storey, 1998). As in the case of freezing, ROS formation was supposed to be decreased only during dehydration, due to the hypoxic/ischemic condition. However, levels of GSSG in liver, as well as the GSSG:GSH ratio, increased during dehydration by 80-90%. These results were regarded as a failure in the GSSG recycling mechanism, as in the case of wood frogs under freezing or leopard frogs under anoxia. On the other hand, this redox imbalance in liver could be (from the current point of view) a sign for increased ROS formation during severe dehydration.

In summary, there is a wide range of species that had signs of redox imbalance and increased levels of oxidative markers when exposed to low oxygen stress (anoxia, hypoxia, air exposure, freezing and dehydration). In most cases, these observations were somehow unexpected due to the lack of O_2 availability. Thus, due to the assumption of an assured reduction of ROS formation during oxygen restriction, the perturbations in redox balance parameters as well as the increases in oxidative stress markers (Table 4) were left without explanation or explained by "non-radical" hypotheses. It was not until 2005 (Almeida et al., 2005) that an explanation different from the idea of a "non-radical" messenger was made and set the base to a more comprehensive explanation of the "preparation for oxidative stress".

4. Estivation

Another adaptation to stressful environmental condition that is related to modulation of the antioxidant capacity is estivation in snails, fish and anurans. Estivating land snails *O. lactea* deeply depress their metabolic rate and continue to rely on aerobic catabolism. As a result of the dormancy phenotype, oxygen partial pressure decreases in the hemolymph of estivating snails (Barnhart, 1986). Thus, mitochondrial respiratory chain may function at "low speed" due to restricted provision of NADH and/or $FADH_2$ in a hypoxic intracellular environment. Therefore, in the mid 90s, it was considered that mitochondrial ROS formation would be low under estivation (Hermes-Lima et al., 1998). On the other hand, there is a bout in oxygen consumption (Herreid, 1977) that could increase mitochondrial ROS formation and oxidative stress during early moments of arousal.

The activities of several antioxidant enzymes in *O. lactea* increase after 30 days estivation (Hermes-Lima and Storey, 1995). This occurs with total SOD and GPX in hepatopancreas and total SOD, catalase and GST in foot muscle, which could be a preparatory mechanism to control oxidative stress during arousal. Moreover, lipid

389 peroxidation (as TBARS) increased in hepatopancreas during the first minutes of arousal. It
390 was suggested that the enhanced antioxidant capacity during estivation functioned in a way to
391 minimize such oxidative stress during arousal (few years after this study, the concept of
392 "preparation for oxidative stress" was proposed).

393 This adaptive mechanism was also reported for land snails *Helix aspersa*, because
394 GPX activity increased in hepatopancreas and foot muscle (by 2-3-fold) during 20-day
395 estivation (Ramos-Vasconcelos and Hermes-Lima, 2003). Levels of GSH increased during
396 estivation in hepatopancreas, but not in foot muscle, suggesting that GSH biosynthesis
397 increases during snail estivation. Furthermore, the GSSG:GSH-eq ratio increased in
398 hepatopancreas during the first moments of arousal, indicating that arousal promotes redox
399 imbalance. Levels of TBARS did rise in hepatopancreas when comparing snails active for 5
400 min versus 30 min. The increase in GSH and GPX levels were regarded as an adaptation to
401 minimize oxidative stress just during arousal, but not during estivation. The same conclusion
402 was made for a study on estivation of the aquatic snail *Biomphalaria tenagophila* in which an
403 increase in GPX activity occurred at 15 days estivation (Ferreira et al., 2003).

404 It was proposed in the studies cited above that during estivation ROS production
405 would be decreased, and that an alternative "non-radical" mechanism would activate
406 antioxidant defenses. However, when re-examining these studies, a number of evidences
407 indicate that ROS formation increases in estivating snails, compared to active snails. In the
408 case of *O. lactea*, the increase in GSSG levels in hepatopancreas and foot muscle and the
409 increase in GSSG:GSH ratio in foot muscle alone during estivation (Hermes-Lima and
410 Storey, 1995) are evidences of a redox imbalance. The hypoxic condition in between breaths
411 in *O. lactea*, as well as the increase in oxygen input during breaths, could increase
412 mitochondrial ROS formation during estivation.

In the case of *H. aspersa*, there is clear evidence for increased ROS formation and consequent oxidative stress during estivation, even though this was not concluded in the original article. When compared to aroused active snails, estivating animals had increased TBARS and lipid peroxides levels in hepatopancreas, increased carbonyl protein levels in foot muscle and increased GSSG concentration in hepatopancreas (Ramos-Vasconcelos and Hermes-Lima, 2003). These findings strongly suggest that oxidative stress increases during 20-day winter estivation in *H. aspersa*. No changes in markers of oxidative stress and redox balance happened in summer estivation in this snail species (Ramos-Vasconcelos et al., 2005).

In addition, a Polish study on *Helix pomatia* revealed an increase in lipid peroxidation (determined as TBARS) in muscle and kidney, but not in hepatopancreas, during winter torpor. Moreover, these land snails increase their enzymatic antioxidant activities, especially catalase and glutathione-related enzymes during torpor (Nowakowska et al., 2009). The results obtained with *H. pomatia* in the field roughly agree with those from estivating *O. lactea* and *H. aspersa* performed in the laboratory.

The up-regulation of antioxidant proteins, such as catalase and thioredoxin peroxidase, as well as some chaperones (small heat shock protein and protein disulfide isomerase), was also reported in the freshwater apple snail *Pomacea canaliculata* after 30 days of estivation (Sun et al., 2013). Furthermore, other authors also found increased TBARS concentration in total soft tissue or in foot and kidney, indicating oxidative stress in apple snails after 45 days of estivation at 23-25°C (Giraud-Billoud et al., 2011; Giraud-Billoud et al., 2013). Under these conditions uric acid was possibly used as non-enzymatic antioxidant in hepatopancreas and kidney (Giraud-Billoud et al., 2013). Furthermore, when metabolic depression was induced at 13°C these snails showed an increase in both TBARS

437 concentration (in hepatopancreas, kidney and foot) and in antioxidant defenses (SOD and
438 GSH) in foot muscle (Giuffrida et al., 2013).

439 Estivation and oxidative stress were also investigated in anurans. The first study
440 analyzed desert spadefoot toads *Scaphiopus couchii* and compared animals that had been
441 estivating for two months with active animals (Grundy and Storey, 1998). The majority of the
442 activities of antioxidant enzymes and concentrations of GSH were decreased in several
443 tissues of toads under estivation (exceptions are shown in Table 3). Moreover, in most organs
444 lipid peroxidation parameters, as well as GSSG:GSH ratio increased during estivation (Table
445 4).

446 A study with the striped burrowing frog *Cyclorana alboguttata* showed that
447 superoxide scavenging capacity (possibly SOD activity) increased in both iliofibularis and
448 gastrocnemius muscles during estivation. Carbonyl protein levels increased in the iliofibularis
449 muscle, but not in the gastrocnemius muscle, after 6 months of estivation (Young et al.,
450 2013). Another study with these animals showed that the mRNA levels for glutamate
451 cysteine ligase regulatory subunit and GST-O2 increased after 4 months of estivation (Reilly
452 et al., 2013). Furthermore, H_2O_2 formation by permeabilised gastrocnemius frog muscle was
453 decreased after 4 months estivation, while it remained unchanged in cardiac muscle when
454 compared to muscles from active controls (Reilly et al., 2014). These observations as a whole
455 indicate that there is a response of the redox metabolism (ROS formation, oxidative damage
456 and expression of antioxidants) during estivation in green-striped frogs and such response is
457 dependent on muscle type.

458 In the case of lungfish *Protopterus dolloi* increased activity/protein levels of
459 antioxidant enzymes were observed in brain (MnSOD, CuZnSOD, catalase and GR) and
460 heart (GPX) after 60 days of estivation. However, there was no increase in the levels of
461 indicators of oxidative stress, except for nitrotyrosine levels in brain (Page et al., 2010).

Because the ratio GSSG:GSH was not measured, one cannot conclude if a redox imbalance took place during estivation of *P. dolloi*.

Similar to what was observed for low oxygen stresses there are many examples of estivating animals that had increased oxidative stress markers, and enhanced antioxidant defenses. These observations indicate that ROS generation increase at some point of estivation in anurans, gastropods and lungfish. Such increase in ROS production would in turn trigger mechanisms that would ultimately result in increased expression of endogenous antioxidants. ROS-mediated activation of Nrf2 and FoxO1 transcription factors (Malik and Storey, 2009; Malik and Storey, 2011; Reilly et al., 2013) could be one of these mechanisms.

5. Mechanisms of ROS formation under hypoxia – evidence in mammalians and invertebrates

The effects of hypoxia on mammalian models have been long and extensively studied under the premise that ROS production is directly proportional to oxygen concentration. But it is not until the early 1970s, with the radiobiological studies by Hall (Hall, 1973), that we find the first reports providing a deeper insight on the levels of free radical production under limiting oxygen conditions. Rao et al. (Rao et al., 1983) later reported that coronary occlusion elicited a 55% increase in free radical levels in dog ventricular tissue when compared to normoxia. Such observations constituted a key finding that opened a completely new perspective on what was known at the time on ROS formation under limited oxygen concentrations and set the bases for later works.

The impact of the earlier works by Rao et al. (Rao et al., 1983) is demonstrated by the growing number of studies showing similar increases in ROS upon hypoxic exposure in a wide diversity of mammalian cell lines, cells types and tissues (Table 5). Rao et al. (Rao et al., 1983) measured free radical formation through electron spin resonance, so far the only

analytical method for direct detection of free radicals (Malanga and Puntarulo, 2011), but the theory of increased ROS formation under hypoxia has also been widely supported by data obtained using fluorescent specific probes for detecting ROS. Imaging cellular events is by no means an easy task, and much controversy has arisen on the use of such techniques (Forman et al., 2015; Kalyanaraman et al., 2012). Further studies have however confirmed hypoxic-ROS production through the application of RNA interference techniques in conjunction with the use of protein-based fluorescence resonance energy transfer (FRET) sensors (HSP-FRET). Guzy et al. (Guzy et al., 2005) applied such a ratiometric probe, consisting in the fusion of two fluorescent peptides (one yellow, YFP, and one cyan, CFP) linked to a redox-sensitive bacterial heat shock protein, to provide further direct and reliable evidence for increased ROS formation in the cytosol under hypoxia.

Diving mammals such as whales or seals constitute a valuable model in the study of the physiological effects of hypoxia-reoxygenation events. These animals are obliged to make subsequent dives in order to feed, exposing themselves to cyclic bouts of ischemia and reperfusion and the negative consequences that the later entails. Vázquez-Medina et al. (Vázquez-Medina et al., 2012) have recently reviewed the current knowledge regarding how such diving species are able to avoid oxidative damage. This and similar reports led the authors to hypothesize that hypoxia-induced ROS production in diving mammals may be involved in the induction of antioxidant mechanisms and other protective pathways relevant for hypoxic adaptation. This would occur through the activation of HIFs (e.g. (Johnson et al., 2005) reviewed by (Zenteno-Savín et al., 2011)), heterodimeric transcription factors present in the cytoplasm which through their activation a wide variety of hypoxia adaptive cell responses are regulated (Chandel et al., 1998; Klimova and Chandel, 2008; Semenza, 2000; Semenza and Wang, 1992).

A great body of information also comes from the field of cardiology and what it is currently known as "ischaemic preconditioning" (IPC), that is, the process by which subsequent ischemic-reperfusion events increases cell resistance (Murry et al., 1986). The involvement of ROS in IPC has been demonstrated through indirect manners, such as through the use of antioxidants (which reduce the cardioprotective effects of IPC) (Tanaka et al. 1994) and prooxidants (which under normoxia induced IPC-like protection as shown by Baines et al. (Baines, 1997) or Vanden Hoek et al. (Vanden Hoek et al., 1998)).

But when does such a ROS increase occur in mammalian cells? Reports agree that this ROS peak can occur only some minutes after the hypoxic insult. Recent investigations conducted on bovine and human endothelial cells under acute hypoxia (1-2% O_2) showed that ROS formation peaks around 10-20 min of hypoxic exposure (Hernansanz-Agustín et al., 2014), agreeing with previous studies (Table 5). In contrast to mammalian cells, fewer studies have assessed ROS production in animals that are naturally exposed to low oxygen stress. A study using *in vivo* staining with the non-specific ROS detector C-H_2DFFDA, evidenced that the marine platyhelminthes *Macrostomum lignano* ROS formation follows the typical oxygenation-dependent pattern (Rivera-Ingraham et al., 2013a). However, superoxide formation (DHE staining) remained constant across oxygenation conditions (ranging from hyperoxia (40 kPa) to near-anoxia), and only reoxygenation caused a 2-OH-E^+:DHE ratio increase. Even though these contrasting results are difficult to interpret, this work was the first in comparative biology to demonstrate that superoxide formation under near-anoxia remains unchanged in comparison to normoxia and suggests that it could be contributing as a signaling mechanism in hypoxic acclimation.

Another study by the same group evidenced that, in the hypoxia tolerant mussel *Mytilus edulis*, levels of carbonyl proteins in gills increase after 48 h under near-anoxia (<0.6% air saturation) even though ROS formation decreases (as determined *ex-vivo* by DHE

and C-H_2DFFDA staining) (Rivera-Ingraham et al., 2013b). Shorter periods of near-anoxia exposure were not assessed, but it is possible that ROS production increases in gill tissues before the investigated 48 h time point. Indeed, as discussed above, increased ROS formation occurs during the first 10-20 minutes from the onset of hypoxia exposure in mammalian cells, decreasing after that period (Hernansanz-Agustín et al., 2014). Future quantification of ROS at shorter incubation times might help verify if, as observed in mammalian cells, increased ROS formation under hypoxia also applies to invertebrates.

It is also of major interest to review the current knowledge related to the molecular mechanism of hypoxic ROS production since most of the results come from mammalian cell studies. It is within mammalian cells that it is long known that mitochondria produce ROS (Jensen, 1966) and that mitochondria are the main ROS producers (e.g. (Cadenas and Davies, 2000; Turrens, 2003). Thus, many works have attempted to determine the exact source of the hypoxia-induced ROS formation. Both pharmacological and genetic methods have been extensively applied, the later being mostly related to the analysis of the activation of HIFs. Chandel et al. (Chandel et al., 1998) demonstrated in Hep3 cells that functional mitochondria are necessary to produce the hypoxia-induced ROS that are required for IPC. This is expected, since as the same author correctly points out, other ROS-regenerating systems such as cytochrome P450 or NADPH oxidase would decrease ROS production under hypoxic conditions. In a more recent study, Hernansanz-Agustín et al. (Hernansanz-Agustín et al., 2014) further demonstrated that the hypoxia-derived ROS largely requires a mitochondrial oxidative phosphorylation system. But where exactly are such ROS produced? Complexes I, II and III are the main mitochondrial sources of $O_2^{\bullet-}$ (Poyton et al., 2009; Turrens, 2003). Located in the inner side of the inner mitochondrial membrane, complexes I and II generate $O_2^{\bullet-}$ which is released in the mitochondrial matrix. Complex III, on the other hand, is a transmembrane complex, and, thus, not only generates $O_2^{\bullet-}$ in the matrix, but also to the

intermembrane space from where they can be carried to the cytoplasm via voltage-dependent anion channels (Han et al., 2003) and be potentially available for HIF activation and participation in other signaling pathways. Even though the exact mechanism through which mitochondrial ROS are involved in HIF activation is not established, the important role of complex III must be highlighted. This has been further supported by several other groups working with a variety of cells lines (e.g. (Bell et al., 2007; Guzy et al., 2005; Mansfield et al., 2005; Waypa et al., 2001). Chandel et al. (Chandel et al., 1998) proposed that this occurs due to an accumulation of electrons in the proximal areas of the respiratory chain and their further leakage to form $O_2^{\bullet-}$ when there is limited oxygen available to the terminal cytochrome c oxidase. Later, studies allowed the refinement of such model and suggested that, under hypoxia, the mitochondrial complex III suffers a conformational change that would facilitate the interaction between O_2 and ubisemiquinone, resulting in an increase of $O_2^{\bullet-}$ formation (Guzy et al., 2005). Others also consider complex II as relevant for ROS formation during hypoxia exposure (Paddenberg et al., 2003). It was proposed that this complex switches its catalytic activity from succinate dehydrogenase to fumarate reductase at diminished oxygen levels. This would not only cause succinate to accumulate but additionally will cause ROS generation because fumarate reductase has been demonstrated to be a powerful $O_2^{\bullet-}$ generator (e.g. (Imlay, 1995; Messner and Imlay, 2002; Turrens, 1987). This change in complex II would then be modulating the directionality of the electron flow because not only O_2 would be the final electron acceptor, but also fumarate (Chouchani et al., 2014). Further research is required to consolidate knowledge on the role of each of the mitochondrial complexes in the induction of hypoxia adaptation across the different tissues and cell models.

6. Redox sensitive transcription factors and low oxygen stress

The description of transcription factors that regulate the expression of genes coding for antioxidant proteins in animals exposed to low oxygen stress is limited. A strong indication that a transcription factor acts in any particular gene is the presence of a consensus binding sequence for such specific factor in the promoter region of the gene. In this case, the promoter region of the gene must be known. Data about the promoter sequences of the antioxidant genes from the organisms cited the present review are scarce. Therefore, the evidence of the action of any particular transcription factor on the induction of antioxidant defense genes in the animals discussed herein is rather indirect.

The nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) regulates genes involved in the biosynthesis of glutathione and NADPH as well as genes coding for catalase, CuZnSOD, peroxiredoxin, thioredoxin, GPXs and GSTs (Banning et al., 2005; Chan and Kan, 1999; Ishii et al., 2000; Kim, 2001; Kobayashi and Yamamoto, 2006; Suzuki et al., 2005). Its stability and activity can increase upon exposure to H_2O_2 through oxidation of its inhibitory protein Keap1 (Fourquet et al., 2010). Nrf2 also controls a number of genes involved in intermediate metabolism that may also contribute to survival under reduced oxygen tensions (Hayes and Dinkova-Kostova, 2014). In the African clawed frog *X. laevis*, the exposure to dehydration resulted in increased expression of GST isoforms in several organs (Malik and Storey, 2009). GST-P1 was induced in liver, heart and skin by 2- to 9-fold, whereas GST-M1 and GST-M3 increased in muscle, kidney and skin. These results were related with increased Nrf2 expression at both protein and mRNA levels (Malik and Storey, 2009).

The protein p53 is reported to control the expression of MnSOD and GPXs (Mai et al., 2010; Tan et al., 1999). Both up and down-regulation of antioxidant gene expression can result from p53 action depending on its intracellular concentration (Dhar et al., 2010). This protein may also provide antioxidant protection under hypoxia by up-regulating the

610 mitochondrial glutaminase 2 (GSL2) gene. Glutaminase 2 catalyzes the hydrolysis of
611 glutamine to glutamate, which is a precursor of GSH. Activation of p53 increases the level of
612 glutamate and GSH and decreases ROS levels in cells *in vitro*. In addition, the human GSL2
613 gene contains a p53 consensus DNA-binding element and this element may also be present in
614 GSL2 gene from other organisms (Hu et al., 2010).

615 The hypoxia inducible factor 1 (HIF-1) is the main transcription factor involved in the
616 response to hypoxia. The role of HIF in animal adaptation to hypoxia has been subject of
617 innumerous studies since the 1990s (Hochachka and Somero, 2002). There is an indication
618 that HIF-1 regulates the expression of GPX3 in human plasma (Bierl et al., 2004). HIF-1
619 subunits have been cloned from some non-mammalian species, including shrimp, oyster, and
620 fish (Mohindra et al., 2013; Piontkivska et al., 2011; Soñanez-Organis et al., 2009). A
621 putative HRE has been located in intron 2 of the lactate dehydrogenase B gene from the
622 killifish *Fundulus heteroclitus*. Putative HREs may also be present in the genes coding for *F.*
623 *heteroclitus* antioxidant enzymes. This indicates that HIF-1 or its homologs may be involved
624 in mediating the effects of environmental hypoxia in other animals (Rees et al., 2001).

625 The genes coding for MnSOD and catalase are direct transcriptional targets of
626 forkhead box O (FoxO) transcription factors (Greer and Brunet, 2005; Kops et al., 2002). The
627 role FoxOs in the induction of antioxidant defenses has been demonstrated in studies in
628 species facing low oxygen stress. The liver of African clawed frogs, *X. laevis*, exposed to
629 dehydration had increased FoxO1 abundance in nucleus, increased FoxO1 DNA binding
630 activity and reduced levels of phosphorylated FoxO1 (Malik and Storey, 2011). This
631 activation of the FoxO1 pathway was related to the increase of two antioxidant enzymes,
632 MnSOD and catalase, at both protein and mRNA levels in the liver (Malik and Storey, 2011).
633 Furthermore, activation of FoxO1 and FoxO3 transcription factors has been also

demonstrated in the anoxia-tolerant turtle *Trachemys scripta elegans* (Krivoruchko and Storey, 2013).

The influence of NF-κB proteins on ROS levels occurs via increased expression of MnSOD, CuZnSOD, GST, and GPX (Morgan and Liu, 2011). In mice neonatal cardiac myocytes and adult myocardial endothelial cells the migration of NF-κB and AP-1 to the nucleus is associated with increased enzyme activity and amount of MnSOD protein under condition of anoxia/reoxygenation preconditioning. The mouse MnSOD gene contains putative bind sites for NF-κB and AP-1 in its promoter region. The use of NF-κB and AP-1 artificial inhibitors indicates a direct action of these transcription factor on MnSOD gene expression (Rui and Kvietys, 2005).

An explanation was proposed on how antioxidant enzymes are increased under conditions of low oxygen availability (Almeida and Di Mascio, 2011; Welker et al., 2013). The proposal assumes that ROS formation increases during hypoxia in aquatic animals, and that such increased ROS formation would activate transcriptional factors that regulate the expression of antioxidant enzymes. Candidate transcription factors are Nrf2, p53, HIF-1, NF-κB proteins and FoxO proteins (Figure 3). Herein, we expand the idea on the role of such transcription factor in animals during estivation and situations of low oxygen stress, including freezing, dehydration in both terrestrial and aquatic species. In a next section, the role of ROS-derived electrophiles in the activation of transcription factors will also be considered.

