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Hypometabolism as the ultimate defense in stress response: how the comparative approach helps understanding of medically relevant questions

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Hypometabolism: Concepts and Translation

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Abstract

First conceptualized from breath-hold diving mammals, later recognized as the ultimate cell-autonomous survival strategy in anoxia-tolerant vertebrates and burrowing or hibernating rodents, hypometabolism is typically recruited by resilient organisms to withstand and recover from otherwise life-threatening hazards. Through the coordinated down-regulation of biosynthetic, proliferative and electrogenic expenditures at times when little ATP can be generated, a metabolism turned "down to the pilot light" allows the re-balancing of energy demand with supply at a greatly suppressed level in response to noxious exogenous stimuli or seasonal endogenous cues. A unifying hallmark of stress-tolerant organisms, the adaptation effectively prevents lethal depletion of ATP, thus delineating a marked contrast with susceptible species. Along with disengaged macromolecular syntheses, attenuated trans-membrane ion shuttling and pO_2-conforming respiration rates, the metabolic slowdown in tolerant species usually culminates in a non-cycling, quiescent phenotype. However, such a reprogramming also occurs in leading human pathophysiologies. Ranging from microbial infections through ischaemia-driven infarcts to solid malignancies, cells involved in these disorders may again invoke hypometabolism to endure conditions nonpermissive for growth. At the same time, their reduced activities underlie the frequent development of a general resistance to therapeutic interventions. On the other hand, a controlled induction of hypometabolic and/or hypothermic states by pharmacological means has recently stimulated intense research aimed at improved organ preservation and patient survival in situations requiring acutely administered critical care. The current review article therefore presents an up-to-date survey of concepts and applications of a coordinated and reversibly down-regulated metabolic rate as the ultimate defense in stress responses.

Keywords:

Hypoxia, hypometabolism, ATP turnover, oxy-conformance, dormancy, quiescence, hibernation, hypothermia, carbon monoxide, nitric oxide, hydrogen sulfide, critical care model, *Drosophila*, *C. elegans*, mammalian neonate, *Carassius, Chrysemys*, *Trachemys*, naked mole rat, arctic ground squirrel, hypoxia-ischaemia encephalopathy, sudden cardiac arrest.

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Prelude: Biomedical context and review rationale

Inadequate availability of oxygen, a condition known as hypoxia, is central to the pathophysiological progression of the three main causes of death by noncommunicable diseases (NCD), i.e. cardiovascular disease (46%), cancer (22%) and respiratory dysfunction (11%), including asthma and chronic obstructive pulmonary disease (COPD) (percentages: 2012 WHO reported fraction of NCD deaths by given pathology; http://www.who.int/gho/ncd/mortality_morbidity/en/). Even when regarding ischaemia-driven tissue destruction, as in most myocardial infarctions, stroke and infectious or inflammatory pathologies such as COPD or gastrointestinal colitis, it is the local tissue hypoxia that is commonly viewed as main etiological factor for the potentially life-threatening loss of cells (Eltzschig and Carmeliet, 2011, Cannon, 2013, Ramakrishnan et al., 2014). Beyond this influence, at a societal scale, hypoxia irreversibly affects additional lives across all stages of human development, including: a) adults with obstructive sleep apneas, chronic bronchitis, emphysema; b) babies succumbing to sudden infant death syndrome; c) newborns with recurrent apneas, and d) fetal developments experiencing intra-uterine growth restriction (Semenza, 2011, Semenza, 2014). This extensive impact of inadequate oxygenation in worsening the outcome of some of today's deadliest maladies makes efforts to improve treatments of these hypoxia-aggravated pathologies major ongoing academic, health and economic imperatives for many countries in the Western world (Luengo-Fernandez et al., 2013).

The intent of this review article, then, is a comprehensive portrayal - from biological concepts to medical applications - of the diverse suite of responses by cells and organisms that do not succumb to, but manage to survive, the challenges of low (hypoxia) or no (anoxia) oxygen, or of an insufficient supply of both blood born nutrients and oxygen (ischaemia). Due to the ample literature already available, the review makes, however, no attempt surveying hypoxia-associated human pathophysiologies and will only touch upon the O_2-mediated control and function of the hypoxia-induced signaling by HIF-1 and HIF-2 transcription factors (i.e see (Semenza, 2011, Keith et al., 2012, Myllyharju, 2013, Semenza, 2014, Bishop and Ratcliffe, 2015) for recent reviews). Neither will the article dwell on the role of mRNA translational checkpoints to slow protein synthesis as key

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energy sparing survival strategy in response to hypoxic, anoxic or ischaemic perils, as this point will be addressed in an upcoming review (unpublished data). Rather, the focus now will be on *adaptations at the cellular and organismal levels* that aim at reprogramming metabolic activities upon sensing any of the above noxious cues. Yet, a proper understanding of these cellular or organismal defenses critically hinges on a clear concept of one of their prime signals: hypoxia. This concept of hypoxia should robustly apply to different cell backgrounds (primary, transformed), stress coping modes (hypoxia-sensitive, hypoxia-tolerant) and should involve both known and unknown roles of the HIF pathway.

This review will thus start by elaborating on key concepts (i.e. hypoxia, hypoxia tolerance) before it leads to the theory of the ultimate coping mechanism in severe O_2 deprivation: the coordinated slow-down of metabolic activities to reach a new ATP steady state (i.e. hypometabolism). Subsequently, we will look at select examples of hypometabolic defenses across a highly diverse biology (from "bugs to babies") to demonstrate the widespread nature of this trait. Although much of what we know today about mechanisms of metabolic dormancy was unraveled when examining hypoxia- and anoxia-tolerant phenotypes, hypometabolism does by no means unfold only during exposures to little or no O_2. Manifest in hibernating mammals during torpor, hypometabolism also occurs in fully aerobic settings. Moreover, the defense is prompted in animals during breath-hold dives, temporary summer or nocturnal torpor, aestivation, diapause, anhydrobiosis and cold-hardiness. Underlying mechanisms of hypometabolic states are, as we will see, largely conserved irrespective of phenotypes, and operate, whether in hypoxic or aerobic contexts, through ways of enormous clinical potential. Yet, of the many life-saving facets of hypometabolism in endothermic hibernators, only hypothermia has so far matured into a mainstay treatment for enhanced organ-, and particularly, neuro-protective effects in human individuals challenged by ischaemia-reperfusion injuries. Accordingly, protective principles, first elicited in stress-tolerant and/or hibernating animal specialists, *can* be translated, via mammalian models, to newborn and even adult human patients. Besides a controlled cooling, pharmacological inhibitors of respiration and heat production (e.g. H_2S and sulfide application) are to date increasingly utilized in small and large animal models to assess their organ-protective efficacy.

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Revealing this conceptual bridge of metabolic dormancy from deoxygenated cells and torpid hibernators to H_2S and sulfide-treated models and future human therapies is the main goal of the text´s second section.

1. Concepts

1.1. Hypoxia

Let us begin with a fundamental question: *what is hypoxia* or, in other words: how does a cell "know" it is hypoxic? Contrary to public opinion, hypoxia does *not* correspond to any particular numerical value of a reduced O_2 tension (e.g. 1% oxygen), nor is there an absolute or universal oxygen level that can be called hypoxic (Nikinmaa, 2013, Wenger et al., 2015). Understanding hypoxia at the level of cells may best be approached from a biological perspective. Most, if not all, vertebrate (Wilson et al., 1979, Pörtner et al., 1985, Wilson and Erecinska, 1986), nematode, crustacean or insect species (see (Gorr et al., 2006) and references therein) consume oxygen across a wide range of partial pressures (pO_2) in a more or less constant, so-called oxyregulated, fashion (Dejours, 1981, Prosser, 1991). Isolated mitochondria and cells of oxyregulating species, including primary platelets (Arthur et al., 1999), cardiomyocytes (Pörtner and Grieshaber, 1993) and hepatocytes (Jones and Kennedy, 1982), as well as various cancer cell lines (e.g. Ehrlich ascites cells (Froese, 1962), neuroblastoma (Robiolio et al., 1989)), of human or rodent origin, also display pO_2 independent respiration above a critical oxygen threshold (i.e. P*c*) (Pörtner and Grieshaber, 1993, Gnaiger et al., 1995, Buchner et al., 2001). For all these oxyregulating systems, responses to hypoxia via the transcription factor HIF or other cascades tend to peak around their s*pecific* critical threshold P_C. Generally speaking, this threshold separates the aerobe-oxyregulated from the anaerobe-oxyconforming physiological state of a given cell. Under standard culture conditions the critical oxygen threshold usually occurs for primary or transformed mammalian cells within the ~0.15%–1.5% O_2 range (Froese, 1962, Robiolio et al., 1989), thus emphasizing that P_C thresholds of different cell types can vary by more than one order of magnitude. Closely superimposed with the P_C, HIF´s preserved maximal activity, as assessed by *in*

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vitro binding to specific hypoxia response (cis) elements (HREs) of HIF target genes and/or accumulation of HIF-α protein levels, usually resides for cultured mammalian cells at ~0.5-2.0% O_2 (Jiang et al., 1996, Ebbesen et al., 2004). From mammals to teleosts, and in *Drosophila melanogaster*, the crustacean *Daphnia magna*, the nematode *Caenorhabditis elegans*, the cnidarian polyps *Acropora millepora* and *Nematostella vectensis*, and even the simplest animal *Trichoplax adhaerens*, a functional, O_2-regulated HIF system exists widely, perhaps ubiquitously, across metazoans (Gorr et al., 2006, Hampton-Smith and Peet, 2009, Hoogewijs et al., 2007, Loenarz et al., 2011). Wherever known, the expression-activating functional HIF complex forms within minutes to hours of low oxygen as a heterodimer of homologous alpha and beta subunits, both members of the family of basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS; Per-Arnt-Sim first reported PAS domain proteins) transcription factors. Once generated in the nucleus, the α-β heterodimer controls expression of hypoxia-sensitive target genes by binding to aforementioned HREs. Regulation of HIF abundance and activity by oxygen occurs primarily, in cultured cells at least, at protein level through the enzymatic hydroxylation of specific α-subunit prolyl and asparaginyl residues, respectively (see (Semenza, 2011, Keith et al., 2012, Myllyharju, 2013, Semenza, 2014, Bishop and Ratcliffe, 2015) for recent reviews).

Regarding an insect model, Figure 1 illustrates the transition from oxyregulation into oxyconformance by presenting a composite of the O_2 consumption rate (pink line) of *Drosophila melanogaster* fruitflies and the HIF activity profile in Schneider`s cell line 2 (S2) from late stage *D. melanogaster* embryos (gray line). As "genuine" insects, resting fruitflies display regulated O_2-uptake as long as ambient pO_2 levels are fully sufficient to sustain oxidative metabolism (see Fig.1 caption for details (Csik, 1939, Chadwick and Gilmour, 1940, Krishnan et al., 1997, Ma et al., 1999)). Yet, once pO_2 measures have fallen sufficiently to approach the *D. melanogaster* P_C value of ~1.6 to 3.0% O_2 (Csik, 1939, Chadwick and Gilmour, 1940), adult flies respond with poor coordination, and, eventually, enter a reversible state of complete immobilization (i.e. stupor). This behavioral switch is accompanied by the transition from aerobe → anaerobe metabolism (lactate build-up) and from oxyregulated → oxyconforming respiration. During the latter state, O_2 uptake decreases drastically

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and proportionally with falling O_2 tensions. Still deeper into hypoxia, the insect, particularly the fly embryo, proceeds into a metabolic arrested state as indicated by cell cycle arrest and condensed chromatin (Foe and Alberts, 1985, Wingrove and O'Farrell, 1999, DiGregorio et al., 2001, Douglas et al., 2001, Douglas et al., 2005). Superimposed with these physiological data is the pO_2 range of the HRE binding activity of S2 HIF (inset in Fig. 1 and (Gorr et al., 2004b)). Exemplified by *Drosophila*, HIF activity peaks *in vitro* (Gorr et al., 2004b) and *in vivo* (Lavista-Llanos et al., 2002) where it matters the most: around the P_C. Thus, the state of *hypoxia* in cells represents a *relative measure* of deoxygenation. Hypoxia, in oxyregulating systems, is best described by $pO_2 \leq P_C$ and best illustrated by *peaking HIF activity*. The co-occurrence of these metabolic and respiratory switches at $pO_2 \leq P_C$ on the one hand, and maximal HIF activation on the other, suggests HIF's sentinel, and perhaps primordial, function to lie within safeguarding the cells' transition from an aerobic ($pO_2 > P_C$) to an anaerobic-glycolytic mode of ATP synthesis ($pO_2 \leq P_C$) (Webster, 1987, Firth et al., 1994, Iyer et al., 1998, Seagroves et al., 2001).

1.2. O_2 homeostasis

Oxygen tensions in normal, healthy organs are in a near-perfect homeostasis of supply and demand (reviewed: (Hochachka, 1999)). The exquisite equilibrium between metabolite delivery and metabolic output works so well that most healthy human organs remain physiologically aerobic even at maximal workloads (Krogh, 1919). Thus, median pO_2 values of organs, e.g. ~66 in the spleen and ~20 mmHg in the brain cortex and heart myocardium (Vaupel et al., 1989, Vanderkooi et al., 1991), do not fall far from the 40 mmHg partial O_2 pressure of venous blood (Carreau et al., 2011) (7.35 mmHg ≈ 1% O_2). Although oxygen partial pressures of any solid organ are always reflective of the tissue`s own oxygen consumption rate and it´s local distance to the nearest capillaries, the general constancy of oxygenation in resting or stimulated tissue has made adult mammals (e.g. mice (Duffy et al., 1972), rats (Erecinska and Silver, 2001), humans (Ramirez et al., 2007)) highly susceptible to even slight disturbances in O_2 supply. Beyond the vulnerable central nervous system (e.g. (Hansen, 1985,

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Leblond and Krnjevic, 1989, Erecinska and Silver, 2001)), other organs with high O_2 and ATP requirements in vertebrate endotherms, including the kidneys and liver, cease functioning after minutes-long exposure of the animal to hypoxic, anoxic or ischaemic perils (e.g. (Jaeschke et al., 1988, Lefebvre et al., 1993, Rosenberger et al., 2005)). However, recovery of "mice and men" from hypoxia and ischaemia can be as detrimental as the stress itself (Galli and Richards, 2014). Regarding humans, sudden cardiac death accounts for more than half of all cardiovascular deaths in the United States. These deaths are largely attributed to cardiac arrest that occurs after an ischaemic insult (Ramirez et al., 2007).

