Hypoxia tolerance of jumbo squids (*Dosidicus gigas*) in the Eastern Pacific oxygen minimum zones: Physiological and biochemical mechanisms

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Hypoxia Toleranz der Riesen-Flugkalmare (*Dosidicus gigas*) in den Sauerstoffminimumzonen des östlichen Pazifiks: Physiologische und biochemische Mechanismen

Dissertation

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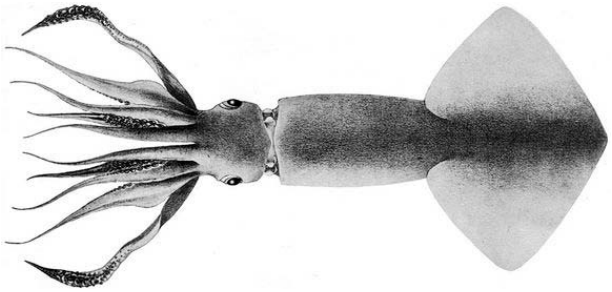

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Summary

Marine hypoxia has become one of the major concerns of the world, as oceanic dead zones continue expanding horizontally and vertically, a phenomenon primarily caused by global warming and anthropogenic eutrophication. As consequence, drastic changes in community structures, predator-prey relationships (i.e. uncoupling) and/or habitat compression are expected followed by severe impacts on food-webs, ecosystems and fisheries. Moreover, habitat compression is aggravated by the synergistic effects of climate change, as elevated temperature and PCO_2 will narrow the habitat from above. The jumbo squid, *Dosidicus gigas*, undergoes diel vertical migrations into oxygen minimum zones (OMZs) off the Eastern Tropical Pacific, where he plays an important ecological role both as predator and prey. In fact, this species can easily remove more than 4 million tons of food per year from the pelagic food web and is an important component in the diets of birds, fishes, and mammals. Besides its ecological role, the jumbo squid also plays an important economically role being target of the world's largest cephalopod fishing industry with around 14% of world's total squid catch and landings estimated at 818,000 tons in 2006. However, the main problem that arises with hypoxia is a reduced gradient that drives O_2 uptake via diffusion pathways. At some point, the critical O_2 partial pressure (P_{crit}), the reduced diffusion gradient cannot support the metabolic demand fully aerobically, and has to be supplemented by anaerobic pathways and/or compensated by a reduction in metabolic rate. Commonly, aquatic animals respond to hypoxia by first attempting to maintain O_2 delivery, as aerobic metabolism is much more efficient, followed by conserving energy expenditure and reducing energy turn over and finally by enhancing energetic efficiency of those metabolic processes that remain and derive energy from anaerobic sources. A further problem that vertical migrators of OMZs have to face is the elevated production of radical oxygen species (ROS) during the reoxygenation phase while ascending, as non-neutralized ROS formation can damage biological macromolecules (i.e. lipids, proteins and DNA) resulting in severe functional alterations in cells and tissues. To determine the cost and benefits of such diel vertical migrations, I investigated biochemical and physiological mechanisms in juvenile *D. gigas* off the Gulf of California with a focus on ventilation, locomotion, metabolism and antioxidant defense.

The respiratory regulation in *D. gigas* was unpredictably high and is mirrored in maximized oxygen extraction efficiencies (EO_2) at early (EH, < 160 min, 1 kPa O_2) and late hypoxia (LH, > 180 min, 1 kPa O_2). EO_2 at EH was maximum 82% and achieved via (1) deep-breathing mechanism with more powerful contractions and an enlarged inflation period, and (2) reduction in the relaxed mantle diameter to favor diffusion. At LH, EO_2 was still 40%, despite all other ventilatory mechanisms were drastically reduced, probably by using the collar-flap system (uncoupling of locomotory and ventilatory mechanisms) and a further reduction in the relaxed mantle diameter. Moreover, the drastic change in locomotion between EH and LH (onset of lethargy) was accompanied by a switch in the energy source of anaerobic pathways. At EH, anaerobic energy equivalents (AEE) primarily arrived via rapid energy reserve depletion (ATP, phospho-L-arginine), and, under LH, was mainly obtained via fermentative pathways (mainly octopine). As octopine formation simultaneously creates protons, intracellular acidosis and acid-base disturbances under progressing hypoxia are expected, which might negatively impact squid's energy household and expenditures from locomotion towards more important cellular processes (i.e. ion regulation). Energy reserve depletion might even trigger lethargic behavior to conserve energy and extend hypoxia residence time. At EH, in contrast, deep-breathing behavior enabled *D. gigas* to pass the same amount of water through the mantle cavity per period of time and thereby could maintain a stable ventilatory volume per min, which explains its high level of activity observed under such extreme conditions.

Moreover, *D. gigas* suppressed its metabolism (45-60%) at severe hypoxia (below P_{crit}), as the reduction in O_2 consumption rate (70-80%) could not be compensated by an upregulation in anaerobic energy production (70%). Cephalopods primarily feed on proteins and their glycogen storage potential is low (< 0.4% of body weight). Therefore anaerobic protein degradation came into focus as strategy in hypoxia tolerant species. Yet, total protein concentration in muscle tissue of *D. gigas* did not vary significantly under severe hypoxia, but the reduced protein expression of heat shock protein 90 (Hsp90) and α-actinin indicates that, at least under progressing hypoxia, jumbo squids might degrade specific muscle proteins anaerobically. Moreover, the lower α-actinin expression at LH might be related to a decreased protection via the Hsp90 chaperon machinery resulting in increased ubiquitination and subsequent degradation. Therefore, the ubiquitin-proteasome system seems to play an important role in hypoxia tolerance, but further investigations are necessary to discover its full potential and pathways.

Antioxidant enzyme activities in *D. gigas* were generally low and in the range of other squid species, but malondialdehyde concentrations (indicative of cellular damage) did not

significantly change between normoxic and hypoxic conditions, demonstrating an efficient antioxidant defense system. Moreover, superoxide dismutase and catalase activities were enhanced under normoxia that seem to constitute an integrated stress response at shallower depths by buffering increased ROS formation, and, in addition, might even be a strategy to cope with the reoxygenation/recovery process. Moreover, heat shock protein 70 concentration was significantly increased under severe hypoxia (1 kPa O_2), which may constitute a preparation for the reoxygenation phase during squid's upward migration.

Accordingly, the present thesis demonstrates that *D. gigas* evolved a variety of adaptive mechanisms and strategies to cope with hypoxia and the imposed challenges of diel vertical migrations. *D. gigas* might even actively descent into OMZs to suppress metabolism and escape from high metabolic demands at surface waters. Especially the high O_2 uptake capacity and respiratory regulation were surprising taking into account cephalopods physiological and anatomical restraints. Therefore, *D. gigas* seems well-adapted to hypoxic conditions and might even out-compete less hypoxia tolerant species under hypoxia expansion, but the synergistic impacts of climate change, in turn, might endanger its survival.

Zusammenfassung

Die vertikale und horizontale Ausbreitung mariner Hypoxie hat sich zu einem der grössten Umweltprobleme der Welt entwickelt, ein Phänomen, dass hauptsächlich durch die globale Erwärmung und anthropogene Eutrophierung verursacht wird. Als Konsequenz sind drastische Veränderungen in der Zusammensetzung und dem Aufbau mariner Tier- und Pflanzengemeinschaften, sowie Änderungen in Räuber-Beute Beziehungenen (z.B. durch Entkopplung) und/oder eine Komprimierung der Lebensräume zu erwarten, was erhebliche Einflüsse auf die Nahrungsnetze, Ökosysteme und Fischerei zur Folge haben wird. Zudem wird die Komprimierung der Habitate durch die synergistischen Effekte der Klimaveränderung verschärft, da erhöhte Temperaturen und PCO_2-Werte die Lebensräume zusätzlich von oben her einengen. Der Riesen-Flugkalmar, *Dosidicus gigas*, unternimmt tägliche Vertikalwanderungen in die Sauerstoffminimumzonen (OMZs) des östlichen tropischen Pazifiks, wo er eine wichtige ökologische Rolle als Räuber und Beute spielt. Tatsächlich kann diese Art mit Leichtigkeit mehr als 4 Millionen Tonnen Nahrung pro Jahr aus dem pelagischen Nahrungsnetz entfernen und ist ein wichtiger Bestandteil auf dem Speiseplan von Vögeln, Fischen und Säugetieren. Neben seiner ökologischen Stellung nimmt der Riesen-Flugkalmar auch eine bedeutende wirtschaftliche Rolle ein, da er eine der begehrtesten Zielscheiben der Fischereiindustrie für Kopffüssler ist und dabei alleine 14% des gesamten weltweiten Tintenfischfangs abdeckt mit geschätzten Anlandungen von 818,000 Tonnen im Jahr 2006. Das Hauptproblem der Hypoxie ist ein verringerter Gradient, der die Sauerstoffaufnahme über Diffusionswege steuert. Erreicht dieser Gradient den kritischen Sauerstoffpartialdruck (P_{crit}) können die Anforderungen des Stoffwechsels nicht mehr alleine durch Atmungsprozesse abgedeckt werden, und muss daher mit Hilfe anaerober Stoffwechselwege ergänzt und/oder mit einer Reduktion des Stoffwechsels kompensiert werden. Weil der aerobe Stoffwechsel viel energiereicher ist versuchen aquatische Organismen unter Hypoxie als erstes den Sauerstofftransport aufrecht zu erhalten, gefolgt von der Konservierung von Energieausgaben, einem verringertem Energieumsatz, und letztendlich durch die Erhöhung der Energieeffizienz von Stoffwechselprozessen, welche Energie aus anaeroben Quellen beziehen und aufrechterhalten. Ein weiteres Problem, das sich Vertikalwanderer in OMZs stellen müssen,

ist die erhöhte Produktion von Sauerstoffradikalen (ROS) während der Reoxygenierungsphase beim Aufsteigen, da nicht neutralisierte ROS Formierungen biologische Makromoleküle (wie z.B. Lipide, Proteine und DNA) beschädigen können, was wiederum erhebliche funktionelle Veränderungen in Zellen und Geweben hervorrufen kann. Um die Kosten und Vorteile solcher Vertikalwanderungen bestimmen zu können, habe ich ihm Rahmen meiner Doktorarbeit die biochemischen und physiologischen Mechanismen juveniler Riesen-Flugkalmare aus dem Golf von Kalifornien untersucht, und mich dabei auf die Atmung, die Bewegung, den Stoffwechsel und die Antioxidansabwehr fokussiert.

Die respiratorische Regulierung in *D. gigas* war wiedererwartend hoch und wiedergespiegelt in einer erhöhten Sauerstoffaufnahmeeffizienz (EO_2) sowohl unter früher (EH, < 160 min, 1 kPa O_2) als auch unter später Hypoxie (LH, > 180 min, 1 kPa O_2). Die EO_2 unter EH erreichte einen maximalen Wert von 82% und wurde erzielt durch (1) einen Tief-Atmungs-Mechanismus mit kraftvolleren Mantelkontraktionen und einer verlängerten Inflationsperiode, und (2) eine Verringerung des Manteldurchmessers (im Ruhezustand) um die Sauerstoffaufnahme über Diffusion zu steigern. Unter LH, EO_2 betrug weiterhin 40%, obwohl alle anderen Atmungsmechanismen drastisch reduziert waren. Dies wurde vermutlich erzielt durch die Anwendung des sogenannten Mantelkragen-Klapp-Systems (durch Entkopplung der Ventilation von den Bewegungsabläufen) und einer weiteren Reduzierung des Manteldurchmessers (im Ruhezustsand). Desweiteren, war die drastische Änderung der Lokomotion/Atmung zwichen EH und LH (Startpunkt der Lethargie) begleitet von einer Umstellung in der Energieverstoffwechslung unter anaeroben Bedingungen. Während der Grossteil der anaeroben Energieequivalente (AEE) unter EH durch den schnellen Abbau von Energiereserven (ATP, Phospho-L-Arginin) gedeckt werden konnte, wurde unter LH der Hauptanteil über Fermentationswege (hauptsächlich Oktopin) gewonnen. Die Bildung des anaeroben Endproduktes Oktopin aber erzeugt gleichzeitig Protonen, und daher sind intrazelluläre Versauerung und Störungen des Säure-Base Gleichgewichtes zu erwarten. Daher sind Störungen/Änderungen im Energiehaushalt und seinen Ausgaben zu erwarten mit einem erhöhten Fokus auf wichtige zelluläre Prozesse (wie z.B. Ionenregulation) anstelle der Lokomotion. Zusätzlich ist es möglich, dass die Ausbeutung von Energiereserven selbst lethargisches Verhalten auslöst, um Energieausgaben zu konservieren und die Aufenthaltszeit unter hypoxischen Bedingungen zu verlängern. Im Gegensatz dazu, ermöglichte das Tief-Atmungs-Verhalten von *D. gigas* unter EH einen konstanten Wassertransport durch die Mantelhöhle pro Zeitintervall wie unter normoxischen Bedingungen, was den hohen Aktivitätsgrad unter solchen extremen Bedingungen erklären könnte.

Zusätzlich zeigte *D. gigas* unter Hypoxie eine aktive Absenkung seines Stoffwechsels (45-60%), da die verringerte Atmungsrate (70-80%) nicht durch die Aktivierung anaerober Stoffwechselwege kompensiert werden konnte (AEE 70% erhöht). Kopffüssler unter normoxischen Bedingungen beziehen ihre Energie weitgehend aus Proteinen und ihr Potential Glykogen zu speichern ist äusserst begrenzt (< 0.4% des Körpergewichts). Daher könnnte der anaerobe Abbau von Proteinen eine wichtige Rolle in der Hypoxietoleranz von Kopffüsslern spielen, als weitere Strategie, um die Aufenthaltszeit in OMZs zu verlängern. Die Proteinkonzentration im Muskelgewebe von *D. gigas* allerdings variierte nicht signifikant unter dem Einfluss von Hypoxie (1 kPa O_2), trotzdem konnte eine reduzierte Proteinexpression des Hitzeschockproteins 90 (Hsp90) und α-actinin entdeckt werden, was zumindest unter fortschreitender Hypoxie darauf schliessen lässt, dass *D. gigas* spezifische Muskelproteine anaerobisch verstoffwechselt. Weiterhin scheint die verringerte Expression von α-actinin unter LH mit einem reduzieten Schutz der Hsp90 Chaperon-Maschinerie zusammenzuhängen, was wiederum eine erhöhte Ubiquitinierung mit anschliessender Degradierung zur Folge hat. Dies lässt darauf schliessen, dass das Ubiquitin-Proteasom-System eine entscheidende Rolle in der Hypoxietoleranz spielt, aber weitere Untersuchungen sind notwending um das gesamte Potential und seine Pfade erfassen zu können.