7. Is there a role for post translational modifications of antioxidant enzymes during low oxygen stress?

In addition to transcription factors, post-translational modification of proteins is a mechanism to regulate protein function with the advantage to be rapid and ATP-inexpensive, which meets the condition of hypometabolism. Indeed, protein phosphorylation has been

659 shown to regulate the activity of enzymes involved in energy metabolism in animals under
660 low oxygen stress, for example, arginine kinase and glutamate dehydrogenase in crayfish
661 under severe hypoxia (Dawson and Storey, 2011; Dawson and Storey, 2012), lactate
662 dehydrogenase in turtles under anoxia (Xiong and Storey, 2012), creatine kinase and
663 hexokinase in frogs exposed to freezing conditions (Dieni and Storey, 2009; Dieni and
664 Storey, 2011). Moreover, there are evidences that reversible phosphorylation and other post-
665 translational modifications are mechanisms that control the activity (in terms of V_{max}) of
666 antioxidant enzymes. However, only one study has analyzed the specific role of post-
667 translation regulation of an antioxidant enzyme in animals under low oxygen stress (Dawson
668 et al., 2015). The altered ROS production in organisms under low oxygen stress could trigger
669 signaling pathways leading to post-translational modifications of antioxidant enzymes and
670 related proteins.

671 Protein phosphorylation is widely recognized as a post-translational modification that
672 modulates the activity of enzymes in general. In addition to the switch on and off effect, by
673 the action of kinases and phosphatases on proteins, reversible phosphorylation may also alter
674 enzyme properties and the interaction between enzymes and other proteins (Storey, 2004).
675 Although not an antioxidant enzymes itself, glucose 6-phosphate dehydrogenase (G6PDH) is
676 an important enzyme that fuels glutathione and thioredoxin systems by producing reducing
677 potential in the form of NADPH. Reversible phosphorylation regulates G6PDH enzymatic
678 properties in response to hypometabolism in land snails (Ramnanan and Storey, 2006), to
679 freezing in wood frogs (Dieni and Storey, 2010) and to anoxia in periwinkles (Lama et al.,
680 2013). Snails estivating for 10 days have higher levels of phosphorylated G6PDH resulting in
681 increased G6PDH activity in comparison to active animals (Ramnanan and Storey, 2006). On
682 the other hand, phospho-G6PDH levels are reduced in wood frogs exposed to freezing

683 conditions for 24 h, leading to a reduced affinity for its substrates in the frozen state (Dieni
684 and Storey, 2010).

685 Regarding antioxidant enzymes, H_2O_2-related enzymes (catalase, glutathione
686 peroxidase and peroxiredoxins) have been shown to have their activities regulated by
687 phosphorylation in response to oxidative stress mediated by H_2O_2 in mammalian cells (Rhee
688 et al., 2005). Two important tyrosine kinases c-Abl and Arg are activated upon H_2O_2
689 treatment and phosphorylates catalase (Cao et al., 2003a) and GPX1 (Cao et al., 2003b)
690 leading to increased activities. Phosphorylation of several peroxiredoxins occurs *in vitro* and
691 this modification leads to reduced activity of peroxiredoxin I, which is phosphorylated *in vivo*
692 (Chang et al., 2002). The antioxidant-related enzymes glutamate-cysteine ligase (GCL) and
693 glutathione transferase P1 (GSTP1) are also subject of reversible phosphorylation. While
694 GCL have its activity reduced (Sun et al., 1996), GSTP1 presents higher catalytic efficiency
695 when phosphorylated by different kinases (Lo et al., 2004; Okamura et al., 2009; Singh et al.,
696 2010). Phosphorylation has also been reported to regulate MnSOD activity *in vivo* and *in*
697 *vitro* in mammalian cells exposed to radiation, in such a manner that phosphorylation by
698 CyclinB1/Cdk1 increases its activity (Candas et al., 2013).

699 In addition to reversible phosphorylation, antioxidant proteins are targets of other
700 covalent modifications that may alter their activities, including acetylation (Kim et al., 2006)
701 and glutathionylation (Manevich et al., 2004). There is a broad range of proteins known to be
702 modified by reversible acetylation (the transfer of an acetyl group from acetyl coenzyme A to
703 a protein) resulting in the regulation of many cellular processes (Norris et al., 2009; Spange et
704 al., 2009). A large number of mitochondrial proteins have been found to be acetylated,
705 including enzymes involved in the energetic metabolism and stress response – e.g. MnSOD,
706 CuZnSOD, thioredoxin and isocitrate dehydrogenase 2 (Kim et al., 2006).

Several studies have shown that MnSOD activity is affected by deacetylation in response to different stresses (Ozden et al., 2011; Zhu et al., 2012). Specifically, the deacetylation of MnSOD by the mitochondrial sirtuin Sirt3 (a NAD+-dependent protein deacetylase) increases SOD activity (Chen et al., 2011; Qiu et al., 2010). Increased MnSOD expression (by 6-fold) results in a slight (10%) decrease of ROS levels in mammalian cells (Qiu et al., 2010). However, ROS levels are strongly suppressed (90%) when SIRT3 and SOD2 are coexpressed. Moreover, the expression of a modified deacetylated SOD2 alone also reduces ROS levels by 90% (Qiu et al., 2010). Thus, the overexpression of SOD has little effect on ROS levels unless deacetylation occurs (Chen et al., 2011; Qiu et al., 2010; Tao et al., 2010).

To our knowledge, there is a single study about post-translational modification of antioxidant enzymes in animals in response to low oxygen stress (Dawson et al., 2015). Muscular MnSOD was purified from control and frozen *Rana sylvatica* frogs. Freezing induces increased relative phosphorylation levels of MnSOD resulting in greater stability (assessed by resistance to urea denaturation) and increased affinity (lower K_m) of the enzyme for $O_2^{\bullet -}$. However, no effect on V_{max} was observed as a result of phosphorylation (Dawson et al., 2015).

It is tempting to raise the role of post translational modifications on the activity of antioxidant enzymes for several reasons. First, there is a wide range of intermediary metabolism enzymes regulated by phosphorylation, as well as others post-translational modifications, in these animals (Storey and Wu, 2013). Second, many studies have shown post-translational modification of antioxidant enzymes in other systems (including in response to increased ROS) and sites for modifications other than phosphorylation have been identified in these enzymes. Finally, the trigger for these modifications to occur during hypoxia exposure could be the increased ROS formation, which, for example, is known to

alter the activities of protein kinases, phosphatases and sirtuins. Thus, one could speculate that reversible covalent modifications of antioxidant enzymes play an important role in hypoxia tolerant animals under low oxygen stress. For example, a hypothetical Sirt3-mediated activation of MnSOD in response to elevated levels of H_2O_2 under hypoxia (which could happen in the hypoxic condition that antecedes full anoxia), could be an alternative explanation for the increase in SOD activity in anoxia-exposed garter snakes reported by Hermes-Lima and Storey (Hermes-Lima and Storey, 1993).

Besides the direct effects on antioxidant enzymes, many studies have reported the occurrence of the post-translational modifications addressed above on redox-sensitive transcription factors. For example, the acetylation of several transcription factors has been reported, including FoxOs, HIF-1α, NF-κB, Nrf2 and p53 (Bell et al., 2011; Spange et al., 2009; Sun et al., 2009; Tseng et al., 2013). Furthermore, the roles of reversible phosphorylation and acetylation of p53 (Zhang et al., 2013) and reversible phosphorylation of FoxO1 and FoxO3 (Krivoruchko and Storey, 2013) were investigated in *T. scripta elegans* turtle exposed to anoxia. For example, in liver and muscle, several phosphorylated forms of p53 increase in *T. scripta elegans* exposed to anoxia (Zhang et al., 2013).

The discussion above highlights the importance of the employment of methodologies to specifically detect antioxidant enzymes in their active forms (e.g. enzymatic activity or selective antibodies against the active form of the enzyme). Due to the multiple layer control of gene expression, mRNA levels do not always match protein levels, and protein levels do not necessary reflect active protein levels (Feder and Walser, 2005). Moreover, when analyzing antioxidant enzymes one should take care to avoid *in vitro* protein modification during sample handling, for example, adding phosphatase inhibitors to the sample at the time of homogenization. Furthermore, proteins involved in these post-translational modifications

(e.g. Sirt3) are candidates to be regulated and investigated in animals under low oxygen stress and during metabolic depression.

8. The role of reactive nitrogen species

Reactive nitrogen species (RNS) have also been demonstrated to play important physiological roles in a wide range of taxa. Nitric oxide (NO), for example, is an evolutionarily conserved intercellular messenger involved in multiple biological processes, ranging from defense in bacteria (Hausladen et al., 1998) to mitochondrial biogenesis in mammals (Nisoli et al., 2003). NO is directly involved in regulating respiration rates (Poderoso et al., 1996), essential for prolonging survival upon hypoxic periods. This molecule is more stable under very low environmental oxygen (0.5-1.5 μM O_2) and acts as a multi-site inhibitor of the mitochondrial respiratory chain (Cassina and Radi, 1996). Cytochrome oxidase has higher affinity for NO when compared to O_2, making complex IV (responsible for most of the oxygen consumption) the most sensitive site to this inhibition (Cleeter et al., 1994; Poderoso et al., 1996). But the reversible nature of this inhibition (Brown, 1999) is probably the key adaptive response to subsequent ischemia-reperfusion events, since a decrease in mitochondrial respiration prevents an excess of ROS production upon reoxygenation. Moreover, NO-derived metabolites (nitrite and, due to the low pH in tissues during hypoxia, also the strong S-nitrosylating agent N_2O_3) can interact with complex I through S-nitrosylation, also slowing down the electron flow at the respiratory chain, and thus mitigating any ROS burst (Fago and Jensen, 2015).

Studies analyzing RNS-derived biochemical markers in estivating or low oxygen stressed animals are scarce. In addition to the well-known products nitrite and nitrate, RNS may react with a range of cellular components producing, for example, iron-nitrosyl (FeNO), S-nitroso (SNO), and N-nitroso (NNO) compounds (Challis and Kyrtopoulos, 1979; Joshi et

al., 2002; Kelm, 1999). Jensen et al. (Jensen et al., 2014) reported that, in the anoxia-tolerant red-eared slider turtle *T. scripta*, NO metabolites (FeNO and NNO) increased in response to a 9-day exposure to anoxia in all analyzed tissues. Other metabolites such as SNO also increased during anoxia but decreased shortly after O_2 reintroduction, which makes it a good candidate molecule for it to be involved in S-nitrosylation of complex I and, thus, controlling ROS formation. Nitrite, which has also been demonstrated to be an important cytoprotector upon ischemia-reperfusion events (Dezfulian et al., 2007), increased in a tissue-specific way in turtles exposed to anoxia (Jensen et al., 2014). Animal species that estivate are also interesting study models. A study in the lungfish *Protopterus dolloi* showed an increase in NOS activity in heart and kidney after 40 days estivation, suggesting that NO is involved in the adjustment of these organs (Amelio et al., 2008). Such long term estivation had no effect in heart nitrotyrosine levels in the pulmonate snail *Achatina fulica* (subject to four-week estivation) (Salway et al., 2010) or the lungfish *P. dolloi* (60-day estivation) (Page et al., 2010), although levels increased in other tissues such as brain (Page et al., 2010). After an estivation period of 6 months (Chng et al., 2014), another lungfish, *Protopterus annectens*, also had increased levels of nitrite and nitrate in liver. These and other examples not reviewed here indicate some similarities regarding NO-derived metabolism among taxa.

At the transcriptional level, NO is also involved in the regulation of hypoxia-related genes. Several studies focused on the role of NO and its derivatives in stabilization of HIF, a key component in hypoxic acclimation. HIFs are not only stabilized by a decrease in O_2, but it also requires S-nitrosylation of certain pathway components as recently reviewed (Ho et al., 2012; Poyton and Hendrickson, 2015), highlighting the important role of RNS in hypoxic acclimation.

9. Lipid peroxidation and hypoxia studies

9.1. Molecular mechanisms for increased lipid peroxidation under hypoxia

A number of studies have shown increased lipid peroxidation products, including TBARS and lipid hydroperoxides, in tissues of many animal species under low oxygen stress (Table 4). However, molecular oxygen (O_2) is a critical substrate for the propagation of the lipid peroxidation cascade (Yin et al., 2011), playing a role, for instance, in the reaction of alkyl radicals with O_2. Therefore, if O_2 is essential for the lipid peroxidation cascade, how can it be enhanced in organisms exposed to hypoxic conditions?

As discussed above, mitochondria may increase ROS formation (specifically $O_2^{\bullet-}$ and H_2O_2) under low oxygen stress. Furthermore, H_2O_2 can undergo a heterolytic reduction in the presence of iron ions or heme-containing proteins giving rise to hydroxyl radicals. This radical, in turn, can abstract hydrogen atoms from unsaturated lipids initiating lipid peroxidation reactions (Hermes-Lima, 2004). However, the burst of free radicals produced under hypoxia explains only the formation of alkyl radicals, but fails to explain the formation of peroxyl radicals, a crucial step that requires the reaction with O_2. In this context, Hernansanz-Agustín and co-workers (Hernansanz-Agustín et al., 2014) showed that the burst of free radical production – measured by superoxide detection with DHE – lasts for 30-60 min in cells exposed to hypoxia (Hernansanz-Agustín et al., 2014). If this hypoxia-induced superoxide production occurs for such a short period in most animal species, how can we explain the increased lipid peroxidation observed over the course of hours or days under hypoxia?

One hypothesis to explain this increased oxidative damage in animals exposed to hypoxia is related to the chemistry of O_2 and its solubility in membranes (Scheme 1). Since oxygen is a non-polar molecule, its solubility in the non-polar core of lipid membranes is higher than in aqueous media (Dzikovski et al., 2003; Windrem and Plachy, 1980).

Therefore, regardless of the overall O_2 availability in tissues/cells, the pO_2 in the hydrophobic portion of the membrane should be higher than the pO_2 in the aqueous phase of cytosol. Such behavior ensures that there would be enough O_2 in the membrane to allow the occurrence of lipid peroxidation even under hypoxia.

Scheme 1. *Chemical equilibrium of oxygen in cells.*

Once alkyl radicals are generated in the initial burst of free radicals (equation 1 in scheme 2) the following reactions of the cascade can occur for hours (even days) with the residual O_2 in the membrane. This relatively high abundance of O_2 in the membrane during hypoxia could be a key aspect to explain the increased levels of lipid peroxidation products in a number of studies discussed in this article (Table 4). Noteworthy, this reaction (equation 2 in scheme 2) is favorable from both a thermodynamic and a kinetic point of view (Yin et al., 2011). Peroxyl radicals, in turn, generate several other oxidizing species – including lipid hydroperoxides – that could induce the oxidative damage observed in animals exposed to hypoxia (**scheme 2**). The production of such peroxyl-derived oxidizing species would be independent from the pO_2 inside the cell, which could also be related to the increase in lipid peroxidation during hypoxia. Moreover, besides their involvement in free radical reactions, lipid hydroperoxides can also affect membrane organization and cell-signaling effects, leading to increased cellular protection, apoptosis or necrosis (Girotti, 1998; Miyamoto and Di Mascio, 2014).

$$^{\bullet}OH + LH \rightarrow L^{\bullet} + H_2O \qquad \text{(equation 1)}$$

$$L^{\bullet} + O_2 \rightarrow LOO^{\bullet} \qquad \text{(equation 2)}$$

□□□• + LH → LOOH + L• (equation 3)

2 LOO• → LOOOOL → LO + LOH + $^1O_2$ (equation 4)

LOOH + Fe^{2+} → LO• + OH^- + Fe^{3+} (equation 5)

LO• + LH → LOH + L• (equation 6)

Scheme 2*. Simplified lipid peroxidation cascade showing the requirement of O_2 and formation of oxidizing species.* ***Equation 1*** *represents the initiation of the lipid peroxidation cascade promoted by •OH (generated in the burst of free radicals).* ***Equation 2*** *represents the critical step that requires O_2.* ***Equations 3 to 6*** *represent reactions in the peroxidation cascade that produces oxidizing species, including peroxyl and alkoxyl radicals and singlet molecular oxygen ($^1O_2$)* (Miyamoto and Di Mascio, 2014; Miyamoto et al., 2003). *Excited carbonyl species can also be formed as a product of the Russel mechanism* (Miyamoto et al., 2003). *Such species, in turn, could lead to oxidative damage.*

The lipid peroxidation cascade ends with the production of ketones and aldehydes, including α,β-unsaturated species such as 4-hydroxynonenal (HNE) (Hermes-Lima 2004). These species have been shown elevated in cells exposed to hypoxia (Cervellati et al., 2014) and also conjugated to GSH in livers of a fish under hypoxic conditions (Bastos et al., 2013). Due to the chemical nature of species such as HNE, it can covalently modify several amino acids in the cell through either a Michael addition (addition to the double bond) or a Schiff base mechanism (formation of an imine bond) (Isom et al., 2004). Indeed, lysine, histidine, cysteine and arginine residues were modified in proteins modified by HNE (Isom et al., 2004). Such modifications are able to alter protein structure and function, which leads to protein malfunction, oxidative stress, signaling effects and cell death (West and Marnett, 2006).

Overall, the observed increase in oxidative stress could be related to the residual oxygen levels found in the membrane even after long periods under hypoxic conditions. In addition, the electrophiles produced over the course of the lipid peroxidation could also modulate Nrf2 activity (see following topic) and, therefore, the enzymatic antioxidant response of the organisms under hypoxia.

9.2. Lipid peroxidation products as signaling molecules

A new line of thought to explain the activation of antioxidant response in organisms exposed to long periods of hypoxia is based on the effects of electrophiles in cells. As discussed above, lipid peroxidation is a process that can occur "independently" from the pO_2 in the cell and may last for hours (even days). In such scenario, lipid peroxidation products – as electrophilic aldehydes – could be produced hours (or days) after the beginning of the hypoxia. These molecules, such as HNE, may play a pivotal role in triggering the antioxidant defense in organisms. Some reports show that HNE can react with amino acid residues in the Keap1-Nrf2 complex, modulating its activity (Higdon et al., 2012; Kansanen et al., 2012). Although the exact mechanism remains unclear, Kansanen and co-workers (Kansanen et al., 2012) suggest that HNE covalently modifies specific cysteine residues in the Keap1 domain (Kansanen et al., 2012). These modifications decrease the affinity between Keap1 and Nrf2, which allows Nrf2 translocation to the nucleus where it activates the antioxidant response (Higdon et al., 2012; Kansanen et al., 2012). Moreover, treatment with oxidized LDL also led to an activation of Nrf2 pathway, corroborating the finding that lipid peroxidation products modulate antioxidant response (Ishii et al., 2004). In addition to non-specific lipid peroxidation products (as HNE), enzymatic lipid peroxidation products, such as prostaglandins, have been shown to modulate Nrf2 pathway, increasing antioxidant response (Figure 4; (Higdon et al., 2012)).

9.3. Challenges for lipid peroxidation measurements in comparative biology

A major limitation of our study (and the comparative biology field) is that most the available data on lipid peroxidation is based on TBARS, xylenol orange and determinations of lipid hydroperoxides by conjugated dienes. All these methods have been publicly criticized due to their lack of specificity, which could lead to a misinterpretation of the actual levels of lipid peroxidation. Therefore, one of the great challenges of the field is to improve the analytical measurements of lipid peroxidation. Methods as TBARS should not be used when the matrix is complex, which is the case of all studies of the comparative biology field (Forman et al., 2015). Future researchers should consider replacing such methods by more precise methods of detection, such as the detection of F2-isoprostanes by mass spectrometry (Liu et al., 2009; Milne et al., 2007).

One can also argue that the observed increase in lipid peroxidation markers in tissues of hypoxia-exposed animals could be due to post-mortem effects. However, since the handling of control (normoxia) and hypoxic groups were equal, the post-mortem effects were also equal, which would only increase the baseline for both groups without affecting the difference already present.

10. Conclusions, limitations and perspectives

10.1 Free radical formation under low oxygen stress and estivation produce redox imbalance and activate antioxidant defenses

As mentioned before, it was recently proposed that increased ROS generation during hypoxia – in comparison with normoxia – is responsible for the activation of transcriptional factors involved in the up-regulation of antioxidant enzymes (Welker et al., 2013). This mechanism potentially explains how antioxidant enzymes can be activated under estivation and low oxygen stress: anoxia/hypoxia (in which ROS formation could be higher in the

hypoxic phase that precedes full anoxia), dehydration, freezing, and air-exposure of water breathing animals. Thus, ROS-mediated activation of redox-sensitive transcription factors and pathways leading to post translational modifications are - according to our proposal - key components of the molecular POS mechanism.

Furthermore, the increase in GSH oxidation (towards GSSG), lipid peroxidation, protein oxidation and DNA damage - reported in many works throughout this review - can be explained by an augment in ROS formation. One example is the increased levels of lipid peroxidation after 6 days under anoxia in marine gastropods (Pannunzio and Storey, 1998). In another example, the putative low pO_2 in internal organs of land snails during estivation could be the reason for an increased ROS formation, leading to mild oxidative stress (increased lipid peroxidation, protein oxidation and GSSG levels; Ramos-Vasconcelos and Hermes-Lima, 2003). Thus, the augment in endogenous antioxidant defenses may minimize oxidative damage under (i) low oxygenation and (ii) also in the following condition: normoxic recovery, which is expected to increase ROS generation.

In summary, we propose that the following events underlie the increased expression of endogenous antioxidants in response to oxygen restriction known as "preparation for oxidative stress" (Figure 4):

(i) Once animals are exposed to low oxygen stress, oxygen concentration begins to drop and, at some point, pO_2 reaches a threshold level, in which electrons accumulate at the mitochondrial electron transport chain and, thus, the generation of superoxide radicals and H_2O_2 increases temporarily;

(ii) This increment in ROS levels under low oxygenation may: (a) cause redox imbalance, increasing the GSSG/GSH-eq ratio; (b) oxidize cellular components directly or participate in reactions that produce other oxidizing species (e.g. peroxynitrite and lipid hydroperoxides), increasing the levels of oxidative stress markers (e.g. conjugated dienes,

protein carbonyls and 8-oxodGuo); (c) trigger the activation of redox-sensitive transcription factors (e.g. FoxOs, HIF-1, NF-κB, Nrf2 and p53) resulting in an increased expression of antioxidant defenses; and (d) activate signaling pathways (e.g. Sirt3 and specific kinases) that cause post translational modifications in both antioxidant enzymes and redox-sensitive transcription factors. The overall result would be an enhanced antioxidant system.

(iii) After some period of time the burst in ROS generation will eventually decrease and so will its effects. However, electrophile lipid peroxidation products may further extend the signal for the expression of antioxidants by acting on transcription factors (e.g. Nrf2; section 9.2). This should be important to maintain the "POS response" in long-term hypoxia.