In stark contrast to the situation in healthy and homeostatic organs, the concentrations of oxygen in solid cancers often reveal spatially and temporarily highly heterogeneous profiles. While O_2 diffusion gradients are also prevalent within avascular (e.g. articulated cartilage), hypovascular (e.g. dermal sub cutis), wounded (e.g. diabetic ulcers, venous ulcers, burns), chronically inflamed (e.g. ulcerative colitis, rheumatoid arthritis) or re-modelled tissues (e.g. scar around ischaemic or trauma-mediated infarct) (reviewed: (Wattel and Mathieu, 2005, Schreml et al., 2010, Eltzschig et al., 2014, Manresa et al., 2014)), cause and consequence of these gradients have been particularly well elucidated within neoplastic masses. In growing cancers the loss of homeostasis typically results from an increasing mismatch between the erratic oxygen supply by poorly functional and structurally aberrant vasculature on the one hand, and the high demand for oxygen by strongly proliferating epithelia on the other (e.g. (Brown and Wilson, 2004, Dewhirst et al., 2008, Rademakers et al., 2008, Jubb et al., 2010)). Landmark studies by the British radiologists Gray and Thomlinson in the 1950s first established the direct link between a tumor`s O_2 tension and its susceptibility to irradiation treatment (Gray et al., 1953, Thomlinson and Gray, 1955). Through measuring histological sections of lung cancers, the authors identified viable hypoxic cells in the ~150μm-spanning layer of the tumors' concentric cord structure, directly adjacent to the necrotic core. There is little doubt today that radiobiological hypoxia (i.e. level of hypoxia where significant resistance to radiation is observed; usually at $pO_2 \leq 2.5$ mmHg) develops locally in the majority of advanced tumors, including brain, lung, breast, pancreatic, cervical, prostate and head and neck carcinomas, as well as soft tissue

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sarcomas (reviewed: (Vaupel et al., 1998, Brown and Wilson, 2004, Vaupel and Mayer, 2007, Rademakers et al., 2008)). The clinical consequences of this breach in the O_2 supply-demand equilibrium in tumor tissue are severe, and often manifested by the fact that hypoxia *per se* causes general resistance to chemotherapeutical and radiological treatment along with a stronger prevalence for the disease to disseminate to remote organs (e.g. (Okunieff et al., 1993, Höckel et al., 1996, Brizel et al., 1997, Nordsmark et al., 2005)). Moreover, extensive changes in gene expression, partly mediated by run-away HIF activity, further promote cancer cells to become refractory towards blood born poisons or irradiation (e.g. (Unruh et al., 2003, Moeller et al., 2004, Williams et al., 2005)). At very low pO_2, radiotherapy becomes ineffective in killing cells because O_2-derived radicals are generated at insufficient levels. Since biosynthetic and cell cycling activities also decline in the severely hypoxic, perinecrotic areas of model spheroids or growing malignancies (Freyer, 1994, Zölzer et al., 1999), non-cycling dormant or occult cancer cells - now appreciated as critical stage for the asymptomatic lag phase of many tumor entities (Enderling et al., 2013) - are effectively protected from conventional treatments (Masunaga et al., 2002, Masunaga et al., 2006). Once reactivated, e.g. by anti-cancer interventions, formerly dormant cell survivors are prone to reseed nascent tumors and metastases, and thus might drive much of the post-therapeutic relapse of the malignancy (Bragado et al., 2012, Zhang et al., 2014).

Due to the inherent difficulties in effectively targeting hypoxic tumor cells, including dormant stages, therapies designed specifically to affect the altered pathways underlying the tumors` *hypoxia tolerance* may actually represent especially promising approaches for producing a therapeutic gain (Wouters et al., 2004). By targeting hypoxia tolerance mechanisms, more selective anti-cancer therapies can be anticipated since almost all healthy tissues are homeostatic in their oxygenation (above) and, arguably, either do not recruit metabolism-slowdown strategies upon deoxygenation, or do so to a lesser extent. The only problem before realizing that objective is to determine which players and pathways are key in the metabolic reprogramming that lies at the heart of a hypoxia-tolerant phenotype.

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1.3. Hypoxia survival responses

Our prevalent use of transformed cells and hypoxia-sensitive animal models has shaped our notion of "typical" mammalian responses to oxygen or substrate scarcity to include mainly the induced activation of angiogenic, erythropoietic, glycolytic and apoptotic components. In comparison, our physiological concept of hypoxia tolerance, together with its underlying mechanisms and key regulatory elements (e.g. role if HIF-1, HIF-2), remains relatively poorly described – a fact that directly results from the complex nature of a hypoxia tolerant phenotype and that greatly limits the concept's impact on mainstream clinical thinking and practice. Most tolerant cells or species, when challenged by declining O_2 supplies, will utilize not one but several defenses as a function of stress severity. As summarized in Figure 2, these defenses can be arranged along a spectrum. Mild degrees of O_2 deprivation (i.e. $pO_2 \geq P_C$; note O_2 gradient in Fig. 2) activate physiological avoidance strategies in invertebrates and ectotherms, such as behavioral hypoxia-induced hypothermia (reviewed: (Wood, 1991, Wood, 1995)), to trigger a reduction of metabolic activities. Endothermic species, challenged by mild levels of O_2-deprivation, also employ, as a preemptive line of defense, the marked reduction of their oxygen demand through the cessation of endogenous heat production (Branco et al., 2014). Such a stress-responsive intentional drop in body temperature (i.e. anapyrexia) marks a cornerstone within the heightened stress resilience of mammalian pups (see below) and of species undergoing transient nocturnal torpor (e.g. red deer, wapiti) or daily torpor (e.g. bats, hummingbirds).

At slightly deeper or more persistent moderate hypoxia, responses next aim to maintain the O_2 carrying capacity of the respective system, for example through augmented FGF-mediated sprouting of tracheal ends as in *Drosophila* (e.g. (Jarecki et al., 1999, Centanin et al., 2008, Centanin et al., 2010)) or through the HIF-driven induction of globin genes as in *Daphnia* (e.g. (Gorr et al., 2004a, Colbourne et al., 2011, Gerke et al., 2011)). Physiologically speaking, these responses form the invertebrate equivalent to the induced synthesis of erythropoietin (EPO) and augmented production and maturation of erythrocytes in mammals, arguably the best described molecular event of reduced pO_2 values in "mice and men" (i.e. see: (Jelkmann, 2007, Wenger and Kurtz, 2011) for recent reviews). As exemplified by these responses in *Daphnia*, *Drosophila* and mammals, organisms aim to

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maintain their respective O_2 carrying capacities as long as a reasonable reduction in pO_2 justifies it. Still deeper into hypoxia, however, these HIF-guided metabolic (aerobe → anaerobe) and respiratory (oxyregulated → oxyconforming) transitions (also Fig. 1) eventually give way to different coping mechanisms of severely deoxygenated *tolerant* cells or organisms (i.e. $pO_2 << P_C$), now aiming to re-balance ATP demand with supply at a new, deeply depressed, steady state (i.e. hypometabolism). Exposure to near-anoxic levels of O_2 scarcity eventually sees the waning of HIF signaling, in favor of truly anoxic cascades (e.g. signaling mediated by: p53, ATF4; Fig. 2). In reflection of that, the homolog of the O_2-regulated HIF alpha subunit ensures survival of both *C. elegans* and *Drosophila* during hypoxic, but not anoxic or normoxic, exposures (Jiang et al., 2001, Lavista-Llanos et al., 2002, Shen and Powell-Coffman, 2003, Centanin et al., 2005). Taking together, the concept of hypoxia tolerance does not rely on a "one size fits all" universal strategy. Rather, it entails multiple responses that are activated appropriately at any given stress level, and that include HIF-dependent as well as HIF-independent signaling events.

1.4. Turning down metabolism

As emphasized above (Fig. 2), tolerance towards severe hypoxia ($pO_2 << P_C$) or anoxia ($pO_2 \rightarrow 0$) in organisms is commonly achieved through a hypometabolic state. Enhanced survival through hypometabolism was first conceptualized by the founding father of diving research, the Swedish physiologist Per Fredrik Scholander (Scholander, 1940). Best demonstrated during his studies on seals undergoing forced submersion, Scholander noticed the incurrence of an oxygen debt during the breath-hold dive, which was neither fully repaid by glycolysis during submergence (i.e. low lactate accumulation during dive *per se*; less-than-expected lactate washout into blood upon re-surfacing) nor by excess oxygen uptake during recovery (i.e. absence of O_2 uptake spike upon re-surfacing). He consequently inferred the oxygen consumption (metabolic) rate to be reduced from pre-submersion levels during the breath-hold period. This metabolic slowdown turned out to be an integral component of the so-called dive response of mammalian (e.g. seals, porpoises) and avian (e.g. penguins, ducks)

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"apnea divers". Parallel physiological changes during prolonged dives of these animals include a pronounced bradycardia and body (brain) cooling, constriction of peripheral blood vessels and blood shift to vital organs, and the reliance on greatly increased intramuscular, myoglobin-based O_2 stores (e.g (Hochachka and Somero, 2002, Ponganis, 2013)). The concept of hypometabolism as the ultimate survival strategy beyond apneic animal divers was later recognized and extended by Hochachka (Hochachka et al., 1996), Boutilier (Boutilier and St-Pierre, 2000), Lutz (Lutz and Milton, 2004) and others to mature into arguably the central stress defense paradigm of today's comparative biologists.

A "metabolism turned down to the pilot light" (citation Kjell Johansen; see (Hochachka et al., 1996)), along with an associated inactivity (dormancy), is also manifest in species undergoing hibernation, temporary torpor, aestivation, diapause, anhydrobiosis and cold-hardiness (Storey and Storey, 2004). In all these cases, hypometabolism refers to a moderate to severe reduction of the organism's resting metabolic rate (RMR), i.e. of the energy expenditure utilized to sustain basic vital functions while the organism is at complete rest (existing in neutrally temperate environment and in a post-absorptive state) (see Table 1). This form of metabolic depression is to be strictly distinguished from the essentially ametabolic categories of cryptobiosis (hidden life; reviewed: (Withers and Cooper, 2010)), including anhydrobiotic (stressor: desiccation), cryobiotic (stressor: freezing) and osmobiotic states (stressor: high solute concentration) (see Supplemental Figure 1 for details).

The concept of hypometabolism is best understood by comparing the principal adaptations in hypoxia-sensitive versus tolerant contexts. As introduced in section 1.2, operation within a tightly regulated O_2 homeostasis necessitates hypoxia-sensitive organs of adult, endothermic mammals (e.g. brain, kidney, liver) to primarily invoke *energy compensating* defenses during hypoxia via HIF in the attempt to maintain or extend the pre-existing coupling of O_2 supplies (induced vasodilation, angiogenesis and erythropoiesis) and ATP demands (elevated glycolytic substrate flux). Regarding the latter adaptation, Louis Pasteur was the first to document in 1861 that glucose consumption via glycolysis in yeast was positively regulated by hypoxia (i.e. Pasteur effect: (Pasteur, 1861)). More than a century would elapse, however, before major hypoxia research laboratories incontrovertibly described HIF-1 as the prime mediator of the Pasteur effect (Webster, 1987, Firth et al., 1995, Iyer et

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al., 1998, Seagroves et al., 2001). HIF-1-driven transactivation of most glycolytic enzyme genes is key in switching a mammalian cell from aerobic to fermentative metabolism. However, too strong a flux of glycolysis quickly depletes finite stores of fermentable substrate (e.g. glycogen) and amasses toxic levels of end products in sensitive, hypoxic cells, often resulting in cell death (Storey, 1985, Schmidt and Kamp, 1996). For hypoxia tolerance to emerge, other mechanisms, in concert with a *moderate and sustained* Pasteur effect, must be present. Many lower organisms evolved the ability to severely reduce their metabolic rate and enter a state of suspended animation in response to reduced oxygen tensions. This strategy enables *Drosophila* embryos (≤ 8d) and even adult flies (≤ 4h) to fully withstand periods of no oxygen (respective anoxia survival times at full recovery (Krishnan et al., 1997, Wingrove and O'Farrell, 1999)). Some primary mammalian cells bear vestigial remnants of this response and can depress metabolism, e.g. chronically hypoxic astrocytes (Vega et al., 2006, Schmid-Brunclik et al., 2008) or noncontracting hibernating cardiomyocytes (Casey and Arthur, 2000, Casey et al., 2002).

Physiological hypoxia tolerance in animals primarily utilizes *energy conservation*, not compensation as in sensitive species, as a long-term survival strategy. This remarkable resilience is achieved through the controlled, yet fully reversible metabolic rate suppression down to a new steady-state of proportionately reduced ATP supply and demand (reviewed: (Gorr et al., 2006)). Sustained throughout the entire stress, manifest hypometabolism prevents lethal falls in cellular ATP levels and is considered the single most protective and *unifying feature* of hypoxia-tolerant tissues (Hochachka et al., 1996, Boutilier and St-Pierre, 2000, Boutilier, 2001). While in this state of dormancy, O_2 consumption rates equally drop to a small percentage of normoxic uptake and become dependent of ambient pO_2, or oxy-conforming (Fig. 1). To match with the declining ATP production in O_2-depleted cells, hypometabolism requires the immediate and coordinated down-regulation of every major ATP-utilizing function in the cell (Rolfe and Brown, 1997), including 1) macromolecular, notably protein and DNA, syntheses (Land et al., 1993), 2) protein degradation (Land and Hochachka, 1994); 3) ion-motive ATPases, notably Na^+/K^+-ATPase, along with a reduced influx of ions (Buck and Hochachka, 1993); and 4) gluconeogenesis (Rolfe and Brown, 1997). The further these activities can be

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suppressed, the better and longer are cells and organisms able to withstand O_2 deprivations (Guppy and Withers, 1999). In shifting remaining energy resources towards truly vital functions, inhibition of the majority of protein synthesis (*translational arrest*) and degradation allows, as a hallmark of tolerance, electrochemical gradients across membranes to be maintained at reduced permeabilities (*channel arrest*) (Buck and Hochachka, 1993, Krumschnabel et al., 2000). As a further advantage of hypometabolic states, glycolytic fluxes need only be elevated and provide ATP for as much as the residual energy expenditures require (i.e., weak to absent Pasteur effect during a hypometabolic depression of energy expenditure (Storey, 1985, Schmidt and Kamp, 1996)). Hence, this defense spares fermentable fuel and reduces metabolic waste. Along with hypometabolism, cells also enter the non-replicative, non-proliferative "silent S-state" of quiescence (DiGregorio et al., 2001, Douglas et al., 2001, Padilla and Roth, 2001, Padilla et al., 2002, Douglas et al., 2005). Together, hypometabolism, defined as pO_2-conforming O_2 uptake rates, disengaged protein and DNA synthesis and a non-cycling, quiescent phenotype, underlies the enormous resistance of numerous invertebrate and certain ectothermic vertebrate species in response to a large variety of life-threatening hazards.