Die antioxidantischen Enzymaktivitäten in *D. gigas* zeigten generell niedrige Werte im Bereich anderer Tintenfischarten, wobei die Malondialdehydkonzentrationen (Anzeiger für Zellschäden) keine signifikanten Veränderungen zwischen Normoxie und Hypoxie aufzeigte, was wiederum einen effizienten Antioxidansabwehrmechanismus aufzeigt. Zudem waren die Enzymaktivitäten von Superoxiddismutase und Katalase unter Normoxie gesteigert, was mit einer integrierten Stressantwort im Oberflächenwasser zusammenzuhängen scheint, und möglicherweise sogar selbst eine Strategie darstellt, um mit der Reoxygenierung/ Erholungsphase umzugehen, um die erhöhte ROS Produktion abzupuffern. Die signifikante Erhöhung der Hitzeschockprotein 70 Konzentration unter Hypoxie (1 kPa O_2) scheint dabei eine zusätzliche Vorsorgemassnahme bezüglich der Reoxygenierungsphase in aufsteigenden Riesen-Flugkalmaren darzustellen.

Die Ergebnisse meiner Doktorarbeit zeigen, dass *D. gigas* eine Vielzahl von adaptiven Mechansimen und Strategien entwickelt hat, welche ihm ermöglichen mit hypoxischen Bedingungen und den Herausforderungen der Vertikalwanderungen umzugehen. *D. gigas* sucht dabei möglicherweise absichtlich OMZs auf, um seinen hohen Energieverbrauch aktiv zu unterdrücken, um vor seinen hohen Stoffwechselanforderungen im Oberflächenwasser zu flüchten. Besonders die erhöhte Sauerstoffaufnahmeeffizienz und respiratorische

Regulation waren überraschend, vor allem unter dem Aspekt der physiologischen und anatomischen Beinträchtigungen die Kopffüssler typischerweise charakterisieren. Daher scheint *D. gigas* sehr gut an hypoxische Bedingungen angepasst zu sein und kann vermutlich bei weiterer Hypoxieausbreitung weniger tolerante Arten verdrängen. Trotzalledem könnten die synergistischen Einflüsse des Klimawandels das Überleben von *D. gigas* drastisch beinträchtigen.

Content

Abbreviations

AEC	adenylate energy charge
AEE	anaerobic energy equivalents
AMR	active metabolic rate
AOX	antioxidant
CAT	catalase
EH	early hypoxia
EO_2	oxygen extraction efficiency
ETA	Eastern Tropical Atlantic
ETP	Eastern Tropical Pacific
GST	glutathione-S-transferase
H^+	proton
Hsp70	heat shock protein 70
Hsp90	heat shock protein 90
HSR	heat shock response
IMR	inactive metabolic rate
L-Arg	L-arginin
LH	late hypoxia
MDA	malondialdehyde
MR	metabolic rate
MR_{max}	maximum metabolic rate
O_2	oxygen
OCR	oxygen consumption rate

OMZ	oxygen minimum zone
P_{crit}	critical oxygen partial pressure
PLA	phospho-L-arginin
RMR	routine metabolic rate
ROS	reactive oxygen species
SOD	superoxide dismutase
UPS	ubiquitin-proteasome-system

1 Introduction

1.1. Hypoxia

The phenomenon hypoxia, defined as dissolved oxygen (O_2) less than 2.8 mg O_2 l^{-1} (equivalent to 2 ml O_2 l^{-1} or 91.4 µM; Diaz and Rosenberg, 1995), affects thousands of km^2 of marine waters all over the world, and has caused mass mortality of marine animals, with consequent declines in fisheries production (Diaz and Rosenberg, 1995; Lu and Wu, 2000; Wu, 2002; Chan *et al.*, 2008). The severity, frequency of occurrence and spatial scale of hypoxia have increased in the last few decades and, therefore, it has become one of the major ecological concerns in the world (GESAMP, 1990; Goldberg, 1995; Wu, 1999 and 2002).

1.1.1. Anthropogenic eutrophication

Eutrophication is caused by excessive input of nutrients and organic matter into coastal ecosystems (Nixon, 1995; Diaz and Rosenberg, 1995, 2001 and 2008) and typically occurs where human population or agricultural production is high (Rabalais, 2004, 2009). Consequently, phytoplankton growth becomes excessive resulting in a further accumulation of organic matter, as the metabolic capacity of metazoan consumers is exceeded (Rabalais, 2009; Fig. 1.1). The remaining organic matter settles to the sediments or in stratified water bodies (i.e. pycno- and thermoclines), where it is decomposed mainly by heterotrophic bacteria. This decay process depletes the dissolved O_2 at a rate faster than it is resupplied from the well-oxygenated surface waters (Levin *et al.*, 2009; Fig. 1.1).

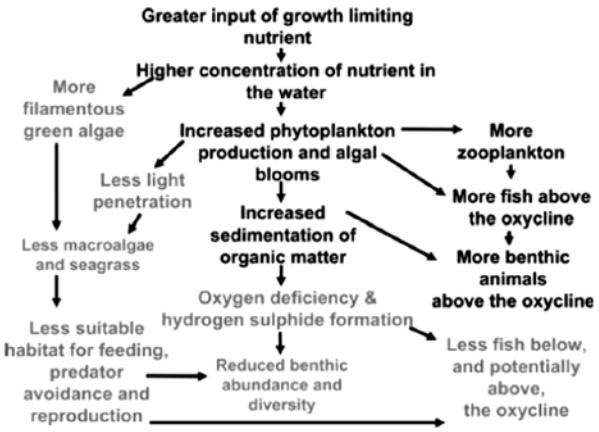

Figure 1.1 - Schematic representation of the cascading effects of increasing nutrients in a coastal ecosystem. The harmful effects of nutrient overenrichment are presented in grey letters (after Rabalais, 2009).

Ocean stratification is a natural occurring process resulting from strong thermal or salinity gradients, including the formation of freshwater lenses (i.e. due to intense rainfall during monsoons and/or excessive river runoff from land; Levin *et al.*, 2009). Today, global warming, caused by increased anthropogenic greenhouse gases levels in the atmosphere, forces the formation of thermoclines and, in addition, enhances freshwater runoff into coastal systems (Wu, 2002). This favors nutrient loading and the formation of haloclines (Justic *et al.*, 2001). Increased stratification means less deep water formation, and excess nutrient input, which in turn, lead to an extension of hypoxic areas especially in coastal, estuarine and semi-enclosed water systems. It is worth noting that the world population continues rising (especially near the coastal zone), and therefore further nutrient loadings into neritic waters are expected, as it seems unlikely that construction of sewage treatment facilities will catch up with such a rapid population growth (Wu, 2002). Additionally, the use of fertilizers, deforestation and release of nitrogen oxides into the atmosphere are expected to increase (Nixon, 1990). Therefore, global warming will trigger the spread/extension of hypoxia and aggravate its deleterious effects on coastal ecosystems and economies (Stramma *et al.*, 2008; Rabalais, 2009; see Fig. 1.1).

1.1.2. Oxygen minimum zones

In regions and at water depths where well-oxygenated currents prevail, hypoxia is rapidly dissipated. The interaction of such currents (e.g. the California Current, the Humboldt Current and the Benguela Current) with strong upwelling that supports high primary production and high subsurface O_2 demand creates sharp natural O_2 gradients along the coast and continental margin – the permanent oxygen minimum zones (OMZ) (Kamykowski and Zentara, 1990; Olson *et al*., 1993; Karstensen *et al*., 2008). These OMZs are hundreds of meters deep and thousands of kilometers wide and occupy large volumes of the intermediate-depth Eastern Tropical oceans (Stramma *et al.*, 2008; Garcia *et al.*, 2010; Hofman *et al.*, 2011; see Fig. 1.2). Thereby the horizontal and vertical extent of the OMZ, as well as the intensity of the hypoxia found there, varies considerably between ocean basins, but as much as 8% of the volume of the entire ocean contains less than 20 μmol kg^{-1} (~ 2 kPa; 2% O_2) (Paulmier and Ruiz-Pino, 2009). The O_2 content of a water mass is dependent on several factors, including the O_2 concentration when it was last in contact with air, the time elapsed since that point, and the rate of biological O_2 consumption. These factors, in turn, depend on temperature, air-sea gas exchange, ocean circulation and biology (Paulmier and Ruiz-Pino, 2009; Keeling *et al*., 2010). Nowadays, such oxygen-poor waters are expanding (Grantham *et al.*, 2004; Chan *et al.*, 2008) both horizontally and vertically with a steady decrease in O_2 levels at a rate of 0.09-0.34 μmol kg^{-1} yr^{-1} over the past 50 yr

(Stramma *et al.*, 2008). Recent analysis (from 1984 to 2006) of the California Cooperative Fisheries Investigation (CalCOFI) oxygen time series off southern California conducted by Bograd *et al.* (2008) revealed a decline in O_2 concentration by 20-30% within the 200 to 300 m layer, with a shoaling of the hypoxic boundary (1.42 ml l^{-1}) of up to 90 m within inshore regions of the southern California Current system. Also in the Northern Gulf of Mexico the hypoxic area has increased from 9000 km^2 (in 1985-1992) to 16,000–20,000 km^2 (in 1993–1999) and in the East China Seas likewise from less than 1000 km^2 in 1980 to 13,700 km^2 in 1999 (Rabalais, 2001). The substantial loss of dissolved O_2 and shoaling of hypoxia throughout much of the ocean (Emerson *et al.*, 2004; Whitney *et al.*, 2007; Bograd *et al.*, 2008; Stramma *et al.*, 2008) is assumed to be related to climate change (i.e. Bopp *et al.*, 2002; Keeling and Garcia, 2002; Oschlies *et al.*, 2008) because: (1) oxygen is less soluble in warm water and (2) global warming is increasing upper ocean stratification, which increases productivity in surface waters, fueling increased O_2 demand at depth and simultaneously reducing O_2 supply to greater depths (Keeling *et al.*, 2010). Therefore severe behavioral, biochemical and physiological challenges for fish and invertebrate communities inhabiting the OMZs are expected with consequent deleterious effects on food web structures, pelagic and benthic ecosystems and fisheries (Grantham *et al.*, 2004; Stramma *et al.*, 2008).

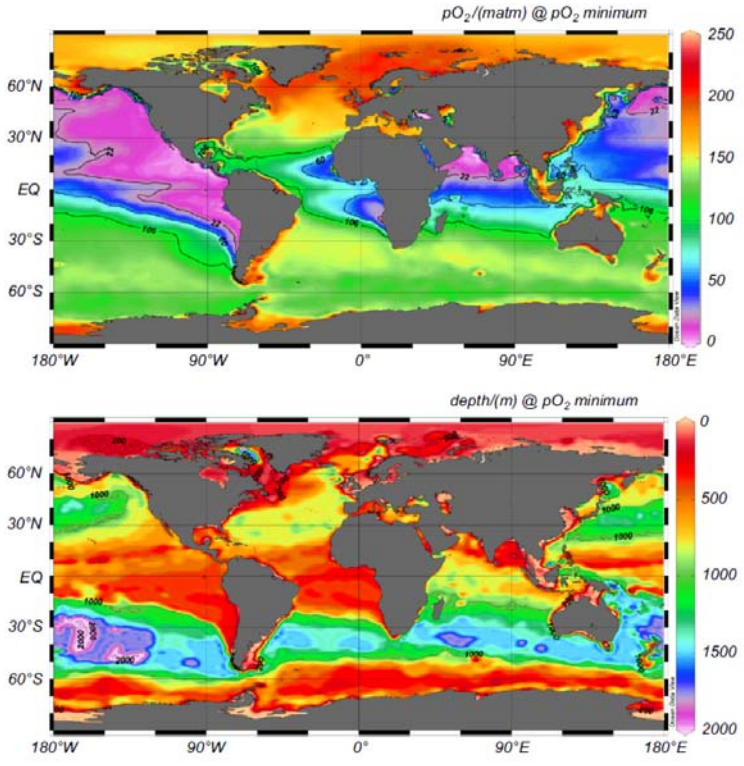

Figure 1.2 – Bathyal oxygen minimum zones (from Hofman *et al.*, 2011): pO_2 minimum values (top panel), and depth at which pO_2 minimum is attained (bottom panel). Data from the World Ocean Atlas 2009 oxygen climatology (Garcia *et al.*, 2010).

1.2. Biological impacts in different levels of organization

Hypoxia came into focus of marine science as it elicits deleterious impacts on the marine biota, at different levels of organization, namely at: (1) molecular level (e.g. up- and

downregulation of genes: Wu, 2002; Bruick, 2013), (2) biochemical level (e.g. metabolism, protein synthesis, reactive O_2 species formation: Hand, 1998; Guppy and Withers, 1999; Cooper *et al.*, 2002; Hochachka and Somero, 2002; Romero *et al.*, 2007), (3) physiological level (e.g. ventilation frequency, circulatory capacity, acid-base regulation: Hochachka and Mommsen, 1983; Grieshaber *et al.*, 1994; Childress and Seibel, 1998; Seibel, 2011), (4) organismal level (e.g. feeding, growth, fecundity, mortality: Diaz and Rosenberg, 1995; Keckeis *et al.*, 1996; Chabot and Dutil, 1999; Thetmeyer *et al.*, 1999; Zhou, 2001), (5) the population level (e.g. immigration, emigration: Rosenberg *et al.*, 1991; Long *et al.*, 2008), and (6) community level (e.g. biodiversity, species abundance, trophic interactions: Diaz *et al.*, 1992; Diaz and Rosenberg, 1995; Breitburg *et al.*, 1997; Taylor and Eggleston, 2000). Therefore, the specific adaptive potential of marine organisms, especially that of vulnerable early life-cycle stages (Keckeis *et al.*, 1996; Levin *et al.*, 2009), is expected to determine competitiveness, food-web structures and survival in benthic and pelagic ecosystems.

1.3. Physiological and biochemical impacts

Numerous studies have been carried out on physiological and biochemical responses of aquatic animals to hypoxia, especially on fish (e.g. Holton and Randall, 1967; Burggren and Randall, 1978; Woo and Wu, 1984; Wu and Woo, 1985; Dunn and Hochachka, 1986; Ip *et al.*, 1991; Val *et al.*, 1995). The main problem that arises with hypoxia is a reduced gradient to drive O_2 uptake via diffusion (i.e. in gills, skin, respiratory appendages) from the environment to mitochondria, meaning that it is not the quantity of O_2 in the environment that is limiting but its availability. As the energy yield of aerobic metabolism is much higher than the anaerobic one, there is strong selection for mechanisms to take up O_2 more effectively from low ambient partial pressures (Childress and Seibel, 1998; Seibel, 2011). However, at some point, the reduced diffusion gradient cannot fully support the metabolic demand aerobically, and has to be supplemented by anaerobic metabolism or compensated by a reduction in metabolic rate (Childress and Seibel, 1998). Therefore, aquatic animals generally respond to hypoxia by first attempting to maintain O_2 delivery, then by conserving energy expenditure and reducing energy turn over, and finally by enhancing energetic efficiency of those metabolic processes that remain and derive energy from anaerobic sources (Holton and Randall, 1967; Burggren and Randall, 1978; Van den Thillart and Smit, 1984; Wu and Woo, 1985; Dunn and Hochachka, 1986; Boutilier *et al.*, 1988; Chew and Ip, 1992; Randall *et al.*, 1992; Dalla Via *et al.*, 1994; Hochachka, 1997).