To some extent, our proposal is a simplification of a complex process that may be affected by the action of RNS (section 8), protein chaperones (Storey and Storey, 2011; Trübenbach et al., 2014), uncoupling proteins (UCPs 2 and 3 seen to control mitochondrial ROS formation; Issartel et al., 2009) as well as the presence of non-enzymatic compounds such as ascorbate (Rice et al., 2002) or uric acid (Giraud-Billoud et al., 2011).

10.2 Limitations on the POS mechanistic proposal

The biggest limitation of our POS hypothesis is that actually there is no direct evidence that mitochondrial ROS generation increase during estivation or under low oxygen stress. The few works that had measured ROS levels using chemical probes indicate that indeed ROS formation still occurs during oxygen deprivation (even in animals under anoxia) (Milton et al., 2007; Rivera-Ingraham et al., 2013a; Rivera-Ingraham et al., 2013b), but, so far, there is no report of increased ROS in such situations. On the other hand, there are many pieces of indirect evidence that indicates that ROS levels should rise. The disturbed redox balance, increased levels of oxidative stress markers and the increase in antioxidant defenses itself points toward a conditions of increased ROS generation. Thus, the hypothesis that a

burst in ROS formation occurs in hypoxia-tolerant and estivating animals is still to be experimentally tested.

Another relevant limitation of our proposal is that we can not predict (or even estimate) exactly when ROS production is expected to increase (and then activate antioxidant defenses) once exposure to low oxygen stress begins. The reason is that available data on the activation of endogenous in oxygen-restricted and metabolic depressed animals varies enormously in terms of exposure time. If we look at the reports of increased antioxidant defenses only, the response times ranged from 5 h to 24 h for freezing; 12 h to 1 week for dehydration; 10 min to 21 days for hypoxia/anoxia; 1 to 18 h for air exposure; and 6 to 180 days for estivation. Moreover, the respiratory physiology of a given animal is also expected to affect the exact time in which the proposed phenomena (i.e. increased ROS formation and antioxidant response) take place.

What is common among all these cases is that so many different animals had increased antioxidants - at some point - when exposed to low oxygen stress. This process was observed in six animal phyla: cnidarians (corals), annelids (polychaetes), tardigrades, mollusks (bivalves and gastropods), arthropods (crustaceans and insects) and vertebrates (fish, amphibians and reptiles) (Figure 5). Such widespread distribution is also observed for hypoxia tolerance phenotypes (p. 108, Hochachka and Somero, 2002).

10.3 Historical perspective

Our present article described the scientific path that led to a biochemical/molecular explanation on how animals respond to low oxygen stress. It has been observed by a great number of authors since the 1990s that many animals increase their antioxidant defenses during estivation and under low oxygen stress and this was interpreted as a way to protect themselves against the potential danger of reoxygenation or reoxygenation-like stress. For

quite a number of years researchers could not go beyond a biological/physiological explanation for the POS phenomenon. This phenomenon was regarded as an adaptive strategy for hypoxia/reoxygenation survival, with ecological relevance for animals facing intermittently oxygen restriction in nature [1] (Costantini, 2014). The observations that mitochondrial ROS formation could be increased under hypoxia in mammalian cells shed some light on the potential molecular pathways to induce the POS phenomena (section 5). Evidences for that started to emerge in the 1980s (Table 5), and for many years, in the words of Thomas Clanton in an editorial, "*scientists have been hesitant to embrace the idea that conditions of hypoxia induce ROS in the absence of reoxygenation*" (Clanton, 2005). Therefore, according to our understanding, ROS formed at an early phase of estivation or low oxygen stresses could both (i) activate endogenous antioxidants and (ii) inflict oxidative damage to biomolecules in anoxia/hypoxia tolerant animals. Other reactive species, such as 4-HNE and prostaglandins (lipid peroxidation products), as well as RNS, may also play relevant roles in the POS process.

The key aspect of the present article is that the original POS proposal has not changed to "something else", but it has evolved from a simplistic theory to an explanation with molecular mechanisms based on several direct and indirect evidences. The "danger" of reoxygenation (well recognized since the 1980s) is still there (Chouchani et al., 2014). The understanding of the mechanisms that allow animals to respond to low oxygen stress should pave the road for further experiments that now can be based on a firm rational and on a hypothesis that can be tested, confirmed or dismissed. Because most of the studies about low oxygen stress was performed in the laboratory, the occurrence of the mechanisms proposed herein is yet to be verified in animals in the wild. This is the next frontier.

[1] In contrast to intermittent oxygen availability, animals chronically exposed to hypoxia may present increased fitness when compared to animals in normoxia. This is the case of the fingernail clams (Sphaerium sp.), in which a population living further in the swamp (low dissolved O_2) presents higher number of animals, decreased levels of oxidative damage to nucleic acids and increased reproductive success when compared to clams living in normoxia closer to the stream (Joyner-Matos and Chapman, 2013).

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Figures captions.

Figure 1. The old explanation of how endogenous antioxidants would become enhanced in animals exposed to low oxygen stress. Because the formation of reactive oxygen species was expected to decrease during oxygen deprivation, a "non-radical messenger" would be responsible for the activation of antioxidants. This explanation was first published in a study with garter snakes exposed to freezing and anoxia stresses (Hermes-Lima and Storey 1993) and then referred to by many works in the comparative biology field.

Figure 2. Current view of how changes in oxygen availability and ROS levels would modulate the preparation for oxidative stress. As oxygen concentration declines from normoxia to anoxia, cellular hypoxia occurs. At some point during the hypoxic phase, mitochondrial ROS formation increases temporarily. During reoxygenation, as oxygen concentration rises from anoxia to normoxia (reoxygenation) ROS formation also increases. In both moments, increased ROS levels are expected to cause oxidative damage and activate antioxidant defenses. Thus, ROS are the signaling molecules involved in the preparation for oxidative stress. This figure was based on Welker et al. (2013) and on references therein. Note that this figure has no quantitative meaning; it is just an illustrative expression of how ROS generation would behave during hypoxia/anoxia exposure. Moreover, we cannot predict the specific pO2 where the burst of ROS should happen - this should vary considerably within the many animal species that are tolerant to low oxygenation.

Figure 3. The general proposed mechanism used by organisms to tolerate estivation or low oxygen stress. Low oxygen stress includes: hypoxia (including anoxia), freezing, aerial exposure of water breathing animals, and severe dehydration. The hypoxic nature of such stresses leads to mitochondrial ROS overproduction that causes oxidative damage to biomolecules and activates redox sensitive transcription factors (FoxO, Nrf2, p53, HIF-1α and NF-κB). These transcription factors promote activation of antioxidant defenses (such as catalase, SOD, glutathione transferase, glutathione peroxidase, thioredoxin and peroxiredoxins). Mitochondrial ROS overproduction might also promote post translational modification of antioxidant enzymes. Activation of antioxidant defenses may function as a negative feedback and reduce ROS levels.

Figure 4. The overall view of the mechanisms that participates in the preparation for oxidative stress (POS). When some animal species are exposed to an environmental situation that induce a short term hypoxia exposure, we propose that reactive oxygen species (ROS)

formation increases causing the oxidation of biomolecules (e.g., GSH, proteins, and membrane lipids) and the activation of redox-sensitive transcription factors (Nrf2, HIF, etc). These transcription factors shall induce augmented expression of antioxidant enzymes. Moreover, ROS-mediated covalent modification of in antioxidant proteins may also increase their activities, contributing to the "POS response". The formation of electrophilic products of lipid peroxidation (such as HNE) could also activate Nrf2 and thus contribute to the "POS response" under long-term hypoxia.

Figure 5. Animals from six different phyla when exposed to low oxygen stresses (anoxia, hypoxia, freezing, dehydration and air exposure) or during estivation enhance their antioxidant defenses. The examples are distributed within the following groups: Anthozoa (corals), Polychaeta, Bivalvia (mussels and clams), Gastropoda (land and aquatic snails), Eutardigrada ("water bears"), Malacostraca (amphipods, shrimps, and crabs), Maxillopoda (barnacles), Insecta, Actinopterygii (ray-finned fish), Dipnoi (lungfish), Amphibia (frogs and toads), and Repitilia (snakes and turtles).

Preparation for oxidative stress under hypoxia and metabolic depression: revisiting the proposal two decades later

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Abstract

Organisms that tolerate wide variations in oxygen availability, especially to hypoxia, usually face harsh environmental conditions during their lives. Such conditions include, for example, lack of food and/or water, low or high temperatures and reduced oxygen availability. In contrast to an expected strong suppression of protein synthesis, a great number of these animals present increased levels of antioxidant defenses during oxygen deprivation. These observations have puzzled researchers for more than twenty years. Initially, two predominant ideas seemed to be irreconcilable: on one hand, hypoxia would decrease reactive oxygen species (ROS) production, while on the other the induction of antioxidant enzymes would require the overproduction of ROS. This induction of antioxidant enzymes during hypoxia was viewed as a way to prepare animals for oxidative damage that may happen ultimately during reoxygenation. The term "preparation for oxidative stress" (POS) was coined in 1998 based on such premise. However, there are many cases of increased oxidative damage in several hypoxia tolerant organisms under hypoxia. In addition, over the years, the idea of an assured decrease in ROS formation under hypoxia was challenged. Instead, several findings indicate that the production of ROS actually increases in response to hypoxia. Recently, it became possible to provide a comprehensive explanation for the induction of antioxidant enzymes under hypoxia. The supporting evidence as well as the limitations of the POS idea are extensively explored in this review as we discuss results from research on estivation and situations of low oxygen stress, such as hypoxia, freezing exposure, severe dehydration, and air exposure of water-breathing animals. We propose that, under some level of oxygen deprivation, ROS are overproduced and induce changes leading to hypoxic biochemical responses. These responses would occur mainly through the activation of specific transcription factors (FoxO, Nrf2, HIF-1, NF-κB and p53) and post-translational mechanisms; both mechanisms leading to enhanced antioxidant defenses. Moreover, reactive nitrogen species are candidate modulators of ROS generation in this scenario. We conclude by drawing out the future perspectives in this field of research, and how advances in the knowledge of the mechanisms involved in the POS strategy will offer new and innovative study scenarios of biological and physiological cellular responses to stress.

Keywords: anoxia, dehydration, estivation, freeze tolerance, hypoxia tolerance, ischemia.

1. Introduction

Oxygen is essential for the majority of organisms on Earth. For most of them, oxygen restriction is highly deleterious. However, many animal species are able to survive long periods of oxygen deprivation, including a variety of invertebrates and vertebrates. In nature, oxygen deprivation ranges from mild hypoxia to complete anoxia (Welker et al., 2013). Information on the physiological processes and biochemical mechanisms to cope with hypoxia, in special metabolic depression, has been amassed for the past 30-40 years, and several mechanisms have been unveiled (Storey and Wu, 2013). One of the issues that have interested researchers since the 1990s is the role of free radicals in the process of natural hypoxia tolerance, and our aim is to discuss the interplay between reactive oxygen species and this process. Additional natural conditions that involve reduced oxygen delivery to organs, such as freezing stress and severe dehydration will also be covered in this article.

One well-known mechanism used to survive low oxygen levels is the severe depression of the metabolic rate during oxygen deprivation in association with lower rates of ATP production via fermentative pathways. Such ability to severely slow down many energy-consuming pathways is a key strategy for survival. It includes reduction of key metabolic enzymes activities via post translational modifications, or via decreased transcription and/or translation. Changes in expression/activity of several kinases and phosphatases, transcription factors and microRNAs participate in such responses (Biggar and Storey, 2015; Staples and Buck, 2009; Storey and Storey, 2007; Storey and Storey, 2012). Next, the modulation of free radical metabolism during oxygen deprivation in hypoxia tolerant animals is presented.

In the early 1990s it was well established that ischemia and reperfusion episodes in mammalian organs elicited intense formation of reactive oxygen species (ROS) during the recirculation of oxygenated blood (Zhu et al., 2007; Zweier et al., 1987). There were unequivocal evidences that increased formation of ROS during reperfusion was one of the

key factors for cell damage under these conditions. Mitochondria were already recognized as the major source of excess ROS formation during reoxygenation. Based on the knowledge about ischemia/reperfusion injury it was reasonable to argue that hypoxia tolerant animals would be adapted to endure the potential dangers of reoxygenation. At that time, it was hypothesized by one of us that these animals would rely on high levels of antioxidant defenses to control the effects of a putative overproduction of ROS during reoxygenation.

The first examination of this hypothesis was performed using garter snakes *Thamnophis sirtalis parietalis*, which are naturally tolerant to anoxia for a few days, and freezing for several hours. The activities of garter snake antioxidant enzymes, such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GPX) and glutathione S-transferase (GST) in liver, muscle and lung were determined. Unexpectedly, garter snake enzyme activities (in tissues from control animals) were much lower than those reported, for example, in mice and rats. On the other hand, it was also unexpected that some of the activities increased during exposure to anoxia for 10 h at 5°C or freezing for 5 h (with 40-50% of the total body water frozen at −2.5°C) (Hermes-Lima and Storey, 1993). This increase was observed for SOD in muscle and liver from anoxia-exposed snakes, as well as for catalase in muscle and lung and for GPX in muscle from frozen animals. In both conditions the increase in enzyme activities was higher than 100% (liver SOD and muscle GPX) when compared to controls. Moreover, levels of reduced glutathione (GSH) increased by about 1.6-fold in muscle of anoxia-exposed garter snakes (Hermes-Lima and Storey, 1993).

In the light of these results, it was suggested that garter snakes activated their antioxidant defenses during conditions when ROS production would be low (freezing) or absent (anoxia). The reasoning at that time was that free radical formation would always be dependent on and directly proportional to oxygen availability. Thus, under the hypoxic

114 condition of freezing oxygen radicals production would be low. This assumption hampered
115 the understanding of how antioxidant defenses were being activated. In those days, it was
116 already known that up-regulation of genes for antioxidant defenses happens under oxidative
117 stress conditions, i.e. rises in ROS formation should stimulate mechanisms that result in
118 increased expression of antioxidant enzymes. Because garter snakes under anoxia or freezing
119 would be under severe oxygen limitation, it was improbable (at 1990s) that ROS formation
120 would increase under these conditions. Therefore, it was proposed that these snakes enhanced
121 their antioxidant capacity in order to prepare them for a putative increase in ROS generation
122 and oxidative stress during reoxygenation (i.e. increasing the defenses before oxidative
123 stress). The conclusion of the study (Hermes-Lima and Storey, 1993) was:

124 "*The antioxidant defenses are built when oxyradical formation is not likely to occur*
125 *(under the frozen or anoxic condition) in anticipation of their need when the*
126 *perfusion of oxygenated blood is reinitiated. A nonradical messenger must stimulate*
127 *these enzyme systems at either transcriptional, translational, or posttranslational*
128 *levels.*"

129 Since then, many studies reported that when hypoxia-tolerant animals are exposed to
130 low oxygen stress there is an increase in their antioxidant defense capacity (Hermes-Lima
131 and Zenteno-Savín, 2002; Hermes-Lima et al., 2001; Storey, 1996; Welker et al., 2013). Low
132 oxygen stress situations include anoxia, hypoxia, freezing, severe dehydration and aerial
133 exposure of water-breathing animals. Estivation is another situation related to the adaptation
134 to harsh environmental conditions (and, in the case of gastropods, decreased O_2 availability in
135 tissues) in which endogenous antioxidants were found increased. In the late 1990s, this
136 process was coined as "preparation for oxidative stress" (POS) (Hermes-Lima et al., 1998).

137 Although there were growing evidences of increased antioxidant levels in animals
138 under low oxygen stress (i.e. POS), a biochemical explanation on the molecular basis of this

mechanism was still missing. The first observation of POS dates back to 1993 and only in 2005 the first attempt to explain the molecular mechanisms underlying antioxidant activation appeared in the literature (Almeida et al., 2005). The present work describes how we put together the puzzle about the POS mechanism and discuss a rational for the understanding of this process.

2. Preparation for oxidative stress (POS) under low oxygenation

After the pioneering work on garter snakes (Hermes-Lima and Storey, 1993) many studies reported that anoxia or hypoxia exposure in several other organisms induces increased activity or gene expression of antioxidant enzymes. Leopard frogs (*Rana pipiens*) under 30 h anoxia presented increased activities of catalase in muscle and heart, GPX in heart and brain, as well as GST in brain (Hermes-Lima and Storey, 1996). Goldfish (*Carassius auratus*) under anoxia showed increased GPX activity in brain and catalase activity in liver (Lushchak et al., 2001). Crabs (*Chasmagnathus granulata*) under anoxia showed increased catalase and GST activities in both anterior and posterior gills (de Oliveira et al., 2005). Anoxia exposure for six days of the marine gastropod (*Littorina littorea*) caused an increase in GSH levels, although the activity of antioxidant enzymes were reduced (Pannunzio and Storey, 1998). Furthermore, pupae of the Caribbean fruit fly, *Anastrepha suspensa*, presented higher GPX and MnSOD activities, while the activities of catalase and CuZnSOD remained unchanged in response to 1 h anoxia (Lopez-Martinez and Hahn, 2012).

Similar results have been observed in several animals exposed to hypoxia. Common carps (*Cyprinus carpio*) under hypoxia for 5 h presented increased catalase and GPX activities in brain (Lushchak et al., 2005). Pacific oysters (*Crassostrea gigas*) under hypoxia (from 3 to 24 days) showed increased mRNA expression for GPX in mantle, gill and hepatopancreas (David et al., 2005). Moreover, microarray analysis of these oysters under

hypoxia (for 20 days) also showed increased expression of peroxiredoxin-5 in hepatopancreas (Sussarellu et al., 2010). In the disc abalone, *Haliotis discus discus*, hypoxia for 8 h prompted increased expression (mRNAs) of MnSOD, SeGPX, catalase and thioredoxin (De Zoysa et al., 2009). Exposure of amphipods *Monoporeia affinis* to hypoxia for 5 or 9 days induced a rise in the activities of catalase and SOD (Gorokhova et al., 2010; Gorokhova et al., 2013). In a study with *Balanus amphitrite* barnacles under anoxia, severe hypoxia and mild hypoxia (24 h) resulted in a sharp increase in catalase and SOD activities in larvae and adults (Desai and Prakash, 2009). In another crustacean, the shrimp *Litopenaeus vannamei*, hypoxia exposure for 4 hours elicited an up-regulation of the genes coding for GPX, cytoplasmic MnSOD and peptide-methionine (R)-S-oxide reductase (Kniffin et al., 2014). The later enzyme participates in the repair of oxidatively damaged proteins, specifically methionyl residues. In the case of the subterranean amphipod *Niphargus rhenorhodanensis*, an increase in GPX activity was observed after exposure to either anoxia (24 h) or hypoxia (10 days) (Lawniczak et al., 2013). Other cases of increased antioxidant levels in response to anoxia or hypoxia are presented in Tables 1 and 2.

In addition to hypoxia/anoxia, other conditions are also related to functional low oxygenation, such as freezing stress, severe dehydration and aerial exposure of water-breathing animals. We and other authors observed that leopard frogs under dehydration (Hermes-Lima and Storey, 1998), wood frogs, garter snakes and turtle hatchlings under freezing (Hermes-Lima and Storey, 1993; Joanisse and Storey, 1996; Krivoruchko and Storey, 2010b), and crabs and bivalves under aerial exposure (Almeida and Bainy, 2006; Freire et al., 2011a; Romero et al., 2007) presented increased activities of antioxidant enzymes. The endogenous antioxidants that were found to be increased in response to freezing, dehydration or air exposure are presented below (Tables 1 and 2).

In the majority of the studies the enhancement of antioxidant defenses was regarded as an important adaptation to deal with the recovery/reoxygenation phase from oxygen restriction, in which a sharp ROS overproduction is expected to occur. The trigger to such response, however, was unknown and few attempts were made to explain at the molecular level this phenomenon. If one looks closely, there are clues that indicate a putative trigger to the increase in endogenous antioxidant. These clues are the observations of altered redox balance and increased oxidative stress during low oxygen stress discussed in the next section.

Not all animals respond to oxygen restriction by increasing activity/expression of endogenous antioxidants. Examples are salamanders under anoxia exposure (Issartel et al., 2009), three fish species under hypoxia (Leveelahti et al., 2014), golden gall fly larvae exposed to freezing (Joanisse and Storey, 1998), and in the fish *Heteropneustes fossilis* exposed to air exposure (Paital, 2013; Paital, 2014). Thus, as mentioned in previous works (Hermes-Lima and Zenteno-Savín, 2002), the process of "preparation for oxidative stress" (POS) is not a universal adaptive mechanism in animals for dealing with the stress of low oxygenation. However, POS is present in a great number of species that evolved under the pressure of low oxygen stress.

3. Redox imbalance and oxidative stress under low oxygen stress and metabolic depression

Following the proposal that a "non-radical" messenger would trigger the activation of endogenous antioxidant defenses (Hermes-Lima and Storey, 1993) (Figure 1), there were many observations indicating oxidative stress and/or a redox imbalance during low oxygen stress and estivation. The most relevant examples are discussed below for studies on the stresses of anoxia, hypoxia, freezing, severe dehydration, aerial-exposure of aquatic animals and estivation.

3.1. Anoxia

A 1990s study on the free radical metabolism in leopard frogs (*R. pipiens*) under anoxia indicated that levels of disulfide glutathione (GSSG) were increased in muscle and liver after 30 h anoxia exposure (Hermes-Lima and Storey, 1996). Moreover, the ratio GSSG:GSH-eq was also increased in muscle after 30 h anoxia and in liver at 10 and 30 h of anoxia, followed by a decrease in the GSSG:GSH-eq ratio during recovery. The elevation in this ratio was an indicative of redox imbalance, indicating that GSSG accumulation occurred at 10 and 30 h anoxia. These results were interpreted as a consequence of diminished capacity of anoxic frogs to recycle GSSG, possibly by a reduction in the carbon flux via the pentose pathway, that provides NADPH for glutathione reductase-catalyzed reactions. However, such interpretation did not explain why GSH was still being oxidized under anoxia.