Entry into oxy-conforming respiration, in anoxia-tolerant fish, frogs and turtles, though following different paths of silencing the mitochondrial electron transport onto oxygen as final acceptor, always results in a clear down-regulation of the organism's respiratory capacity during anoxia (reviewed: (Galli and Richards, 2014)). To assess if a hypoxia-tolerant insect model would similarly attenuate electron transport chain activity upon entering pO_2-conformance *Drosophila* S2 cells were exposed to graded hypoxia. Molecular transitions of S2 cells along a falling O_2 trajectory as delineated in Fig. 1 were recorded with cells kept either in normoxia (room air, ~15% O_2 in medium) or challenged for 16h at 4% or 1% or 0.2% O_2 to approximate oxy-regulatory ($pO_2 > P_C$, 4% O_2), aerobe-anaerobe transitory ($pO_2 \sim P_C$, 1% O_2) and hypometabolic ($pO_2 << P_C$, 0.2% O_2) physiological states of the fly, respectively (note green and red X-axis labels in Fig. 1). Next, RNA expression profiles of gene products relevant for the electron transport efficacy among OXPHOS complexes I-IV, ATP synthesis (OXPHOS complex V), scavenging of reactive oxygen species (ROS) in mitochondria and various other mitochondrial enzyme activities, were examined by Northern blot analyses (Figure 3), and re-

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assessed by microarray data (see Supplemental Figure 2 for details). Representative fold-expression changes in hypoxia (i.e. 0.2 – 1 – 4 lanes, Fig. 3), normalized with the constitutively expressed mRNA RpL29 (blue labels), are shown as expression ratios underneath each corresponding Northern blot lane relative to the normoxic (N) reference values set to 1 (Fig. 3). All OXPHOS transcripts, comprising both nuclear (ATPsynγ) and mitochondrially encoded products (mt:ND5, mt:CoI), show a marked ~2-fold down-regulation at 1% and 0.2% O_2, yet were completely refractory during the oxy-regulated state at 4% O_2 or during exposure to hypoxia mimetics (cobalt (C), desferrioxamine (D)). Similar profiles of decreased mRNA abundance during 1% and 0.2% O_2 were noticed for mitochondrial antioxidant (Sod2), TCA (Idh), and β-oxidation enzymatic activities (Thiolase) as well as for mtDNA replication engaging proteins (mtSSB). On the other hand, steady-state mRNA quantities of glutamate dehydrogenase (Gdh) demonstrated induced transcription and/or elevated mRNA stability specifically at $pO_2 \leq P_C$ (1% O_2, 0.2% O_2). Clearly, these transcriptional footprints suggest the emergence of oxyconformity and coordinately reduced ATP turnover (e.g. reduced Idh expression → slows TCA cycle; Thiolase → β-oxidation; OXPHOS subunits → ATP synthesis; mtSSB → mtDNA replication) at hypoxic pO_2 levels that are non-limiting for mitochondrial function (i.e. at pO_2 well in excess of the P_{50} of cytochrome c oxidase (COX), the O_2 reducing OXPHOS complex IV). For further energetic balancing these organelles presumably harness extra ATP and GTP through induced Pepck and Gdh activities (Fig. 3; (Grieshaber et al., 1994)). Hypoxia-stimulated Pepck expression might, in fact, be reflective of an active phosphoenolpyruvate (PEP) branchpoint to enable a sufficient fermentative production of succinate via a counterclockwise running of the TCA, as seen in facultative marine anaerobes of the tidal zone, including cnidarians, littoral bivalves, annelids and sipunculids (light-blue arrows in TCA cycle – Fig. 3; see also (De Zwaan, 1983, De Zwaan and Putzer, 1985, Grieshaber et al., 1994)). Remarkably, the strong induction of the CG7224 mRNA (Fig. 3), with its early onset of responsiveness (4% O_2), its marked sensitivity to DFO (but not cobalt) and its peaking induction at 1% O_2, deviates from the above responses and rather follows the ranking of relative S2 HIF activities (hypoxia > DFO >> normoxia ≅ cobalt.; (Gorr et al., 2004b, Gorr et al., 2006)), suggesting activation of this gene to be HIF-mediated. If future studies indeed

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corroborate the CG7224 peptide to act as OXPHOS complex II (SDH) assembly factor (Van Vranken et al., 2014), induction of this transcription also needs to be assessed in the light of a possible TCA flux reversal in hypoxic fly cells.

2. Hypometabolism translated

The following arbitrary list of topics was chosen to highlight applied facets of hypometabolism. While making no claim to completeness, the aim here is to illustrate the adaptations` biological breath and its conceptual bridge, mentioned in the onset of this survey, that spans from the basic discoveries on the metabolic depression seen in dormant cells or stress-tolerant and hibernating animal specialists to H_2S- and sulfide-treated models and putative future therapies of human patients. Importantly, medical interventions centered on hypometabolism can be utilized in antagonistic or promoting ways: to inhibit and eradicate dormant cells, for example during potentially fatal infections (i.e. antagonizing hypometabolic physiology), or to help save lives and salvage organ function in situations requiring critical care (i.e. inducing hypometabolic physiology). The following section will start out describing applied hypometabolism through a brief digression into the world of dormant microbial parasites.

2.1. Dormant Parasites

2.2. From quiescent embryos to resilient neonates

2.3. Anoxic carp and turtles

2.4. Mammals under-ground and in hibernation

2.5. Organ protection and metabolic depression by hydrogen sulfide and other gases

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2.1. Dormant Parasites

The ability of microorganisms to persist in metabolically inactive states enables survival during conditions nonpermissive for growth, and may contribute to microbial diversity by facilitating taxonomic richness in harsh environments (Dworkin and Shah, 2010). Unfavorable conditions known to induce metabolic depression in bacterial cells include, but are not limited to, nutrient or oxygen deprivation, high and low extremes of temperature or solute concentrations, desiccation, heavy metals and UV light (Gilbert et al., 1990, Li et al., 2014). First noticed with environmental isolates of *Vibrio cholerae* several decades ago (Xu et al., 1982, Roszak and Colwell, 1987) and *Vibrio vulnificus* (Oliver et al., 1995), this stress-response transition results in a declining culturability of bacterial cells instead of cell lethality. Such 'viable but non-culturable' (VBNC) cells fail to grow (i.e. number of colony forming units below detection limit) on routine agar but are nonetheless alive, as reflected by intact cell membranes, undamaged chromosomal or episomal DNA, active transcription and maintenance of a stress-specific coat of cell surface markers (Li et al., 2014). On the other hand, this kind of cell persistence across microbial taxa is accompanied by a strong depression of overall metabolism and emergence of quiescence, as earlier exemplified, for instance, by the significant aquaculture pathogen *Pasteurella piscicida* (Magariños et al., 1994). While other bacterial pathogens, i.e. *Bacillus anthracis* and *Clostridium difficile*, are notorious for their ability in producing extremely resistant endospores (Stephenson and Lewis, 2005, McKenney et al., 2013), VBNC states also occur in non-sporulating taxa. Similar to spores, the hypometabolism in VBNC-cells can persist over a remarkable period of time. For example, Mycobacteria, including with the tubercle bacillus (*M. tuberculosis)* the most significant bacterial killer on Earth (i.e. in 2013: ~1.5 million TB-related deaths worldwide; http://www.cdc.gov/tb/statistics/), can enter into a low replicative VBNC state that is estimated to aid decade-long survival as latent infection in ~2 billion people, or ⅓ of humanity (Keep et al., 2006, Barry et al., 2009, Oliver, 2010, Boon and Dick, 2012).

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Beyond the tubercle bacillus, an ever-increasing list of bacterial pathogens (i.e. currently up to 85 species) is able to enter quiescent VBNC states. The majority of these species (67 of 85) are known to cause infections in human hosts (e.g. *EHEC serotype of Escherichia coli, Vibrio cholerae, Listeria monocytogenesis, Salmonella enterica, Helicobacter pylori etc.*) (reviewed: (Oliver, 2010, Li et al., 2014) and references therein). The ability of bacterial cells in persisting as hypometabolic state (e.g. (Guppy and Withers, 1999)) has been appreciated as an essential characteristic for microbial pathogens to actually cause disease. This is because VBNC cells generally exhibit a far greater resistance to antibiotic treatment while retaining virulence; i.e. are able to initiate infection upon being resuscitated to their active state (Oliver, 2010, Li et al., 2014, Ayrapetyan et al., 2015). Moreover, VBNC or persistent states of, for example, the notorious animal and human pathogens among the genus *Chlamydia*, are actually inducible by antimicrobials such as gamma-interferon and β-lactam-based penicillins (*Clamydiae* infection reviews: (Hogan et al., 2004, Wyrick, 2010, Schoborg, 2011, Borel et al., 2014)). In some cases, the resulting metabolic slow-down can correlate with the development of multidrug resistance (Ramamurthy et al., 2014, Postnikova et al., 2015). Regarding other cases, benzylpenicillin, piperacillin, and gentamicin are known to act as potent inhibitors of peptidoglycan or protein synthesis in VBNC cells of *Enterococcus faecalis* and can impede the cells resuscitation. Similarly, biofilms of *Staphylococcus aureus* can cause recurrent infection once cells have entered the VBNC state in the presence of vancomycin or the combination of quinupristin and dalfopristin (Ramamurthy et al., 2014). Thus, many bacteria adopt a VBNC state as a metabolically depressed survival strategy in response to ambient or chemical challenges. Future studies need to focus on the emerging link between VBNC and antibiotic resistance and on the production and validation of anti-dormancy drugs to effectively target hypometabolic states of pathogenic species.

Among obligate intracellular protozoan parasites with reiterated dormancy during the life cycle of the organism, we encounter with the Apicomplexans *Toxoplasma gondii* and *Plasmodium sp.* the infectious agents of Toxoplasmosis and Malaria, respectively. These parasitic diseases, in their acute and chronic expression, comprise some of the most common human infections. According to the latest

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WHO estimates, every year malaria causes 200-300 million infections and over 0.4 million deaths, thus producing a situation in which half of the world's population is at risk for acquiring this disease (i) http://www.who.int/mediacentre/factsheets/fs094/en/; ii) http://malaria.jhsph.edu/about_malaria/). *T. gondii*, on the other hand, can infect essentially any warm-blooded vertebrate and is found, in its latent form, in nearly one third of humans. Arguably, this makes it the world's most successful zoonotic parasite (Jones et al., 2001, Reid et al., 2012). As a hallmark of *T. gondii* infection, a fraction of the multiplying tachyzoite-called stage can convert in response to various noxious stimuli into the dormant stage of bradyzoites (Zhang et al., 2013a). Bradyzoites, in turn, can form tissue cysts, predominantly in the brain, heart, and skeletal muscles. Analogously to VBNC bacteria, *Toxoplasma* tissue cysts remain largely quiescent for many years and even throughout the entire life of the host. However, in immune-compromised patients, i.e. those with AIDS or chemotherapeutically-treated subjects with neoplastic diseases and organ transplants, bradyzoit cysts can reactivate and may trigger a life-threatening toxoplasmic encephalitis (Suzuki et al., 2010, Flegr, 2013, Zhang et al., 2013a). Learning on how to overcome microbial dormancy may thus widely assist future antibiotic paradigms.

2.2. From quiescent embryos to resilient neonates

Pre-adult developmental stages are known to exhibit an increased resilience to O_2 deprivation relative to fully developed adult individuals of the same species. Such hardiness of immature life forms, covered in this section, ranges from a completely paused development, once early invertebrate and vertebrate embryos face sharp and acute falls in pO_2, to reduced oxygen uptake rates and relinquished heat production by newborn mammalian pups as preemptive hypoxia-coping strategy. Thus, immature stages reflect a remarkable plasticity of stress responses that are typically not seen in adults.

Nematodes lack both specialized respiratory systems and complex circulatory organs, and, for that reason, rely on diffusion to supply their tissues with oxygen (Atkinson, 1980, Paget et al., 1987). Nonetheless, wild-type (N2 strain) *C. elegans* are known to oxyregulate at near-normal metabolic rates down to a P_C of ~3.6kPa (~3.65% O_2). Anoxia, in contrast, readily immobilizes the worms and

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diminishes their CO_2 output to 5% of normoxic controls (Van Voorhies and Ward, 2000). *C. elegans* is able to withstand 24 h of anoxia with negligible mortality across all stages of development (Van Voorhies and Ward, 2000). The morphologically distinct and essentially "ageless" dauer diapause of *C. elegans*, however, is particularly well-endowed of withstanding even days of anoxia (Anderson, 1978, Padilla et al., 2002), as well as starvation, desiccation, heat and oxidative stresses (Fielenbach and Antebi, 2008, Sommer and Ogawa, 2011). Genetic dissection of the dauer has revealed TGF-β and insulin-IGF1 pathways, and their convergence on steroid hormone receptor-driven transcription, as key in governing entry and maintenance of this hypometabolic stage (reviewed: (Fielenbach and Antebi, 2008, Lant and Storey, 2010, Sommer and Ogawa, 2011)). In contrast, *hif-1* mutant nematodes show completely unaffected anoxia-arrested development, and are able to persist in this dormancy for days (Padilla et al., 2002, Miller and Roth, 2009). HIF-signaling, therefore, while strictly required to ensure hypoxic survival, becomes dispensable for the suspended animation in oxygen-free milieus.