1.3.1. Ventilatory mechanisms

In general, the respiratory regulation in aquatic invertebrates is poor or even absent, especially in marine species that normally live in well-aerated waters with relatively constant O_2 supply (Schmidt-Nielsen, 1997a). Most epipelagic predators (i.e. large squids, sharks, marlin and swordfish) show low hypoxia tolerance (Brill, 1994 and 1996; Prince and Goodyear, 2006; Vetter *et al.*, 2008; Nasby-Lucas *et al.*, 2009; Stramma *et al.*, 2012) and, therefore, actively avoid such areas. Prolonged exposure to O_2 deprivation in such high-performance animals will result in irreversible membrane damage and loss of cellular ion homeostasis in vital organs (i.e. heart, brain; Boutilier, 2001) followed by death. Nevertheless, some lower organisms are quite tolerant to O_2 shortage like bivalves that can close their shells and, in the absence of ventilation, utilize anaerobic metabolic processes and/or metabolic suppression (Schmidt-Nielsen, 1997a).

Other hypoxia-tolerant organisms, instead, developed highly effective O_2 uptake mechanisms that enable survival of short-term O_2 deprivation (i.e. vertical migrators of OMZs) or even chronic hypoxic exposure (i.e. permanent OMZ residents). Accordingly, most permanent hypoxia residents are oxyconformers and regulate their O_2 consumption rate in a nearly linear function to the minimum O_2 levels encountered in their environments (Prosser and Brown, 1961; Grieshaber *et al.*, 1994; Childress, 1995; Childress and Seibel, 1998; Boutilier, 2001; see Fig. 1.3). In other words, their critical oxygen partial pressure (P_{crit}) values are generally lower than the minimum O_2 level they encounter to favor diffusion pathways and O_2 uptake. Even gelatinous zooplankton (e.g. medusa and ctenophores) appear capable of regulation to very low PO_2 despite an apparent lack of O_2 transport systems or specific gas exchange surfaces. In such cases, weak diffusion gradients may simply be sufficient to meet low O_2 demand in species that are 95% water and whose actively metabolizing tissues are located towards the exterior of the animal (Thuesen *et al.*, 2005). However, crustaceans, fishes and cephalopods that permanently inhabit OMZs, commonly maximize their O_2 extraction potential by showing the following

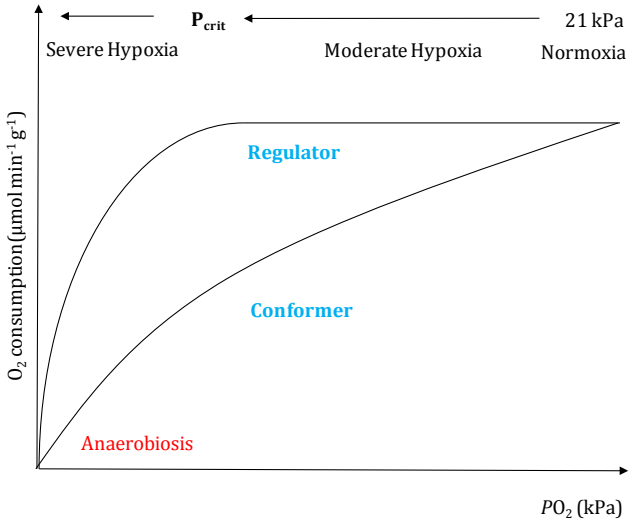

Figure 1.3 – General scheme O_2 consumption at different ambient partial pressures of oxygen (PO_2, kPa). Moderate hypoxia is compensated for by physiological mechanisms, whereas anaerobiosis commences during severe hypoxia and anoxia below the critical PO_2 (P_{crit}) (after Grieshaber *et al.* 1992).

adaptations: (1) high ventilatory volume and circulatory capacity (Belman and Childress, 1976), (2) thin blood-to-water diffusion distances across the gills including high gill surface areas (e.g. Childress, 1969; Antezana, 2002), and (3) high O_2 binding capacity of respiratory proteins via high pH sensitivity (large Bohr effect; Seibel and Walsh, 2003) and high O_2 binding affinity (low P_{50}; Seibel *et al.*, 1999).

Contrarily, diel vertical migrators that are able to survive severe hypoxic conditions for just a few hours, are usually oxyregulators and maintain their O_2 consumption rates until a certain P_{crit} by upregulating: (1) ventilatory (i.e. ventilation frequency, stroke volume: Grieshaber *et al.*, 1994) and circulatory (i.e. heart rate, blood flow: Jørgensen *et al.*, 1982; McMahon, 1988; Grieshaber *et al.*, 1994) performance, (2) gill diffusion capacity (i.e. elevation of perfused gill lamellae number; Randall, 1970, 1982; Randall and Daxboeck, 1984; Wu and Woo, 1985), (3) O_2 transport capacity (i.e. increased blood cell number; Randall, 1982; Soldatov, 1996), and (4) O_2 extraction efficiency from the ventilatory stream (Childress and Seibel, 1998).

But it is worth noting that the ventilatory strategies are highly species specific and dependent on species' overall potential to regulate ventilation and O_2 uptake. For instance, in vertical migrating krill, elevated gill surface area relative to species living in more oxygenated regions was discovered, suggesting enhanced O_2 extraction capabilities without the necessity to downregulate their metabolism, feeding or swimming performance (Antezana, 2002). Some high-performance predators (e.g. big-eye tuna, swordfish) that undergo vertical excursions into OMZs have managed to minimize the trade-offs inherent between high performance and hypoxia tolerance mainly due to the development of a highly pH dependent high O_2 affinity respiratory protein (Lowe *et al.*, 2000; Seibel, 2011). Moreover, O_2 storage ("breath-holding"), an attractive option for mammalian species, has been hypothesized for few fish and invertebrates (i.e. fish, nautilus and gelatinous organisms: Boutilier *et al*., 1996; Love *et al*., 2004; Thuesen *et al*., 2005), but this strategy for O_2 provision during migrations over greater depths into OMZs seems limited (Seibel, 2011). Anyhow, below P_{crit}, O_2 consumption rates start to fall rapidly in oxyregulators and conformers (Grieshaber *et al.*, 1994) resulting in reduced O_2 flow to tissues and activation of anaerobic pathways (see Fig. 1.3) to make up the difference between aerobic capacity and total metabolic demand (Childress and Seibel, 1998).

1.3.2. Metabolic strategies

Under O_2 deprivation, pre-existing energy demands cannot be sustained for more than a few minutes or hours as fermentable substrates are limitative and deleterious end products

accumulate (e.g. protons [H^+]; Boutilier, 2001). To cope with the severe impacts of hypoxia acting on energy production, hypoxia-tolerant marine organisms developed two different metabolic strategies.

The common strategy in permanent hypoxia residents (usually oxyconformers) is the maintenance of a low, routine metabolism, which is achieved by an effective O_2 extraction efficiency that permits continued reliance on aerobic metabolism (Childress and Seibel, 1998). In fact, their metabolic rates are usually orders of magnitudes lower than that of shallower-living species while showing similar gill diffusion capacities to those seen in active fishes and squids (Childress and Seibel, 1998; Wegner *et al.*, 2010). Marine animals with such reduced energy expenditures usually show low locomotory activity (i.e. *Vampyroteuthis infernalis*), decreased aerobic and anaerobic enzyme activity (Seibel *et al.*, 1998; Gonzales and Quinones, 2002), and enhanced pH sensitivity (e.g. Bohr coefficient) of their respiratory protein to facilitate sufficient O_2 release to the tissues (Childress and Seibel, 1998; Seibel, 2011). However, typical permanent OMZ residents are predominantly zooplankton species, including copepods, amphipods, chaetognaths and a variety of gelatinous species (Childress and Seibel, 1998; Seibel, 2011).

The second strategy, metabolic suppression, is a prerequisite for the survival of prolonged bouts of O_2 limitation to conserve energy expenditure (Guppy and Withers, 1999; Hochachka and Somero, 2002; Bickler and Buck, 2007), and its extent depends on species metabolic demand, phylogenetically available biochemical pathways, and its capacity for O_2 transport (Seibel, 2011). Commonly, organisms suppress their metabolism by 50–95% and supplement the remaining energy demand using a combination of available O_2 and anaerobic metabolic pathways (Rosa and Seibel, 2008, 2010; Seibel, 2010). The energy turnover downregulation is primarily achieved by shutting down expensive cellular processes (Hand, 1998), such as biosynthesis (Kwast and Hand, 1996), ion pumping (Buck and Hochachka, 1993), or the downregulation and/or modification of certain regulatory enzymes of anaerobic and aerobic pathways (Rahman and Storey, 1988; Storey, 1988; Dalla Via *et al.*, 1994; Hochachka, 1997). Such biochemical adjustments enable animals to enter a hypometabolic state. Thereby, ATP turnover rates and demands can be down regulated by more than an order of magnitude, and metabolic rates reduced by 5–20fold during hypoxia (Storey, 1996; Hochachka, 1997; Rosa and Seibel, 2008, 2010). As consequence, the arrest of protein synthesis, mainly controlled via phosphorylation and dephosphorylation of translation components (i.e. initiation and elongation factors; Grieshaber *et al.*, 1994), has to be coordinated with a blockage of protein decomposition in order to maintain structural integrity of the organism to make an instantaneous recovery possible when O_2 becomes

available again (Grieshaber *et al.*, 1994). A further task of energy limitation is the inability to maintain sensitive ion gradients over cell membranes, in particular the maintenance of the sodium (Na^+)/potassium (K^+) gradient, leading to a breakdown of the membrane potential, followed by cell damage or even cell death in hypoxia-sensitive tissues (Hochachka, 1986). However, in hypoxia-tolerant organisms (i.e. lower vertebrates, diving mammals) hypoxia-induced membrane destabilization is either slow to develop or might not occur at all as a result of adaptive decreases in membrane permeability (i.e. ion "channel arrest") that dramatically reduce the energetic costs of ion-balancing ATPases (Hochachka, 1986). Another important strategy to conserve energy under O_2 shortage is a reduction in the locomotory performance, for example by reducing activity frequencies, lowering speed or even turning into a lethargic state (Fischer *et al.*, 1992; Schurmann and Steffensen, 1994; Eriksson and Baden, 1997; Rosa and Seibel, 2008 and 2010). For example in vertically migrating shrimps and copepods of OMZs, a reduction in basal metabolism was found accompanied by lower locomotory activity (Svetlichny *et al.*, 2000; Cowles, 2001). The majority of organisms that use metabolic suppression (or depression) as strategy for hypoxia tolerance are diel vertically migrators of OMZs (mesopelagic micronecton species i.e. myctophids, euphausids; Seibel, 2011) and immobile benthic or intertidal species with low metabolic rates (i.e. bivalves, polychaetes and sipunculids; Pörtner *et al.*, 1984, 1986 and 1987; Oeschger, 1990; DeZwaan *et al.*, 1996; Abele, 2002). Nevertheless, metabolic suppression is a strategy to prolong the tolerance of low O_2 levels, but its potential is limited.

1.3.3. Anaerobic pathways

Under routine conditions in well-oxygenated waters, the energy demand of marine animals is met aerobically via mitochondrial oxidative phosphorylation. Under severe hypoxia (below P_{crit}), O_2 uptake is insufficient and has to be supplemented by anaerobic ATP production (Seibel, 2011; see Fig. 1.3). Thereby, the main biochemical substrate in fermentative pathways is glycogen (von Brand, 1946; Blazka, 1958; Hochachka and Somero, 1973), but its energy yield compared to oxidative phosphorylation is always modest (Hochachka and Somero, 2002). Some anaerobic metabolic pathways (i.e. those resulting in succinate and propionate accumulation) are more efficient than others (i.e. lactate or octopine), although still nowhere nearly as efficient as oxidative metabolism. Therefore, the extended utilization of anaerobic metabolic pathways requires a substantial supply of food or large reserve stores of fermentable substrate. A further consequence of anaerobic metabolism is the accumulation of deleterious end products. Anaerobic glycolysis results in the accumulation of protons as well as organic compounds such as lactate and octopine

(Hochachka and Somero, 2002), so consideration must be given to acid–base disturbances and intracellular acidosis (Hochachka and Mommsen, 1983). Some anaerobic pathways also produce volatile end-products like ethanol, acetate or propionate that can be excreted directly across the gills, allowing their continued use without risk of acid–base disturbance (Stecyk *et al.*, 2004), but, counterproductively, their remaining energy is lost and cannot be recycled (Seibel, 2011). Thus, despite the disadvantages accompanied by fermentative pathways, anaerobic metabolic capacity is elevated at severe hypoxia.

However, the metabolic strategy to catabolize glycogen under hypoxia has been discovered in bottom animals like gastropods, bivalves and sluggish fish (Shulman *et al.*, 2002), with major carbohydrate reserves and glycogen stores as high as 5% of total body weight (i.e. bivalves; Giese, 1969). Active pelagic organisms like pelagic fish and cephalopods, in turn, have low glycogen storage potential (i.e. < 0.4% in squids; Rosa *et al*., 2005), and therefore a few minutes of activity can significantly deplete them (Storey and Storey, 1983; Shulman *et al.*, 2002).

Fast moving organisms (i.e. pelagic fishes) generally fuel their Krebs cycle with lipids and carbohydrates, but the carbon input from both sources is essentially limited at a single point, the addition of acetyl-CoA to oxaloacetate (O'Dor and Wells, 1986). Under extreme conditions like at late stages of salmon migrations, fishes switch to fuel their metabolism with proteins (Mommsen *et al.,* 1980) like less active animals typically do (Fischer, 1970; Shulman and Love, 1999). Cephalopods maximally catabolize proteins (Campbell and Bishop, 1970; Hochachka *et al.*, 1975; Storey and Storey, 1978; O'Dor and Wells, 1986; Shulman *et al.*, 1984 and 1993; Lamarre *et al*., 2012), which is probably a prerequisite from developing from slow moving mollusks. Moreover, available evidence does indicate that squids, at least, under extreme conditions like at the end of their migrations show high levels of muscle proteolysis due to fasting and exercise (O'Dor *et al*., 1984; Shulman *et al*., 2002).

Proteins have much to recommend as an energy reserve, as: (1) the energy yield per unit weight is higher than that of carbohydrates (4.7 vs. 4.0 kcal g^{-1}; Brett and Groves, 1979) in animals that produce ammonia as the primary metabolic end product, and (2) when produced in the form of muscle proteins it is an active aid to locomotion rather, than an inert hindrance (O'Dor and Wells, 1978). It has been shown that one of the most important mechanisms of adaptations, which provide normal existence of planktonic crustaceans and fish in oxygen deficiency, may be anaerobic utilization of protein in energy metabolism. These data are supported by physiological research on whole organism (Schmidt-Nielsen, 1975; Douglas *et al*., 1976; Shulman *et al*., 1993; Svetlichny *et al*., 1998), as well as

subcellular and molecular studies (Hochachka *et al*., 1973). A similar phenomenon was noted for some predatory fish after hunting (Sukumaran and Kutty, 1977). Under well-oxygenated conditions the nektonic squid *Sthenoteuthis oualaniensis* even uses a considerable amount of the protein substrates (up to 50%) anaerobically due to "overfeeding", an adaptation that was linked to hypoxia tolerance (Shulman *et al*., 2002). This example of a peculiar functional hypoxia was connected, not with O_2 deficiency in the external environment, but with the necessity to utilize "endogenous" O_2, which is formed during catabolism of reserve substances and probably from tissue destruction (Shulman *et al*., 1993). Moreover, many invertebrates are known to use the large free amino acid pool as an important potential source of energy under hypoxia as it represents astonishing energetic advantages over lactic acid formation (Hochachka *et al.*, 1973). Nevertheless, the full picture of how the complete mix of the amino acids produced can be used for energy and the extent to which protein and amino acid catabolism fuels muscle itself is still not available (O'Dor and Webber, 1986).