Indeed, in the earlier garter snake study (Hermes-Lima and Storey, 1993), it was observed that GSSG levels increased in muscle from animals exposed to anoxia for 10 h, but not in liver and lung. This was explained as a consequence of the elevation in total-glutathione under anoxia. This increase was possibly due to activation of GSH biosynthesis, because the GSSG:GSH-eq ratio was maintained and levels of GSH increased under anoxia in muscle. This interpretation however failed to explain why oxidation of GSH to GSSG increased under anoxia. Oxidation of GSH by GPX-catalyzed reaction requires H_2O_2 or other hydroperoxides. How would hydroperoxides still be available (or formed) in muscle tissue after 10 h under anoxia? Other intriguing observations were made in a study with marine gastropods *Littorina littorea* under anoxia (Pannunzio and Storey, 1998) showing that lipid peroxidation (determined by two different methods) increased in foot muscle after 6 days anoxia, returning to control levels following recovery. No explanation could be given as how lipids would undergo peroxidation under anoxia. Recently, it was suggested that there might be an increase in mitochondrial ROS formation during the hypoxic phase that preceded full

anoxia (Welker et al., 2013). Figure 2 shows a proposed connection between ROS formation under anoxia/hypoxia exposure and the activation of endogenous antioxidant defenses.

In addition, a Brazilian study with crabs (*C. granulata*) under 8 h anoxia showed an increase in lipid peroxidation in hepatopancreas (de Oliveira et al., 2006). Lipid peroxidation was determined by two methods (conjugated dienes and TBARS) and in both methods the levels increased after 8 h anoxia and diminished upon recovery. The authors explained that by the presence of residual O_2 in the internal tissues after exposure to anoxia, which could trigger ROS formation. However, the general view in those days was that under hypoxia there would be less ROS formation. As discussed in more detail in following sections, since the late 1990s there are amounting evidences for increased ROS formation, at least in mammalian cells, under hypoxia.

3.2. Hypoxia

A Ukrainian study with carps exposed to hypoxia reported an increase in liver lipid peroxidation, determined as TBARS, while no changes were observed in brain, kidney and muscle (Lushchak et al., 2005). At that time, it was expected that low oxygenation would cause a decrease or no change in lipid peroxidation, since it was assumed that ROS formation would be lower. In fact, levels of lipid peroxides (measured as cumene hydroperoxide equivalents by the xylenol orange method), initial products of peroxidation, decreased in carp liver and brain under hypoxia (Lushchak et al., 2005). Based on these findings, the authors speculated that non-radical molecules would be responsible for the activation of antioxidants (Lushchak and Bagnyukova, 2006). However, in their next publication, an increase in oxidative stress markers was observed in rotan fish *P. glenii* exposed to hypoxia (Lushchak and Bagnyukova, 2007). Protein oxidation (as carbonyl protein) increased in brain, liver and muscle after 2 to 6 h of hypoxia exposure. Lipid peroxides concentration also increased in

brain and liver after 2 h of hypoxia. Based on such findings, authors suggested that mitochondrial ROS production could be increased under hypoxia (Lushchak and Bagnyukova, 2007). This series of publications by Lushchak et al. illustrates the transition from the idea of a non-radical messenger (Figure 1) to the current view of increased ROS production during oxygen deprivation that the field has gone through (Figure 2).

An interesting study by UK researchers showed that erythrocytes from carps exposed to hypoxia for 30 days present similar levels of DNA damage when compared to carps exposed to hyperoxia (Mustafa et al., 2011). This result was obtained using three different protocols of the Comet assay. It is well known that hyperoxia induces ROS formation and oxidative stress, therefore the authors concluded that ROS formation also increased under hypoxia, possibly by "electron scape" from the mitochondrial respiratory chain. Therefore, fish oxidative stress would result from either excess or diminished oxygen availability. Since 2007-2008, researchers in general studying hypoxia tolerant animals begun to be aware of the possibility of increased mitochondrial ROS formation under hypoxia (see (Bickler and Buck, 2007)).

A study with two species of subterranean mole rats (*Spalax galili* and *S. judaei*) revealed that these hypoxia tolerant species have increased constitutive expression levels (mRNA) of many genes related to antioxidant protection when compared to laboratory rats (Schülke et al., 2012). This observation was interpreted as a way to counteract the effects of ROS formation under hypoxia: *"The permanent subterranean lifestyle of Spalax most probably requires constant protection against hypoxia-generated ROS injury. If O_2 tension suddenly drops even more, e.g. by flooding of the soil in the rainy season (Shams et al., 2005), immediate prophylactic protection by high antioxidant levels is required."* (Schülke et al., 2012). As far as we know, this is the first study in comparative biology to treat increased ROS formation during hypoxia as an assured phenomenon. Accordingly, the idea that

289 mitochondrial ROS formation is increased under hypoxia in mammalian cells is presented in
290 presented in the 2008 edition of the Lenhinger text-book.
291
292 **3.3. Air exposure**
293 Aerial exposure of aquatic animals, that causes functional hypoxia to internal tissues,
294 has been the subject of many studies (Freire et al., 2011b). Many water breathing sessile
295 animals are periodically subjected to air exposure as a consequence of tidal height variations.
296 In this context, bivalves compose a group of extensively studied animals. Mussels *Perna*
297 *perna* exposed to air for 18 h showed 1.5-fold increase in hepatopancreas GST activity
298 (Almeida et al., 2005). Moreover, when the same species was exposed to air for 24 h
299 increased lipid peroxidation (as TBARS) in gills and hepatopancreas was observed. They also
300 had increased DNA damage in gills measured as levels of 8-oxodGuo (Almeida et al., 2005).
301 The authors originally proposed the idea (quoted below) that increased mitochondrial ROS
302 formation during hypoxia would modulate antioxidant defenses in mussels. This was the first
303 biochemical explanation by which the POS process functions and was also discussed in
304 following publications (Almeida and Di Mascio, 2011; Almeida et al., 2007).
305 *"The decrease in cytochrome oxidase V_{max} during hypoxia is responsible for an*
306 *increase in mitochondrial redox state (Chandel and Schumacker, 2000), which, in*
307 *turn, accelerates ROS generation during hypoxia, triggering the activation of*
308 *different transcriptional factors involved in numerous cellular hypoxia responses.*
309 *Despite its modulator effect, such increase in ROS production would be also*
310 *accounted for increases in lipid and DNA damage in cells."*
311 As in mussels, stone crabs *Paralomis granulosa* exposed to air for 3 to 24 h presented
312 increased protein oxidation in gills, measured as carbonyl protein, while no changes happen
313 in muscle or hepatopancreas (Romero et al., 2007). Moreover, lipid peroxidation, determined

314 as levels of lipid peroxides, increases in muscle and hepatopancreas after 6 to 24 h of aerial
315 exposure. This indicative of increased ROS formation could be a trigger for regulation of
316 several antioxidant enzymes in the tissues of *P. granulosa*.

317 In cnidarians, the air exposure of corals *Veretillum cynomorium* for 2.5 h resulted in
318 no change in TBARS levels, but a sharp increase (by 10-fold) in TBARS levels occurs after
319 30 min re-immersion (Teixeira et al., 2013). Catalase and GST activities increased during air
320 exposure, and SOD activity shows no changes. Interesting, catalase and GST activities
321 returned to control levels upon re-immersion, while SOD activity increased upon re-
322 immersion. In spite of a probable ROS overgeneration upon return to water, an increase in
323 ROS of smaller magnitude may have happened under air exposure, inducing catalase and
324 GST without an increase in oxidative damage.

325

326 **3.4. Freezing and Dehydration**

327 In the mid 1990s, a Canadian study (Joanisse and Storey, 1996) described the
328 alterations in the redox metabolism in a cycle of freeze and thaw in the wood frog *R.*
329 *sylvatica*. The main observation was the increase in GPX activity in several tissues (muscle,
330 liver, kidney, brain and heart) after 24 h of freezing exposure at −2.5°C. Overall lipid
331 peroxidation levels (determined as TBARS and total lipid peroxides) remained stable in all
332 tested tissues after thawing (30 min, 90 min and 4 h). Moreover, GSSG levels remained
333 stable after 24 h thawing in five tissues. It was concluded that thawing would cause no
334 oxidative stress because tissues had increased antioxidant capacity during freezing, in a way
335 to prepare tissues for potentially deleterious effects of ROS. This was in agreement with
336 previous observations in garter snakes under freezing stress (Hermes-Lima and Storey, 1993).
337 However, a couple of observations indicated a redox imbalance in kidney and brain during
338 freezing in wood frogs: the increase in GSSG levels and GSSG:GSH-eq ratio. These changes

were not observed in muscle, liver and heart. The unexpected increase in GSSG and GSSG:GSH-eq ratio were interpreted by the authors as a consequence of reduced capacity (due to hypometabolism) for glutathione recycling (Joanisse and Storey, 1996), the same explanation presented in the study with leopard frogs under anoxia (Hermes-Lima and Storey, 1996). Alternatively, we can currently consider that an increase in ROS formation during freezing could cause GSH oxidation to GSSG in the two frog organs (kidney and brain), even though this was unable to induce lipid peroxidation.

Severe dehydration is a condition that resembles the effect of freezing on internal organs. Dehydration tolerant anurans may endure up to 60% loss of body water. The consequences of this condition are reduced blood volume and increased blood viscosity, which induces a severe loss in aerobic cardiovascular capacity, including a decline in pulse rate and oxygen consumption. Therefore, internal organs become hypoxic during severe dehydration. Restoration of body fluids in dehydrated anurans is comparable to reoxygenation/reperfusion, in which an increase in ROS formation would be expected (Hermes-Lima and Zenteno-Savín, 2002; Hermes-Lima et al., 2001).

The observed increase in the activity of antioxidant enzymes in liver and muscle (and levels of hepatic GSH) during severe dehydration (50% body water loss) in leopard frogs was considered a preventive process to counteract the effects of ROS during rehydration (Hermes-Lima and Storey, 1998). As in the case of freezing, ROS formation was supposed to be decreased only during dehydration, due to the hypoxic/ischemic condition. However, levels of GSSG in liver, as well as the GSSG:GSH ratio, increased during dehydration by 80-90%. These results were regarded as a failure in the GSSG recycling mechanism, as in the case of wood frogs under freezing or leopard frogs under anoxia. On the other hand, this redox imbalance in liver could be (from the current point of view) a sign for increased ROS formation during severe dehydration.

In summary, there is a wide range of species that had signs of redox imbalance and increased levels of oxidative markers when exposed to low oxygen stress (anoxia, hypoxia, air exposure, freezing and dehydration). In most cases, these observations were somehow unexpected due to the lack of O_2 availability. Thus, due to the assumption of an assured reduction of ROS formation during oxygen restriction, the perturbations in redox balance parameters as well as the increases in oxidative stress markers (Table 4) were left without explanation or explained by "non-radical" hypotheses. It was not until 2005 (Almeida et al., 2005) that an explanation different from the idea of a "non-radical" messenger was made and set the base to a more comprehensive explanation of the "preparation for oxidative stress".

4. Estivation

Another adaptation to stressful environmental condition that is related to modulation of the antioxidant capacity is estivation in snails, fish and anurans. Estivating land snails *O. lactea* deeply depress their metabolic rate and continue to rely on aerobic catabolism. As a result of the dormancy phenotype, oxygen partial pressure decreases in the hemolymph of estivating snails (Barnhart, 1986). Thus, mitochondrial respiratory chain may function at "low speed" due to restricted provision of NADH and/or $FADH_2$ in a hypoxic intracellular environment. Therefore, in the mid 90s, it was considered that mitochondrial ROS formation would be low under estivation (Hermes-Lima et al., 1998). On the other hand, there is a bout in oxygen consumption (Herreid, 1977) that could increase mitochondrial ROS formation and oxidative stress during early moments of arousal.

The activities of several antioxidant enzymes in *O. lactea* increase after 30 days estivation (Hermes-Lima and Storey, 1995). This occurs with total SOD and GPX in hepatopancreas and total SOD, catalase and GST in foot muscle, which could be a preparatory mechanism to control oxidative stress during arousal. Moreover, lipid

389 peroxidation (as TBARS) increased in hepatopancreas during the first minutes of arousal. It
390 was suggested that the enhanced antioxidant capacity during estivation functioned in a way to
391 minimize such oxidative stress during arousal (few years after this study, the concept of
392 "preparation for oxidative stress" was proposed).

393 This adaptive mechanism was also reported for land snails *Helix aspersa*, because
394 GPX activity increased in hepatopancreas and foot muscle (by 2-3-fold) during 20-day
395 estivation (Ramos-Vasconcelos and Hermes-Lima, 2003). Levels of GSH increased during
396 estivation in hepatopancreas, but not in foot muscle, suggesting that GSH biosynthesis
397 increases during snail estivation. Furthermore, the GSSG:GSH-eq ratio increased in
398 hepatopancreas during the first moments of arousal, indicating that arousal promotes redox
399 imbalance. Levels of TBARS did rise in hepatopancreas when comparing snails active for 5
400 min versus 30 min. The increase in GSH and GPX levels were regarded as an adaptation to
401 minimize oxidative stress just during arousal, but not during estivation. The same conclusion
402 was made for a study on estivation of the aquatic snail *Biomphalaria tenagophila* in which an
403 increase in GPX activity occurred at 15 days estivation (Ferreira et al., 2003).

404 It was proposed in the studies cited above that during estivation ROS production
405 would be decreased, and that an alternative "non-radical" mechanism would activate
406 antioxidant defenses. However, when re-examining these studies, a number of evidences
407 indicate that ROS formation increases in estivating snails, compared to active snails. In the
408 case of *O. lactea*, the increase in GSSG levels in hepatopancreas and foot muscle and the
409 increase in GSSG:GSH ratio in foot muscle alone during estivation (Hermes-Lima and
410 Storey, 1995) are evidences of a redox imbalance. The hypoxic condition in between breaths
411 in *O. lactea*, as well as the increase in oxygen input during breaths, could increase
412 mitochondrial ROS formation during estivation.

In the case of *H. aspersa*, there is clear evidence for increased ROS formation and consequent oxidative stress during estivation, even though this was not concluded in the original article. When compared to aroused active snails, estivating animals had increased TBARS and lipid peroxides levels in hepatopancreas, increased carbonyl protein levels in foot muscle and increased GSSG concentration in hepatopancreas (Ramos-Vasconcelos and Hermes-Lima, 2003). These findings strongly suggest that oxidative stress increases during 20-day winter estivation in *H. aspersa*. No changes in markers of oxidative stress and redox balance happened in summer estivation in this snail species (Ramos-Vasconcelos et al., 2005).

In addition, a Polish study on *Helix pomatia* revealed an increase in lipid peroxidation (determined as TBARS) in muscle and kidney, but not in hepatopancreas, during winter torpor. Moreover, these land snails increase their enzymatic antioxidant activities, especially catalase and glutathione-related enzymes during torpor (Nowakowska et al., 2009). The results obtained with *H. pomatia* in the field roughly agree with those from estivating *O. lactea* and *H. aspersa* performed in the laboratory.

The up-regulation of antioxidant proteins, such as catalase and thioredoxin peroxidase, as well as some chaperones (small heat shock protein and protein disulfide isomerase), was also reported in the freshwater apple snail *Pomacea canaliculata* after 30 days of estivation (Sun et al., 2013). Furthermore, other authors also found increased TBARS concentration in total soft tissue or in foot and kidney, indicating oxidative stress in apple snails after 45 days of estivation at 23-25°C (Giraud-Billoud et al., 2011; Giraud-Billoud et al., 2013). Under these conditions uric acid was possibly used as non-enzymatic antioxidant in hepatopancreas and kidney (Giraud-Billoud et al., 2013). Furthermore, when metabolic depression was induced at 13°C these snails showed an increase in both TBARS

437 concentration (in hepatopancreas, kidney and foot) and in antioxidant defenses (SOD and
438 GSH) in foot muscle (Giuffrida et al., 2013).

439 Estivation and oxidative stress were also investigated in anurans. The first study
440 analyzed desert spadefoot toads *Scaphiopus couchii* and compared animals that had been
441 estivating for two months with active animals (Grundy and Storey, 1998). The majority of the
442 activities of antioxidant enzymes and concentrations of GSH were decreased in several
443 tissues of toads under estivation (exceptions are shown in Table 3). Moreover, in most organs
444 lipid peroxidation parameters, as well as GSSG:GSH ratio increased during estivation (Table
445 4).

446 A study with the striped burrowing frog *Cyclorana alboguttata* showed that
447 superoxide scavenging capacity (possibly SOD activity) increased in both iliofibularis and
448 gastrocnemius muscles during estivation. Carbonyl protein levels increased in the iliofibularis
449 muscle, but not in the gastrocnemius muscle, after 6 months of estivation (Young et al.,
450 2013). Another study with these animals showed that the mRNA levels for glutamate
451 cysteine ligase regulatory subunit and GST-O2 increased after 4 months of estivation (Reilly
452 et al., 2013). Furthermore, H_2O_2 formation by permeabilised gastrocnemius frog muscle was
453 decreased after 4 months estivation, while it remained unchanged in cardiac muscle when
454 compared to muscles from active controls (Reilly et al., 2014). These observations as a whole
455 indicate that there is a response of the redox metabolism (ROS formation, oxidative damage
456 and expression of antioxidants) during estivation in green-striped frogs and such response is
457 dependent on muscle type.

458 In the case of lungfish *Protopterus dolloi* increased activity/protein levels of
459 antioxidant enzymes were observed in brain (MnSOD, CuZnSOD, catalase and GR) and
460 heart (GPX) after 60 days of estivation. However, there was no increase in the levels of
461 indicators of oxidative stress, except for nitrotyrosine levels in brain (Page et al., 2010).

462 Because the ratio GSSG:GSH was not measured, one cannot conclude if a redox imbalance
463 took place during estivation of *P. dolloi*.
464 Similar to what was observed for low oxygen stresses there are many examples of
465 estivating animals that had increased oxidative stress markers, and enhanced antioxidant
466 defenses. These observations indicate that ROS generation increase at some point of
467 estivation in anurans, gastropods and lungfish. Such increase in ROS production would in
468 turn trigger mechanisms that would ultimately result in increased expression of endogenous
469 antioxidants. ROS-mediated activation of Nrf2 and FoxO1 transcription factors (Malik and
470 Storey, 2009; Malik and Storey, 2011; Reilly et al., 2013) could be one of these mechanisms.
471
472 **5. Mechanisms of ROS formation under hypoxia – evidence in mammalians and**
473 **invertebrates**
474 The effects of hypoxia on mammalian models have been long and extensively studied
475 under the premise that ROS production is directly proportional to oxygen concentration. But
476 it is not until the early 1970s, with the radiobiological studies by Hall (Hall, 1973), that we
477 find the first reports providing a deeper insight on the levels of free radical production under
478 limiting oxygen conditions. Rao et al. (Rao et al., 1983) later reported that coronary occlusion
479 elicited a 55% increase in free radical levels in dog ventricular tissue when compared to
480 normoxia. Such observations constituted a key finding that opened a completely new
481 perspective on what was known at the time on ROS formation under limited oxygen
482 concentrations and set the bases for later works.
483 The impact of the earlier works by Rao et al. (Rao et al., 1983) is demonstrated by the
484 growing number of studies showing similar increases in ROS upon hypoxic exposure in a
485 wide diversity of mammalian cell lines, cells types and tissues (Table 5). Rao et al. (Rao et
486 al., 1983) measured free radical formation through electron spin resonance, so far the only

analytical method for direct detection of free radicals (Malanga and Puntarulo, 2011), but the theory of increased ROS formation under hypoxia has also been widely supported by data obtained using fluorescent specific probes for detecting ROS. Imaging cellular events is by no means an easy task, and much controversy has arisen on the use of such techniques (Forman et al., 2015; Kalyanaraman et al., 2012). Further studies have however confirmed hypoxic-ROS production through the application of RNA interference techniques in conjunction with the use of protein-based fluorescence resonance energy transfer (FRET) sensors (HSP-FRET). Guzy et al. (Guzy et al., 2005) applied such a ratiometric probe, consisting in the fusion of two fluorescent peptides (one yellow, YFP, and one cyan, CFP) linked to a redox-sensitive bacterial heat shock protein, to provide further direct and reliable evidence for increased ROS formation in the cytosol under hypoxia.

Diving mammals such as whales or seals constitute a valuable model in the study of the physiological effects of hypoxia-reoxygenation events. These animals are obliged to make subsequent dives in order to feed, exposing themselves to cyclic bouts of ischemia and reperfusion and the negative consequences that the later entails. Vázquez-Medina et al. (Vázquez-Medina et al., 2012) have recently reviewed the current knowledge regarding how such diving species are able to avoid oxidative damage. This and similar reports led the authors to hypothesize that hypoxia-induced ROS production in diving mammals may be involved in the induction of antioxidant mechanisms and other protective pathways relevant for hypoxic adaptation. This would occur through the activation of HIFs (e.g. (Johnson et al., 2005) reviewed by (Zenteno-Savín et al., 2011)), heterodimeric transcription factors present in the cytoplasm which through their activation a wide variety of hypoxia adaptive cell responses are regulated (Chandel et al., 1998; Klimova and Chandel, 2008; Semenza, 2000; Semenza and Wang, 1992).

A great body of information also comes from the field of cardiology and what it is currently known as "ischaemic preconditioning" (IPC), that is, the process by which subsequent ischemic-reperfusion events increases cell resistance (Murry et al., 1986). The involvement of ROS in IPC has been demonstrated through indirect manners, such as through the use of antioxidants (which reduce the cardioprotective effects of IPC) (Tanaka et al. 1994) and prooxidants (which under normoxia induced IPC-like protection as shown by Baines et al. (Baines, 1997) or Vanden Hoek et al. (Vanden Hoek et al., 1998)).

But when does such a ROS increase occur in mammalian cells? Reports agree that this ROS peak can occur only some minutes after the hypoxic insult. Recent investigations conducted on bovine and human endothelial cells under acute hypoxia (1-2% O_2) showed that ROS formation peaks around 10-20 min of hypoxic exposure (Hernansanz-Agustín et al., 2014), agreeing with previous studies (Table 5). In contrast to mammalian cells, fewer studies have assessed ROS production in animals that are naturally exposed to low oxygen stress. A study using *in vivo* staining with the non-specific ROS detector C-H_2DFFDA, evidenced that the marine platyhelminthes *Macrostomum lignano* ROS formation follows the typical oxygenation-dependent pattern (Rivera-Ingraham et al., 2013a). However, superoxide formation (DHE staining) remained constant across oxygenation conditions (ranging from hyperoxia (40 kPa) to near-anoxia), and only reoxygenation caused a 2-OH-E^+:DHE ratio increase. Even though these contrasting results are difficult to interpret, this work was the first in comparative biology to demonstrate that superoxide formation under near-anoxia remains unchanged in comparison to normoxia and suggests that it could be contributing as a signaling mechanism in hypoxic acclimation.