Among vertebrates, a similar resilience towards discontinued oxygen supply is seen in cleavage-segmentation stage embryos of the zebrafish *Danio rerio* (Padilla and Roth, 2001). Remarkably, nematode and zebrafish embryos both enter, when challenged by anoxic surroundings, a recoverable state of dormancy dubbed "suspended animation", where all microscopically observable movement ceases, including cell division, developmental progression, and motility (Padilla and Roth, 2001, Padilla et al., 2002). Nematodes in developmental suspension are nonfeeding and discontinue egg laying (Padilla and Ladage, 2012). Zebrafish embryos, when dormant, also arrest their heartbeat during hour-long exposure to anoxia (Padilla and Roth, 2001). This physiological standstill resembles embryos of the annual killifish *Austrofundulus limnaeus* whose development has been halted by endogenous, rather than exogenous, cues at diapause II, following somitogenesis but prior to major organogenesis phases. Remarkably, diapause II killifish embryos subjected to an oxygen-free medium display a lethal time to 50% mortality (LT_{50}) of ~65 days at 25°C – rendering them by far the most anoxia tolerant pre-adult vertebrates known (Podrabsky et al., 2007, Podrabsky and Culpepper, 2012).

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Although complete quiescence and suspended animation have yet to be reported for the ontogeny of higher vertebrates, the fetus and newborns of mammals nonetheless exhibit a number of reactions that bear striking similarity to adaptations seen in hypoxia-tolerant animals. Understanding potential and limitations of this neonatal tolerance to hypoxia is key for advancing current approaches towards placental pathologies, including severe forms of early-onset preeclampsia and intrauterine growth restriction (IUGR), which both result from sustained gestational hypoxia (Burton, 2009, Hutter et al., 2010, Maltepe and Fisher, 2015). The birth weight of human infants falls as a function of the altitude where gestation occurred (Monge and Leon-Velarde, 1991), while stunted growth is common in children with cyanotic heart diseases (Mortola et al., 2000, Mortola, 2003). For a better comprehension of such pathophysiological extremes, let us first recapitulate the challenging O_2 milieu pertinent even for a normal human pregnancy (reviewed: (Burton, 2009, Hutter et al., 2010, Maltepe and Fisher, 2015)).

The oxygen level in the uterus at the time of implantation is low in many species: rabbit 24 mmHg, rhesus monkey 11–14 mmHg and human 15-19 mmHg (Fischer and Bavister, 1993). As the maternal circulation to the placenta is not fully established until the end of the first trimester (= gestational weeks 9-13), early human embryonic development (i.e. organogenesis) proceeds under a physiologically low oxygen environment within a feto-placental unit nourished by histotrophic secretions from endometrial glands (Burton, 2009). Such a hypoxic milieu is essential for normal embryonic (e.g. active angiogenesis) and placental development (e.g. cytotrophoblast expansion), and premature onset of blood flow can contribute to pregnancy failure (Hustin et al., 1990, Jauniaux et al., 2003). Once maternal circulation to the placenta becomes fully functional, the human fetus is faced with a major oxidative challenge as the oxygen tension within the intervillous space rises from ~18 mmHg (≈ 2.5% O_2) at 8 weeks to ~60 mmHg (8.5%) at 12 weeks (Jauniaux et al., 2000, Jauniaux et al., 2003). Through increasing feto-placental O_2 consumption, the mean pO_2 eventually drops again from ~60 to ~40 mmHg at term (Soothill et al., 1986). Thus, embryonic and early fetal development of mammals, including humans, commences at an initial pO_2 range of ~20-40mmHg that corresponds to 6000-8000m altitude (referred to as “Everest in utero”; (Barcroft, 1946)). Evidently, the adaptation

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of the fetus to weeks-long hypoxia and quickly rising oxygenation is the basis of a successful pregnancy.

Given this unstable milieu, it comes as small surprise to see hypoxia defenses in operation during neo- and peri-natal mammalian development, including human infancy, that are no longer recruited in adulthood (Mortola, 2004). In conditions of hypoxia, the pO_2-proportional decrease in oxygen consumption rates (= $\dot{V}O_2$) for mammals is particularly apparent in the neonatal period, as seen, for example, in newborn kittens and puppies (Pedraz and Mortola, 1991, Gautier, 1998, Rohlicek et al., 1998), or for early stage chicken embryos (Mortola et al., 2012). Several pieces of evidence, including the lack of an oxygen debt in hypoxic avian embryos or mammalian neonates (Hill, 1959, Sidi et al., 1983, Fahey and Lister, 1989, Frappell et al., 1991), suggest this to be a controlled oxy-conforming mode of O_2 uptake, rather than simply reflecting the passive response to a decreasing O_2 availability (Mortola, 2001). Thus, it is generally agreed that entry into oxy-conforming respiration mirrors a true hypometabolic state that mammalian neonates and human infants actively acquire in response to O_2 scarcity (Singer, 1999, Mortola et al., 2012). Since hypoxic hypometabolism in newborns commonly occurs even at thermoneutrality (Mortola, 2004), hypothermia cannot be the cause, but must be a consequence, of metabolic suppression (see below). Moreover, as heat is produced by an active, oxygen-requiring electron transfer chain in mitochondria, the inhibition of heat production in hypoxic newborns is thought to occur in order to prevent futile and potentially life-endangering increases in energy expenditure (Hill, 1959, Eales and Small, 1985, Mortola, 2004, Beaudry and McClelland, 2010). The body-heat of newborns largely, or solely, arises via non-shivering thermogenesis (NST) by brown adipose tissue (BAT). Additional defenses in perinatal mammals towards acute or chronic hypoxia comprise bradycardia, shunting of blood flow from peripheral to central organs, diminished cerebral vulnerability, and inhibited tissue growth plus differentiation (reviewed: (Singer, 1999, Mortola, 2004)). Utilization of such defenses enable neonatal rats to survive up to 50 min in pure nitrogen, while adult individuals succumb after less than 5 min (Singer, 1999).

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As with most seasonal hibernators (section 2.4), hypothermia adds a protective layer, but is not a causative prerequisite for the hypoxia hypometabolism in newborn mammals (e.g. (Pedraz and Mortola, 1991)). In fact, a reduction in ambient temperature has been noted for more than a century to be one of the most effective ways of prolonging the survival time of asphyxiated mammalian neonates (Bert, 1870, Fazekas et al., 1941, Britton and Kline, 1945). As hypoxia itself acts to suppress NST, the perinatal change in thermal environment leads to a lowered body temperature, which favors hypoxic survival by reducing, instead of increasing, the metabolic rate. In translation of these findings, deliberate cooling for brief periods has, despite its varying outcome among institutions, contributed to the progress in infant heart surgery in some studies (Drury et al., 2013, Haydin et al., 2013). Moreover, three randomized controlled trials support the concept of total body cooling or selective head cooling to reduce cardiac dysfunction and brain injury in term infants with moderate to severe hypoxic-ischaemic encephalopathy (HIE) after perinatal asphyxia (Vannucci and Perlman, 1997, Jacobs et al., 2007, Liu et al., 2013). To date, many countries and hospitals have implemented hypothermia as standard care for term infants with HIE (Thoresen, 2011). Since xenon inhalation, when added to cooling, was able to double neuroprotection in both small (Hobbs et al., 2008) and large (Chakkarapani et al., 2010) newborn brain injury models, studies on human infants with post-asphyxia neonatal encephalopathy currently test the feasibility of this combinatorial protocol for an improved efficacy (Dingley et al., 2014). Of note in this context, FDG-PET scans of healthy human volunteers have linked general xenon anesthesia to a marked and global depression of cerebral glucose metabolism (Rex et al., 2006), suggesting the primary anesthetic and neuroprotective effect of xenon to not be transduced via its alleged antagonism of the glutaminergic N-methyl-D-aspartate receptors (NMDAR), whose overactivation in ischaemia-reperfusion (I-R) injury (e.g. HIE) results in massive cell death of neurons. Rather, and unlike canonical NMDAR antagonistic anesthetics (ketamine, N_2O), xenon´s neuroprotection seemingly involves a tuning down of neuronal activity via a diminished glucose turnover (Rex et al., 2006, Rex et al., 2008, Jordan and Wright, 2010).

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2.3. Anoxic cyprinids and turtles

The ability of certain *adult* fish, amphibians, and reptiles to survive extremes of oxygen availability (reviewed: (Galli and Richards, 2014)) derives from a core triad of adaptations: profound metabolic suppression, tolerance of increased levels of metabolic by-products (e.g. protons), and capacity for avoiding or repairing free-radical injury during reoxygenation (reviewed: (Bickler and Buck, 2007, Galli and Richards, 2014)). For long-term anoxic survival, enhanced storage of glycogen in critical tissues provides added benefit. Yet, highly anoxia-tolerant painted turtles and crucian carp meet the challenge of profound oxygen fluctuations, again as adult specimen, in fundamentally different ways. Turtles undergo near-suspended animation, with profound peripheral vasoconstriction, blunted autonomic control and 80% decreases in heart rate and cardiac output (e.g. (Nilsson and Lutz, 2004) and references therein). In sharp contrast, the carp remains an active swimmer and responsive in the absence of oxygen. Functionality of ventilation and circulatory systems are maintained at normal levels during anoxic exposure. Despite such activity, the crucian carp (*Carassius carassius*) can endure months in oxygen-free waters if temperatures are low (Nilsson and Renshaw, 2004). Its cousin, the common goldfish (*Carassius auratus*) exhibits half-lethal times of 45h under anoxia at 5°C and 22h at 20°C. Compared to turtles, both species of *Carassius* rely on a moderately reduced metabolism (*C. auratus*: anoxic heat production ≈ ⅓ of normoxic level; (Nilsson and Lutz, 2004)), and avoidance of lactic acidosis by converting lactate to CO_2 and ethanol that can be excreted via the gills. Both species of *Carassius* also produce haemoglobin with an extreme, myoglobin-like affinity for oxygen (e.g. p50 of 0.35 kPa = 2.6 mmHg), allowing these fishes to maintain their oxy-regulated rate of O_2 uptake down to water oxygen levels of 5-10% of air saturation (Sollid et al., 2003). Furthermore, cold acclimation (8°C) of crucian carp augmented HIF-1α protein levels in the liver, heart and gills, and the factors DNA binding activity in the heart and gills of both normoxic and transiently hypoxic (6h; $[O_2]$ = 0.7 mg L^{-1} ≈ 6-8% air saturation) fish (Rissanen et al., 2006). Another remarkable adaptation of the crucian carp, and unique among vertebrates, involves the marked enlargement of the respiratory surface area of its gills during hypoxic challenges (Sollid et al., 2003). Beyond the *Carassius* species, the Californian blind goby (*Typhlogobius californiensis*) manages to

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withstand 80h of anoxia even at 15°C (Nilsson and Lutz, 2004), while the epaulette shark (*Hemiscyllium ocellatum*), a benthic reef elasmobranch of tropical waters, enters metabolic depression to withstand O_2 levels of 0.34 mg O_2 L^{-1} (5% of normal levels) for 120 min at 25°C (Mulvey and Renshaw, 2009). According to these examples, anoxia tolerance in adult fish can emerge without being associated with the cold tolerance necessary for overwintering in water temperatures close to 0°C (Nilsson and Renshaw, 2004).

Certain northern latitude freshwater turtles (e.g. painted turtles, red-eared sliders, snapping turtles) manage to repeatedly survive up to 4–5 months without O_2 and food intake during their breath-holding mode of hibernation in ice-locked lakes (reviewed: (Jackson and Ultsch, 2010, Krivoruchko and Storey, 2015)). This survival is even more remarkable if one considers that the metabolic rate of the turtle's brain, corrected for temperature, is similar to that of mammals (Lutz et al., 2003). The key adaptation of particularly anoxia-tolerant species of the genus *Chrysemys* (e.g. *C. picta bellii*) and *Trachemys* (*T. scripta elegans*) focuses on the remarkable metabolic depression by as much as 95% or even 99% when submerged in anoxic water, relative to air-breathing control animals kept at 20°C (Herbert and Jackson, 1985). Due to this magnitude of depression, which is extreme for any adult vertebrate, anoxic turtles actually reveal a decreased activity of key glycolytic enzymes and a progressively slowed flux through the glycolytic pathway with lengthening exposure to anoxia (Kelly and Storey, 1988, Storey, 1996, Krivoruchko and Storey, 2015). In anoxic or reoxygenating *Trachemys* turtles these hypometabolic cellular adaptations can be supplemented via a greatly stimulated nitrite-to-nitric oxide ($NO^{\bullet}$) conversion that is mediated by the nitrite reductase activity of the species` deoxygenated haemoglobin (Jacobsen et al., 2012, Fago and Jensen, 2015).

The coordinated reduction in both ATP production and ATP utilization is instrumental in cold anoxic turtles for achieving a new energetic steady state. On the utilization side, a drastically reduced protein synthesis (Land et al., 1993) and breakdown rate (Land and Hochachka, 1994) along with lessened trans-membrane ion pumping secondary to reduced ion channel leak pathways (Buck and Hochachka, 1993, Hochachka et al., 1996) contribute to slowing the rate of ATP hydrolysis (Bickler and Buck, 2007). The fact that *Chrysemys* and *Trachemys* turtles are true champions in re-balancing energy

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supply with demand is reflected by their ATP levels being maintained, or even increased, after 5h of anoxia at 18°C in different tissues (for *T. scripta*: (Kelly and Storey, 1988)). Yet, another key element underlying this stability of ATP concentrations during O_2 deprivation derives from the fact that the activity of F_1F_O-ATPsynthase (OXPHOS Complex V) in permeabilised cardiac fibers and isolated mitochondria of *T. scripta* was profoundly down-regulated, but not reversed into ATP hydrolysis, in response to chronic submergence in O_2-free waters (Galli et al., 2013, Galli and Richards, 2014). We also noted for hypoxic fly S2 cells a reduced abundance of the ATPsynγ steady state mRNA (Fig. 3). Whether this reflects, also for *Drosophila* cells, a protective inactivation of F_1F_O-ATPsynthase to prevent pump reversal remains to be seen. In any event, O_2-limited mitochondria of mammals adapt differently as they seek to keep reversal of proton pumping by Complex V at bay through interaction with inhibitory factor 1 (Campanella et al., 2009, Solaini et al., 2010). Yet, when overpowered by stress, the mammalian organelles still run the risk of transforming the dominant producer of ATP (i.e. ATPsynthase) into a consuming machinery (i.e. ATPase), only to precipitate ATP depletion and cell death (Galli et al., 2013).