1.3.4. Antioxidant defense

In aerobic organisms, oxidative stress occurs when reactive oxygen species (ROS) cause damage that cannot be balanced by the organism's antioxidant defense system. ROS are molecules derived from oxygen, such as the superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^{\cdot}$), with the latter one being the most reactive and destructive one (Pannunzio and Storey, 1998). ROS species are mostly formed in mitochondria during O_2 reduction in the electron transport chain and can damage biological macromolecules (i.e. lipids, proteins and DNA; for review see Halliwell, 2006; Halliwell and Gutteridge, 2006), resulting in functional alterations in cells and tissues. The most frequent cellular injury caused by non-neutralized ROS formation is called "peroxidation", which is the reaction of ROS with organism's lipids, especially membrane-associated ones (Lesser, 2012). One of the expected effects of hypoxia is the generation of ROS promoted by the transition between hypoxia and reoxygenation states (i.e. during upward migrations from OMZs or incoming tides), as reoxygenation increases O_2 consumption with each cell generating about 0.1% ROS per molecule O_2 consumed (Fridovich, 2004). Many molecules, like reduced glutathione (GSH), vitamins, heat shock proteins and antioxidant (AOX) enzymes, can eliminate or change the molecular configuration of ROS and by that protect organisms from cellular damage (Tremblay *et al.*, 2010).

The most important enzymes to promote an efficient AOX defense system are: (1) superoxide dismutase (SOD), which transforms $O_2^{\cdot-}$ to H_2O_2, (2) catalase (CAT), which converts H_2O_2 into H_20 and O_2 independently of any substrate and thereby inhibits its

accumulation in cells and tissues, (3) glutathione reductase (GR), which furnishes cells with the antioxidant glutathione (GSH, i.e. by reacting with $O_{2}^{\cdot-}$ and $OH^{\cdot}$) and further supplies the enzyme activity of glutathione peroxidase (GPx) and glutathione-S-transferase (GST), (4) GPx, which also eliminates H_2O_2 using GSH as substrate, and (5) GST, which, in association with GSH, transforms xenobiotics into other conjugates as part of a detoxification route (Lesser, 2006).

The expression of the heat shock protein 70 (Hsp70) has also been reported to be induced under hypoxic conditions (Cheng *et al.*, 2003) by functioning as molecular chaperones and preventing protein unfolding among other important roles (Iwama *et al.,* 1998 and 1999). Thereby, Hsp70 can be elevated several hundred times compared to non-stress conditions (Chuang *et al.*, 2007).

However, several studies have shown that, during oxygen-restricted periods, some aquatic organisms (e.g. marine worms, gastrotrichs and turbellarians: Lesser, 2006; crustaceans: Romero *et al.*, 2007; Desai and Prakash, 2009; fishes: Cooper *et al.*, 2002; Hermes-Lima and Zenteno-Savín, 2002;) activate antioxidant enzymes and heat shock proteins (Cheng *et al.*, 2003; Teixeira *et al.*, 2013) as a biological tool to minimize post-hypoxic oxidative damage resultant from the reoxygenation.

1.4. Cephalopods

In the aquatic environment, fish can be considered the group of animals that reached the highest degree of complexity, in terms of sensory and locomotive ability. Among all invertebrates there is no phylum that has reached a comparable complexity, except for the cephalopods. Cephalopods have a highly developed neural system with very efficient sensory organs (i.e. lens eyes, chemo-receptors, balance receptors and the ability to detect under water sound; Budelmann *et al.*, 1997; Hu *et al.*, 2009). It is believed that these features were derived from the competition of cephalopods and fish since the so called "Cambrian explosion" around 500 million years ago (O'Dor and Webber, 1986; Smith and Caron, 2010). Although many features (e.g. vision, activity and metabolism) of these two groups have been described as convergent, several anatomical and physiological features of cephalopods constrain their competition with fish. For example, the typical way of locomotion in cephalopods, jet propulsion, enables a high degree of mobility but at high energetic costs compared to undulatory swimming movements of fish (Wells and O'Dor, 1991; Webber *et al.*, 2000; O'Dor, 2002). In fact, there is no way around this fundamental inefficiency of the jet propulsion system. Although they evolved some compensations

(i.e. lower drag, because squid can maintain a streamlined shape unlike undulatory fish), the best performing squid studied to date still uses more than twice as much energy to travel half as fast as an average fish (Webber and O'Dor, 1985; O'Dor and Webber, 1986; Webber and O'Dor, 1986; O'Dor and Webber, 1991). In addition, ventilatory and locomotory systems in cephalopods are closely tied, resulting in a fundamental incompatibility between efficient O_2 uptake and efficient jet propulsion (Wells and O'Dor, 1991). Moreover, the oxygen-carrying capacity in cephalopods is limitative relative to that of fishes, because of (1) low venous oxygen reserve storage (Pörtner, 2002), (2) viscosity-related constraints (Pörtner, 2002), and (3) an extracellular, low-oxygen-affinity respiratory protein (Pörtner, 2002). Although considerable evolutionary refinements have developed in the respiratory pigment of cephalopods, it is only able to carry about half the O_2 of the cellular hemoglobin of vertebrates (Brix *et al.*, 1989). A major improvement of the O_2 transport efficiency in cephalopod hemocyanins is its high pH dependency (i.e. large Bohr-effect; Pörtner, 1990), a property, combined with its low O_2 affinity, that facilitates fast O_2 release to the demanding tissues (Pörtner, 2002; Seibel, 2011, 2013). Moreover, the fact that cephalopod hemocyanins are highly pH sensitive requires tightly regulated blood pH homeostasis in organisms that have strong metabolic rate fluctuations (Pörtner, 1994; Pörtner and Zielinski, 1998). Octopine formation during exercise and hypoxia is clearly part of the molluscan heritage (Gäde, 1980) and accompanied by H^+ production causing acid-base and blood pH disturbances that presumably interferes with efficient O_2 uptake at the gills (Pörtner, 1994, 2002; Melzner *et al.*, 2007; Seibel, 2011, 2013).

However, the remarkable prevalence of cephalopods in the world oceans and their large fraction to the total biomass highlights their successful life strategy: Live fast, die young. Reliable data on squid numbers are not available, but it has been estimated that sperm whales alone eat about 100 million tons of squid per year (Gosline and DeMont, 1985), which is more than the total catch of fish by the world's commercial fishermen ($\sim$ 90 million tons; FAO, 2012).

1.5. Ecophysiology of *Dosidicus gigas*

Dosidicus gigas (jumbo or Humboldt squid) is the largest ommastrephid squid (up to 2.5 m length and 50 kg in mass; Nesis, 1983) and has one the highest metabolic rates of any animal in the ocean (Rosa and Seibel, 2008). The jumbo squid is endemic to the Eastern Pacific, and particularly abundant in the highly productive waters of the California and Peru Current systems (Nigmatullin *et al.*, 2001) and the Costa Rica Dome (Ichii *et al.*, 2002; Waluda and Rodhouse, 2006), where he plays a crucial role both as predator (Markaida and Sosa-

Nishizaki, 2003) and prey (Clarke and Paliza, 2001; Abitía-Cárdenas *et al.*, 2002; Ruiz-Cooley *et al.*, 2004). In fact, this species can easily remove more than 4 million tons of food per year from the pelagic food web (Shulman *et al.*, 2002). Besides its ecological role, the Humboldt squid also plays an important economically role being target of the world's largest cephalopod fishing industry (Rodhouse *et al.*, 2006) with around 14% of world's total squid catch and landings estimated at 818,000 tons in 2006 (FAO, 2010). In addition to seasonal horizontal migrations (Markaida *et al.*, 2005), *D. gigas* undergoes diel vertical migrations into mesopelagic depths (250-300m) during day time (Gilly *et al.*, 2006; Trueblood and Seibel, 2013), assumingly to follow their preys (Markaida and Sosa-Nishizaki, 2003; Markaida *et al.*, 2008). In this way they also escape from: (1) unfavorable warmer sea surface temperatures, and (2) elevated predation pressure and resource (food) competition, as most active fish predators (i.e. marlin, swordfish and sharks) are excluded from the OMZ (Prince and Goodyear, 2006). While at depth *D. gigas* encounters the permanent OMZ of the Eastern Tropical Pacific with O_2 levels often less than 0.5 kPa O_2 (Kamykowski and Zentara, 1990; Morrison *et al.*, 1999). In fact, jumbo squid spend more than 70% of the day below 200 m (≤ 2% O_2 = 2 kPa = ~ P_{crit}) (Gilly *et al.*, 2006; Trueblood and Seibel, 2013; Seibel, 2013) and might even actively descent into such oceanic dead zones to escape from his high metabolic demands (Seibel, 2013). One might expect that the presence of active, muscular squids in hypoxic zones would be precluded (Pörtner, 2002), as consequence of their physiological and anatomical restraints (Pörtner, 1994, 2002; Melzner *et al.*, 2007). Recently, a highly temperature and pH sensitive high O_2-affinity hemocyanin was discovered in *D. gigas,* which facilitates O_2 uptake in cold, deep waters where O_2 is scarce and enhances O_2 release in warmer surface waters where O_2 and O_2 demand are elevated (Seibel, 2013). Further, metabolic suppression has been suggested as strategy to overcome the counterproductive demands of high-performance and hypoxia tolerance (Rosa and Seibel, 2008, 2010). Nevertheless, little is known about the biochemical and physiological mechanisms *D. gigas* uses to lead such an extraordinary life.

1.6. Objectives and hypotheses

Hence, the aim of my thesis is to identify physiological and biochemical mechanisms that *D. gigas* uses to tolerate the harsh conditions prevailing in OMZs. As oceanic dead zones are currently expanding horizontally and vertically with a steady decrease in O_2 level, the habitat of *D. gigas* might be endangered. Therefore, the potential to tolerate hypoxia and to adapt to a changing ocean is important to forecast its future survival and competitiveness.

a) Can D. gigas *regulate its ventilatory mechanisms to increase the O_2 uptake capacity?*

Aquatic animals respond to hypoxia by first attempting to maintain O_2 delivery (Boutilier *et al.*, 1988; Chew and Ip, 1992; Randall *et al.*, 1992; Dalla Via *et al.*, 1994; Hochachka, 1997), as the energy yield of aerobic metabolism is much higher compared to anaerobic metabolic pathways (Hochachka and Somero, 2002). Pelagic muscular squids that routinely depend on jet propulsion, are characterized by a low O_2 extraction efficiency (5–10%; Wells *et al.*, 1988; Pörtner, 1994), as the mechanisms of locomotion and ventilation are closely tied via jet-propulsion (Wells and O'Dor, 1991), and their potential to elevate O_2 uptake from the ventilatory stream according to falling ambient O_2 levels or exercise are low (15–20%; Wells *et al.*, 1988; Wells, 1990; Pörtner, 1994). Moreover, squids are thought to live chronically 'on the edge of O_2 limitation', as their oxygen-carrying capacity is limitative and therefore are not well poised to adapt to low ambient O_2 levels (Pörtner, 2002). In order to determine the aerobic regulatory capacity of juvenile *D. gigas* diverse ventilatory parameters and mechanisms were examined.

b) Does D. gigas *use metabolic suppression as strategy to survive prolonged descents into severe hypoxia?*

At severe hypoxia (below P_{crit}), aquatic animals are not able to maintain routine metabolic rates solely aerobically, and have to supplement their energy demand using anaerobic pathways (Childress and Seibel, 1998; Seibel, 2011). As fermentable substrates are limitative and its degradation causes deleterious end product accumulation (e.g. protons; Boutilier, 2001), a common strategy for the survival of prolonged bouts of O_2 limitation is metabolic suppression (Guppy and Withers, 1999; Hochachka and Somero, 2002; Bickler and Buck, 2007). Recent studies suggest that *D. gigas* uses metabolic suppression during daily excursions into oceanic dead zones (Rosa and Seibel, 2008, 2010) and might even actively descent into such mesopelagic depths to escape from its high metabolic demands. In order to understand the potential of *D. gigas* to suppress its metabolism under severe hypoxia, both aerobic and anaerobic ATP production pathways were thoroughly investigated.

c) Does D. gigas *use anaerobic (muscle) protein degradation as mechanism to increase the energy output and extend hypoxia exposure time?*

In well-oxygenated waters, cephalopods maximally catabolize proteins as primary energy substrate (Campbell and Bishop, 1970; Hochachka *et al.*, 1975; Storey and Storey, 1978; O'Dor and Wells, 1987; Shulman *et al.*, 1984, 1993). Furthermore, under well-oxygenated

conditions some nektonic squids (e.g. *S. oualaniensis*) are known to use considerable amount of the protein substrates (up to 50%) anaerobically due to "overfeeding", an adaptation that was linked to its increased hypoxia tolerance (Shulman *et al*., 2002). It is known that many invertebrates use the large free amino acid pool as an important potential source of energy under hypoxia as it represents astonishing energetic advantages over lactic acid/octopine formation (Hochachka *et al.*, 1973), but the full picture of how the complete mix of the amino acids produced can be used for energy and the extent to which amino acid catabolism fuels muscle itself is still not available (O'Dor and Webber, 1986). In order to know the potential of *D. gigas* to use anaerobic (muscle) protein degradation, protein expression profiles and protein identification techniques (e.g. MALDI-TOF/TOF analysis) were performed.

d) Does D. gigas *reduce its level of activity as strategy to conserve energy under severe hypoxia although locomotion and respiration in cephalopods are closely tied?*

In previous studies, a certain periodicity in the rate of O_2 consumption (activity) was discovered in juvenile jumbo squids (cycle length ~20 min) that disappeared at severe hypoxia accompanied by the onset of lethargy (Rosa and Seibel, 2008, 2010). Reduction of locomotory activity is a common strategy employed to safe energy under hypoxia. In the Atlantic cod fish *Gadus morhua* for example the motility was reduced by 60% when the dissolved O_2 concentration fell below 3 mg O_2 l^{-1} (Schurmann and Steffensen, 1994), whereas the eelpout *Zoarces viviparous* rested nearly motionless at the bottom when the O_2 level dropped below 2.9 mg O_2 l^{-1} (Fischer *et al.*, 1992) and the Norway lobster *Nephrops norvegicus* ceased its digging behavior when O_2 was reduced to 2.5 mg O_2 l^{-1} (Eriksson and Baden, 1997). Gilly and colleagues (2006), via acoustic tagging, discovered a rhythmic diving pattern in jumbo squids at day time that has been as robust as under normoxic conditions, a behavior that was linked to active forages. Contrarily, submersible observations by Seibel (unpublished) revealed that *D. gigas* is lethargic in the OMZ but, at least under the bright lights of the submersible, is capable of capturing prey during the day in severe hypoxia. In order to identify different activity levels in juvenile jumbo squids, individual respiratory runs and video tapes were analyzed and synchronized under normoxic and severe hypoxic conditions.

e) Does D. gigas *possess an efficient antioxidant defense mechanism to prevent cellular damage during the reoxygenation phase while ascending?*

In diel vertical migrators of OMZs, the formation of reactive oxygen species (ROS) is expected during (1) upward migrations (the transition between hypoxia and reoxygenation states), as temperature and concomitantly O_2 consumption increases with each cell generating about 0.1% ROS per molecule O_2 consumed (Fridovich, 2004), and (2) hypoxia exposure itself, as low O_2 supply changes the mitochondrial redox state resulting in reduced electron transport in the lower part of the respiratory chain (Brand, 2000; Hochachka and Lutz, 2001; Schumacker, 2003). Many molecules, like reduced glutathione (GSH), vitamins C and E, heat shock proteins and antioxidant (AOX) enzymes, are known to eliminate or change the molecular configuration of ROS and by that protect organisms from cellular damage (Tremblay *et al.*, 2010). Several studies have shown that, during oxygen-restricted periods, some aquatic organisms (e.g. marine worms, gastrotrichs and turbellarians: Lesser, 2006; crustaceans: Romero *et al.*, 2007; Desai and Prakash, 2009; fishes: Cooper *et al.*, 2002; Hermes-Lima and Zenteno-Savín, 2002) activate antioxidant enzymes and heat shock proteins (Cheng *et al.*, 2003; Teixeira *et al.*, 2013) as a biological tool to minimize post-hypoxic oxidative damage resultant from the reoxygenation. Here, I quantified the antioxidant defense system of juvenile jumbo squids, namely the different AOX enzyme activities and heat shock response.