Another study by the same group evidenced that, in the hypoxia tolerant mussel *Mytilus edulis,* levels of carbonyl proteins in gills increase after 48 h under near-anoxia (<0.6% air saturation) even though ROS formation decreases (as determined *ex-vivo* by DHE

and C-H_2DFFDA staining) (Rivera-Ingraham et al., 2013b). Shorter periods of near-anoxia exposure were not assessed, but it is possible that ROS production increases in gill tissues before the investigated 48 h time point. Indeed, as discussed above, increased ROS formation occurs during the first 10-20 minutes from the onset of hypoxia exposure in mammalian cells, decreasing after that period (Hernansanz-Agustín et al., 2014). Future quantification of ROS at shorter incubation times might help verify if, as observed in mammalian cells, increased ROS formation under hypoxia also applies to invertebrates.

It is also of major interest to review the current knowledge related to the molecular mechanism of hypoxic ROS production since most of the results come from mammalian cell studies. It is within mammalian cells that it is long known that mitochondria produce ROS (Jensen, 1966) and that mitochondria are the main ROS producers (e.g. (Cadenas and Davies, 2000; Turrens, 2003). Thus, many works have attempted to determine the exact source of the hypoxia-induced ROS formation. Both pharmacological and genetic methods have been extensively applied, the later being mostly related to the analysis of the activation of HIFs. Chandel et al. (Chandel et al., 1998) demonstrated in Hep3 cells that functional mitochondria are necessary to produce the hypoxia-induced ROS that are required for IPC. This is expected, since as the same author correctly points out, other ROS-regenerating systems such as cytochrome P450 or NADPH oxidase would decrease ROS production under hypoxic conditions. In a more recent study, Hernansanz-Agustín et al. (Hernansanz-Agustín et al., 2014) further demonstrated that the hypoxia-derived ROS largely requires a mitochondrial oxidative phosphorylation system. But where exactly are such ROS produced? Complexes I, II and III are the main mitochondrial sources of $O_2^{\bullet-}$ (Poyton et al., 2009; Turrens, 2003). Located in the inner side of the inner mitochondrial membrane, complexes I and II generate $O_2^{\bullet-}$ which is released in the mitochondrial matrix. Complex III, on the other hand, is a transmembrane complex, and, thus, not only generates $O_2^{\bullet-}$ in the matrix, but also to the

intermembrane space from where they can be carried to the cytoplasm via voltage-dependent anion channels (Han et al., 2003) and be potentially available for HIF activation and participation in other signaling pathways. Even though the exact mechanism through which mitochondrial ROS are involved in HIF activation is not established, the important role of complex III must be highlighted. This has been further supported by several other groups working with a variety of cells lines (e.g. (Bell et al., 2007; Guzy et al., 2005; Mansfield et al., 2005; Waypa et al., 2001). Chandel et al. (Chandel et al., 1998) proposed that this occurs due to an accumulation of electrons in the proximal areas of the respiratory chain and their further leakage to form $O_2^{\bullet-}$ when there is limited oxygen available to the terminal cytochrome c oxidase. Later, studies allowed the refinement of such model and suggested that, under hypoxia, the mitochondrial complex III suffers a conformational change that would facilitate the interaction between O_2 and ubisemiquinone, resulting in an increase of $O_2^{\bullet-}$ formation (Guzy et al., 2005). Others also consider complex II as relevant for ROS formation during hypoxia exposure (Paddenberg et al., 2003). It was proposed that this complex switches its catalytic activity from succinate dehydrogenase to fumarate reductase at diminished oxygen levels. This would not only cause succinate to accumulate but additionally will cause ROS generation because fumarate reductase has been demonstrated to be a powerful $O_2^{\bullet-}$ generator (e.g. (Imlay, 1995; Messner and Imlay, 2002; Turrens, 1987). This change in complex II would then be modulating the directionality of the electron flow because not only O_2 would be the final electron acceptor, but also fumarate (Chouchani et al., 2014). Further research is required to consolidate knowledge on the role of each of the mitochondrial complexes in the induction of hypoxia adaptation across the different tissues and cell models.

585 **6. Redox sensitive transcription factors and low oxygen stress**

586 The description of transcription factors that regulate the expression of genes coding
587 for antioxidant proteins in animals exposed to low oxygen stress is limited. A strong
588 indication that a transcription factor acts in any particular gene is the presence of a consensus
589 binding sequence for such specific factor in the promoter region of the gene. In this case, the
590 promoter region of the gene must be known. Data about the promoter sequences of the
591 antioxidant genes from the organisms cited the present review are scarce. Therefore, the
592 evidence of the action of any particular transcription factor on the induction of antioxidant
593 defense genes in the animals discussed herein is rather indirect.

594 The nuclear factor-erythroid 2 p45-related factor 2 (Nrf2) regulates genes involved in
595 the biosynthesis of glutathione and NADPH as well as genes coding for catalase, CuZnSOD,
596 peroxiredoxin, thioredoxin, GPXs and GSTs (Banning et al., 2005; Chan and Kan, 1999; Ishii
597 et al., 2000; Kim, 2001; Kobayashi and Yamamoto, 2006; Suzuki et al., 2005). Its stability
598 and activity can increase upon exposure to H_2O_2 through oxidation of its inhibitory protein
599 Keap1 (Fourquet et al., 2010). Nrf2 also controls a number of genes involved in intermediate
600 metabolism that may also contribute to survival under reduced oxygen tensions (Hayes and
601 Dinkova-Kostova, 2014). In the African clawed frog *X. laevis*, the exposure to dehydration
602 resulted in increased expression of GST isoforms in several organs (Malik and Storey, 2009).
603 GST-P1 was induced in liver, heart and skin by 2- to 9-fold, whereas GST-M1 and GST-M3
604 increased in muscle, kidney and skin. These results were related with increased Nrf2
605 expression at both protein and mRNA levels (Malik and Storey, 2009).

606 The protein p53 is reported to control the expression of MnSOD and GPXs (Mai et
607 al., 2010; Tan et al., 1999). Both up and down-regulation of antioxidant gene expression can
608 result from p53 action depending on its intracellular concentration (Dhar et al., 2010). This
609 protein may also provide antioxidant protection under hypoxia by up-regulating the

610 mitochondrial glutaminase 2 (GSL2) gene. Glutaminase 2 catalyzes the hydrolysis of
611 glutamine to glutamate, which is a precursor of GSH. Activation of p53 increases the level of
612 glutamate and GSH and decreases ROS levels in cells *in vitro*. In addition, the human GSL2
613 gene contains a p53 consensus DNA-binding element and this element may also be present in
614 GSL2 gene from other organisms (Hu et al., 2010).

615 The hypoxia inducible factor 1 (HIF-1) is the main transcription factor involved in the
616 response to hypoxia. The role of HIF in animal adaptation to hypoxia has been subject of
617 innumerous studies since the 1990s (Hochachka and Somero, 2002). There is an indication
618 that HIF-1 regulates the expression of GPX3 in human plasma (Bierl et al., 2004). HIF-1
619 subunits have been cloned from some non-mammalian species, including shrimp, oyster, and
620 fish (Mohindra et al., 2013; Piontkivska et al., 2011; Soñanez-Organis et al., 2009). A
621 putative HRE has been located in intron 2 of the lactate dehydrogenase B gene from the
622 killifish *Fundulus heteroclitus*. Putative HREs may also be present in the genes coding for *F.*
623 *heteroclitus* antioxidant enzymes. This indicates that HIF-1 or its homologs may be involved
624 in mediating the effects of environmental hypoxia in other animals (Rees et al., 2001).

625 The genes coding for MnSOD and catalase are direct transcriptional targets of
626 forkhead box O (FoxO) transcription factors (Greer and Brunet, 2005; Kops et al., 2002). The
627 role FoxOs in the induction of antioxidant defenses has been demonstrated in studies in
628 species facing low oxygen stress. The liver of African clawed frogs, *X. laevis,* exposed to
629 dehydration had increased FoxO1 abundance in nucleus, increased FoxO1 DNA binding
630 activity and reduced levels of phosphorylated FoxO1 (Malik and Storey, 2011). This
631 activation of the FoxO1 pathway was related to the increase of two antioxidant enzymes,
632 MnSOD and catalase, at both protein and mRNA levels in the liver (Malik and Storey, 2011).
633 Furthermore, activation of FoxO1 and FoxO3 transcription factors has been also

demonstrated in the anoxia-tolerant turtle *Trachemys scripta elegans* (Krivoruchko and Storey, 2013).

The influence of NF-κB proteins on ROS levels occurs via increased expression of MnSOD, CuZnSOD, GST, and GPX (Morgan and Liu, 2011). In mice neonatal cardiac myocytes and adult myocardial endothelial cells the migration of NF-κB and AP-1 to the nucleus is associated with increased enzyme activity and amount of MnSOD protein under condition of anoxia/reoxygenation preconditioning. The mouse MnSOD gene contains putative bind sites for NF-κB and AP-1 in its promoter region. The use of NF-κB and AP-1 artificial inhibitors indicates a direct action of these transcription factor on MnSOD gene expression (Rui and Kvietys, 2005).

An explanation was proposed on how antioxidant enzymes are increased under conditions of low oxygen availability (Almeida and Di Mascio, 2011; Welker et al., 2013). The proposal assumes that ROS formation increases during hypoxia in aquatic animals, and that such increased ROS formation would activate transcriptional factors that regulate the expression of antioxidant enzymes. Candidate transcription factors are Nrf2, p53, HIF-1, NF-κB proteins and FoxO proteins (Figure 3). Herein, we expand the idea on the role of such transcription factor in animals during estivation and situations of low oxygen stress, including freezing, dehydration in both terrestrial and aquatic species. In a next section, the role of ROS-derived electrophiles in the activation of transcription factors will also be considered.

7. Is there a role for post translational modifications of antioxidant enzymes during low oxygen stress?

In addition to transcription factors, post-translational modification of proteins is a mechanism to regulate protein function with the advantage to be rapid and ATP-inexpensive, which meets the condition of hypometabolism. Indeed, protein phosphorylation has been

659 shown to regulate the activity of enzymes involved in energy metabolism in animals under
660 low oxygen stress, for example, arginine kinase and glutamate dehydrogenase in crayfish
661 under severe hypoxia (Dawson and Storey, 2011; Dawson and Storey, 2012), lactate
662 dehydrogenase in turtles under anoxia (Xiong and Storey, 2012), creatine kinase and
663 hexokinase in frogs exposed to freezing conditions (Dieni and Storey, 2009; Dieni and
664 Storey, 2011). Moreover, there are evidences that reversible phosphorylation and other post-
665 translational modifications are mechanisms that control the activity (in terms of V_{max}) of
666 antioxidant enzymes. However, only one study has analyzed the specific role of post-
667 translation regulation of an antioxidant enzyme in animals under low oxygen stress (Dawson
668 et al., 2015). The altered ROS production in organisms under low oxygen stress could trigger
669 signaling pathways leading to post-translational modifications of antioxidant enzymes and
670 related proteins.

671 Protein phosphorylation is widely recognized as a post-translational modification that
672 modulates the activity of enzymes in general. In addition to the switch on and off effect, by
673 the action of kinases and phosphatases on proteins, reversible phosphorylation may also alter
674 enzyme properties and the interaction between enzymes and other proteins (Storey, 2004).
675 Although not an antioxidant enzymes itself, glucose 6-phosphate dehydrogenase (G6PDH) is
676 an important enzyme that fuels glutathione and thioredoxin systems by producing reducing
677 potential in the form of NADPH. Reversible phosphorylation regulates G6PDH enzymatic
678 properties in response to hypometabolism in land snails (Ramnanan and Storey, 2006), to
679 freezing in wood frogs (Dieni and Storey, 2010) and to anoxia in periwinkles (Lama et al.,
680 2013). Snails estivating for 10 days have higher levels of phosphorylated G6PDH resulting in
681 increased G6PDH activity in comparison to active animals (Ramnanan and Storey, 2006). On
682 the other hand, phospho-G6PDH levels are reduced in wood frogs exposed to freezing

683 conditions for 24 h, leading to a reduced affinity for its substrates in the frozen state (Dieni
684 and Storey, 2010).

685 Regarding antioxidant enzymes, H_2O_2-related enzymes (catalase, glutathione
686 peroxidase and peroxiredoxins) have been shown to have their activities regulated by
687 phosphorylation in response to oxidative stress mediated by H_2O_2 in mammalian cells (Rhee
688 et al., 2005). Two important tyrosine kinases c-Abl and Arg are activated upon H_2O_2
689 treatment and phosphorylates catalase (Cao et al., 2003a) and GPX1 (Cao et al., 2003b)
690 leading to increased activities. Phosphorylation of several peroxiredoxins occurs *in vitro* and
691 this modification leads to reduced activity of peroxiredoxin I, which is phosphorylated *in vivo*
692 (Chang et al., 2002). The antioxidant-related enzymes glutamate-cysteine ligase (GCL) and
693 glutathione transferase P1 (GSTP1) are also subject of reversible phosphorylation. While
694 GCL have its activity reduced (Sun et al., 1996), GSTP1 presents higher catalytic efficiency
695 when phosphorylated by different kinases (Lo et al., 2004; Okamura et al., 2009; Singh et al.,
696 2010). Phosphorylation has also been reported to regulate MnSOD activity *in vivo* and *in*
697 *vitro* in mammalian cells exposed to radiation, in such a manner that phosphorylation by
698 CyclinB1/Cdk1 increases its activity (Candas et al., 2013).

699 In addition to reversible phosphorylation, antioxidant proteins are targets of other
700 covalent modifications that may alter their activities, including acetylation (Kim et al., 2006)
701 and glutathionylation (Manevich et al., 2004). There is a broad range of proteins known to be
702 modified by reversible acetylation (the transfer of an acetyl group from acetyl coenzyme A to
703 a protein) resulting in the regulation of many cellular processes (Norris et al., 2009; Spange et
704 al., 2009). A large number of mitochondrial proteins have been found to be acetylated,
705 including enzymes involved in the energetic metabolism and stress response – e.g. MnSOD,
706 CuZnSOD, thioredoxin and isocitrate dehydrogenase 2 (Kim et al., 2006).

Several studies have shown that MnSOD activity is affected by deacetylation in response to different stresses (Ozden et al., 2011; Zhu et al., 2012). Specifically, the deacetylation of MnSOD by the mitochondrial sirtuin Sirt3 (a NAD+-dependent protein deacetylase) increases SOD activity (Chen et al., 2011; Qiu et al., 2010). Increased MnSOD expression (by 6-fold) results in a slight (10%) decrease of ROS levels in mammalian cells (Qiu et al., 2010). However, ROS levels are strongly suppressed (90%) when SIRT3 and SOD2 are coexpressed. Moreover, the expression of a modified deacetylated SOD2 alone also reduces ROS levels by 90% (Qiu et al., 2010). Thus, the overexpression of SOD has little effect on ROS levels unless deacetylation occurs (Chen et al., 2011; Qiu et al., 2010; Tao et al., 2010).

To our knowledge, there is a single study about post-translational modification of antioxidant enzymes in animals in response to low oxygen stress (Dawson et al., 2015). Muscular MnSOD was purified from control and frozen *Rana sylvatica* frogs. Freezing induces increased relative phosphorylation levels of MnSOD resulting in greater stability (assessed by resistance to urea denaturation) and increased affinity (lower K_m) of the enzyme for $O_2^{\bullet -}$. However, no effect on V_{max} was observed as a result of phosphorylation (Dawson et al., 2015).

It is tempting to raise the role of post translational modifications on the activity of antioxidant enzymes for several reasons. First, there is a wide range of intermediary metabolism enzymes regulated by phosphorylation, as well as others post-translational modifications, in these animals (Storey and Wu, 2013). Second, many studies have shown post-translational modification of antioxidant enzymes in other systems (including in response to increased ROS) and sites for modifications other than phosphorylation have been identified in these enzymes. Finally, the trigger for these modifications to occur during hypoxia exposure could be the increased ROS formation, which, for example, is known to

732 alter the activities of protein kinases, phosphatases and sirtuins. Thus, one could speculate
733 that reversible covalent modifications of antioxidant enzymes play an important role in
734 hypoxia tolerant animals under low oxygen stress. For example, a hypothetical Sirt3-
735 mediated activation of MnSOD in response to elevated levels of H_2O_2 under hypoxia (which
736 could happen in the hypoxic condition that antecedes full anoxia), could be an alternative
737 explanation for the increase in SOD activity in anoxia-exposed garter snakes reported by
738 Hermes-Lima and Storey (Hermes-Lima and Storey, 1993).

739 Besides the direct effects on antioxidant enzymes, many studies have reported the
740 occurrence of the post-translational modifications addressed above on redox-sensitive
741 transcription factors. For example, the acetylation of several transcription factors has been
742 reported, including FoxOs, HIF-1α, NF-κB, Nrf2 and p53 (Bell et al., 2011; Spange et al.,
743 2009; Sun et al., 2009; Tseng et al., 2013). Furthermore, the roles of reversible
744 phosphorylation and acetylation of p53 (Zhang et al., 2013) and reversible phosphorylation of
745 FoxO1 and FoxO3 (Krivoruchko and Storey, 2013) were investigated in *T. scripta elegans*
746 turtle exposed to anoxia. For example, in liver and muscle, several phosphorylated forms of
747 p53 increase in *T. scripta elegans* exposed to anoxia (Zhang et al., 2013).

748 The discussion above highlights the importance of the employment of methodologies
749 to specifically detect antioxidant enzymes in their active forms (e.g. enzymatic activity or
750 selective antibodies against the active form of the enzyme). Due to the multiple layer control
751 of gene expression, mRNA levels do not always match protein levels, and protein levels do
752 not necessary reflect active protein levels (Feder and Walser, 2005). Moreover, when
753 analyzing antioxidant enzymes one should take care to avoid *in vitro* protein modification
754 during sample handling, for example, adding phosphatase inhibitors to the sample at the time
755 of homogenization. Furthermore, proteins involved in these post-translational modifications

(e.g. Sirt3) are candidates to be regulated and investigated in animals under low oxygen stress and during metabolic depression.

8. The role of reactive nitrogen species

Reactive nitrogen species (RNS) have also been demonstrated to play important physiological roles in a wide range of taxa. Nitric oxide (NO), for example, is an evolutionarily conserved intercellular messenger involved in multiple biological processes, ranging from defense in bacteria (Hausladen et al., 1998) to mitochondrial biogenesis in mammals (Nisoli et al., 2003). NO is directly involved in regulating respiration rates (Poderoso et al., 1996), essential for prolonging survival upon hypoxic periods. This molecule is more stable under very low environmental oxygen (0.5-1.5 μM O_2) and acts as a multi-site inhibitor of the mitochondrial respiratory chain (Cassina and Radi, 1996). Cytochrome oxidase has higher affinity for NO when compared to O_2, making complex IV (responsible for most of the oxygen consumption) the most sensitive site to this inhibition (Cleeter et al., 1994; Poderoso et al., 1996). But the reversible nature of this inhibition (Brown, 1999) is probably the key adaptive response to subsequent ischemia-reperfusion events, since a decrease in mitochondrial respiration prevents an excess of ROS production upon reoxygenation. Moreover, NO-derived metabolites (nitrite and, due to the low pH in tissues during hypoxia, also the strong S-nitrosylating agent N_2O_3) can interact with complex I through S-nitrosylation, also slowing down the electron flow at the respiratory chain, and thus mitigating any ROS burst (Fago and Jensen, 2015).

Studies analyzing RNS-derived biochemical markers in estivating or low oxygen stressed animals are scarce. In addition to the well-known products nitrite and nitrate, RNS may react with a range of cellular components producing, for example, iron-nitrosyl (FeNO), S-nitroso (SNO), and N-nitroso (NNO) compounds (Challis and Kyrtopoulos, 1979; Joshi et

al., 2002; Kelm, 1999). Jensen et al. (Jensen et al., 2014) reported that, in the anoxia-tolerant red-eared slider turtle *T. scripta*, NO metabolites (FeNO and NNO) increased in response to a 9-day exposure to anoxia in all analyzed tissues. Other metabolites such as SNO also increased during anoxia but decreased shortly after O_2 reintroduction, which makes it a good candidate molecule for it to be involved in S-nitrosylation of complex I and, thus, controlling ROS formation. Nitrite, which has also been demonstrated to be an important cytoprotector upon ischemia-reperfusion events (Dezfulian et al., 2007), increased in a tissue-specific way in turtles exposed to anoxia (Jensen et al., 2014). Animal species that estivate are also interesting study models. A study in the lungfish *Protopterus dolloi* showed an increase in NOS activity in heart and kidney after 40 days estivation, suggesting that NO is involved in the adjustment of these organs (Amelio et al., 2008). Such long term estivation had no effect in heart nitrotyrosine levels in the pulmonate snail *Achatina fulica* (subject to four-week estivation) (Salway et al., 2010) or the lungfish *P. dolloi* (60-day estivation) (Page et al., 2010), although levels increased in other tissues such as brain (Page et al., 2010). After an estivation period of 6 months (Chng et al., 2014), another lungfish, *Protopterus annectens*, also had increased levels of nitrite and nitrate in liver. These and other examples not reviewed here indicate some similarities regarding NO-derived metabolism among taxa.

At the transcriptional level, NO is also involved in the regulation of hypoxia-related genes. Several studies focused on the role of NO and its derivatives in stabilization of HIF, a key component in hypoxic acclimation. HIFs are not only stabilized by a decrease in O_2, but it also requires S-nitrosylation of certain pathway components as recently reviewed (Ho et al., 2012; Poyton and Hendrickson, 2015), highlighting the important role of RNS in hypoxic acclimation.

9. Lipid peroxidation and hypoxia studies

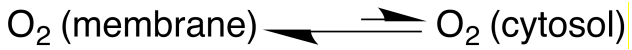
9.1. Molecular mechanisms for increased lipid peroxidation under hypoxia

A number of studies have shown increased lipid peroxidation products, including TBARS and lipid hydroperoxides, in tissues of many animal species under low oxygen stress (Table 4). However, molecular oxygen (O_2) is a critical substrate for the propagation of the lipid peroxidation cascade (Yin et al., 2011), playing a role, for instance, in the reaction of alkyl radicals with O_2. Therefore, if O_2 is essential for the lipid peroxidation cascade, how can it be enhanced in organisms exposed to hypoxic conditions?

As discussed above, mitochondria may increase ROS formation (specifically $O_2^{\bullet-}$ and H_2O_2) under low oxygen stress. Furthermore, H_2O_2 can undergo a heterolytic reduction in the presence of iron ions or heme-containing proteins giving rise to hydroxyl radicals. This radical, in turn, can abstract hydrogen atoms from unsaturated lipids initiating lipid peroxidation reactions (Hermes-Lima, 2004). However, the burst of free radicals produced under hypoxia explains only the formation of alkyl radicals, but fails to explain the formation of peroxyl radicals, a crucial step that requires the reaction with O_2. In this context, Hernansanz-Agustín and co-workers (Hernansanz-Agustín et al., 2014) showed that the burst of free radical production – measured by superoxide detection with DHE – lasts for 30-60 min in cells exposed to hypoxia (Hernansanz-Agustín et al., 2014). If this hypoxia-induced superoxide production occurs for such a short period in most animal species, how can we explain the increased lipid peroxidation observed over the course of hours or days under hypoxia?