2.4. Mammals under-ground and in hibernation

Fossorial animals experience chronic challenges by being permanently subjected to O_2 levels as low as 6–14% and CO_2 levels as high as 6–10% (Arieli, 1979, van Aardt et al., 2007). African naked mole-rats (*Heterocephalus glaber*) spend their entire life in subterranean burrows with similar hypoxic and hypercapnic milieus. These rodents are most unusual since they are the only mammal that secondarily turned thermoconformer (i.e., animals adopt ambient temperature (*T*a) as own body temperature (*T*b)) thus avoiding the need for internal thermoregulation (Buffenstein and Yahav, 1991). They are also the longest-lived rodent species, with recorded lifespans exceeding 30 years, negligible signs of senescence and no reported development of any spontaneous neoplasms (Buffenstein, 2008). They live in their burrows in an eusocial community similar to ants and bees (Jarvis, 1981). In the lab, naked mole rats (NMRs) are known to survive 3% O_2 (Nathaniel et al.,

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2009). Consistent with living in a chronically oxygen-poor environment with an abandoned production of endogenous heat, the basal metabolic rate of normoxic NMRs is ~6fold lower (i.e. 0.27 ml O_2/g/h; (Buffenstein and Yahav, 1991, Nathaniel et al., 2013)) compared to a mouse (i.e. 1.65 ml O_2/g/h; (Schmidt-Nielsen 2002)). This low basal metabolic rate of NMR is marginally depressed further during experimental hypoxia (3% O_2), while *T*b in normoxic and hypoxic animals was maintained at ~28°C (Nathaniel et al., 2013).

Not surprisingly, the stress resilience of the NMR extends to its neurons (reviewed: (Nathaniel et al., 2013, Larson et al., 2014)). In agreement with attenuated excitotoxic signaling in anoxic turtle brains, calcium accumulation during hypoxia was similarly dampened in brain slices from NMRs compared with age-matched mice (Peterson et al., 2012). Interestingly, postnatal (day 6) mice or NMRs exhibited significantly less calcium influx into hypoxic hippocampal neurons relative to adult individuals of that species (see also section 2.2), suggesting the brains of aging specimens to progressively wane in their ability to cope with stress and capacity to recuperate (Larson et al., 2014). It is currently discussed whether NMR´s robust hypoxia tolerance is actually based on traits acquired through the species´ retention of juvenile characteristics into adulthood (neoteny hypothesis; (Nathaniel et al., 2013, Larson et al., 2014)).

In coping with seasonal food scarcity, different mammalian and bird species hibernate during cold northern winters or tropical drought periods in a similar nonfeeding state (reviewed: (Ruf and Geiser, 2015)). These include several dwarf lemur taxa as exclusive primate representatives (e.g. (Dausmann et al., 2004)), and examples in six avian families (e.g. common poorwill (*Phalaenoptilus nuttallii*); (Woods and Brigham, 2004)). Although hibernating mammals mirror strategies seen in apnea diving species, hibernators can employ a far deeper hypometabolism than any other endotherm. Transient (hour-long) forms of reduced energy expenditure along with phases of substantial peripheral cooling in winter nights or concurrently with frigid-warming temperature oscillations at subtropical latitudes ("daily torpor"; reviewed: Ruf and Geiser, 2015) have been reported for a) northern ungulates (e.g. red deer, moose, wapiti; Arnold et al., 2004), b) subtropical bats (Dietz and Kalko, 2006, Geiser et al., 2011) and c) roosting hummingbirds of higher altitudes (Calder, 1994). However, periods of energy-

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saving inactivity and cooling in mammals are typically measured in days, weeks or months, not hours. For such seasonal hibernators (e.g. rodents) common adaptations during onset and maintenance of torpor normally include i) profound overall depression of metabolism with the minimum rate corresponding to 5–30% of euthermic basal metabolism; ii) reduced oxygen consumption (down to 2-3% of euthermic uptake) with intermittent ventilation and a heart rate as low as 3-10 beats/min (reference: 200-300 beats/min in awake animals); iii) temporary heterothermy as a consequence of hypometabolism, with *T*b decreasing enough to approach ambient values; iv) slow-down of cerebral blood flow, v) declining numbers of circulating lymphocytes and neutrophils, partly mediated by falling plasma levels of sphingosine-1-phosphate in response to low *T*b, and v) physical immobility (Heldmaier et al., 2004, Andrews, 2007, Bouma et al., 2011, Dave et al., 2012, Bouma et al., 2013, Ruf and Geiser, 2015). In the hibernating arctic ground squirrel (AGS), *Urocitellus parryii*, torpor metabolism may even drop to 1-2% of the resting rate in awake animals (Larson et al., 2014). During the hypometabolic-hypothermic state of deep torpor, transcription, translation and protein turnover are largely suspended (van Breukelen and Martin, 2001, van Breukelen and Martin, 2002, Storey and Storey, 2004), as is cell division in proliferative intestinal epithelia (Carey et al., 2003). To fulfill the remaining energy requirements, hibernators normally switch to lipids as major oxidative fuel and cease the flow of glycolytic intermediates into the TCA cycle by inducing pyruvate dehydrogenase kinase 4 (PDK4), for the effective inactivation of the pyruvate-to-acetyl CoA conversion by pyruvate dehydrogenase (Carey et al., 2003, Andrews, 2007, Staples and Brown, 2008). As oxidation of fatty acid substrates consumes large amounts of oxygen, respiratory quotients (i.e. RQ = moles $CO_{2\ eliminated}$ / moles $O_{2\ consumed}$) of near 0.7 are typical for torpid stages (Carey et al., 2003), thus illustrating the strict requirement to sustain residual torpor metabolism by aerobic means. Nonetheless, ground squirrels and brown bats markedly up-regulate HIF-1α protein levels in skeletal muscle upon entering torpor (*T*b: 5-8°C), relative to euthermic controls (*T*b: 34-37°C) (Maistrovski et al., 2012). Signals for prompting this enrichment of HIF-1α protein in aerobic tissues of torpid hibernators might involve cold acclimation as seen in tissues of the crucian carp (section 2.3; Rissanen et al., 2006) and/or chemical cues of unknown nature. Moreover, solid evidence today also supports the accumulation and

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activation of HIF-1α by reactive nitrogen and/or oxygen species (RNS, ROS), cytokines, and growth factors in completely oxygenated mammalian cell types (reviewed: (Brüne and Zhou, 2003)).

When common dormice (*Glis glis*) enter hibernation, the rapid depression of their metabolic rate clearly precedes the development of hypothermia (Heldmaier et al., 2004, Staples and Brown, 2008). In parallel with the declining metabolic rate, cerebral blood flow (CBF) can drop as much as 10-fold (Frerichs et al., 1994). Mammals that hibernate show unprecedented capacities to tolerate cerebral ischaemia (Dave et al., 2012). While a 10% residual CBF would very quickly and irrevocably lead to death or disability in humans and most other endotherms, it has virtually no effect on neuronal viability and function in brains of hibernators (Dave et al., 2012). This cerebral resilience is all the more remarkable since the vast majority of small mammalian hibernators (i.e. dormice, ground squirrels, marmots, bats etc.), when cooled to a Tb <30°C, interrupt prolonged bouts of torpor by brief, albeit regular periods of euthermy (i.e. interbout arousals, with Tb returning to 35–37°C; (Geiser and Ruf, 1995, Dausmann et al., 2004)). Hibernating bears, in contrast, moderately suppress their metabolism during dormancy yet do not display repeat arousals. Instead their Tb shows multiday 30°C-36°C oscillations (Heldmaier, 2011, Tøien et al., 2011). Although speculative at this point, the extent of the metabolic rate depression seems to associate with the need for repeat arousals.

Transitions in and out of torpor are even more challenging for the brain than the extreme metabolic suppression, steady state hypoperfusion and cold body temperatures of torpor *per se*. Yet, interruptions of deep torpor states by spikes of arousal trigger no evidence of cerebral I-R injury in small rodent hibernators (Frerichs et al., 1994, Ma et al., 2005), but some restorative processes instead (von der Ohe et al., 2006, Weltzin et al., 2006). Moreover, animals such as AGS, when emerging from torpor, are known to encounter a significant fall of paO_2 (i.e hypoxaemia) during these spikes of euthermy (Ma et al., 2005). The 10–100-fold increase in metabolic rate during arousals is initially caused by massive sympathetic activation of NST based on abundant thoracic and interscapular stores of BAT. Subsequent muscular shivering may raise Tb to euthermic levels within hours (Lyman et al., 1982, Heldmaier et al., 2004). Additional hibernation-based neuroprotective principles, include the delayed ischaemic depolarization of cells, restricted glutamate-induced calcium influx, attenuated

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excitotoxicity plus cell death upon oxygen-glucose deprivation (OGD)-triggered glutamate release and fewer functional NMDA receptors (i.e. "channel arrest") in the plasma membrane of AGS neurons (Larson et al., 2014).

As the mammalian specialists highlighted here are able to suppress systemic and brain metabolic activities by actively reducing oxygen consumption and re-balancing energy supply with demand, research on these species might prove very valuable for the ultimate goal of suppressing metabolic demand in human subjects undergoing stroke and/or cardiac arrest. Of particular interest in this regard could be the exploration of "pharmacological means to induce metabolic suppression in non-hypoxia tolerating species" (Nathaniel et al., 2013) (see section 2.5). More advanced to date, however, is the clinical application of therapeutic hypothermia as neuro- and cardio-protective measure against I-R-related brain and heart injuries (Yenari et al., 2008, Dave et al., 2012). On average, hypothermia reduces brain oxygen consumption by ~5% for every degree Celcius body cooling within the range of 22-37°C (Erecinska et al., 2003).

Known for its survival-aiding effects in hypoxic newborns (section 2.2) and mammalian hibernators (above), mild hypothermia (i.e. deliberate cooling of core *T*b to 32-35°C) has been applied by many pre-clinical studies to ameliorate I-R-triggered neuronal tissue damage or to harness its benefits during cardiothoracic surgery and organ transplantation (Krieger, 2004, Krieger and Yenari, 2004, Lyden et al., 2006, Aslami and Juffermans, 2010). Yet, translating hypothermia as therapy to save human lives by acutely preserving brain function in victims of circulatory arrest was, perhaps, particularly encouraged by the well-documented, now legendary, accident of Anna Bågenholm in 1999 in Northern Norway (Gilbert et al., 2000). Until that day, no adult patient had ever survived to discharge such an extreme case of deep hypothermia *and* without any long-term neurological deficits. Ms Bågenholm, then 29, suffered a skiing accident that trapped her under a layer of ice in the freezing waters of a fast-flowing gully for 80 min before her, then motionless, body was freed by a rescue team. Under non-stop delivery of cardiopulmonary resuscitation (CPR) and oxygen ventilation the patient was flown to Tromsø University Hospital. Upon arrival almost 3 hours after her accident the patient appeared clinically dead, as she showed no signs of spontaneous respiration or circulation (i.e.

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cardiac arrest for >2 hours), her dilated pupils were unresponsive to light and her electrocardiography produced isoelectric signals. Nonetheless, full cardiopulmonary bypass (CBP) blood flow was established 30 min later, at which point her rectal temperature had fallen to a world-record low of 13.7°C. Upon the gradual rewarming of the CBP flow a sinus rhythm was seen shortly thereafter, followed by a return to rectally measured 36°C. Ms. Bågenholm eventually made a full recovery with back-to-normal mental functions. Similar success stories have been reported from other near drowning incidents of children and adults in icy sea waters (e.g. (Romlin et al., 2015, Claret et al., 2013, Husby et al., 1990)). The life-saving benefit, besides continued reanimation, in these cases is seen in the fact that the circulatory arrest occurred subsequent to the cooling of the victim`s body. Under this directive (i.e. first cooling-then ceasing (of circulation)), a torpid state can well translate from small mammalian hibernators to adult human beings and exhibit a profound protection of the subject`s nervous system.

Landmark trials a few years after the Bågenholm case report showed indeed a beneficial effect of deliberate head and torso cooling on survivors with persistent coma after resuscitation from out-of-hospital cardiac arrest. After the return of spontaneous circulation *en route* to hospital, randomly assigned treatment with vigorous hypothermia (e.g. core *T*b reduced to 33°C in 2 hours and maintained for 12 hours) resulted in significantly better survival and neurological outcome in these critical care patients, compared to the normothermia control cohort (Bernard et al., 2002, Hypothermia after Cardiac Arrest Study, 2002, Arrich et al., 2009). Mild hypothermia (core *T*b 32-33°C) has also been found to reduce ischaemic brain edema in the setting of massive ischaemic strokes (Schwab et al., 1998, Schwab et al., 2001) and to benefit thrombolytic therapies (Naritomi et al., 1996, Shimizu et al., 1997, Krieger et al., 2001) as well as those delivered in cases with acute lung or kidney disease and gut ischaemia (Aslami and Juffermans, 2010). Clinical trials of therapeutic hypothermia in neonates with hypoxic encephalopathy also suggest a benefit in this patient population (section 2.2). On the other hand, serious complications of therapeutic hypothermia can include thrombocytopenia, bradycardia, hypotension, pneumonia, coagulopathy, and cardiac arrest (Yenari et al., 2008, Yenari and Hemmen, 2010). Further, hypothermia may not always be applicable, for

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example with stroke patients that are awake and do not tolerate cooling (Yenari et al., 2008, Yenari and Hemmen, 2010). The rebound effect of increased intracranial pressure during rewarming is another concern, especially as the mechanisms that facilitate the rewarming process are not fully understood (Schwab et al., 2001). Combining hypothermia with other approaches might address some of the challenges.