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RESEARCH ARTICLE

Ventilation rates and activity levels of juvenile jumbo squid under metabolic suppression in the oxygen minimum zone

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SUMMARY

The Humboldt (jumbo) squid, *Dosidicus gigas*, is a part-time resident of the permanent oxygen minimum zone (OMZ) in the Eastern Tropical Pacific and, thereby, it encounters oxygen levels below its critical oxygen partial pressure. To better understand the ventilatory mechanisms that accompany the process of metabolic suppression in these top oceanic predators, we exposed juvenile *D. gigas* to the oxygen levels found in the OMZ (1% O_2, 1 kPa, 10°C) and measured metabolic rate, activity cycling patterns, swimming mode, escape jet (burst) frequency, mantle contraction frequency and strength, stroke volume and oxygen extraction efficiency. In normoxia, metabolic rate varied between 14 and 29 μmol O_2 g^{-1} wet mass h^{-1}, depending on the level of activity. The mantle contraction frequency and strength were linearly correlated and increased significantly with activity level. Additionally, an increase in stroke volume and ventilatory volume per minute was observed, followed by a mantle hyperinflation process during high activity periods. Squid metabolic rate dropped more than 75% during exposure to hypoxia. Maximum metabolic rate was not achieved under such conditions and the metabolic scope was significantly decreased. Hypoxia changed the relationship between mantle contraction strength and frequency from linear to polynomial with increasing activity, indicating that, under hypoxic conditions, the jumbo squid primarily increases the strength of mantle contraction and does not regulate its frequency. Under hypoxia, jumbo squid also showed a larger inflation period (reduced contraction frequency) and decreased relaxed mantle diameter (shortened diffusion pathway), which optimize oxygen extraction efficiency (up to 82%/34%, without/with consideration of 60% potential skin respiration). Additionally, they breathe 'deeply', with more powerful contractions and enhanced stroke volume. This deep-breathing behavior allows them to display a stable ventilatory volume per minute, and explains the maintenance of the squid's cycling activity under such O_2 conditions. During hypoxia, the respiratory cycles were shorter in length but increased in frequency. This was accompanied by an increase in the number of escape jets during active periods and a faster switch between swimming modes. In late hypoxia (onset ~170±10 min), all the ventilatory processes were significantly reduced and followed by a lethargic state, a behavior that seems closely associated with the process of metabolic suppression and enables the squid to extend its residence time in the OMZ.

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Key words: hypoxia, OMZ, jet propulsion, ventilation, jumbo squid, *Dosidicus gigas*, metabolic suppression.

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INTRODUCTION

Dosidicus gigas (jumbo or Humboldt squid) is the largest ommastrephid squid (up to 2.5 m length and 50 kg in mass) (Nesis, 1983) and has one the highest metabolic rates of any animal in the ocean (Rosa and Seibel, 2008). It is endemic to the Eastern Pacific, and particularly abundant in the highly productive waters of the California and Peru Current systems (Nigmatullin et al., 2001) and the Costa Rica Dome (Ichii et al., 2002; Waluda and Rodhouse, 2006), where it plays a crucial role both as prey (Clarke and Paliza, 2001; Abitía-Cárdenas et al., 2002; Ruiz-Cooley et al., 2004) and predator (Markaida and Sosa-Nishizaki, 2003). In addition to its ecological role, the Humboldt squid is an economically important species and the target of the world's largest cephalopod fishing industry (Rodhouse et al., 2006).

In addition to seasonal horizontal migrations (Markaida et al., 2005), *D. gigas* undergoes diel vertical migrations into mesopelagic depths (around 250 m) during the daytime (Gilly et al., 2006) (Trueblood and Seibel, 2012), similar to the migration pattern of their primary prey – myctophid fishes (Markaida and Sosa-Nishizaki, 2003; Markaida et al., 2008). While at depth, *D. gigas* encounters the permanent oxygen minimum zone (OMZ) of the Eastern Tropical Pacific during the day, with oxygen levels often less than 5 μmol l^{-1} (~0.5% O_2) (Kamykowski and Zentara, 1990; Morrison et al., 1999). Other top pelagic predators (e.g. tuna, swordfish, marlin and sharks) (Brill, 1994; Prince and Goodyear, 2006; Vetter et al., 2008; Nasby-Lucas et al., 2009; Stramma et al., 2012) seem to avoid these hypoxic depths, as their tolerance to hypoxic conditions is low [<150 μmol l^{-1} O_2 (Brill, 1994; Brill, 1996); ~45 μmol l^{-1} O_2, big-eye tuna (Lowe et al., 2000)]. One might expect that the presence of active, muscular squid in these hypoxic zones would be precluded, as, in other active squid: (i) the primary mode of locomotion, jet propulsion, is energetically inefficient (Webber et al., 2000); (ii) the oxygen-carrying capacity is limitative relative to that of fishes, requiring that cephalopods use most of the O_2 in the blood on each

cycle, leaving little venous oxygen reserve behind (Pörtner, 2002); (iii) the oxygen-carrying capacity is low because of viscosity-related constraints and an extracellular low-affinity respiratory protein (Pörtner, 2002); and (iv) ventilatory and locomotory systems are closely tied. Regarding the last of these, two different strategies evolved in these mollusks to deal with this restriction. Pelagic squid (like *D. gigas*) that routinely depend on jet propulsion optimize their locomotory apparatus by pushing a large water volume through their mantle cavity, reducing oxygen extraction efficiency (5–10%) (Pörtner, 1994; Wells et al., 1988). In contrast, nekton-benthic cephalopods (i.e. cuttlefish and octopus) developed an efficient oxygen uptake system (40–50%) (Wells and Wells, 1985) by minimizing their ventilatory volume to increase diffusion (Wells et al., 1988).

Squid are thought to live chronically 'on the edge of oxygen limitation' (Pörtner, 2002) and are not well poised to adapt to low ambient O_2 levels. Yet, the Humboldt squid has managed to minimize the trade-offs between high locomotory performance and hypoxia tolerance *via* metabolic suppression (Rosa and Seibel, 2008; Rosa and Seibel, 2010), coupled with a high-affinity respiratory protein (Seibel, 2012). Here, we exposed juvenile squid to oxygen levels found in the OMZ (1% O_2, 1 kPa, 10°C, severe hypoxia) to investigate the effects on the ventilatory mechanisms that accompany the process of metabolic suppression in *D. gigas*, namely: (i) maximal, active, routine and inactive metabolic rates; (ii) metabolic scope; (iii) activity cycling patterns and swimming mode (with video recording); (iv) escape jet (burst) frequency; (v) mantle contraction frequency and strength; (vi) stroke volume, and ventilatory volume per minute; and (vii) oxygen extraction efficiency (with and without a potential contribution from skin respiration).

MATERIALS AND METHODS

Specimen collection

Juvenile Humboldt squid, *D. gigas* (d'Orbigny 1835), (5.4–13.5 g wet mass) were collected *via* dip net in the Gulf of California (27°N, 111°W; 28°N, 113°W), on the surface at night, in June 2011 (aboard the RV *New Horizon*, Scripps Institute, CA, USA) and were immediately transferred to aquaria containing 10°C seawater (environmental temperature at OMZ) on board the vessel. It is worth noting that all experiments were conducted with juvenile stages, and it is not known whether juvenile and adult jumbo squid display similar diel vertical migration behavior (i.e. whether they encounter the same minimum oxygen levels during descent).

Experimental procedure

Animals were placed in a flow-through respirometry set up (270 ml volume, Loligo Systems, Tjele, Denmark) (Rosa and Seibel, 2008; Rosa and Seibel, 2010), and allowed to acclimate for 8–12 h before measurements of oxygen consumption were started. Respirometers were immersed in a large thermostatically controlled waterbath (Lauda, Lauda-Königshofen, Germany) at 10°C, a temperature approximating that found at 250 m in the OMZ (Rosa and Seibel, 2010). Filtered (0.2 µm) and treated (50 mg l^{-1} streptomycin) seawater was pumped from a water-jacketed, gas-equilibration column through the respirometers at a constant flow rate (average 120 ml min^{-1}). The water in the column was bubbled continuously to maintain incoming water at high (21% O_2, 21 kPa,) or low P_{O_2} (certified gas mixture with 1% O_2, 1 kPa). Once the final 1% O_2 level in the respiration chambers had been reached, the first 30 min were excluded, as they may represent an acclimatory phase after adjusting to a new oxygen concentration. Oxygen concentrations were recorded at the entrance and the exit of each chamber with two Clarke-type O_2 electrodes connected to a 928 Oxygen Interface (Strathkelvin Instruments, North Lanarkshire, UK). The system was calibrated using air- and nitrogen-saturated seawater and checked for electrode drift and for microbial oxygen consumption before and after each trial. All experiments were carried out in darkness and at atmospheric pressure. Video recordings were conducted (Sony DCR-SR78, Lisbon, Portugal) during respiration runs. Afterwards, specimens were immediately weighed on a motion-compensated precision shipboard balance system (Childress and Mickel, 1980). A total of 18 specimens were investigated, with 6 replicates for the normoxic treatment (21% O_2, 21 kPa) and 12 for the hypoxic one (1% O_2, 1 kPa). Because of differences in squid swimming behavior (i.e. mantle contraction frequency per minute) and respiratory profile pattern (i.e. number of cycles) throughout hypoxia, this treatment was subdivided into early hypoxia (EH, hypoxia exposure time 30–160 min, N=6) and late hypoxia (LH, hypoxia exposure time >180 min, N=6). Exposure times are presented in Table 1.

Swimming behavior, metabolic rate and cycling performance

Swimming behavior

Jet propulsion was subdivided into three swimming modes, based on previous studies (Gosline et al., 1983; Bartol, 2001); namely: (1) respiratory movements, (2) steady jetting and (3) bursts/escape jets *via* video footage. Respiratory movements were defined by weak mantle contractions without visible thrust. Thereby, squid could be within the water column or, under LH, even lie at the bottom of the chambers. Steady jetting ranged from slow cruising (weak but visible thrusts) to vigorous jetting (powerful thrusts). Bursts or escape jets were characterized by hyperinflation (≥5% increase of relaxed mantle diameter) followed by maximum mantle contraction (Gosline and DeMont, 1985) and fast movements. The relaxed diameter was assessed under normoxia at inactive metabolic rate (IMR) using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA), as mantle contractions at slow swimming speeds occur without hyperinflation (Gosline and DeMont, 1985). The number of escape jets per minute was quantified *via* video analysis.

Metabolic rate

The number of escape jets per minute and the swimming modes were linked to changes in O_2 consumption during cycling periods. Based on this, inactive, routine, active and maximum active metabolic rate (IMR, RMR, AMR and MR_{max}, respectively) could be distinguished (see Table 2, Fig. 1) with similar results to those of a previous study (Rosa and Seibel, 2008). RMR for normoxia and hypoxia was specified as the average rate for the entirety of

Table 1. Exposure times of *Dosidicus gigas* to normoxic (21% O_2) and hypoxic (1% O_2) conditions

	Exposure time (min)		Onset of late hypoxia (min)
N	Normoxia	Hypoxia	
1	662	199	153
2	762	190	164
3	263	189	182
4	263	202	183
5	560	198	163
6	881	188	153
Mean	582	194	166
s.d.	268	6	13

Values are presented individually and as means and standard deviations. Exposure times for hypoxia include acclimation time (30 min).

Table 2. Different activity levels in *Dosidicus gigas*

	MR_{max}	AMR	RMR	IMR
% RMR	≥35	≥15 to <35		≥ –15
Swimming mode	2–3	1–3	1–3	1 (≥75%)
Burst frequency (bursts min^{-1})	≥4	≥4	<4	<4

Activity levels were defined as inactive, routine, active and maximum active metabolic rate (IMR, RMR, AMR and MR_{max}) and are given in terms of burst frequency (bursts min^{-1}) and swimming behavior (mode and % of time for IMR), resulting in percentage classes of oxygen consumption rate based on RMR (see Rosa and Seibel, 2008). Note that RMRs were specified separately for normoxic and hypoxic conditions and quantified as the average of the entire recording. Swimming modes were classified based on previous criteria (Gosline et al., 1983; Bartol, 2001): 1, respiratory movement; 2, steady jetting; and 3, burst/escape jetting.

each recording. During periods of activity, metabolic rate was ≥15–35% (AMR) and >35–100% (MR_{max}) higher than the routine levels (RMR) with escape jets ≥4 bursts min^{-1}. Thus, the AMR and MR_{max} were quantified by averaging the metabolic rates for all peaks exceeding ≥15% or >35%, respectively. Inactive metabolic rate was defined *via* swimming mode 1 (≥75% of time) and burst events <<4 min^{-1}. These periods of apparent inactivity in the chambers correlated with metabolic rates ≤15% lower than RMR and average rates were calculated. Note that pelagic predators such as *D. gigas* rarely stop swimming in nature and that we did not quantify locomotion continuously for all experiments in its entirety. However, our IMR calculations seem to be a reasonable approximation to the criteria set for standard metabolic rate in mammals and other model organisms. Metabolic rate was quantified as μmol O_2 g^{-1} wet mass h^{-1}. The metabolic scope was calculated as the difference between maximum and minimum O_2 consumption.