One hypothesis to explain this increased oxidative damage in animals exposed to hypoxia is related to the chemistry of O_2 and its solubility in membranes (Scheme 1). Since oxygen is a non-polar molecule, its solubility in the non-polar core of lipid membranes is higher than in aqueous media (Dzikovski et al., 2003; Windrem and Plachy, 1980).

Therefore, regardless of the overall O_2 availability in tissues/cells, the pO_2 in the hydrophobic portion of the membrane should be higher than the pO_2 in the aqueous phase of cytosol. Such behavior ensures that there would be enough O_2 in the membrane to allow the occurrence of lipid peroxidation even under hypoxia.



Scheme 1. *Chemical equilibrium of oxygen in cells.*

Once alkyl radicals are generated in the initial burst of free radicals (equation 1 in scheme 2) the following reactions of the cascade can occur for hours (even days) with the residual O_2 in the membrane. This relatively high abundance of O_2 in the membrane during hypoxia could be a key aspect to explain the increased levels of lipid peroxidation products in a number of studies discussed in this article (Table 4). Noteworthy, this reaction (equation 2 in scheme 2) is favorable from both a thermodynamic and a kinetic point of view (Yin et al., 2011). Peroxyl radicals, in turn, generate several other oxidizing species – including lipid hydroperoxides – that could induce the oxidative damage observed in animals exposed to hypoxia (**scheme 2**). The production of such peroxyl-derived oxidizing species would be independent from the pO_2 inside the cell, which could also be related to the increase in lipid peroxidation during hypoxia. Moreover, besides their involvement in free radical reactions, lipid hydroperoxides can also affect membrane organization and cell-signaling effects, leading to increased cellular protection, apoptosis or necrosis (Girotti, 1998; Miyamoto and Di Mascio, 2014).

$^{\bullet}OH + LH \rightarrow L^{\bullet} + H_2O$ (equation 1)

$L^{\bullet} + O_2 \rightarrow LOO^{\bullet}$ (equation 2)

$LOO^{\bullet} + LH \rightarrow LOOH + L^{\bullet}$ (equation 3)

$2\ LOO^{\bullet} \rightarrow LOOOOL \rightarrow LO + LOH + {}^{1}O_2$ (equation 4)

$LOOH + Fe^{2+} \rightarrow LO^{\bullet} + OH^{-} + Fe^{3+}$ (equation 5)

$LO^{\bullet} + LH \rightarrow LOH + L^{\bullet}$ (equation 6)

Scheme 2*. Simplified lipid peroxidation cascade showing the requirement of O_2 and formation of oxidizing species.* ***Equation 1*** *represents the initiation of the lipid peroxidation cascade promoted by $^{\bullet}OH$ (generated in the burst of free radicals).* ***Equation 2*** *represents the critical step that requires O_2.* ***Equations 3 to 6*** *represent reactions in the peroxidation cascade that produces oxidizing species, including peroxyl and alkoxyl radicals and singlet molecular oxygen ($^{1}O_2$)* (Miyamoto and Di Mascio, 2014; Miyamoto et al., 2003). *Excited carbonyl species can also be formed as a product of the Russel mechanism* (Miyamoto et al., 2003). *Such species, in turn, could lead to oxidative damage.*

The lipid peroxidation cascade ends with the production of ketones and aldehydes, including α,β-unsaturated species such as 4-hydroxynonenal (HNE) (Hermes-Lima 2004). These species have been shown elevated in cells exposed to hypoxia (Cervellati et al., 2014) and also conjugated to GSH in livers of a fish under hypoxic conditions (Bastos et al., 2013). Due to the chemical nature of species such as HNE, it can covalently modify several amino acids in the cell through either a Michael addition (addition to the double bond) or a Schiff base mechanism (formation of an imine bond) (Isom et al., 2004). Indeed, lysine, histidine, cysteine and arginine residues were modified in proteins modified by HNE (Isom et al., 2004). Such modifications are able to alter protein structure and function, which leads to protein malfunction, oxidative stress, signaling effects and cell death (West and Marnett, 2006).

Overall, the observed increase in oxidative stress could be related to the residual oxygen levels found in the membrane even after long periods under hypoxic conditions. In addition, the electrophiles produced over the course of the lipid peroxidation could also modulate Nrf2 activity (see following topic) and, therefore, the enzymatic antioxidant response of the organisms under hypoxia.

9.2. Lipid peroxidation products as signaling molecules

A new line of thought to explain the activation of antioxidant response in organisms exposed to long periods of hypoxia is based on the effects of electrophiles in cells. As discussed above, lipid peroxidation is a process that can occur "independently" from the pO_2 in the cell and may last for hours (even days). In such scenario, lipid peroxidation products – as electrophilic aldehydes – could be produced hours (or days) after the beginning of the hypoxia. These molecules, such as HNE, may play a pivotal role in triggering the antioxidant defense in organisms. Some reports show that HNE can react with amino acid residues in the Keap1-Nrf2 complex, modulating its activity (Higdon et al., 2012; Kansanen et al., 2012). Although the exact mechanism remains unclear, Kansanen and co-workers (Kansanen et al., 2012) suggest that HNE covalently modifies specific cysteine residues in the Keap1 domain (Kansanen et al., 2012). These modifications decrease the affinity between Keap1 and Nrf2, which allows Nrf2 translocation to the nucleus where it activates the antioxidant response (Higdon et al., 2012; Kansanen et al., 2012). Moreover, treatment with oxidized LDL also led to an activation of Nrf2 pathway, corroborating the finding that lipid peroxidation products modulate antioxidant response (Ishii et al., 2004). In addition to non-specific lipid peroxidation products (as HNE), enzymatic lipid peroxidation products, such as prostaglandins, have been shown to modulate Nrf2 pathway, increasing antioxidant response (Figure 4; (Higdon et al., 2012)).

9.3. Challenges for lipid peroxidation measurements in comparative biology

A major limitation of our study (and the comparative biology field) is that most the available data on lipid peroxidation is based on TBARS, xylenol orange and determinations of lipid hydroperoxides by conjugated dienes. All these methods have been publicly criticized due to their lack of specificity, which could lead to a misinterpretation of the actual levels of lipid peroxidation. Therefore, one of the great challenges of the field is to improve the analytical measurements of lipid peroxidation. Methods as TBARS should not be used when the matrix is complex, which is the case of all studies of the comparative biology field (Forman et al., 2015). Future researchers should consider replacing such methods by more precise methods of detection, such as the detection of F2-isoprostanes by mass spectrometry (Liu et al., 2009; Milne et al., 2007).

One can also argue that the observed increase in lipid peroxidation markers in tissues of hypoxia-exposed animals could be due to post-mortem effects. However, since the handling of control (normoxia) and hypoxic groups were equal, the post-mortem effects were also equal, which would only increase the baseline for both groups without affecting the difference already present.

10. Conclusions, limitations and perspectives

10.1 Free radical formation under low oxygen stress and estivation produce redox imbalance and activate antioxidant defenses

As mentioned before, it was recently proposed that increased ROS generation during hypoxia – in comparison with normoxia – is responsible for the activation of transcriptional factors involved in the up-regulation of antioxidant enzymes (Welker et al., 2013). This mechanism potentially explains how antioxidant enzymes can be activated under estivation and low oxygen stress: anoxia/hypoxia (in which ROS formation could be higher in the

hypoxic phase that precedes full anoxia), dehydration, freezing, and air-exposure of water breathing animals. Thus, ROS-mediated activation of redox-sensitive transcription factors and pathways leading to post translational modifications are - according to our proposal - key components of the molecular POS mechanism.

Furthermore, the increase in GSH oxidation (towards GSSG), lipid peroxidation, protein oxidation and DNA damage - reported in many works throughout this review - can be explained by an augment in ROS formation. One example is the increased levels of lipid peroxidation after 6 days under anoxia in marine gastropods (Pannunzio and Storey, 1998). In another example, the putative low pO_2 in internal organs of land snails during estivation could be the reason for an increased ROS formation, leading to mild oxidative stress (increased lipid peroxidation, protein oxidation and GSSG levels; Ramos-Vasconcelos and Hermes-Lima, 2003). Thus, the augment in endogenous antioxidant defenses may minimize oxidative damage under (i) low oxygenation and (ii) also in the following condition: normoxic recovery, which is expected to increase ROS generation.

In summary, we propose that the following events underlie the increased expression of endogenous antioxidants in response to oxygen restriction known as "preparation for oxidative stress" (Figure 4):

(i) Once animals are exposed to low oxygen stress, oxygen concentration begins to drop and, at some point, pO_2 reaches a threshold level, in which electrons accumulate at the mitochondrial electron transport chain and, thus, the generation of superoxide radicals and H_2O_2 increases temporarily;

(ii) This increment in ROS levels under low oxygenation may: (a) cause redox imbalance, increasing the GSSG/GSH-eq ratio; (b) oxidize cellular components directly or participate in reactions that produce other oxidizing species (e.g. peroxynitrite and lipid hydroperoxides), increasing the levels of oxidative stress markers (e.g. conjugated dienes,

protein carbonyls and 8-oxodGuo); (c) trigger the activation of redox-sensitive transcription factors (e.g. FoxOs, HIF-1, NF-κB, Nrf2 and p53) resulting in an increased expression of antioxidant defenses; and (d) activate signaling pathways (e.g. Sirt3 and specific kinases) that cause post translational modifications in both antioxidant enzymes and redox-sensitive transcription factors. The overall result would be an enhanced antioxidant system.

(iii) After some period of time the burst in ROS generation will eventually decrease and so will its effects. However, electrophile lipid peroxidation products may further extend the signal for the expression of antioxidants by acting on transcription factors (e.g. Nrf2; section 9.2). This should be important to maintain the "POS response" in long-term hypoxia.

To some extent, our proposal is a simplification of a complex process that may be affected by the action of RNS (section 8), protein chaperones (Storey and Storey, 2011; Trübenbach et al., 2014), uncoupling proteins (UCPs 2 and 3 seen to control mitochondrial ROS formation; Issartel et al., 2009) as well as the presence of non-enzymatic compounds such as ascorbate (Rice et al., 2002) or uric acid (Giraud-Billoud et al., 2011).

10.2 Limitations on the POS mechanistic proposal

The biggest limitation of our POS hypothesis is that actually there is no direct evidence that mitochondrial ROS generation increase during estivation or under low oxygen stress. The few works that had measured ROS levels using chemical probes indicate that indeed ROS formation still occurs during oxygen deprivation (even in animals under anoxia) (Milton et al., 2007; Rivera-Ingraham et al., 2013a; Rivera-Ingraham et al., 2013b), but, so far, there is no report of increased ROS in such situations. On the other hand, there are many pieces of indirect evidence that indicates that ROS levels should rise. The disturbed redox balance, increased levels of oxidative stress markers and the increase in antioxidant defenses itself points toward a conditions of increased ROS generation. Thus, the hypothesis that a

burst in ROS formation occurs in hypoxia-tolerant and estivating animals is still to be experimentally tested.

Another relevant limitation of our proposal is that we can not predict (or even estimate) exactly when ROS production is expected to increase (and then activate antioxidant defenses) once exposure to low oxygen stress begins. The reason is that available data on the activation of endogenous in oxygen-restricted and metabolic depressed animals varies enormously in terms of exposure time. If we look at the reports of increased antioxidant defenses only, the response times ranged from 5 h to 24 h for freezing; 12 h to 1 week for dehydration; 10 min to 21 days for hypoxia/anoxia; 1 to 18 h for air exposure; and 6 to 180 days for estivation. Moreover, the respiratory physiology of a given animal is also expected to affect the exact time in which the proposed phenomena (i.e. increased ROS formation and antioxidant response) take place.

What is common among all these cases is that so many different animals had increased antioxidants - at some point - when exposed to low oxygen stress. This process was observed in six animal phyla: cnidarians (corals), annelids (polychaetes), tardigrades, mollusks (bivalves and gastropods), arthropods (crustaceans and insects) and vertebrates (fish, amphibians and reptiles) (Figure 5). Such widespread distribution is also observed for hypoxia tolerance phenotypes (p. 108, Hochachka and Somero, 2002).

10.3 Historical perspective

Our present article described the scientific path that led to a biochemical/molecular explanation on how animals respond to low oxygen stress. It has been observed by a great number of authors since the 1990s that many animals increase their antioxidant defenses during estivation and under low oxygen stress and this was interpreted as a way to protect themselves against the potential danger of reoxygenation or reoxygenation-like stress. For

quite a number of years researchers could not go beyond a biological/physiological explanation for the POS phenomenon. This phenomenon was regarded as an adaptive strategy for hypoxia/reoxygenation survival, with ecological relevance for animals facing intermittently oxygen restriction in nature[1] (Costantini, 2014). The observations that mitochondrial ROS formation could be increased under hypoxia in mammalian cells shed some light on the potential molecular pathways to induce the POS phenomena (section 5). Evidences for that started to emerge in the 1980s (Table 5), and for many years, in the words of Thomas Clanton in an editorial, "*scientists have been hesitant to embrace the idea that conditions of hypoxia induce ROS in the absence of reoxygenation*" (Clanton, 2005). Therefore, according to our understanding, ROS formed at an early phase of estivation or low oxygen stresses could both (i) activate endogenous antioxidants and (ii) inflict oxidative damage to biomolecules in anoxia/hypoxia tolerant animals. Other reactive species, such as 4-HNE and prostaglandins (lipid peroxidation products), as well as RNS, may also play relevant roles in the POS process.

The key aspect of the present article is that the original POS proposal has not changed to "something else", but it has evolved from a simplistic theory to an explanation with molecular mechanisms based on several direct and indirect evidences. The "danger" of reoxygenation (well recognized since the 1980s) is still there (Chouchani et al., 2014). The understanding of the mechanisms that allow animals to respond to low oxygen stress should pave the road for further experiments that now can be based on a firm rational and on a hypothesis that can be tested, confirmed or dismissed. Because most of the studies about low oxygen stress was performed in the laboratory, the occurrence of the mechanisms proposed herein is yet to be verified in animals in the wild. This is the next frontier.

[1] In contrast to intermittent oxygen availability, animals chronically exposed to hypoxia may present increased fitness when compared to animals in normoxia. This is the case of the fingernail clams (Sphaerium sp.), in which a population living further in the swamp (low dissolved O_2) presents higher number of animals, decreased levels of oxidative damage to nucleic acids and increased reproductive success when compared to clams living in normoxia closer to the stream (Joyner-Matos and Chapman, 2013).

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Figures captions.

Figure 1. The old explanation of how endogenous antioxidants would become enhanced in animals exposed to low oxygen stress. Because the formation of reactive oxygen species was expected to decrease during oxygen deprivation, a "non-radical messenger" would be responsible for the activation of antioxidants. This explanation was first published in a study with garter snakes exposed to freezing and anoxia stresses (Hermes-Lima and Storey 1993) and then referred to by many works in the comparative biology field.

Figure 2. Current view of how changes in oxygen availability and ROS levels would modulate the preparation for oxidative stress. As oxygen concentration declines from normoxia to anoxia, cellular hypoxia occurs. At some point during the hypoxic phase, mitochondrial ROS formation increases temporarily. During reoxygenation, as oxygen concentration rises from anoxia to normoxia (reoxygenation) ROS formation also increases. In both moments, increased ROS levels are expected to cause oxidative damage and activate antioxidant defenses. Thus, ROS are the signaling molecules involved in the preparation for oxidative stress. This figure was based on Welker et al. (2013) and on references therein. Note that this figure has no quantitative meaning; it is just an illustrative expression of how ROS generation would behave during hypoxia/anoxia exposure. Moreover, we cannot predict the specific pO2 where the burst of ROS should happen - this should vary considerably within the many animal species that are tolerant to low oxygenation.

Figure 3. The general proposed mechanism used by organisms to tolerate estivation or low oxygen stress. Low oxygen stress includes: hypoxia (including anoxia), freezing, aerial exposure of water breathing animals, and severe dehydration. The hypoxic nature of such stresses leads to mitochondrial ROS overproduction that causes oxidative damage to biomolecules and activates redox sensitive transcription factors (FoxO, Nrf2, p53, HIF-1α and NF-κB). These transcription factors promote activation of antioxidant defenses (such as catalase, SOD, glutathione transferase, glutathione peroxidase, thioredoxin and peroxiredoxins). Mitochondrial ROS overproduction might also promote post translational modification of antioxidant enzymes. Activation of antioxidant defenses may function as a negative feedback and reduce ROS levels.

Figure 4. The overall view of the mechanisms that participates in the preparation for oxidative stress (POS). When some animal species are exposed to an environmental situation that induce a short term hypoxia exposure, we propose that reactive oxygen species (ROS)

formation increases causing the oxidation of biomolecules (e.g., GSH, proteins, and membrane lipids) and the activation of redox-sensitive transcription factors (Nrf2, HIF, etc). These transcription factors shall induce augmented expression of antioxidant enzymes. Moreover, ROS-mediated covalent modification of in antioxidant proteins may also increase their activities, contributing to the "POS response". The formation of electrophilic products of lipid peroxidation (such as HNE) could also activate Nrf2 and thus contribute to the "POS response" under long-term hypoxia.

Figure 5. Animals from six different phyla when exposed to low oxygen stresses (anoxia, hypoxia, freezing, dehydration and air exposure) or during estivation enhance their antioxidant defenses. The examples are distributed within the following groups: Anthozoa (corals), Polychaeta, Bivalvia (mussels and clams), Gastropoda (land and aquatic snails), Eutardigrada ("water bears"), Malacostraca (amphipods, shrimps, and crabs), Maxillopoda (barnacles), Insecta, Actinopterygii (ray-finned fish), Dipnoi (lungfish), Amphibia (frogs and toads), and Repitilia (snakes and turtles).

Tables 1-5

Table 1. Activated antioxidant defenses in response to freezing, dehydration and anoxia. Parameters in bold indicate maximal enzyme activity, parameters in italics indicate mRNA levels, underlined parameters indicate protein levels. Note that only increased levels of antioxidant parameters are reported. Even though it is not shown, there are cases in which other antioxidants levels may have been reduced or maintained in the same situation, species or tissue.

Common name	Species	Stress	Duration	Tissue	Antioxidant	Reference
Wood frog	*Rana sylvatica*	Freezing	24 hours	Brain	↑ **GPX,** GSH-eq, GSH	Joanisse and Storey (1996)
				Heart	↑ **SeGPX, GPX**	
				Kidney	↑ **SeGPX, GPX**	
				Liver	↑ **GPX, GST**	
				Muscle	↑ **SeGPX, GPX,** GSH-eq, GSH	
				Muscle[a]	↑ **CuZnSOD,** phospho-MnSOD[a]	Dawson et al. (2015)
Painted turtle	*Chrysemys picta marginata*	Freezing	5 hours	Brain	↑ GSTP1, GSTM1, GSTM3	Krivoruchko and Storey (2010a)
				Gut	↑ GSTM3	
Garter snake	*Thamnophis sirtalis parietalis*	Freezing	5 hours	Lung	↑ **CAT**	Hermes-Lima and Storey (1993)
				Muscle	↑ **CAT, SeGPX**	
Eutardigrade	*Paramacrobiotus richtersi*	Dehydration	20 hours	Whole body	↑ **GPX, SOD,** GSH-eq	Rizzo et al. (2010)
Antarctic midge	*Belgica antarctica*	Dehydration	12 hours	Whole body	↑ *CAT*, *MnSOD*	Lopez-Martinez et al. (2009)
Leopard frog	*Rana pipiens*	Dehydration	90 hours	Liver	↑ **GPX**	Hermes-Lima and Storey (1998)
				Muscle	↑ **CAT**	
African clawed frog	*Xenopus laevis*	Dehydration	162 hours	Heart	↑ GSTP1	Malik and Storey (2009)
				Kidney	↑ GSTK1, GSTA3, GSTT1	
				Liver	↑ GSTP1	
				Lung	↑ GSTM1, GSTM3, GSTA3	
				Muscle	↑ GSTM1, GSTM3, GSTK1, GSTT1	
				Skin	↑ GSTP1, GSTM1, GSTM3	
		Dehydration	6-7 days	Liver	↑ *CAT*, *MnSOD*	Malik and Storey (2011)
				Muscle	↑ CAT	
Marine worm	*Heteromastus filiformis*	Anoxia	6 hours	Whole body	↑ **CAT**	Abele et al. (1998)
			24hours	Whole body	↑ **CAT**	
Pulmonate snail	*Biomphalaria tenagophila*	Anoxia	24 hours	Hepatopancreas	↑ **SeGPX**	Ferreira et al. (2003)
Common periwinkle	*Littorina littorea*	Anoxia	24 hours	Hepatopancreas	↑ GSTT1	Storey et al. (2013)
				Muscle	↑ GSTS1, GSTT1	
		Anoxia	6 days	Hepatopancreas	↑ GSH-eq, GSH	Pannunzio nad Storey (1998)
				Muscle	↑ GSH-eq	
Long-lived bivalve[b]	*Arctica islandica*	Anoxia[c]	3.5 days	Gill	↑ GSH-eq	Philipp et al. (2012)
Midge	*Chironomus riparius*	Anoxia	24 hours	Whole body	↑ **CAT, GCL, GPX, GST**	Forcella et al. (2007)
Caribbean fruit fly	*Anastrepha suspensa*	Anoxia	1 hour	Whole body	↑ **GPX, MnSOD**	López-Martínez and Hahn (2012)
Estuarine crab	*Chasmagnathus granulata*	Anoxia	8 hours	Gill	↑ **CAT, GST**	de Oliveira et al. (2005)
Striped barnacle	*Amphibalanus amphitrite*	Anoxia	24 hours	Whole body	↑ **CAT, SOD**	Desai and Prakash (2009)
Subterranean amphipod	*Niphargus rhenorhodanensis*	Anoxia	24 hours	Whole body	↑ **GPX**	Lawniczak et al. (2013)
Goldfish	*Carassius auratus*	Anoxia	8 hours	Brain	↑ **GPX**	Lushchak et al. (2001)
				Liver	↑ **CAT**	

Table 1. Continued.