2.5. Organ protection and metabolic depression by hydrogen sulfide and other gases

Highlighting some key findings on the commonalities and molecular interplays of hydrogen sulfide with earlier discovered mammalian gasotransmitters will launch this chapter. The primary focus of it lies on reviewing the role of this small and malodorous molecule in triggering a metabolic depression and preserving organ function across different models of acute and/or severe traumatic tissue damage.

A regulated induction of a hypometabolic state, including a hibernation-like depression of metabolism by hypothermia, has been hypothesized to have great medical benefit for a number of acute life-threatening hazards including I-R injury, pyrexia, haemorrhage, septic shock or to exert organ protection during surgery or transplantation procedures (Drew et al., 2001, Carey et al., 2003). Thanks to a pioneering study by Abe and Kimura in 1996, which discovered that hydrogen sulfide (H_2S) facilitated the induction of hippocampal long-term potentiation by enhancing the activity of NMDA receptors, H_2S has emerged as 3rd endogenous gasotransmitter of mammalian cardiovascular and nervous systems ever since (Abe and Kimura, 1996). In addition to carbon monoxide (CO) and the nitric oxide free radical ($NO^{\bullet}$), H_2S potently modulates blood flow along with mitochondrial activity and energy turnover (Szabo, 2007, Wagner et al., 2009, Szabo et al., 2014, Stein et al., 2015). The ability of $NO^{\bullet}$ and CO in triggering states of suspended animation in *Drosophila* (Teodoro and O'Farrell, 2003) and *C. elegans* (Nystul and Roth, 2004)), respectively, was recognized in 2003 and 2004. Soon thereafter, H_2S was found to efficiently induce hypometabolism in mammals (below).

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All three gaseous messengers exhibit vasodilating, pro-angiogenic and cytoprotective properties that, in most heart disease models, are further backed by anti-inflammatory plus anti-apoptotic activities (reviewed: (Whiteman and Winyard, 2011, Polhemus and Lefer, 2014, Kimura, 2014, Shen et al., 2015)). Regarding mitochondria, the "gaseous triumvirate" is known to promote respiration and cellular bioenergetics at low, physiological doses (nM - low μM), while it antagonizes uptake and reduction of oxygen at high, supra-physiological concentrations (high μM – mM). At these high levels, H_2S, its water-soluble sulfide donating derivatives (NaHS, Na_2S), as well as $NO^{\bullet}$ and CO can all reversibly inhibit cytochrome c oxidase (COX) (Szabo, 2007, Wagner et al., 2009, Szabo et al., 2014, Stein et al., 2015), the terminal OXPHOS complex IV in the mitochondrial electron transport chain (Fig. 3), by binding to the enzymes` binuclear haem a_3-Cu_B oxygen reduction site. This inactivation of COX proceeds in a non-competitive fashion (i.e. H_2S; COX binding of O_2 with decreased V_{max}) or, as in the case of ligands exclusively directed at reduced transition metals (Boczkowski et al., 2006), in strict competition with oxygen binding (i.e. CO; apparent K_m for O_2 of COX and respiration are increased) (Cooper and Brown, 2008). Since $NO^{\bullet}$ is able to interact with both ferric (Fe^{3+}) and ferrous (Fe^{2+}) haems, it confers uncompetitive and competitive COX inhibitions, respectively (Cooper and Brown, 2008). Of note, the relative affinity of CO for ferrous haems of haemo- and myoglobin supersedes that for the COX binuclear centre by several orders of magnitude (Motterlini and Otterbein, 2010, Queiroga et al., 2012), while soluble guanylate cyclase (sGC) interacts with physiological levels of $NO^{\bullet}$ with ~50-fold higher sensitivity than cytochrome oxidase (Cooper and Brown, 2008). These primary targets may, thus, limit inhibition of the terminal electron-accepting OXPHOS complex by *endogenously* produced CO or $NO^{\bullet}$ to some extent, perhaps in favor of a H_2S-mediated primary control of respiration under physiological conditions.

Because physiological tissue concentrations of free H_2S (i.e. nM - low μM values; (Kimura, 2015, Beltowski, 2015)) are inversely related to those of O_2 across the physiologically relevant pO_2 range (Olson, 2013), rising concentrations of the endogenous gas molecule may act to modulate COX activity in an O_2-dependent manner. As such, endogenous H_2S, once enriched to supra-physiological levels, might actually confer the slowing of O_2 consumption when it matters most: in hypoxic cells. In

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fact, application of exogenous H_2S and hypoxia have been found to produce essentially identical responses in vessels, nonvascular smooth muscle, chemoreceptors and chromaffin cells from all classes of vertebrates (Olson, 2013). Oddly enough, however, this hypoxia-mimicry by H_2S may or may not extend to the HIF pathway, since accumulation of endogenous sulfides, or parenteral application of sulfides in μM doses, was found to suppress translation, and to enhance degradation, of HIF-1α (Kai et al., 2012, Wu et al., 2012) or HIF-2α (Takano et al., 2014) in certain hypoxic mammalian cell types (reviewed: (Wu et al., 2015)). In contrast, in rat aortic vascular smooth muscle cells H_2S further increased VEGF and HIF-1α expression and HIF-1α DNA-binding activity under hypoxia-mimicking conditions ($CoCl_2$ pre-treatment) (Liu et al., 2010, Yang and Wang, 2015). We are still nowhere near the level of understanding necessary for a robust consensus on the correlation between H_2S and HIF signaling across cell types and species.

The enzymatic production of endogenous H_2S in mammals proceeds primarily by desulfhydration of the amino acid cysteine via the pyridoxal 5′phosphate (vitamin B_6)-dependent enzymes cystathionine γ-lyase (CSE) in the cardiovascular and cystathionine β-synthase (CBS) in the nervous system. Cysteine aminotransferase (CAT) and 3-mercaptopyruvate sulfurtransferase (MST) also produce H_2S under consumption of α-ketoglutarate in hippocampal and cerebellar neurons and astrocytes (Szabo, 2007, Osmond and Kanagy, 2014, Polhemus and Lefer, 2014, Shen et al., 2015). Table 2 summarizes enzymatic syntheses, cardiovascular effects and crosstalk modes of endogenously produced $NO^{\bullet}$, CO and H_2S pathways. Concerning the latter point, feed-forward signaling is known to orchestrate much of the vascular and pulmonary $NO^{\bullet}$-H_2S or CO-H_2S interplay at various levels (Lo Faro et al., 2014). Mice with a global deletion of CSE, and consequently deficient production of vascular H_2S, develop significant hypertension and diminished endothelial vasorelaxation at roughly 8 weeks of age (Wang, 2012). Remarkably, levels of NO metabolites in the blood and heart were also lower in these CSE-knockout animals than in wild-type controls (Polhemus and Lefer, 2014, Kimura, 2015, Shen et al., 2015). To date it is clear that endogenous H_2S effectively increases endothelial nitric oxide synthase (eNOS) activity in arterial or venous endothelial cells either by inducing its phosphorylation on Ser 1177 in an AKT-dependent manner (Predmore et al., 2011, Altaany et al., 2013) or by directly

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inducing Ca^{2+} release from the intracellular storage in the endoplasmic reticulum (Kida et al., 2013) (Table 2). In turn, NO• has been proven to augment H_2S endogenous production by elevating CSE and CBS expression in vascular smooth muscle cells (Zhao et al., 2001, Zhao and Wang, 2002). Although fewer insights are currently available from the CO-H_2S crosstalk (Polhemus and Lefer, 2014), exogenous H_2S application was demonstrated by Zhang et al. to up-regulate the CO system in pulmonary arteries of hypoxic rats (Zhang et al., 2004). In addition, anti-oxidant activities of sulfide donation involving the nuclear translocation of the Nrf2 transcription factor might ultimately yield increased expression of the Nrf2 target haem oxygenase 1 (HO1), and through that, elevated production of CO via the inducible HO1 (Chan et al., 2001, Motohashi and Yamamoto, 2004). Conversely, NO• and CO can stop CBS-mediated H_2S production by binding to the haem moiety of this enzyme and inactivating it (Branco et al., 2014, Kimura, 2015). This negative regulation plays a key role in the hypoxic brain, i.e. when the HO2-mediated production of CO is diminished, which, in turn, de-represses CBS activity in astrocytes surrounding capillaries. CBS-derived H_2S then relaxes the capillaries to recover blood flow and O_2 supply (Branco et al., 2014, Kimura, 2015). Occurrence of endo- or exogenous NO• and CO may, thus, imply CBS-to-CSE-shifted H_2S synthesis.

Prompted by the sulfide-mediated down-regulation of O_2 consumption (Leschelle et al., 2005, Mason et al., 2006, Szabo, 2007, Szabo et al., 2014), the group of M. Roth aimed to assess in their 2005 study whether application of H_2S reduces both metabolic rate and core body temperature, even in non-hibernating species. Despite this lack of hibernating behavior, mice (*Mus musculus*) have long been known to readily undergo daily torpor and reduce their *T*b down to ~20°C, for example in response to fasting (Hudson and Scott, 1979, Schubert et al., 2010). A gradual decline in the body temperature of mice to reach nadir levels over the course of several hours also occurs in response to metabolic inhibitors such as 2-deoxy-d-glucose (Freinkel et al., 1972). However, when adult, awake and normothermic C57BL-6J female mice were placed by Roth and colleagues in an atmosphere containing 80 ppm H_2S, their O_2 consumption and CO_2 production rates fell by ~50-60% in 5 min, and by ~90% in 6h, thus indicating a suspended animation-like state that was fast acting, achievable through inhalation (rather than infusion) and readily reversible upon readmission of air inhalation, and

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that did not produce any aberrant behavior (Blackstone et al., 2005). As in physiological hibernators, the drop in metabolic rate of H_2S-exposed mice was followed by a decline in *T*b to ~2°C above ambient values. Because a linear relationship between *T*a and *T*b was seen over the range of 6°C to 30°C in H_2S-inhaling mice, these data implied H_2S to exert its inhibition of oxidative metabolism in a temperature-independent fashion (Blackstone et al., 2005). Similar to naked mole rats with their fossorial life style (section 2.4), H_2S-breathing mice were simply no longer capable of generating enough internal heat to maintain endothermy. Yet, while being hypothermic, H_2S inhalation has been evidenced to maintain mitochondrial integrity and a higher yield of cellular respiration, and, that way, to enhance the protective effect of body cooling (Baumgart et al., 2010). Along similar lines, Na_2S can preserve mitochondrial O_2 consumption and integrity after myocardial ischaemia (Elrod et al., 2007).

Two years after their initial report, the Roth group documented H_2S pretreatment to be sufficient in protecting lab mice, kept at a 23°C room temperature, from lethal hypoxia. Adult C57BL-6J mice cannot survive in 5% O_2 for more than 15 min (above and (Blackstone and Roth, 2007). However, when mice were first triggered to enter a hibernation-like state by a 20 min inhalation of 150 ppm H_2S and then exposed to low oxygen, they all survived 1h at 5% O_2, and some even up to 6.5h with no apparent harmful effects. Moreover, exposing mice to 20 min H_2S pretreatment followed by 1h preconditioning at 5% O_2, resulted in survival for several hours at oxygen tensions as low as 3% (Blackstone and Roth, 2007). By withstanding otherwise completely lethal degrees of O_2 deprivation, these pretreated mice had reached a metabolic rate of less than 2% of normal and, consistently, acquired a degree of stress resilience similar to that seen in naked mole rats (section 2.4). With regard to a physiological survival benefit promoted by the gas, a recent report implicated endogenous H_2S in lung remodeling during natural hibernation, since both CBS protein expression and H_2S concentration doubled in lung tissue from hibernating hamsters during torpor, and normalized to euthermic levels in late arousal (Talaei et al., 2012).

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Due to these remarkable hypometabolism-inducing and endothermy-inhibiting properties of H_2S or NaHS, these small compounds have shown promise in preventing and/or reducing organ injury in models of acute critical illness, including those of haemorrhagic shock, I-R-based injury, endotoxemia, bacterial sepsis and nonmicrobial inflammation (reviewed: (Wagner et al., 2009, Aslami and Juffermans, 2010, Stein et al., 2015) and references therein). In addition, numerous critical care rodent models (reviewed: (Szabo, 2007, Snijder et al., 2013, Modis et al., 2014)) evidenced protective effects of hypometabolic sulfide concentrations on animal survival, conservation of organ function and dampened inflammatory response; for example, in rats with bilateral renal I-R injury (Bos et al., 2009) or mice with myocardial I-R injury (Snijder et al., 2013). The observation that protection is greatest when sulfide is applied before and during, but much less when given after, the ischaemic stress supports the idea of a reduced O_2 demand during hypoxia as organ protective, anti-inflammatory signal (Bos et al., 2009).

Although the above findings of metabolic suppression have repeatedly been confirmed in small rodents (Blackstone and Roth, 2007, Volpato et al., 2008, Bos et al., 2009), it has been challenging to translate these findings to larger mammals (e.g. (Stein et al., 2015), and references therein). Several studies view the potential benefits of H_2S inhalation for critical care patients with skepticism due to the vast differences by which different mammalian species allocate their energy demands underlying resting metabolism (Leslie, 2008, Derwall et al., 2010, Drabek et al., 2011, Haouzi, 2011, Haouzi, 2012). In mice, as in newborn mammals (section 2.2), the heat produced through NST in brown fat mitochondria accounts for much of the O_2 consumed. Hence, small rodents can save huge amounts of energy (depending on intensity, NST requires >50% to practically all of oxygen uptake in mice; (Cannon and Nedergaard, 2004)) when turning into thermoconformers similar to naked mole rats upon sulfide treatment. When studies by Haouzi and associates documented that larger species, i.e. sheep, completely failed to show reductions in their metabolic rate or their *T*b upon H_2S inhalation, the hype around H_2S-breathing rodents was severely dampened and the critics saw themselves confirmed (e.g. (Haouzi et al., 2008, Haouzi, 2011); but see (Baumgart et al., 2009) for discussion). Yet, the lack of anapyrexia in these sheep may relate to the exposure of the animals at a *T*a of 22°C, a

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value within the thermoneutral zone for this species, particularly for fleece-covered specimen (Blaxter et al., 1959). Under these conditions, basal metabolic rate is already at a minimum and cannot be further reduced by any anapyrexia-inducing agent (Branco et al., 2014). Hence, it is conceivable that sheep or even humans exposed to lower *T*a, or subjected to external body cooling or hypothermia-inducing substances such as adenosine or 3-iodothyronamine (T1AM) (Zhang et al., 2013b), would be more responsive to the anapyrexic effect of H_2S (Branco et al., 2014).