Cycling performance

All control (normoxia) animals and the majority in early hypoxia (5 out of 6) animals showed a distinct periodicity in their rate of oxygen consumption (Fig. 1). These respiratory cycles are characterized by a steep increase in metabolic rate (~first 1/3 of total cycle length, Fig. 1) and video analysis confirmed that this was linked to an elevation in mantle contraction frequency. The remaining cycle length (Fig. 1) consisted of a slow transition from powerful to weaker mantle contractions. Therefore, burst frequency (number of escape jets per minute, calculated over the entire cycle length) and swimming modes were used to describe those respiratory cycles. Periodicity was further investigated by assessing the number of active and maximum active cycles per hour, including their length in minutes (*via* respiratory files).

Mantle contraction frequency and strength

In cephalopods, locomotion and respiration are closely tied and therefore counterproductive in their goals, even though in resting *Sepia* and probably to some extent in squid, ventilatory and locomotory mechanisms can be uncoupled *via* a collar flap system (Bone et al., 1994). However, both ventilatory and locomotory systems are highly dependent on the frequency and strength of mantle contractions, which, in turn, influence the water volume throughput. Mantle contraction frequency (ventilation rate) was quantified as the number of mantle contractions per minute

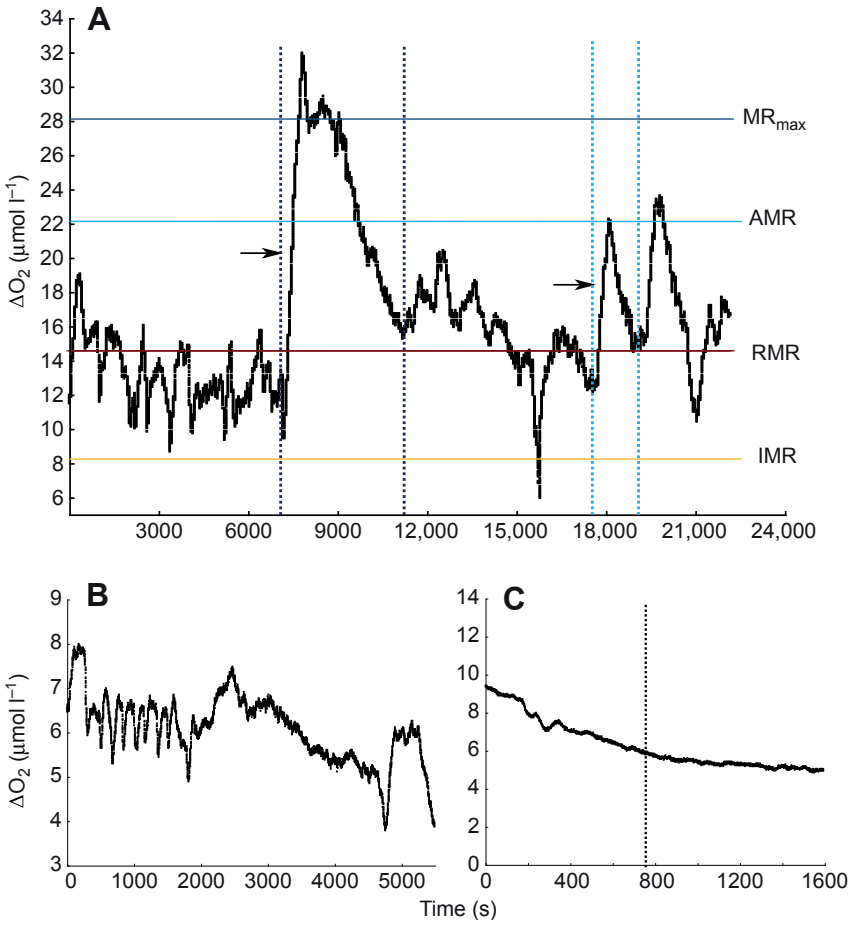

Fig. 1. Oscillations in oxygen levels (ΔO_2) that reflect differences between the values recorded at the entrance and the exit of the chamber in normoxia (A, 10.9 g squid), early hypoxia (B, 1% O_2, 5.4 g squid) and late hypoxia (C, 11.6 g squid) at 10°C. Solid lines in A represent the thresholds for inactive (IMR), routine (RMR), active (AMR) and maximum metabolic rate (MR_{max}) (illustrated in Table 2). Black arrows indicate the acceleration phase of MR_{max} and AMR cycles, and the vertical dashed lines mark the respective cycle lengths. The dashed line in C represents the onset of late hypoxia (170±10 min, *N*=6). Note, the 0 value on the *x*-axis in the hypoxia plots (B,C) does not represent the real onset of hypoxia.

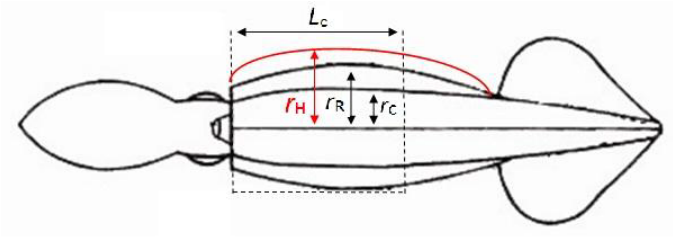

Fig. 2. Schematic drawing of a squid with descriptions of the measurements used to quantify ventilation stroke volume and hyperinflation in *Dosidicus gigas*. Arrows indicate cylinder length (L_c, 0.54 of total mantle length in *D. gigas*), relaxed mantle radius (r_R, recorded during IMR periods in normoxia), contracted mantle radius (r_C) and hyperinflated mantle radius (r_H).

(MC min^{-1}) by video footage analysis. The strength of mantle contraction was calculated as the difference between maximum and minimum mantle diameter (in mm) using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

Hyperinflation and stroke volume quantification

During escape jets, squid powerfully contract their radial muscles and, consequently, induce an increase in the outer mantle diameter (between 5% and 10% of relaxed mantle diameter), a process called hyperinflation (represented by the red line in Fig. 2). The relaxed mantle diameter (Fig. 2) was recorded during the IMR periods in normoxia. Changes in the relaxed diameter were determined as a percentage of the relaxed mantle diameter specified at IMR under normoxia.

Stroke volume (V_w, water throughput per mantle contraction) was quantified based on a modified O'Dor (O'Dor, 1988) equation:

$$V_w = [r_H^2 - (r_C - x)^2]\,(0.54\pi\, L) - (0.15M/d_s)\,, \quad (1)$$

where, r_H represents the maximum (hyperinflated) mantle radius (see also Fig. 2), r_C is the contracted mantle radius, x is the mantle thickness (mm), L is the mantle length (the upper mantle is treated as a cylinder of changing diameter, while the lower region is rigid and independent of the mantle radius; in *D. gigas*, cylinder length L_c=0.54L, see Table 3), M is the wet mass (kg), d_s is the squid density (1055 kg m^{-3}) and 0.15 is the correction factor for visceral mass in *D. gigas* (see Table 3).

The ventilatory volume per minute ($\dot{V}_V$, ml min^{-1}) was quantified by multiplying V_w (ml) by ventilatory frequency (MC min^{-1}). The oxygen extraction efficiency (E_{O_2}, %) was quantified *via* the following equations, based on the theoretical model in *I. illecebrosus* (Pörtner, 1994):

$$E_{O_2} = [(\Delta O_2 \times \dot{V}) / \dot{V}_V)] / [(O_{2,IN} \times \dot{V}) / \dot{V}_V]\,, \quad (2A)$$

$$E_{O_2} = \{[(\Delta O_2 - 0.2\Delta O_2) \times \dot{V}) / \dot{V}_V]\} / [(O_{2,IN} \times \dot{V}) / \dot{V}_V]\,, \quad (2B)$$

$$E_{O_2} = \{[(\Delta O_2 - 0.5\Delta O_2) \times \dot{V}) / \dot{V}_V]\} / [(O_{2,IN} \times \dot{V}) / \dot{V}_V]\,, \quad (2C)$$

$$E_{O_2} = \{[(\Delta O_2 - 0.6\Delta O_2) \times \dot{V}) / \dot{V}_V)] / [(O_{2,IN} \times \dot{V}) / \dot{V}_V]\,, \quad (2D)$$

without (Eqn 2A) and with a potential skin respiration contribution (20%, minimum for resting squid, Eqn 2B; 50%, maximum for resting squid, Eqn 2C; and 60%, for squid under exercise, Eqn 2D). Here, ΔO_2 represents the difference between the oxygen flow in and out of the respiratory chamber (μmol l^{-1}), $\dot{V}$ is the flow rate during respiration measurement (l min^{-1}) and $\dot{V}_V$ is the ventilatory volume per minute (ml min^{-1}).

Statistics

Two-way ANOVA were performed to evaluate significant differences between oxygen treatments (21% and 1% O_2), metabolic rates, mantle contraction frequencies and strengths, % of relaxed diameter and stroke volumes. Subsequently, Tukey HSD *post hoc* tests were conducted. In some ventilatory measurements, no data for the late hypoxia treatment were recorded and, as result, independent Student's *t*-tests were applied. Linear and polynomial regressions were performed to assess the correlations between mantle contraction frequency, contraction strength and metabolic rate at different activity levels under control and hypoxic conditions. ANCOVA were conducted to detect significant differences in the relationship between mantle contraction strength/frequency and metabolic rate. For all statistical analysis, STATISTICA (Tulsa, OK, USA) version 10.0 was used.

RESULTS

The effect of hypoxia on *D. gigas* metabolic rate is shown in Fig. 3A. In control animals, activity level only showed significant differences (P<0.05) between IMR and MR_{max}, increasing from 13.7±6.1 to 28.4±12.0 μmol O_2 g^{-1} wet mass h^{-1} (Fig. 3A; supplementary material Table S1). Exposure to severe hypoxia (1% O_2, 1 kPa) led to a significant decrease in O_2 consumption (≤25% of control values, Fig. 3A). AMR and RMR were significantly reduced in EH (exposure time 30–160 min) and IMR at LH (exposure time >180 min; two-way ANOVA, P<0.05; supplementary material Table S1). MR_{max} was only achieved under normoxic conditions and, overall, activity levels (AMR and MR_{max}) declined with hypoxia exposure from 36% to 15% in EH to 0% in LH (Fig. 3A, Fig. 4). RMR dominated in EH treatments, increasing from 47% (normoxia) to 77%, but was not present in LH (100% IMR; Fig. 4). The metabolic scope also changed dramatically with hypoxia (Fig. 3B), decreasing from 15.0±5.9 μmol O_2 g^{-1} wet mass h^{-1} in normoxia to 1.7±0.7 μmol O_2 g^{-1} wet mass h^{-1} in EH, reaching almost 0 (0.3±0.2 μmol O_2 g^{-1} wet mass h^{-1}) in LH (one-way ANOVA,

Table 3. Species-specific correction factors for visceral mass and cylinder mantle length to determine stroke volume in *D. gigas*

N	Wet mass (g)	*L* (cm)	L_c (cm)	CF_{L_c}	V_T (ml)	V_T–V_{vm} (ml)	CF_{vm}
1	10.3	7.2	3.8	0.52	8.5	7.2	0.15
2	6.0	6.0	3.2	0.53	4.5	4.0	0.11
3	5.9	5.9	3.3	0.56	4.0	3.45	0.14
4	6.6	6.2	3.4	0.55	4.5	3.8	0.16
5	5.6	5.4	2.9	0.54	3.0	2.3	0.23
6	10.5	7.0	3.9	0.55	7.3	6.4	0.12
Mean	7.5	6.3	3.4	0.54	5.3	4.5	0.15
s.d.	2.3	0.7	0.4	0.01	2.1	1.9	0.04

L, mantle length; L_c, cylinder mantle length; V_T, total volume (mantle and viscera); V_{vm}, volume of visceral mass. The correction factor for visceral mass (CF_{vm}) was quantified as the proportion of V_{vm} to V_T, and the correction factor for cylinder mantle length (CF_{L_c}) as the proportion of L_c to L.

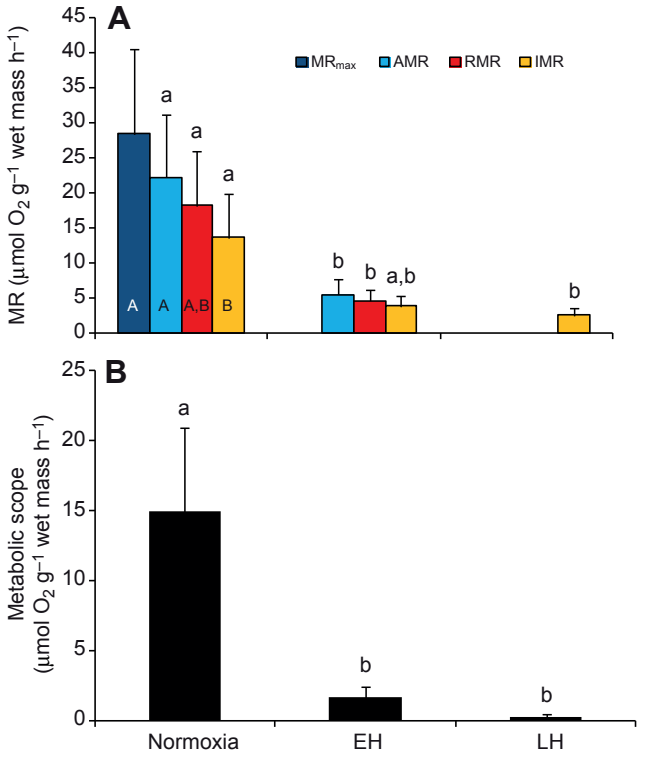

Fig. 3. Effects of hypoxia (1% O_2) on (A) metabolic rate (MR) and (B) metabolic scope (maximum MR–minimum MR) of *D. gigas*. Values are expressed as means and s.d. (*N*=6). EH, early hypoxia; LH, late hypoxia. Significant differences between the level of activity (capital letters) and between treatments (lowercase letters) are indicated (P<0.05, see supplementary material Table S1).

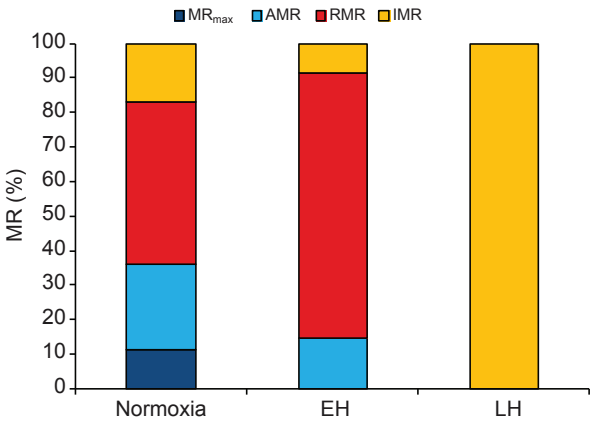

Fig. 4. Changes in IMR, RMR, AMR and MR_{max} of *D. gigas*, under control conditions (21% O_2), and in EH and LH (1% O_2).

F=34.06, P<<0.0001). The number of escape jets per minute increased significantly during AMR from normoxia (11.2 jets min^{-1}) to EH (14.3 jets min^{-1}), but no jets were recorded at LH (Fig. 5A; supplementary material Table S1). Overall (disregarding the activity level), there was a significant decrease in total bursts per hour from normoxia to EH (Fig. 5B, *t*-test, t=5.55, P<0.05).