Common name	Species	Stress	Duration	Tissue	Antioxidant	Reference
Leopard frog	*Rana pipiens*	Anoxia	30 hours	Brain	↑ **SeGPX**	Hermes-Lima and Storey (1996)
				Heart	↑ **CAT, SeGPX**	
				Muscle	↑ **CAT**	
Red-eared slider	*Trachemys scripta elegans*	Anoxia	5 hours	Liver	↑ *CuZnSOD, MnSOD*	Krivoruchko and Storey (2010b)
			5 hours	Liver	↑ *CAT*	Krivoruchko and Storey (2013)
			20 hours	Liver	↑ **GR**	Willmore and Storey (1997b)
				Red muscle	↑ **GR**	
				White muscle	↑ **GRX, GS**	
			20 hours	Liver	↑ *CuZnSOD, MnSOD*	Krivoruchko and Storey (2010b)
			9 days	Plasma	↑ GSH-eq	Jensen et al. (2014)
Garter snake	*Thamnophis sirtalis parietalis*	Anoxia	10 hours	Liver	↑ **SOD**	Hermes-Lima and Storey (1993)
				Muscle	↑ **SOD,** GSH-eq, GSH	

CAT, catalase; GCL, glutamate-cysteine ligase; GPX, glutathione peroxidase; GR, glutathione reductase; GRX, glutaredoxin; GS, glutathione synthase; GSH-eq, total glutathione levels (both reduced and disulfide forms); GSH, reduced glutathione; GST, glutathione transferase; SeGPX, selenium-dependent glutathione peroxidase (activity towards H_2O_2); SOD, superoxide dismutase.

a. Superoxide dismutases were first isolated from frog muscle and then assayed for activity. The phosphorylated form of MnSOD has more affinity for superoxide (i.e. lower K_m).

b. Quahogs from the German bight population.

c. In this study animals were exposed to two kinds of hypoxia, environmentally forced (nitrogen bubbling in water) or self induced (shell closure). This result refers to self-induced anoxia.

Table 2. Activated antioxidant defenses in response to hypoxia and air exposure. Parameters in bold indicate maximal enzyme activity, parameters in italics indicate mRNA levels, underlined parameters indicate protein levels. Note that only increased levels of antioxidant parameters are reported. Even though it is not shown, there are cases in which other antioxidants levels may have been reduced or maintained in the same situation, species or tissue.

Common name	Species	Stress	Duration	Tissue	Antioxidant	Reference
Midge	*Chironomus riparius*	Hypoxia (1.6 mg O_2 L^{-1})	24 hours	Whole body	↑ **GST, CuZnSOD, MnSOD**	Choi et al. (2000)
			48 hours	Whole body	↑ **MnSOD**	
Freshwater clam	*Corbicula fluminea*	Hypoxia (15-25% O_2 saturation)	5 days	Whole body	↑ **CAT, GST**	Vidal et al. (2002)
Chinese scallop	*Chlamys farreri*	Hypoxia (2.66 mg O_2 L^{-1})	12 hours	Serum	↑ **SOD**	Chen et al. (2007)
		Hypoxia (4.57 mg O_2 L^{-1})	12 hours	Serum	↑ **SOD**	
Mediterranean mussel	*Mytilus galloprovincialis*	Hypoxia (2.0 mg O_2 L^{-1})	24 hours	Gill	↑ *CuZnSOD*	Woo et al. (2011)
		Hypoxia (2.0 mg O_2 L^{-1})	24 hours	Muscle	↑ **GST**	Woo et al. (2013)
			48 hours	Muscle	↑ **GST**	
Pacific oyster	*Crassostrea gigas*	Hypoxia (30% O_2 saturation)	7 days	Gill	↑ *GPX*	David et al. (2005)
			7 days	Mantle	↑ *GPX*	
			14 days	Gill	↑ *GPX*	
			14 days	Hepatopancreas	↑ *GPX*	
			14 days	Mantle	↑ *GPX*	
			21 days	Gill	↑ *GPX*	
			21 days	Hepatopancreas	↑ *GPX*	
			21 days	Mantle	↑ *GPX*	
Disk abalone	*Haliotis discus discus*	Hypoxia[a]	1 hour	Gill	↑ *MnSOD, PRX*	De Zoysa et al. (2009)
			2 hours	Gill	↑ *SeGPX, MnSOD, PRX*	
			4 hours	Gill	↑ *SeGPX, MnSOD*	
			8 hours	Gill	↑ *SeGPX, MnSOD*	
Striped barnacle	*Amphibalanus amphitrite*	Hypoxia (1.0 mL O_2 L^{-1})	24 hours	Whole body	↑ **CAT, SOD**	Desai and Prakash (2009)
		Hypoxia (0.5 mL O_2 L^{-1})	24 hours	Whole body	↑ **CAT, SOD**	
Baltic amphipod	*Monoporeia affinis*	Hypoxia (30-34% air saturation)	5-9 days	Whole body	↑ **CAT, SOD**	Gorokhova et al. (2010)
	Monoporeia affinis Reference sediment	Hypoxia (16-20% air saturation)	4 days	Whole body	↑ **CAT, SOD**	Gorokhova et al. (2013)
	Monoporeia affinis Contaminated sediment	Hypoxia (16-20% air saturation)	4 days	Whole body	↑ **CAT, SOD**	
Subterranean amphipod	*Niphargus rhenorhodanensis*	Hypoxia (22.70 µmol O_2 L^{-1})	10 days	Whole body	↑ **GPX**	Lawniczak et al. (2013)
Whiteleg shrimp	*Litopenaeus vannamei*	Hypoxia (1.0 mL O_2 L^{-1})	24 hours	Hepatopancreas	↑ **SOD**	Parrilla-Taylor and Zenteno-Savín (2011)
			24 hours	Muscle	↑ **SOD**	
		Hypoxia (1.5 mg O_2 L^{-1})	6 hours	Gill	↑ **CAT**	Trasviña-Arenas et al. (2013)
			24 hours	Gill	↑ **CAT**	
		Hypoxia (4 kPa)	4 hours	Hepatopancreas	↑ *MnSOD, GPX*	Kniffin et al. 2014
			24 hours	Hepatopancreas	↑ *MnSOD*	
		Hypoxia (4 kPa) Hypercapnic	4 hours	Hepatopancreas	↑ *MnSOD, GPX*	
			24 hours	Hepatopancreas	↑ *MnSOD*	

Table 2. Continued.

Common name	Species	Stress	Duration	Tissue	Antioxidant	Reference
Indian catfish	*Clarias batrachus*	Hypoxia (5.0 - 0.98 mg O_2 L^{-1})	NS [b]	Gill	↑ **SOD**	Tripathi et al. (2013)
		Hypoxia (0.98 mg O_2 L^{-1})	3 hours[b]	Gill	↑ **CAT**	
			6 hours[b]	Blood	↑ **CAT**	
			6 hours[b]	Gill	↑ **CAT**	
			6 hours[b]	Muscle	↑ **CAT**	
			12 hours[b]	Gill	↑ **CAT**	
			12 hours[b]	Muscle	↑ GSH-eq	
Common carp	*Cyprinus carpio*	Hypoxia (<1.0 mg O_2 L^{-1})	8 hours[c]	Brain	↑ **SOD**	Vig and Nemcsók (1989)
			8 hours[c]	Gill	↑ **SOD**	
			8 hours[c]	Liver	↑ **SOD**	
		Hypoxia (0.9 mg O_2 L^{-1})	5.5 hours	Brain	↑ **CAT, SeGPX**	Lushchak et al. (2005)
Characid fish	*Hyphessobrycon callistus*	Hypoxia (≤1.0 mg O_2 L^{-1})	10 minutes[d]	Serum	↑ **GPX, SOD**	Pan et al. (2010)
Piapara fish	*Leporinus elongatus*	Hypoxia (1.92 mg O_2 L^{-1})	14 days	Blood	↑ **SOD,** GSH, GSH-eq	Wilhelm Filho et al. (2005)
			14 days	Liver	↑ **GPX, GST, SOD,** GSH-eq	
		Hypoxia (3.91 mg O_2 L^{-1})	7 days[e]	Blood	↑ GSH-eq	
			7 days[e]	Liver	↑ GSH-eq	
Chinese sleeper	*Perccottus glenii*	Hypoxia (0.4 mg O_2 L^{-1})	2 hours	Muscle	↑ **GST**	Lushchak and Bagnyukova (2007)
			6 hours	Liver	↑ **SOD**, L-SH	
			6 hours	Muscle	↑ **GST**, L-SH	
			10 hours	Liver	↑ L-SH	
			10 hours	Muscle	↑ L-SH	
Gilthead sea bream	*Sparus aurata*	Hypoxia (2.8 mg O_2 L^{-1})	3 hours[f]	Liver	↑ **GR**	Pérez-Jiménez et al. (2012)
			6 hours[f]	Liver	↑ **SeGPX**	
		Hypoxia (18-19% O_2 saturation)	1 hour[g]	Plasma	↑ TAC	Bermejo-Nogales et al. (2014)
Silver catfish	*Rhamdia quelen*	Hypoxia (2.83 mg O_2 L^{-1})	20 days	Gill	↑ **CAT**	Dolci et al. (2014)
Panama lanternfish	*Benthosema panamense*	Hypoxia (≤1 kPa, OMZ)	—[h]	Whole body	↑ **GST**	Lopes et al. (2013)
Mexican lanternfish	*Triphoturus mexicanus*	Hypoxia (≤1 kPa, OMZ)	—[h]	Whole body	↑ **CAT, GST**	
Mole rat	*Spalax judaei*	Hypoxia (6% O_2)	5 hours	Liver	↑ *GST*	Schülke et al. (2012)
				Harderian gland	↑ **GR**	Soria-Valles et al. (2010)
Octocoral	*Veretillum cynomorium*	Air exposure	1.0 hour	Whole body	↑ **GST**	Teixeira et al. (2013)
			1.5 hour	Whole body	↑ **GST**	
			2.0 hours	Whole body	↑ **CAT, GST**	
			2.5 hours	Whole body	↑ **GST**	
Brown mussel	*Perna perna*	Air exposure	4 hours	Hepatopancreas	↑ **SOD**	Almeida and Bainy (2006)
			18 hours	Hepatopancreas	↑ **GST**	Almeida et al. (2005)
Crab	*Callinectes ornatus*	Air exposure	3.0 hours	Muscle	↑ **CAT, GPX**	Freire et al. (2011a)

Table 2. Continued.

Common name	Species	Stress	Duration	Tissue	Antioxidant	Reference
False king crab	*Paralomis granulosa*	Air exposure	3.0 hours	Gill	↑ **CAT**	Romero et al. (2007)
			3.0 hours	Haemolymph	↑ **CAT, SOD**	
			3.0 hours	Hepatopancreas	↑ **SOD**	
			6.0 hours	Gill	↑ **CAT, GST**	
			6.0 hours	Haemolymph	↑ **SOD**	
			6.0 hours	Hepatopancreas	↑ **SOD**	
			6.0 hours	Muscle	↑ **CAT, SOD**	
			12 hours	Haemolymph	↑ **SOD**	
			12 hours	Hepatopancreas	↑ **SOD**	

CAT, catalase; GPX, glutathione peroxidase; GSH-eq, total glutathione levels (both reduced and disulfide forms); GSH, reduced glutathione; GST, glutathione transferase; L-SH, low-molecular mass thiols; PRX, peroxiredoxin; SeGPX, selenium-dependent glutathione peroxidase (activity towards H_2O_2); SOD, superoxide dismutase; TAC, total antioxidant capacity.

a. Hypoxia was achieved by stopping the aeration of the tank. Oxygen levels were not specified.

b. Tank aeration was stopped and by the consumption of O_2 by fish dissolved oxygen decreased from 5.0 to 0.98 mg O_2 L^{-1}. Once this oxygen level was achieved a group of animals was collected (NS). The rest of the fish were maintained under this hypoxic condition for 3, 6 and 12 hours more.

c. Hypoxia was achieved by stopping the aeration of the tank. Oxygen concentration decreased from 5 to <1 mg O_2 L^{-1} within 1.25 hours and was this concentration was kept for more 6.75 hours.

d. Oxygen concentration decreased from 6.5 to <1.0 mg O_2 L^{-1} within 2.5 h and remained at <1.0mg O_2 L^{-1} for additional 10 minutes.

e. Fish were exposed first to 1.92 mg O_2 L^{-1} for 14 days, then a group of fish were subsequently exposed to 3.91 mg O_2 L^{-1} for 7 days more.

f. Water aeration was discontinued and within 1 hour oxygen concentration reached 2.8 mg O_2 L^{-1} (from 7.1 mg O_2 L^{-1}). Once 2.8 mg O_2 L^{-1} was reached it was kept constant for 3 or 6 hours more.

g. Hypoxia advanced gradually from >85% to 18-19% O_2 saturation. Once 18-19% was reached, fish were kept in this condition for 1 hour. Total exposure time was 11 hours.

h. Fish captured at the oxygen minimum zone (OMZ; day time, 300-400m, ≤1 kPa, 10°C) compared to fish captured at the surface (night time, 40-50m, 20 kPa, 20-25°C).

Table 3. Activated antioxidant defenses in response to estivation. Parameters in bold indicate maximal enzyme activity, parameters in italics indicate mRNA levels, underlined parameters indicate protein levels. Note that only increased levels of antioxidant parameters are reported. Even though it is not shown, there are cases in which other antioxidants levels may have been reduced or maintained in the same situation, species or tissue.

Common name	Species	Stress	Duration	Tissue	Antioxidant	Reference
Giant African snail	*Achatina fulica*	Estivation[a]	28 days	Muscle	↑ **CuZnSOD**	Salway et al. (2010)
Freshwater snail	*Biomphalaria tenagophila*	Estivation[a]	15 days	Hepatopancreas	↑ **SeGPX**	Ferreira et al. (2003)
		Estivation[b]	15 days	Hepatopancreas	↑ **SeGPX, GST**	
Land snail	*Helix aspersa*	Estivation[a]	20 days	Hepatopancreas	↑ **SeGPX,** GSH-eq	Ramos-Vasconcelos and Hermes-Lima (2003)
		Winter		Muscle	↑ **SeGPX**	
		Estivation[a]	20 days	Hepatopancreas	↑ **SeGPX**	Ramos-Vasconcelos et al. (2005)
		Summer		Muscle	↑ GSH-eq	
		Estivation[a] Spring	21 days	Muscle	↑ **SeGPX, GPX**	Nowakowska et al. (2014)
		Estivation[b]	21 days	Kidney	↑ **SeGPX, GPX**	
		Spring		Muscle	↑ **SeGPX, GPX**	
Land snail	*Helix pomatia*	Torpor[b]	—	Hepatopancreas	↑ **CAT, GST**	Nowakowska et al. (2009)
		Spring		Kidney	↑ **CAT**	
		Torpor[b] Autumn	—	Hepatopancreas	↑ **GST**	
		Torpor[b]	—	Hepatopancreas	↑ **CAT, GR, GST**	
		Winter		Kidney	↑ **CAT**	
				Muscle	↑ **GPX**	
		Estivation[b]	21 days	Hepatopancreas	↑ **GST**	Nowakowska et al. (2010)
		Estivation[b]	21 days	Muscle	↑ **SeGPX**	Nowakowska et al. (2011)
Land snail	*Otala lactea*	Estivation[a]	30 days	Hepatopancreas	↑ **SeGPX, SOD**	Hermes-Lima and Storey (1995)
				Muscle	↑ **CAT, GST, SOD**	
Apple snail	*Pomacea canaliculata*	Estivation[b]	30 days	Hepatopancreas	↑ <u>CAT</u>, <u>PRX1</u>	Sun et al. (2013)
		Estivation[b]	45 days	Soft tissues	↑ Uric acid	Giraud-Billoud et al. (2011)
		Estivation[b]	45 days	Hepatopancreas	↑ Uric acid	Giraud-Billoud et al. (2013)
				Kidney	↑ Uric acid	
				Muscle	↑ Uric acid	
		Estivation[a]	45 days	Kidney	↑ Uric acid	
African lungfish	*Protopterus dolloi*	Estivation[b]	60 days	Brain	↑ **CAT, SeGPX, GR,** <u>CuZnSOD</u>, **<u>MnSOD</u>**	Page et al. (2010)
				Heart	↑ **CAT, SeGPX**	
African lungfish	*Protopterus annectens*	Estivation[b]	6 days	Liver	↑ *GSTM*	Loong et al. (2012)
		Estivation[b]	6 days	Brain	↑ Asc, tAsc	Ching et al. (2014)
		Estivation[b]	180 days	Brain	↑ *SOD1*	Hiong et al. (2013)
Spadefot toad	*Scaphiopus couchii*	Estivation[c]	60 days	Kidney	↑ **CAT, SeGPX**	Grundy and Storey (1998)
				Liver	↑ **SOD**	
				Muscle	↑ **GPX, SOD**	
Striped burrowing frog	*Cyclorana alboguttata*	Estivation[b]	180 days	Gastrocnemius	↑ **Mit-SSC**	Young et al. (2013)
				Iliofibularis	↑ **Cyt-SSC, mit-SSC**	
		Estivation[b]	120 days	Gastrocnemius	↑ *GCL, GSTO2, Srxn1*	Reilly et al. (2013)

Asc, ascorbic acid; CAT, catalase; cyt-SSC, cytosolic superoxide scavenging activity; GCL, glutamate-cysteine ligase; GPX, glutathione peroxidase; GR, glutathione reductase; GSH-eq, total glutathione levels (both reduced and disulfide forms); GST, glutathione transferase; mit-SSC, mitochondrial superoxide scavenging activity; PRX, peroxiredoxin; SeGPX, selenium-dependent glutathione peroxidase (activity towards H_2O_2); SOD, superoxide dismutase; Srxn, sulfiredoxin; tAsc, total ascorbic acid (ascorbic acid + dehydroascorbic acid).

a. Estivating animals versus 24 h aroused animals.

b. Estivating animals versus control active animals.

c. Estivating animals versus 10 days aroused animals.

Table 4. Increased levels of oxidative stress markers in animals during estivation or exposed to low oxygen stress. Note that only increased levels of oxidative stress markers are reported. Even though it is not shown, there are cases in which other oxidative stress markers levels may have been reduced or maintained in the same situation, species or tissue.

Common name	Species	Stress	Duration	Tissue	Oxidative stress marker	Reference
Intertidal blue mussel	*Mytilus edulis*	Hypoxia (<0.6% air saturation)	48 hours	Gill	↑ PC	Rivera-Ingraham et al. (2013b)
Mediterranean mussel	*Mytilus galloprovincialis*	Hypoxia (2.0 mg O_2 L^{-1})	24 hours	Muscle	↑ TBARS	Woo et al. (2013)
			48 hours	Muscle	↑ TBARS	
Antarctic clam	*Laternula elliptica*	Hypoxia (2 kPa)	16 days	Gill	↑ PC	Clark et al. (2013)
South African abalone	*Haliotis midae*	Hypoxia (6.74 mg O_2 L^{-1})	30 days	Haemocytes	↑ Tail DNA	Vosloo et al. (2013)
Baltic amphipod	*Monoporeia affinis*	Hypoxia (30-34% air saturation)	5-9 days	Whole body	↑ TBARS	Gorokhova et al. (2010)
	Monoporeia affinis Contaminated sediment	Hypoxia (16-20% air saturation)	4 days	Whole body	↑ GSSG(%), TBARS	Gorokhova et al. (2013)
Common carp	*Cyprinus carpio*	Hypoxia (0.9 mg O_2 L^{-1})	5.5 hours	Liver	↑ TBARS	Lushchak et al. (2005)
		Hypoxia (1.8 mg O_2 L^{-1})	30 days	Erythrocytes	↑ Tail DNA	Mustafa et al. (2011)
Longsnout seahorse	*Hippocampus reidi*	Hypoxia (1.5 mg O_2 L^{-1})	8 hours	Erythrocytes	↑ Tail DNA	Negreiros et al. (2011)
Piapara fish	*Leporinus elongatus*	Hypoxia (1.92 mg O_2 L^{-1})	14 days	Blood	↑ GSSG	Wilhelm Filho et al. (2005)
			14 days	Liver	↑ GSSG, TBARS	
		Hypoxia (3.91 mg O_2 L^{-1})	7 days[a]	Blood	↑ GSSG	
			7 days[a]	Liver	↑ GSSG	
Chinese sleeper	*Perccottus glenii*	Hypoxia (0.4 mg O_2 L^{-1})	2 hours	Brain	↑ LOOH	Lushchak and Bagnyukova (2007)
			2 hours	Liver	↑ LOOH, PC	
			6 hours	Brain	↑ PC	
			6 hours	Liver	↑ LOOH, PC	
			6 hours	Muscle	↑ PC	
			10 hours	Brain	↑ PC, TBARS	
			10 hours	Liver	↑ PC	
			10 hours	Muscle	↑ PC	
Gilthead sea bream	*Sparus aurata*	Hypoxia (2.8 mg O_2 L^{-1})	6 hours[b]	Liver	↑ TBARS	Pérez-Jiménez et al. (2012)
Brown mussel	*Perna perna*	Air exposure	24 hours	Gill	↑ 8-oxodGuo, TBARS	Almeida et al. (2005)
			24 hours	Hepatopancreas	↑ TBARS	
False king crab	*Paralomis granulosa*	Air exposure	3.0 hours	Gill	↑ PC	Romero et al. (2007)
			6.0 hours	Gill	↑ PC	
			6.0 hours	Hepatopancreas	↑ LOOH	
			12 hours	Gill	↑ PC	
			12 hours	Hepatopancreas	↑ LOOH	
			24 hours	Gill	↑ PC	
			24 hours	Hepatopancreas	↑ LOOH	
			24 hours	Muscle	↑ LOOH	

Table 4. Continued.