More recent data in sheep and swine subjected to shock, I-R challenge (Simon et al., 2008, Sodha et al., 2008, Sodha et al., 2009, Osipov et al., 2009, Osipov et al., 2010, Simon et al., 2011, Hunter et al., 2012), haemorrhage (Bracht et al., 2012) or burn injury (Esechie et al., 2009), however, did suggest beneficial effects of infusing Na_2S for larger models as well. Many of these benefits seem to be conferred independently of *T*b, as induction of moderate hypothermia often attenuates systemic inflammation rather than lessening the expenditure of a resting metabolism already operating at a (compared to small rodents) sluggish pace (Simon et al., 2011, Bracht et al., 2012). Yet, in other models, including those of anesthetized and ventilated 45 kg swine, intravenous infusion of Na_2S over 10 h reduced the heart rate and subsequently cardiac output after a transient aortic balloon occlusion insult, thereby reducing O_2 uptake and CO_2 production and, ultimately, core temperature, despite the attempt of keeping the animals warm by placing them on heating mattress (Simon et al., 2008, Baumgart et al., 2009, Wagner et al., 2009). While the extent of sulfide-driven organ preservation and functional recovery may be somewhat restricted compared with rodents, larger mammals, including adult humans (section 2.4), can surely utilize metabolic depression and accompanying hypothermia to protect their organs from irreversible damage after disrupted O_2 and/or blood supply (see Table 1 for capacity of metabolic depression (D/RMR ratio) in large mammals).

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3. Conclusions

Hypometabolism, the ultimate cell-autonomous coping mechanism during severe hypoxic, ischaemic or aerobic challenges, saves lives through the coordinated and fully reversible down-regulation of biosynthetic, proliferative and electrogenic expenditures at times when little ATP can be generated. Such a metabolic slowdown allows the re-balancing of energy demand with supply at a greatly suppressed level to withstand, and recover from, these life-threatening hazards. Highlighted by select cases in this review, such a re-programming of energetic expenditures exists, from "bugs to babies", as extremely widespread trait across ontogenetic stages and diverse phylogenetic clades. In microbes and cancer cells, hypometabolism and the accompanying cellular quiescence frequently associate with a much harder-to-treat phenotype. With regard to small mammals undergoing hibernation, one consequence of the pronounced metabolic depression with its reduced oxygen uptake during torpor is the animals` abandonment of endogenous heat production. A similar, secondary thermoconforming phenotype is seen in fossorial mammals such as naked mole rats. Torpor-associated hypothermia in hibernators, or loss of endothermy in life-long burrowing species, was found to act as key organ-protective ingredient, and particularly keeps ischaemia-reperfusion (I-R)-mediated cell damage in the central nervous system of these animals to a minimum. Yet, of the many life-saving facets of the hypometabolic physiology in torpid endotherms (i.e. markedly slowed and pO_2-conforming O_2 consumption rate; pronounced bradycardia and reduced cerebral perfusion; arrested cell proliferation (quiescence) and globally down-regulated protein and RNA and DNA syntheses; HIF activation despite aerobic physiology; lessened immune response; elevated H_2S production etc.), only hypothermia has successfully been translated into mainstay treatments for enhanced organ-, and particular, neuro-protective effects in human individuals once challenged by I-R-injuries. To date, the successful implementation of therapeutic hypothermia for victims of sudden cardiac arrest or in cases of critical heart or brain surgeries has yielded ample clinical evidence for the injury-limiting and recovery-supporting impact of a slowed metabolism in organs of adult patients. A large European multicentre, open-label and randomised, phase III study currently investigates potential benefits of therapeutic hypothermia also on patients with acute ischaemic stroke (http://www.eurohyp1.eu).

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Besides a controlled cooling, pharmacological inhibitors of respiration and heat production are increasingly utilized in small and large animal models to assess their organ-protective efficacy. Application of H_2S and sulfides seem especially worth pursuing as acute, neoadjuvant interventions (e.g. *en route* to hospital or surgery) due to their ease of administration (i.e. inhalation, i.v. injection) and the compounds quick, extensive and fully reversible metabolic depressive effect seen in rodents. While the hype around H_2S-breathing mice was subsequently dampened by studies showing that H_2S inhalation at similar concentrations yielded neither a marked reduction in the metabolic rate nor in the *T*b of larger species, e.g. sheep, one should not overlook the fact that larger mammals are usually sedated for measurement and exposed to an ambient temperature within the species` thermoneutral zone, thus resulting in a metabolism already at basal level that cannot fall much further. Future sulfide applications, then, need to home in on the physiological roles of H_2S-sulfide-signaling in healthy contexts (e.g. torpid hibernators) and on the accurate quantification and bioavailability of the different pools of endogenous H_2S (free vs. molecule-bound) under basal and injury-afflicting conditions of both animal models and human patients. Additionally, dose responses, pharmacokinetics, different ways of administration (i.e. gaseous, i.v., i.a. soluble salt injection, slow *vs*. fast release compounds), tissue- and organ-specific ways of manipulating H_2S levels plus additive or synergistic effects of combinatorial protocols (i.e. H_2S + hypothermia; H_2S + $NO^{\bullet}$or CO; H_2S + hypoxia preconditioning; H_2S + HIF stabilization etc.) for different disease models need to be elucidated far more systematically and with agreed-upon methodology. Also, prior to a complete transition to testing into adult human beings (there are currently 2 cardiovascular H_2S trials, one open, one completed, on clinicaltrials.gov), well-established, large animal models of cardiovascular disease should, perhaps, be more "inspired by cases such as the one of Anna Bågenholm". This is to say that for a more efficient and quickly acting acute care delivery in large mammals, combinatorial treatments of sulfide compounds, including inhalation of H_2S, and either physical or pharmacologically-induced hypothermia should be explored. That way, we will hopefully learn if the tissue-protective effect derived from body and organ cooling can be further ameliorated by sulfide addition or if the sulfide-cold combination might result in the same degree of tissue preservation at lesser *T*b reduction, and thus, fewer cold-related side effects. Despite these current qualms regarding life-saving outcomes of

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sulfide protocols in large-bodied recipients, it should not be forgotten that, regardless of species, newborn mammals with their greater capacity for NST by brown fat depots and, consequently, their heightened energy savings when transitioning into a stress-induced hypometabolic-hypothermic state, may represent a more suitable cohort than adult individuals for sulfide-based or sulfide–supplemented critical care interventions.

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Conflict of Interest

I have no conflict of interest to declare.

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Captions and Figures:

Figure 1: Illustrated by peaking HIF activity, $pO_2 \leq P_C$ defines cellular hypoxia. *Pink line*: synopsis of O_2 uptake and metabolism data from different *Drosophila* species and ontogenetic stages. According to various sources, oxyregulated respiration, aerobic metabolism and ongoing cell cycling in resting *Drosophila* flies ensues between 21 and ~3% oxygen. Resting *D. repleta*, for example, kept at 6% oxygen, exhibited a 78% oxygen consumption rate of air-kept (normoxic) control flies (aerobic "x"; (Chadwick and Gilmour, 1940)). *D. melanogaster* embryos, subjected to ~5% oxygen, displayed unimpeded cell cycle, spindle apparatus formation and cytokinesis relative to control embryos (Douglas et al., 2005). When ambient pO_2 approaches the critical threshold (P_C) at ~1.6 to 3% O_2 adult drosophilids rapidly display poor coordination, and, eventually, complete immobilization (stupor), in which the animals will remain throughout the entire stress (Csik, 1939). At P_C, transition from aerobe → anaerobe metabolism (lactate build-up) and oxyregulated → oxyconforming respiration ensues. Deeper into hypoxia, adult flies proceed into a metabolic arrested state (hypometabolism) with O_2 uptake rate depressed to ~20% of normoxic controls and no signs of an accumulated O_2 debt during recovery (hypoxic "x"; (Krishnan et al., 1997, Ma et al., 1999)). For the fly embryo, the hypometabolic stage is indicated by reversible cell cycle arrest (DiGregorio et al., 2001, Douglas et al., 2001) and condensed chromatin (Foe and Alberts, 1985). *Gray line*: author`s own electrophoretic mobility shift assays (EMSA) analyses of O_2 profile of fly HIF DNA binding activity (inset). Densitometric quantification of these EMSA data (gray graph) has been published previously (Gorr et al., 2004b).

Figure 2: Hypoxia defense strategies according to $[O_2]$. O_2 percentages along gradient are not meant to be taken literally. Their purpose lies solely in discerning responses to mild, moderate and severe O_2 deprivation, respectively. ⬆ = upregulated responses. Key transcriptional cascades are approximated to cover their respective O_2-operative range (vertical lines). See text for more details.

Figure 3: S2 mitochondrial gene expression changes relative to P_C. Representative Northern analyses of hypoxia-responsive transcriptions of ten mitochondrial protein products and one loading control mRNA (RpL29; blue labels). Analyses shown here were reproduced at least once more with independent S2 cell RNA harvests and yielded congruent changes in the banding intensity of said RNA. Northern blot results were also re-assessed, and mostly confirmed, by microarray-based

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determinations of expression changes (see Supplemental Figure 2 for details). Of the experimental genes, eight reading frames of nuclear DNA included: Sod2 (=MnSod: $O_2^{\bullet-}$ scavenging activity); ATPsynγ (= OXPHOS complex V; ATPase F1 subunit); Thiolase (= acetyl-CoA C-acyltransferase: β-oxidation of fatty acids); CG7224 (= Sirup; succinate dehydrogenase (SDH; OXPHOS complex II) assembly factor); mtSSB (= single-strand DNA-binding protein: mtDNA replication); Gdh (= glutamate dehydrogenase: glutamate + $NAD(P)^+$ + H_2O ⇆ α-ketoglutarate* + NAD(P)H + NH_3); Idh (= isocitrate dehydrogenase (NADP+-dependent) of TCA cycle: isocitrate + $NADP^+$ ⇆ α-ketoglutarate* + CO_2 + NADPH); Pepck (= phosphoenolpyruvate carboxykinase: GTP + oxaloacetate ⇆ GDP + phosphoenolpyruvate + CO_2) (* α-ketoglutarate = 2-oxoglutarate). Two additional genes investigated are located within mitochondrial DNA, i.e. mt:ND5 (= mitochondrial NADH-ubiquinone oxidoreductase chain 5; core subunit of OXPHOS complex I), and mt:CoI (= mitochondrial cytochrome c oxidase (COX) subunit I; catalytic COX subunit with haem a and bimetallic CuB-haem a3 reaction center). Northern blots were generated as previously published (Gorr et al., 2004b) and as briefly described in Supplemental Table 1. Loading schemes at each blot: normoxia, N (air, two independent batches of S2 cells); hypoxia, at lanes 0.2, 1 and 4 (16h each; values depict % oxygen levels) and hypoxia mimetics cobalt (C; 100µM, 16h) and DFO (D; 100µM, 16h). Fold-expression changes, normalized to loading control mRNA RpL29, is given underneath each lane, relative to normoxic references set to 1.

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Figure 1:

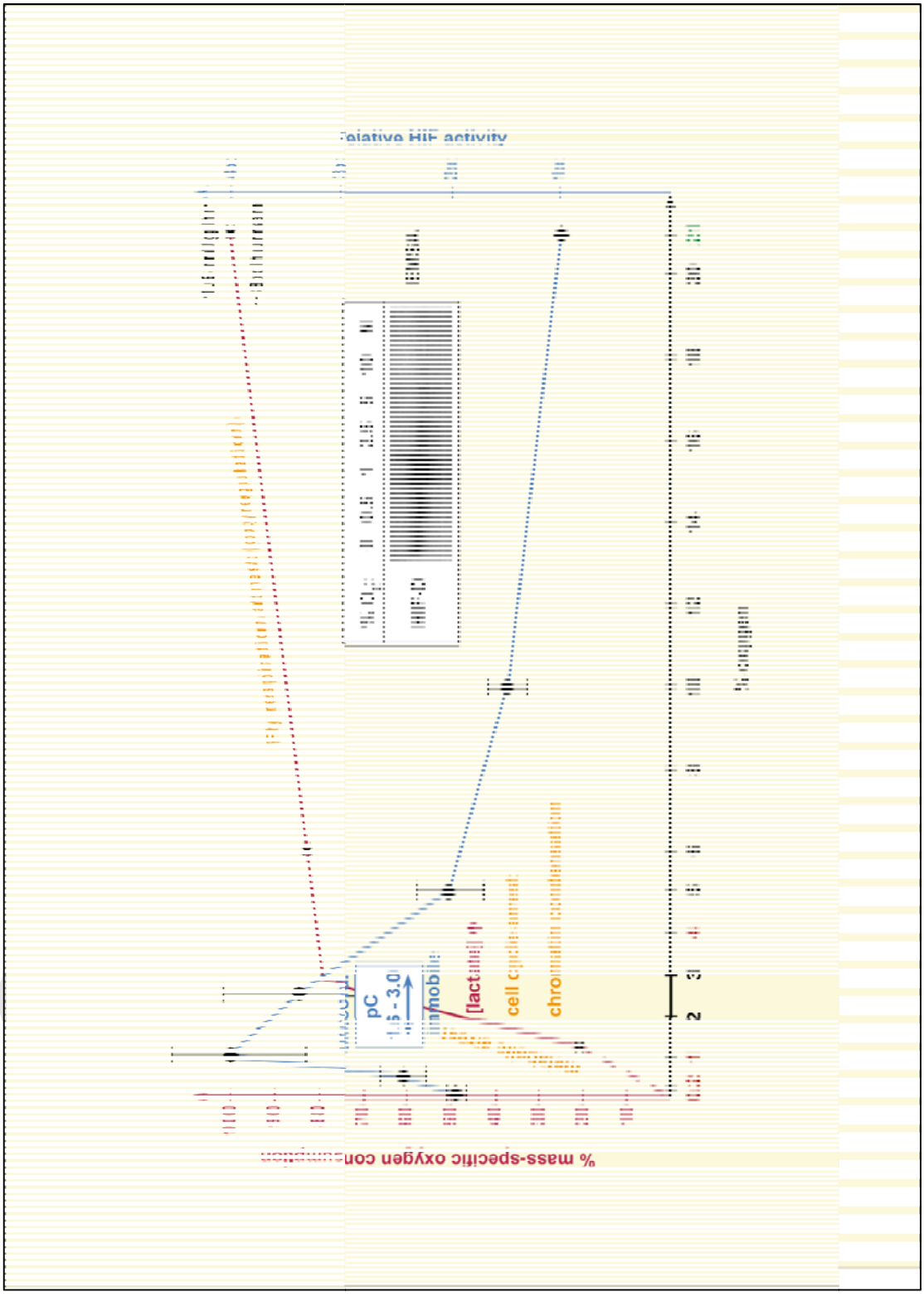

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Figure 2:

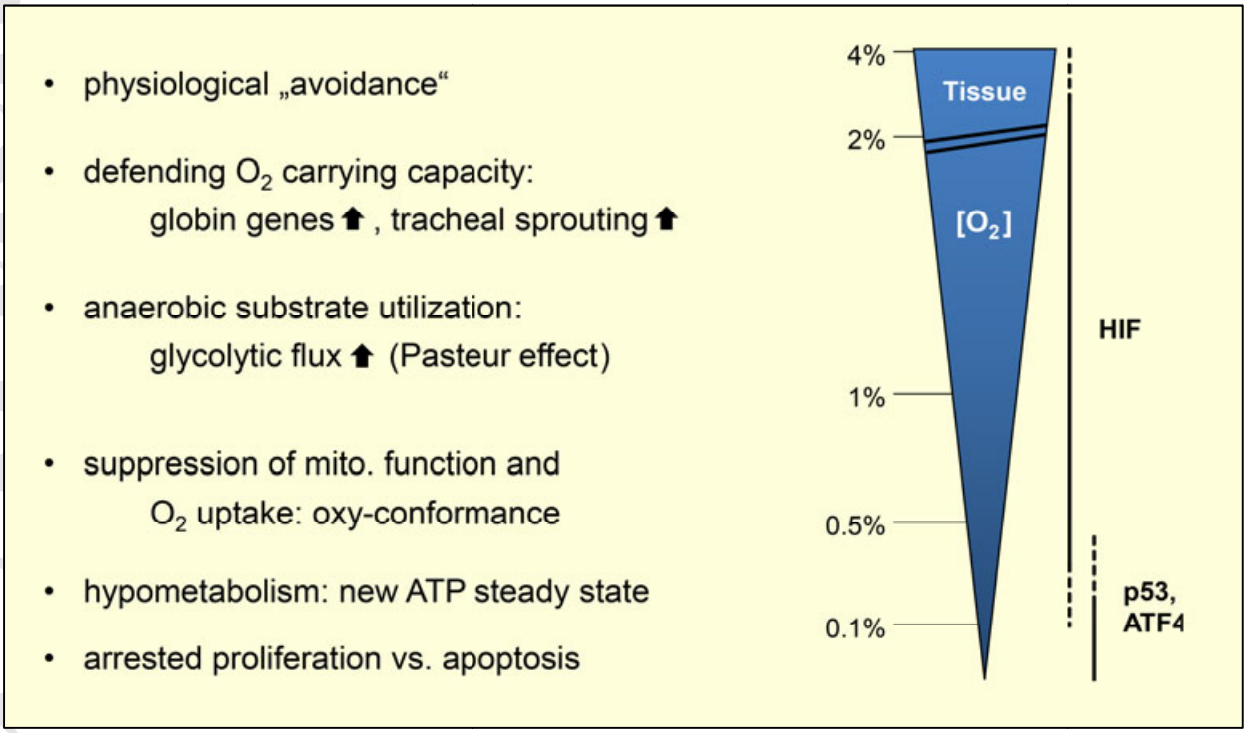

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Figure 3:

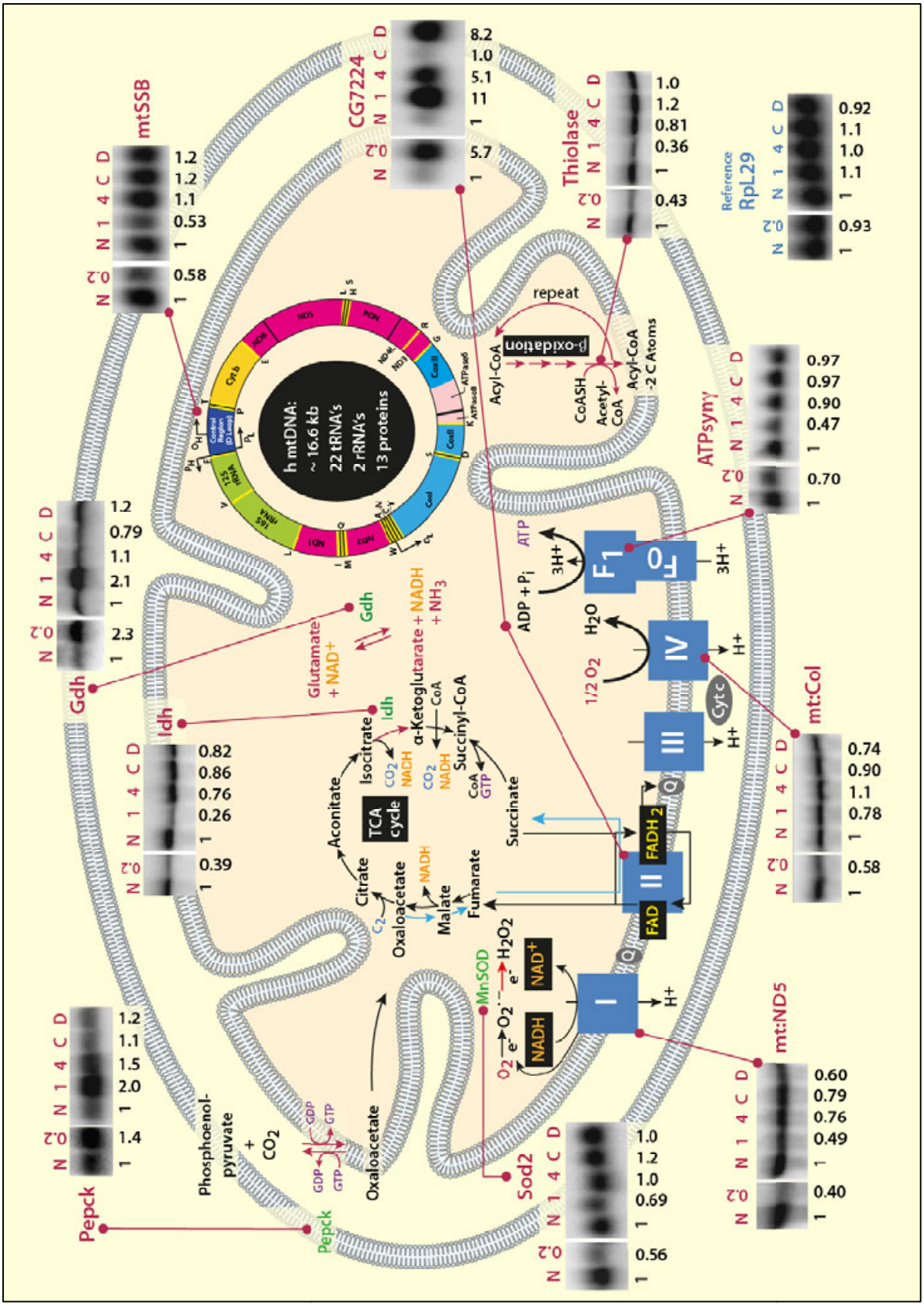

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Table 1:

Table 1

Taxon	D/RMR	State
Crustacean *Artemia spp.*	0	anoxic cyst
Tardigrade *Macrobiotus hufelandi*	0.00004	anhydrobiotic
Leech *Nephelopsis obscura*	0.0023	long-term anaerobic
Fungus *Neurospora tetrasperma*	0.02	dormant spore
Cnidarians	0.047	anaerobic
Insect *Gryllus pennsylvanicus*	0.11	dormant egg
Turtle *Chrysemys scripta*	0.1-0.15	anoxic (comatose)
Crucian carp *Carassius carassius*	~0.33	anoxic (active)
Amphibians and Fish	0.3-0.2	aestivating
Mammals: small (e.g. Alpine marmot)	0.02-0.05	hibernating: torpid state
Mammals: large (e.g. black bear)	0.25	hibernating
Mammals: Northern ungulates (e.g. red deer)	0.4	during winter, at night

Degree of metabolic depression. D/RMR (middle column): ratio between depressed (D) and resting metabolic rate (RMR). RMR is the rate of energy expenditure to sustain basic vital functions while the organism is at rest, i.e. exists at thermo-neutrality and in a post-absorptive state. Rows in dark and light gray denote examples of cryptobiosis (see Supplemental Figure 1), those in dark and light blue examples of hypometabolism (see text). This greatly condensed table version has been adopted from (Guppy and Withers, 1999). Additional data from: (Heldmaier et al., 2004), (Tøien et al., 2011) and (Arnold et al., 2004).

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Table 2:

Table 2

Aspect	NO•	CO	H_2S
Enzymatic sources	eNOS, iNOS, nNOS,	HO-1, HO-2	CBS, CSE, CAT/MST
Substrate modification	Arg oxidation	Heme degradation	Hcy-Cys condensation or Cys oxidation
Half-life *in vivo*	seconds	minutes (mice) – hours (humans)	seconds-minutes
Primary target (maximal affinity)	• sGC / cGMP • K_{Ca} channels • -SH groups	• Hb • Mb	• K_{ATP} channels, • perhaps others
Reaction with Hb: product?	• (deHb + NO•:) HbNO • (HbO_2 + NO•:) Hb^{Fe3+} • binds Fe^{2+} or Fe^{3+} haem	• Hb^{Fe2+}-CO • binds Fe^{2+} haem only	• sulfHb • binds Fe^{2+}-O_2 or Fe^{4+} haem
Exogenous donor: fast bolus vs. slow release	• PROLI NONOate (fast) • DPTA NONOate (slow) • NOR-5 (very slow)	• CO-RM3 (fast) • CO-RMA1 (slow)	• Na_2S, NaHS (fast) • GYY4137 (slow) • S-diclofenac (slow)
Production inhibitor	e.g. L-NAME (all NOS)	e.g. ZnPPNIX	e.g. BCA, PAG (limited potency and selectivity)
Crosstalk (positive)	• NO• ⇨ CSE expression ⬆ in SMCs • NO• ⇨ HO activity ⬆	• (endothelium:) CO ⇨ NOS activity: NO• ⬆ • (liver:) CO ⇨ NOS activity: NO• ⬆ ⇨ HO-1 ⬆	• H_2S ⇨eNOS activity (pSer1177 ⬆) in ECs • H_2S ⇨Nrf2 activity ⇨ HO-1 expression ⬆
Crosstalk (negative)	• NO• ⊣ CBS: H_2S ⬇ in astrocytes	• CO ⊣ CBS: H_2S ⬇ in astrocytes • CO ⊣ NOS activity ⬇	• ?
Cellular energetics effects	LD: ETC ⬆; HD: ⊣ COX (ptl. comp.) + OXPHOS I, III	mainly ⊣ COX (comp.)	LD: ETC ⬆; HD: ⊣ COX (noncomp.) + OXPHOS I, III
Vascular effects	• vasodilation • ⬆ angiogenesis • mainly EC production	• vasodilation • ⬆ angiogenesis • mainly SMC production	• vasodilation • ⬆ angiogenesis • only SMC production
Cardioprotective	Yes	Yes	Yes

Enzymatic synthesis, reaction targets, crosstalk modes and energetic effects of endogenously produced NO•, CO and H_2S pathways. Abbreviations used: NOS: endothelial / inducible / neuronal nitric oxide synthase (eNOS / iNOS / nNOS); HO: haem oxygenase (HO1, HO2); CBS, cystathionine β-synthase; CSE, cystathionine γ-lyase; CAT/MST = cysteine aminotransferase/3-mercaptopyruvate sulfurtransferase; Arg = arginine; Hcy = homocysteine; Cys = cysteine; sGC = soluble guanylyl cyclase; cGMP = cyclic guanosine monophosphate; K_{Ca}: Ca^{2+}-activated K^+ channels; Hb = haemoglobin; Mb = myoglobin; K_{ATP}: potassium-opened ATP channels; deHb = deoxy Hb; HbO_2 = oxy Hb; HbNO = (iron)nitrosyl Hb; CO-RM = CO-releasing molecule; L-NAME: L-N^G-Nitroarginine methyl-ester;; ZnPPNIX: Zn-protoporphyrin-IX; BCA, β-cyanoalanine; PAG, dl-propylargylglycine; EC/SMC: endothelial/smooth muscle cell; ⇨: stimulating interaction; ⊣ : inhibitive interaction; ⬆: increasing level/activity; ⬇: decreasing level/activity; LD/HD: low/high dose; ETC: electron transport chain; COX: Cytochrome c oxidase (OXPHOS complex IV); ptl. comp. = partially competitive (i.e. NO•-based inhibition of COX is largely competitive with the O_2 binding to reduced binuclear heme a_3 (ferrous) / Cu_B (cupric) centre, yet it is also uncompetitive when NO• docks to the oxidized binuclear centre); comp: = competitive inhibition (i.e. CO and O_2 compete for binding to the reduced COX centre); noncomp. = noncompetitive (i.e. H_2S/sulfide-based inhibition of COX is O_2-independent). This table has been mainly adopted from (Szabo, 2007, Polhemus and Lefer, 2014). Additional data from: (Cooper and Brown, 2008, Motterlini and Otterbein, 2010, Wang, 2012, Branco et al., 2014, Lo Faro et al., 2014, Kimura, 2015).

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