All control animals showed a distinct periodicity in oxygen consumption rate (Fig. 1A) and video analysis confirmed that these cycles were correlated with burst frequency and level of activity. Cycles of AMR and MR_{max} differed significantly in length (18±4 and 52±8 min, respectively; Fig. 6A) and frequency (1.5 and 0.2 cycles h^{-1}, respectively; *t*-test, P<<0.001; Fig. 6B). In early hypoxia, periodicity was also observed (in 5 out of 6 animals, illustrated in Fig. 1B), but with a significantly shorter cycle length (5±1 min; Fig. 6A) and higher frequency (4±1 cycles h^{-1}; Fig. 6B; *t*-tests, P<0.001).

The effect of hypoxia (1% O_2, 1 kPa) on ventilation rate, a function of mantle contraction frequency and strength, is presented in Fig. 7. Contraction frequency significantly increased with activity, from 45±7 MC min^{-1} in IMR to 74±3 MC min^{-1} in MR_{max} in normoxia, and from 32±6 MC min^{-1} in IMR to 50±5 MC min^{-1} in AMR during EH (Fig. 7A; supplementary material Table S1). During LH, the contraction frequency decreased to 6±3 MC min^{-1}. Contraction strength (Δ mantle diameter; Fig. 7C) increased significantly with metabolic rate in normoxia and EH (supplementary material Table S1). At IMR, mantle contraction strength was more or less constant (around 1.5 mm) during EH, but then decreased dramatically with duration of exposure (LH) down to ≤0.4 mm (two-way ANOVA, P<0.05; supplementary material Table S1). The correlations between contraction frequency/strength and metabolic rate are given in Fig. 7B,D. In both cases, the relationship is linear for both normoxic (Fig. 7B: R^2=0.977, F=130.3, P=0.001; Fig. 7D: R^2=0.982, F=167.6, P<0.001) and hypoxic treatments (Fig. 7B: R^2=0.850, F=17, P<0.05; Fig. 7D: R^2=0.855, F=17.7, P<0.05). Interestingly, the slope for hypoxia is significantly steeper (F=12.8, P<0.01, frequency; F=5.2, P<0.05, strength) than that for normoxia, indicating that much higher ventilation rates (frequency and strength) are required to achieve even modest metabolic rates during exposure to hypoxia. There was also a significant correlation between mantle contraction frequency and strength (Fig. 8). Under normoxic conditions, squid showed a linear relationship between strength and frequency (R^2=0.993, P<<0.001), whereas under hypoxia (1% O_2, 1 kPa) the relationship was polynomial (R^2=0.988, P=0.01), indicating reduced relative ventilation frequency.

There was a significant increase in the percentage relaxed mantle diameter in AMR and MR_{max} (i.e. hyperinflation) under normoxia (Fig. 9; supplementary material Table S1). However, the opposite trend was observed in EH and LH.

The effect of hypoxia on ventilation stroke volume V_w is shown in Fig. 10A. The highest value was observed during maximum activity (MR_{max}) under normoxia (4.8 ml). During EH, there was a significant increase in V_w, with activity ranging from 2.7 ml (IMR) to 3.6 ml (RMR) to 4.3 ml (AMR; P<0.05). In LH, there was a significant drop (42%) during the inactive state (IMR). By multiplying the V_w (Fig. 10A) by ventilation (contraction) frequency (Fig. 7A), we obtained the ventilatory volume per minute $\dot{V}_V$ (Fig. 10B). As a result, it became evident that in EH the increase in V_w (at AMR and RMR; Fig. 10A) was followed by a decrease in contraction frequency (Fig. 7A), leading to similar values of $\dot{V}_V$ in normoxia and EH (Fig. 10B). In LH, this ventilatory parameter was significantly lower (Fig. 10B, P<0.05; supplementary material Table S1). Oxygen extraction efficiency E_{O_2} under normoxia ranged between 6.4% and 13.4% (2.6% and 5.4%, taking into account 60% potential skin respiration) from IMR to MR_{max} and was significantly elevated as a result of exposure to hypoxia, with a minimum of 43.7% (15.2% including 60% cutaneous uptake) in IMR during LH, and a maximum of 81.5% (33.6%) in AMR during EH (Fig. 10C,D, P<0.05; supplementary material Table S1). In Table 4, E_{O_2} without, and with 20%, 50% and 60% potential skin respiration is presented.

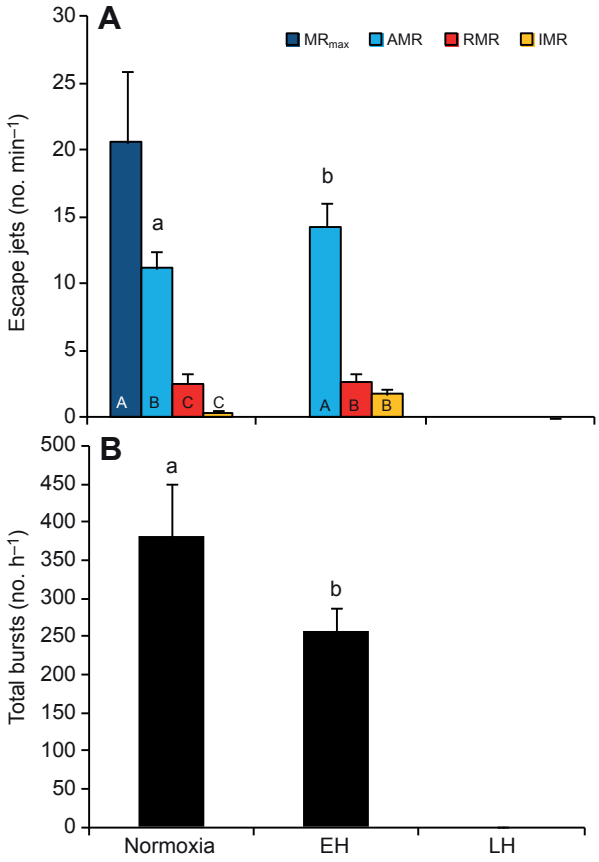

Fig. 5. Effect of hypoxia (1% O_2) on burst frequency in *D. gigas*. (A) Number of escape jets per minute during IMR, RMR, AMR and MR_{max}, and (B) total number of burst events per hour. Values are expressed as means ± s.d. (*N*=6). Significant differences between active and maximum active cycles (capital letters) and between oxygen treatments (lowercase letters) are indicated (*P*<0.05).

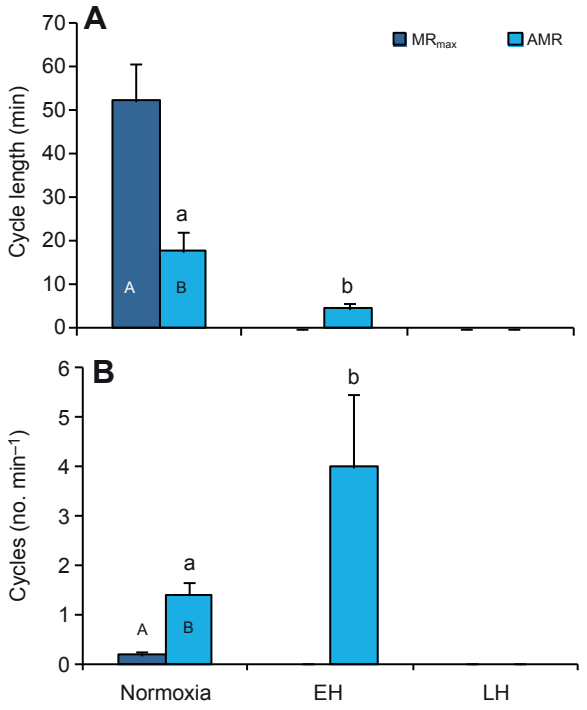

Fig. 6. Effect of hypoxia (1% O_2) on the periodicity of activity cycles in *D. gigas*. (A) Average cycle length for MR_{max} and AMR, and (B) cycle frequency. Values are expressed as means and s.d. (*N*=5; 1 of the 6 was not cycling). Significant differences between AMR and MR_{max} cycles (capital letters) and between oxygen treatments (lowercase letters) are indicated (*P*<0.05).

DISCUSSION

Effect of hypoxia on metabolic rate and metabolic scope

Dosidicus gigas metabolic rate varied between 14 and 29 µmol $O_2\,g^{-1}$ wet mass h^{-1}, depending on the level of activity. These values were in agreement with those from our previous studies (Rosa and Seibel, 2008; Rosa and Seibel, 2010), which also indicated a wide metabolic scope in well-oxygenated water. *Dosidicus gigas* is able to maintain these high metabolic rates down to an ambient O_2 level of ~20 µmol l^{-1} (~2 kPa, ~200 m depth) (Gilly et al., 2006), below which they dramatically drop. In the present experiments, with the temperature and oxygen conditions found in the OMZ, squid metabolic rate dropped more than 75% under exposure to early hypoxia with no further significant reduction with exposure time. Moreover, MR_{max} was not recorded under such conditions and the metabolic scope was significantly decreased (Fig. 1B, Fig. 3B, Fig. 4). In general, organisms suppress their metabolism by 50–95% by shutting down expensive processes (i.e. protein synthesis, muscle activity) (Guppy and Withers, 1999) and supplement the remaining energy demand using a combination of available O_2 and anaerobic metabolic pathways (Seibel, 2011). Cephalopods that routinely depend on jet propulsion extract only a small proportion (5–10%) of the available O_2 from the ventilatory stream, but are able to alter the O_2 extraction efficiency (≤25%) when necessary, including cutaneous respiration, for example (Wells et al., 1988; Wells and O'Dor, 1991; Pörtner, 1994). Yet, the most common strategy among powerful predators that managed to minimize the trade-offs between high performance and hypoxia tolerance is a high-affinity respiratory protein (Lowe et al., 2000; Gilly et al., 2006; Seibel, 2011). Hemocyanin of cephalopods is usually characterized by a low O_2 affinity with high pH sensitivity, properties that facilitate O_2 release to demanding tissues, but presumably interfere with O_2 extraction from hypoxic or CO_2-rich seawater (Pörtner, 1994; Pörtner, 2002; Melzner et al., 2007). Yet, it has been reported (Seibel, 2012) that the respiratory protein in *D. gigas* is both highly temperature and pH sensitive, but, in addition, is characterized by a high O_2-binding affinity. These properties facilitate O_2 uptake in cold, deep waters where O_2 is scarce and enhances O_2 release in warmer surface waters where O_2 and O_2 demand are elevated. Furthermore, Seibel (Seibel, 2012) could link the hypoxia tolerance down to P_{crit} (1.6 kPa, 10°C) (Trueblood and Seibel, 2012) to the optimized hemocyanin function in *D. gigas*, and suggests that below P_{crit} (~170 m, Gulf of California) the onset of metabolic suppression kicks in.

However, metabolic suppression is time limited, as anaerobic glycolysis results in the accumulation of protons (H^+) and organic compounds such as lactate and octopine (Hochachka and Somero, 2002; Rosa and Seibel, 2010) with harmful consequences on acid–base status (Hochachka and Mommsen, 1983). Recent biochemical analysis revealed that under EH ~60% of the depressed metabolism is still achieved aerobically but this drops to ~45% at LH (K.T., unpublished). No significant further drop in metabolic rate between EH and LH could be quantified, indicating a limitation in the potential to suppress metabolism and a possible switch in

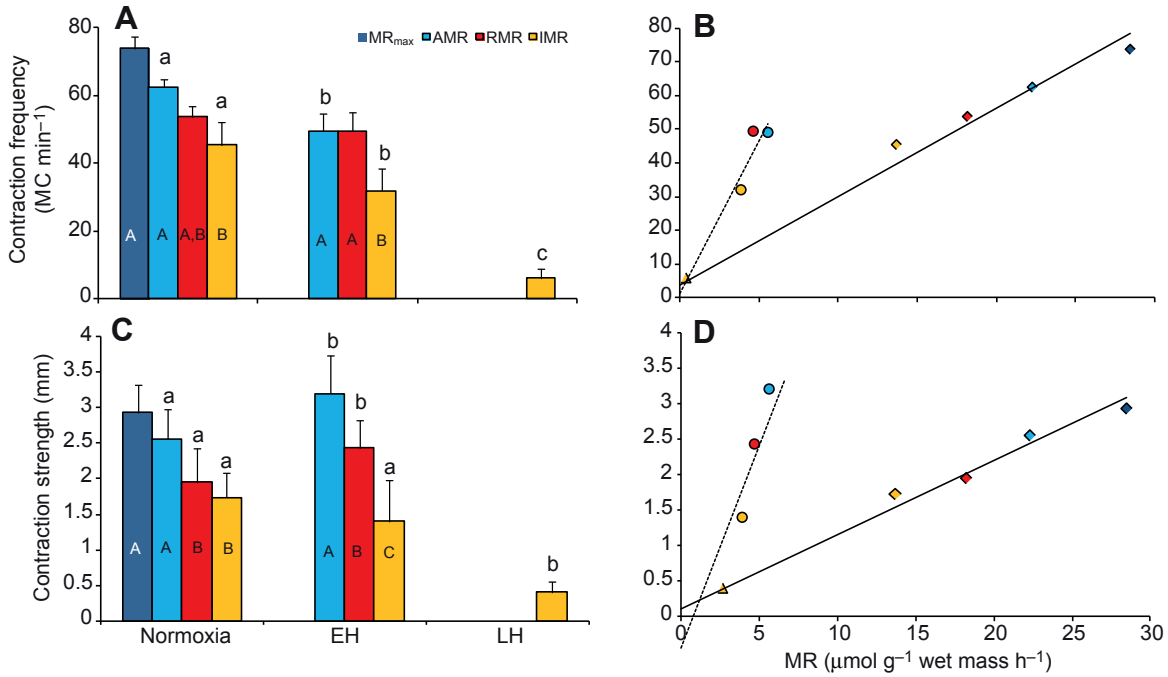

Fig. 7. Effect of hypoxia (1% O_2) on ventilation rates of *D. gigas*. (A) Mantle contraction (MC) frequency, and (C) mantle contraction strength (change in mantle diameter). Significant differences between activity levels (capital letters) and between treatments (lowercase letters) are indicated (P<0.05, see supplementary material Table S1). Values are expressed as means and s.d. (N=6). (B,D) Ventilation frequency and strength, plotted against oxygen consumption. Diamonds, normoxia; circles, EH; triangles, LH. Yellow, IMR; red, RMR; bright blue, AMR; dark blue, MR_{max}.

energy expenditure from muscle activity to acid–base and ion regulation processes.

Vertical migrations and respiratory cycling behavior

Jumbo squid are diel vertical migrators that spend their nights in shallower, well-oxygenated waters (~70 m) and dive into the OMZ during the day (peak depth range 200–300 m; 2–0.2 kPa) (Gilly et al., 2006) (Trueblood and Seibel, 2012). Thereby, jumbo squid spend more than 70% of the day below 200 m (≤2% O_2, 2 kPa) (Gilly et al., 2006) following their main prey (myctophid fishes) (Markaida and Sosa-Nishizaki, 2003; Markaida et al., 2008) and in this way also escape from: (i) unfavorable warmer sea surface temperatures, and (ii) elevated predation pressure and resource (food) competition, as most active fish predators are excluded from the OMZ (Prince and Goodyear, 2006).