Common name	Species	Stress	Duration	Tissue	Oxidative stress marker	Reference
Asian catfish	*Heteropneustes fossilis*	Air exposure	3.0 hours	Brain	↑ PC, TBARS	Paital (2013)
			6.0 hours	Brain	↑ PC, TBARS, H_2O_2	
			12 hours	Brain	↑ PC, TBARS, H_2O_2	
			18 hours	Brain	↑ PC, TBARS, H_2O_2	
			3.0 hours	Muscle	↑ TBARS, H_2O_2	Paital (2014)
			6.0 hours	Muscle	↑ PC, TBARS, H_2O_2	
			12 hours	Muscle	↑ PC, TBARS, H_2O_2	
			18 hours	Muscle	↑ PC, TBARS, H_2O_2	
Goldenrod gall fly	*Eurosta solidaginis*	Freezing	24 hours	Whole body	↑ GSSG, GSSG(%)	Joanisse and Storey (1998)
Wood frog	*Rana sylvatica*	Freezing	24 hours	Brain	↑ GSSG, GSSG(%)	Joanisse and Storey (1996)
				Kidney	↑ GSSG, GSSG(%)	
Leopard frog	*Rana pipiens*	Dehydration	90 hours	Liver	↑ GSSG, GSSG(%)	Hermes-Lima and Storey (1998)
Common periwinkle	*Littorina littorea*	Anoxia	6 days	Hepatopancreas	↑ GSSG	Pannunzio nad Storey (1998)
				Muscle	↑ CD, LOOH	
Estuarine crab	*Chasmagnathus granulata*	Anoxia	8 hours	Hepatopancreas	↑ CD, TBARS	de Oliveira et al. (2006)
Leopard frog	*Rana pipiens*	Anoxia	30 hours	Liver	↑ GSSG, GSSG(%)	Hermes-Lima and Storey (1996)
				Muscle	↑ GSSG, GSSG(%)	
Red-eared slider	*Trachemys scripta elegans*	Anoxia	20 hours	Liver	↑ GSSG, GSSG(%)	Willmore and Storey (1997b)
Garter snake	*Thamnophis sirtalis parietalis*	Anoxia	10 hours	Muscle	↑ GSSG	Hermes-Lima and Storey (1993)
Land snail	*Helix aspersa*	Estivation[c] Winter	20 days	Hepatopancreas	↑ GSSG, LOOH, TBARS	Ramos-Vasconcelos and Hermes-Lima (2003)
				Muscle	↑ PC	
Land snail	*Helix pomatia*	Torpor[d] Autumn	—	Kidney	↑ TBARS	Nowakowska et al. (2009)
		Torpor[d] Winter	—	Muscle	↑ TBARS	
Land snail	*Otala lactea*	Estivation[c]	30 days	Hepatopancreas	↑ GSSG, GSSG(%)	Hermes-Lima and Storey (1995)
			30 days	Muscle	↑ GSSG, GSSG(%)	
Apple snail	*Pomacea canaliculata*	Estivation[d]	45 days	Soft tissues	↑ TBARS	Giraud-Billoud et al. (2011)
		Estivation[d]	45 days	Kidney	↑ TBARS	Giraud-Billoud et al. (2013)
		Estivation[c]	45 days	Kidney	↑ TBARS	
				Muscle	↑ TBARS	
African lungfish	*Protopterus dolloi*	Estivation[d]	60 days	Brain	↑ Nitrotyrosine	Page et al. (2010)
Striped burrowing frog	*Cyclorana alboguttata*	Estivation[d]	180 days	Iliofibularis	↑ PC	Young et al. (2013)
Spadefot toad	*Scaphiopus couchii*	Estivation[d]	60 days	Gut	↑ CD, GSSG, GSSG(%)	Grundy and Storey (1998)
				Heart	↑ CD, GSSG, GSSG(%)	
				Kidney	↑ CD, GSSG(%)	
				Liver	↑ CD, GSSG(%), LOOH	
				Lung	↑ GSSG(%)	
				Muscle	↑ CD, LOOH	

8-oxodGuo, 8-oxo-7,8-dihydro-2'-deoxyguanosine; CD, conjugated dienes; GSSG(%), ratio between disulfide glutathione and the total pool of glutathione; GSSG, disulfide glutathione; LOOH, lipid hydroperoxides; PC, protein carbonyl; Tail DNA, DNA damage assessed by the COMET method; TBARS, thiobarbituric acid reactive substances.

a. Fish were exposed first to 1.92 mg O_2 L^{-1} for 14 days, then a group of fish were subsequently exposed to 3.91 mg O_2 L^{-1} for 7 days more.

b. Water aeration was discontinued and within 1 hour oxygen concentration reached 2.8 mg O_2 L^{-1} (from 7.1 mg O_2 L^{-1}). Once 2.8 mg O_2 L^{-1} was reached it was kept constant for 3 or 6 hours more.
c. Estivating animals versus 24 h aroused animals.
d. Estivating animals versus control active animals.

Table 5. Examples of works reporting hypoxia-induced increase in reactive oxygen species production.

Measurement	Animal model	Tissue or cell	Method	O_2 (%)	Average Δ (hypoxia vs. normoxia) (%)	Duration (min)	Reference
FR	Dog	Ventricular tissue	EPR	n.a.	54.5	15	Rao et al. (1983)
FR	Rat	Ventricular tissue	EPR	n.a.	56.5	10	Maupoil and Rochette (1988)
ROS	Chick	Cardiomyocytes	Fluorescence (DCFH-DA)	1.9	150	10	Vanden Hoek (1998)
ROS	Chick	Cardiomyocytes	Fluorescence (DCFH-DA)	1	300	15	Duranteau et al. (1998)
ROS	Chick	Cardiomyocytes	Fluorescence (DCFH-DA)	1	1100	120	
ROS	Human	Hep3B cells	Fluorescence (DCFH-DA)	1	300	30	Chandel et al. (1998)
ROS	Rat	Pulmonary artery smooth muscle cells	Fluorescence (DCDHFH-DA)	16	500	60	Killilea et al. (2000)
ROS	Rat	Pulmonary artery smooth muscle cells	Fluorescence (DCDHFH-DA)	16	165	10	
ROS	Rabbit	Intrapulmonary artery smooth muscle cells	Fluorescence (DCFH-DA)	1	85	60	Paddenberg et al. (2003)
$O_2^{\bullet-}$	Guinea pig	Ventricular tissue	Fluorescence (DHE)	n.a.	35	1	Kevin et al. (2003)
$O_2^{\bullet-}$	Guinea pig	Ventricular tissue	Fluorescence (DHE)	n.a.	95	20	
ROS	Human	A549 cells	Fluorescence (DCFH-DA)	1	50	120	Goyal et al. (2004)
ROS	Human	HepG2 cells	Fluorescence (DCDHFH-DA)	1	200	1440	Choi et al. (2007)
ROS	Human	Umbilical vein endothelial cells	Fluorescence (C-DHFH-DA)	6.3	15	30	Millar et al. (2007)
ROS	Mouse	Lung tissue	Fluorescence (RoGFP)	1.5	58	5	Desireddi et al. (2010)
$O_2^{\bullet-}$	Cow	Aortic endothelial cells	Fluorescence (DHE)	1	360	10	Hernansanz-Agustín et al. (2014)
$O_2^{\bullet-}$	Human	EA.hy926 cells	Fluorescence (DHE)	1	300	10	
ROS	Cow	Aortic endothelial cells	Fluorescence (C-DCFH-DA)	1	450	30	
$O_2^{\bullet-}$	Human	EA.hy926 cells	Fluorescence (Mito-HE)	1	100	10	
$O_2^{\bullet-}$	Cow	Aortic endothelial cells	Fluorescence (Mito-HE)	1	427	10	
$O_2^{\bullet-}$	Mouse	C57 cells	Fluorescence (DHE)	1	300	10	
$O_2^{\bullet-}$	Mouse	C57 cells lacking functional OXPHOS	Fluorescence (DHE)	1	100	10	

C-DCFH-DA, carboxi-dichlorofluorescein diacetate; C-DHFH-DA, carboxi-dihydrofluorescein diacetate; DCDHFH-DA, dichlorodihydrofluorescein diacetate; DCFH-DA, dichlorofluorescein diacetate; DHE, dihidroethidium; DHFH-DA, dihydrofluorescein diacetate; EPR, electron paramagnetic resonance; Mito-HE, mitohidroethidine; n.a. not available.

Figure 1

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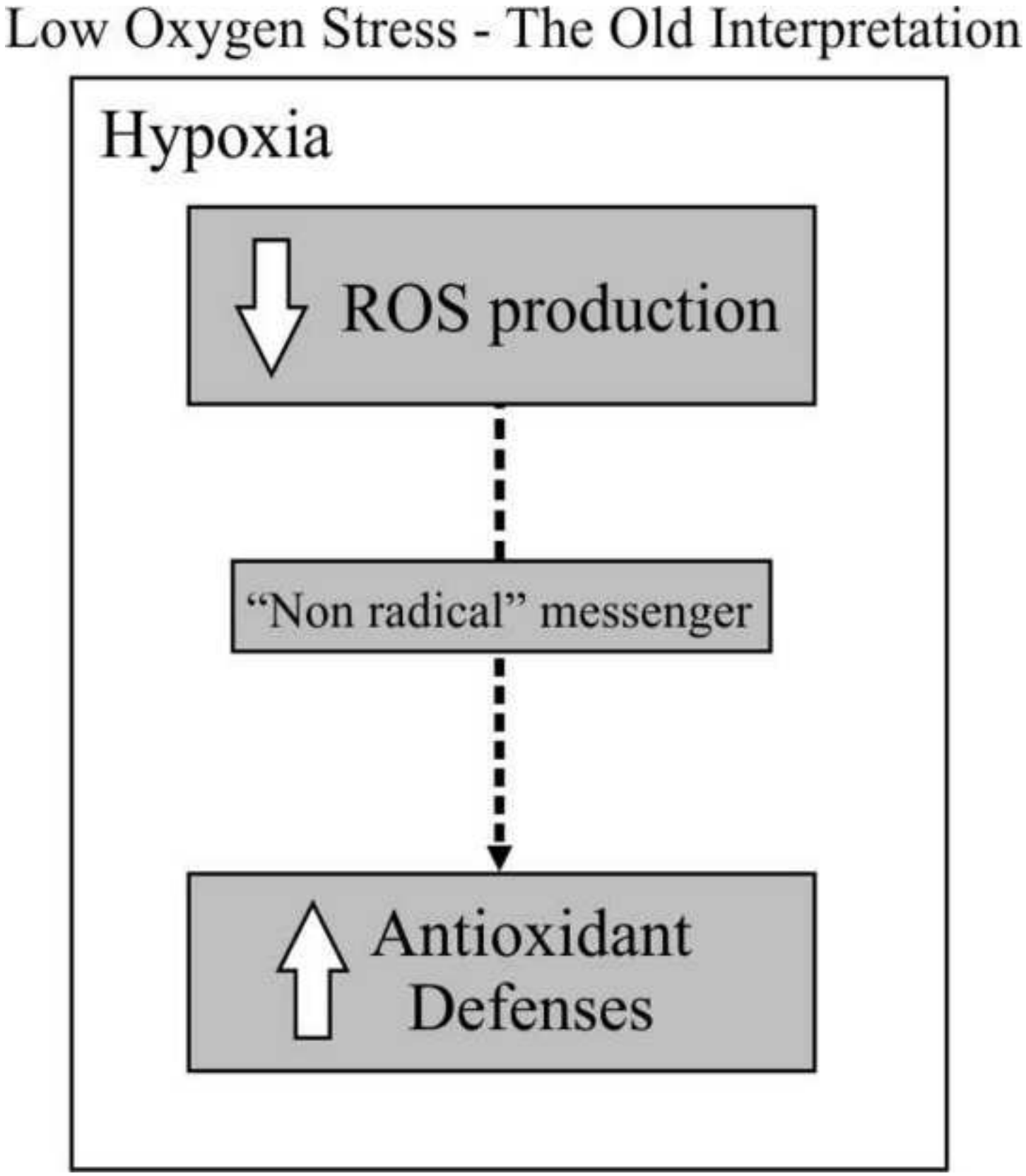

Figure 2

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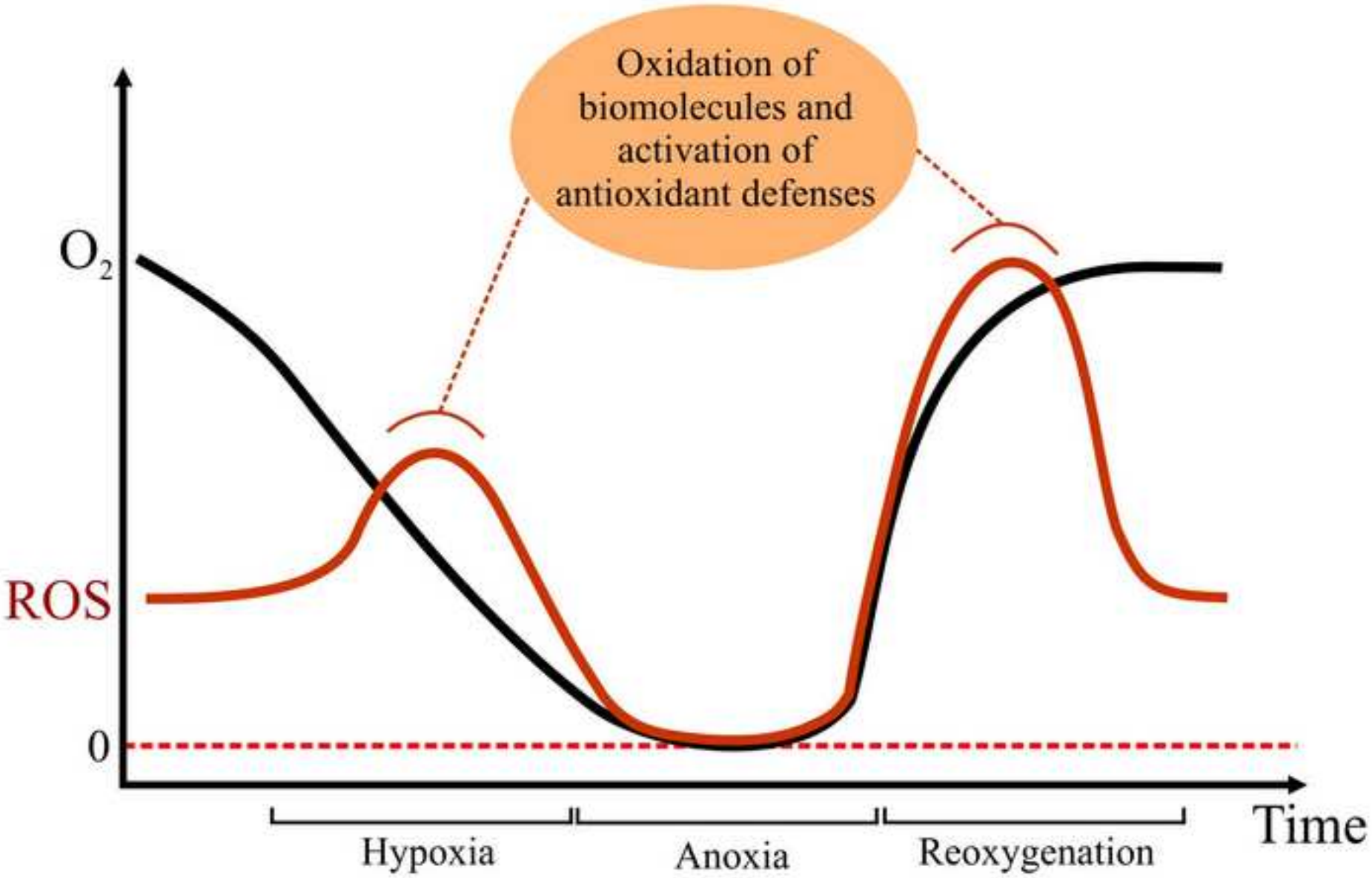

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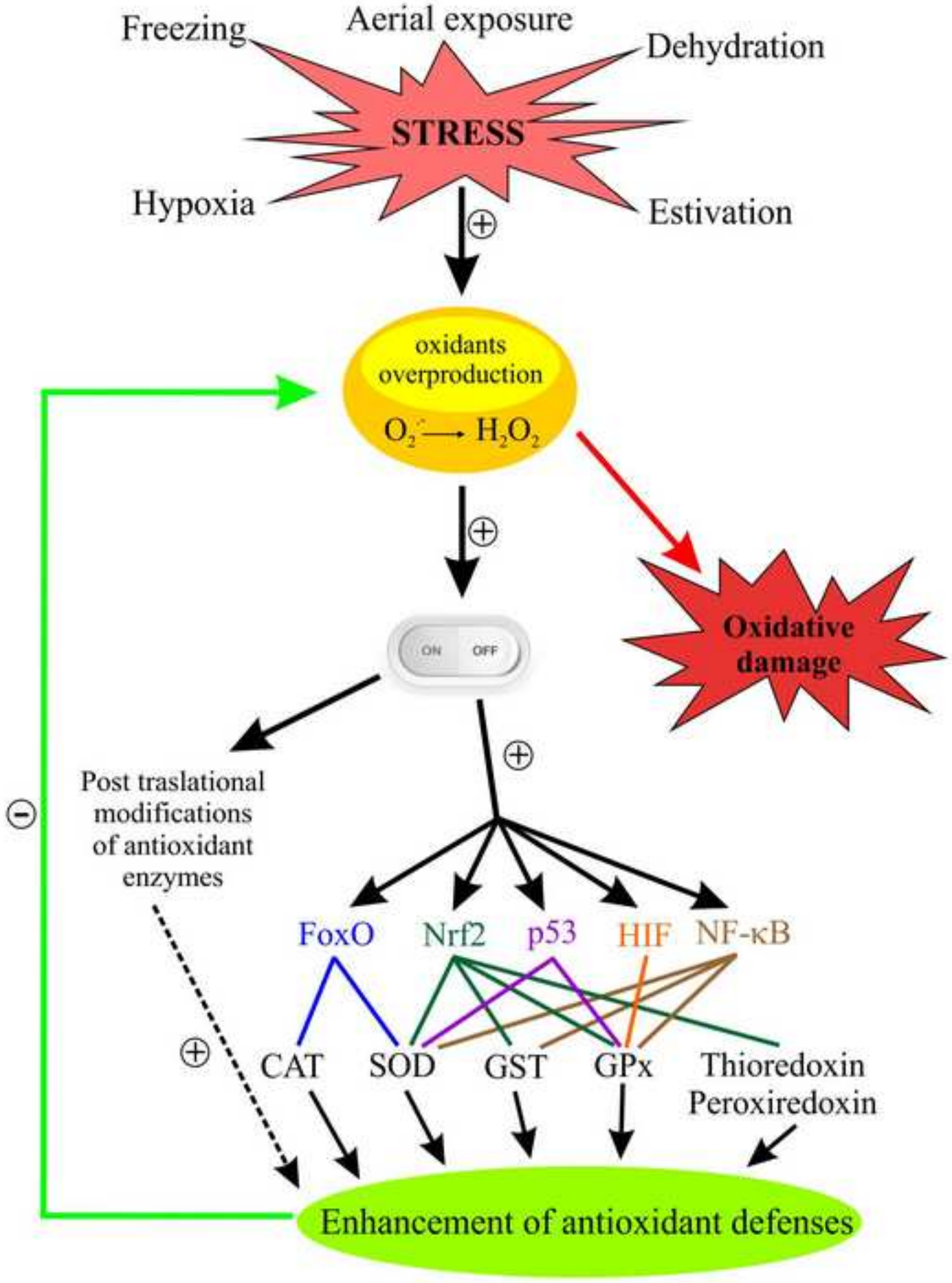

Figure 4

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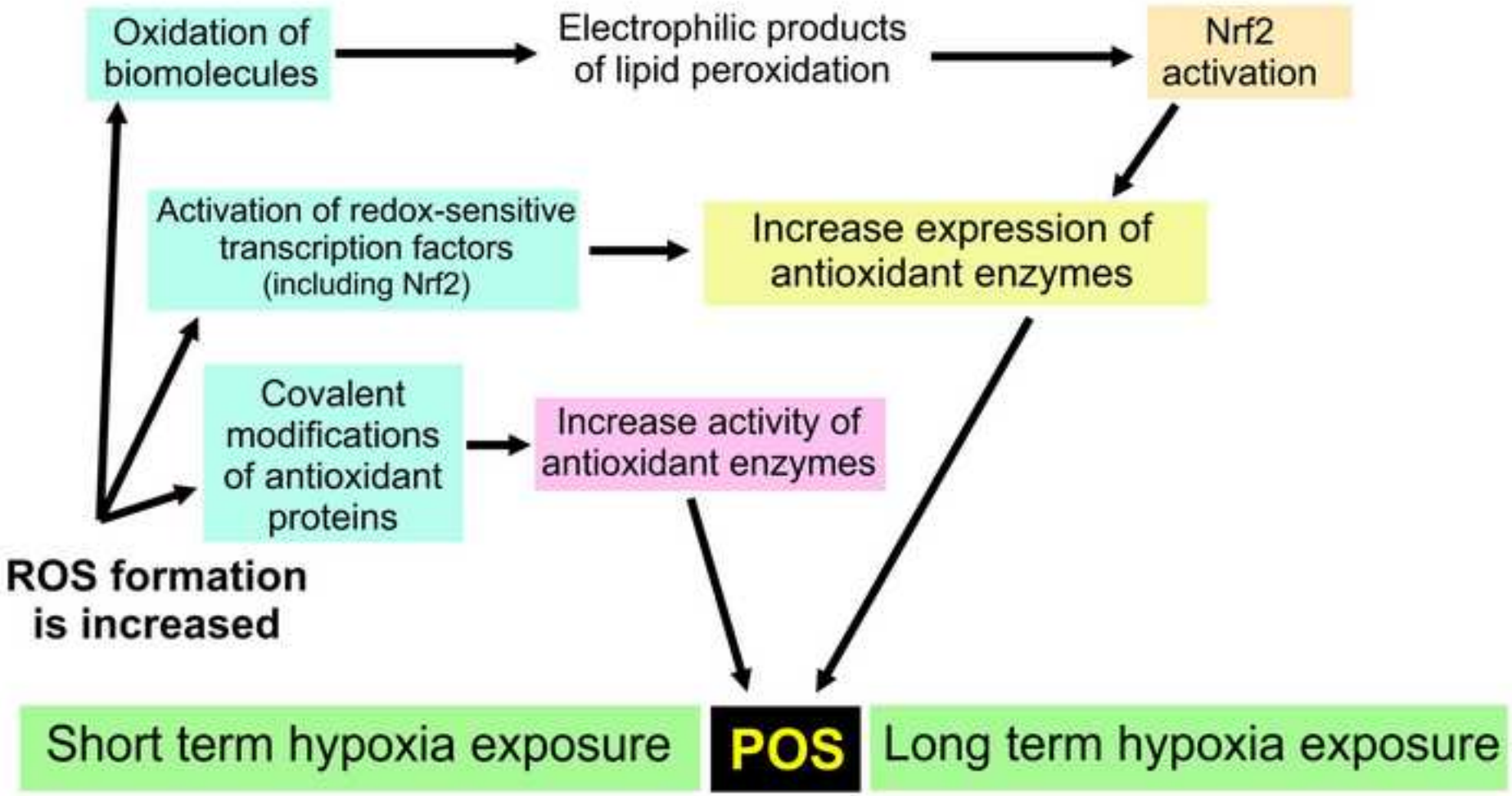

Figure 5

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