Nonetheless, acoustic data revealed short-term cyclic vertical movements (~15–90 min), during day and night time, that have been interpreted as active forages (Gilly et al., 2006). In a previous study, we (Rosa and Seibel, 2010) discovered a similar periodicity in the rate of metabolism in the confines of a respiration chamber (cycle length ~20 min), which was also observed in the present experiments (20/60 min for active/maximum active cycles; Fig. 1A, Fig. 6A). As the frequency and duration of brief vertical movements are similar to the periodicity reported here, we argue that the activity cycles (MR_{max} and AMR) at normoxia may reflect the capacity to perform migratory forays.

During early hypoxia, the respiratory cycles were shorter in length but increased in frequency (Fig. 1B, Fig. 6). This was accompanied by an increase in the number of escape jets in active periods (see AMR in Fig. 5A) and a faster switch between swimming modes (data not shown). As high muscular activity involves the use of anaerobic ATP production, cycling between aerobic and anaerobic swimming phases over short time intervals may reduce the cost of transport and permit a long-term use of anaerobic resources, a strategy that in the long term may imply a maximized use of ambient O_2 (Finke et al., 1996). Finke and colleagues assumed that this oscillation between periods of high and low muscular activity may have been related to the hypoxia tolerance of *Lolliguncula brevis* in coastal waters. It is plausible that *D. gigas* uses the same strategy. Furthermore, it displayed an enhanced usage of its lateral fins under progressive hypoxia, with a peak at the transition from EH to LH (data not shown). This also might contribute to minimize the costs of transport, as lateral fins can push a large mass of water especially at low velocities (O'Dor et al., 1988; Wells and O'Dor, 1991). At LH (onset ~170±10 min),

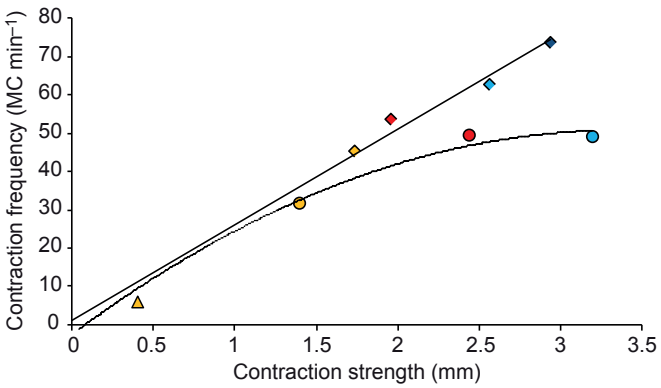

Fig. 8. Relationship between mantle contraction (MC) strength (change in mantle diameter) and frequency (ventilation rate) in *D. gigas* under normoxia and hypoxia (1% O_2). The black straight line and diamonds represent normoxia, the dashed line represents hypoxia, with circles for EH and triangles for LH. Yellow, IMR; red, RMR; bright blue, AMR; dark blue, MR_{max}. Values are expressed as means (N=6).

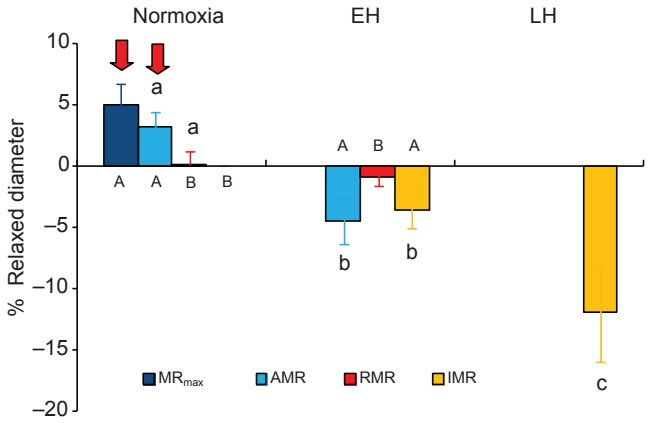

Fig. 9. Changes in the relaxed mantle diameter of IMR, RMR, AMR and MR_{max} in *D. gigas*, under control conditions (21% O_2), EH and LH (1% O_2). Percentage relaxed mantle diameter was calculated based on the relaxed diameter under IMR normoxia. Values are expressed as means and s.d. (*N*=6). Red arrows indicate a hyperinflation mechanism. Significant differences between active and maximum active cycles (capital letters) and between oxygen treatments (lowercase letters) are indicated (P<0.05).

jumbo squid moved into a lethargic state and ventilatory cycles stopped (Fig. 1C, Fig. 6). This lethargic behavior has been associated with metabolic suppression (Rosa and Seibel, 2010). Gilly and colleagues (Gilly et al., 2006) claimed that a substantial number of excursions lasted for many hours (maximum duration of 400/800 min below 300/200 m), during which time rhythmic diving occurred that appeared to be as robust as that observed at well-oxygenated, shallower depths. The majority of these extended dives at 300 m (~1% O_2, 1 kPa) peaked between 140 and 240 min, which is in close agreement with the onset of late hypoxia (~170±10 min) in the present study. Yet, the switch to severe hypoxia within the respiration chambers could have been faster than during average, natural descents [see fig. 11 of Gilly (Gilly et al., 2006)], even though fast migrations (i.e. hunting, escape) in jumbo squid are known, which might have elevated stress. Also the lack of food within the chambers might have accelerated the depletion of anaerobic energy reserves, resulting in the onset of lethargy.

Ventilatory mechanisms and oxygen extraction efficiency during hypoxia

In normoxia, the *D. gigas* mantle contraction frequency and strength were linearly correlated (Fig. 8) and increased significantly with activity level (Figs 7, 8). Additionally, an increased stroke volume and ventilatory volume per minute (Fig. 10) were observed, followed by a hyperinflation process during high activity periods (red arrows in Fig. 9). The squid *Illex illecebrosus* and *L. opalescens*, in contrast, do not vary their ventilation *via* frequency with vigorous jetting, but *via* powerful contractions of their circular fibres (Wells et al., 1988; Webber and O'Dor, 1985). A linear increase in ventilation frequency with speed (<0.6 m s^{-1}) was found in *I. illecebrosus*, but at speeds >0.6 m s^{-1} the correlation was linear with cavity pressure (Webber and O'Dor, 1986). As a result, *I. illecebrosus* and *L. opalescens* seem to be restricted to regulating ventilation strength and frequency at the same time, while *D. gigas* seems to have developed an optimized fine-tuning between locomotion and ventilation, and seems to have the potential to regulate mantle contraction frequency, strength and stroke volume according to activity.

Yet, EH changed the relationship between ventilation strength and frequency from linear to polynomial with increasing activity

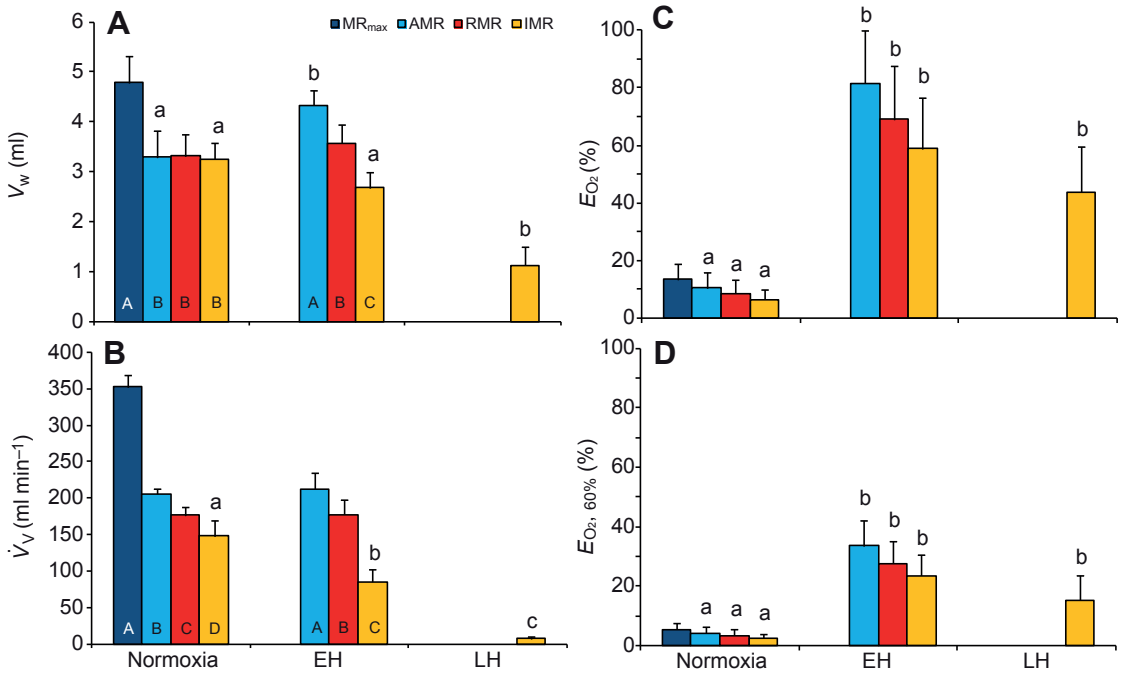

Fig. 10. Changes in (A) stroke volume V_w, (B) ventilatory volume per minute $\dot{V}_V$, (C) O_2 extraction efficiency E_{O_2} and (D) O_2 extraction efficiency with potential skin respiration contribution ($E_{O_2,60\%}$) in IMR, RMR, AMR and MR_{max} of *D. gigas*, under control conditions (21% O_2), EH and LH (1% O_2). Values are expressed as means and s.d. (*N*=6). Significant differences between active and maximum active cycles (capital letters) and between oxygen treatments (lowercase letters) are indicated (P<0.05).

Table 4. Oxygen extraction efficiencies without (0%) and with (20%, 50% and 60%) consideration of potential skin respiration

Treatment	Activity	Skin respiration			
		0%	20%	50%	60%
Normoxia	MR_{max}	13.4±5.4	10.7±4.3	6.7±2.7	5.3±2.1
	AMR	10.5±5.4	8.4±4.4	5.2±2.7	4.2±2.2
	RMR	8.6±4.7	6.9±3.8	4.3±2.4	3.4±1.9
	IMR	6.4±3.4	5.1±2.7	3.2±1.7	2.6±1.4
EH	AMR	81.5±18.3	67.3±16.9	41.9±10.6	33.5±8.5
	RMR	69.1±18.4	55.3±14.7	34.5±9.2	27.6±7.4
	IMR	58.8±17.7	47.1±14.2	29.4±8.9	23.5±7.1
LH	IMR	43.7±16.0	35.0±12.8	19.0±10.6	15.2±8.5

Data (means ± s.d.) are for inactive, routine, active and maximum active levels (IMR, RMR, AMR and MR_{max}) in *D. gigas*, under control conditions (21% O_2), and in early and late hypoxia (1% O_2). Here, 20% skin respiration represents the minimum resting value, 50% the maximum resting value and 60% the cutaneous uptake under exercise in squid (*I. illecebrosus*, theoretical model) (Pörtner, 1994). EH, early hypoxia; LH, late hypoxia (>180 min).

(Fig. 8). This indicates that *D. gigas*, under hypoxic conditions, primarily increases the power of mantle contraction and does not regulate its frequency, as was observed for *I. illecebrosus* and *L. opalescens* under normoxia (Wells et al., 1988).

In EH, jumbo squid showed a larger inflation period leading to reduced contraction frequency and decreased relaxed mantle diameter, which optimize O_2 uptake (i.e. shorter diffusion ways) *via* gills and skin (see Fig. 10C). Additionally, they seem to breathe 'deeply', with more powerful contractions (see different slopes in Fig. 7D) and enhanced stroke volume (Fig. 10A). This deep-breathing behavior allows the squid to have the same amount of water passing through the gills per period of time, i.e. a stable ventilatory volume per minute, and explains the maintenance of the squid's cycling activity under such O_2 conditions.

The O_2 extraction efficiency reached ~82% in EH at active metabolic rates (Fig. 10C) without consideration of cutaneous uptake. Other cephalopods, such as nautilus and squid (*L. brevis*), also alter the effectiveness of oxygen extraction (i.e. cutaneous uptake) from the ventilatory stream as a result of falling ambient O_2 levels or exercise when necessary, but at around 15–20% (once 43% in nautilus) (Wells et al., 1988; Wells, 1990; Pörtner, 1994). Maximum O_2 extraction efficiency (AMR at EH), considering 60% potential skin respiration in squid (theoretical model, *I. illecebrosus* under exercise) (Pörtner, 1994), was still found to be 34% in our study (Fig. 10D). The organization of squid mantle muscle structure (mitochondria-rich inner and outer layer) supports the importance of skin respiration in cephalopods (Gosline and DeMont, 1985). Under hypoxia, P_{O2} gradients drastically decline and therefore do not favor diffusion processes (O_2 uptake *via* skin and gills). Under LH, when jumbo squid cease swimming, cutaneous uptake might even decrease as a result of a lack of convection. Deep-breathing behavior observed at EH, instead, might favour cutaneous uptake (inner and outer mantle), but here we were not able to distinguish between O_2 uptake *via* skin and gills.

In cuttlefish and octopus, organisms that are less dependent on jet propulsion, the fraction of oxygen extraction from the ventilatory stream is higher [50–90% in cuttlefish (Melzner et al., 2006); 45–75% in octopus (Wells and Wells, 1985; Wells, 1990)], and their O_2 uptake can only be enhanced by an elevation in water throughput and increased diffusion gradients at the water–blood threshold (Melzner et al., 2006). Other OMZ residents in the Gulf of California, like *Gnathophausia ingens*, can regulate oxygen uptake mainly *via* an increase in ventilation volume (5–6 times, maximum 8) at constant (or slightly increasing) O_2 extraction efficiencies that can reach 48–60%, with a maximum ~95% (Childress, 1971). However, these large changes in ventilation volume are not possible for squid because of the constraints imposed by the massive collagenous tissues in the mantle walls of muscular squid (Gosline and Shadwick, 1983; Wells et al., 1988).

In contrast, at LH, all the ventilatory processes were significantly reduced, except for O_2 extraction efficiency followed by a lethargic state. Also, Boutilier and colleagues (Boutilier et al., 1996) reported that *Nautilus* cease ventilatory movements, cardiac output and heartbeat to survive prolonged periods of hypoxia. In the majority of investigated squid, ventilation was completely shut down, but we cannot rule out the possibility that there were further very weak contractions that could not be assessed by the method of analysis used. Another explanation could be that *D. gigas* has the potential to uncouple its ventilatory and locomotory mechanisms *via* a collar flap system, as is known for resting sepia and to some extent for squid (Bone et al., 1994). This original respiratory design of cephalopods (Wells, 1988) drives expiration *via* an active inward movement of the collar flaps (and probably *via* stored elastic energy during inhalation) (Gosline and Shadwick, 1983) without the activity of circular muscle fibers (Bone et al., 1994).

Unfortunately, the experiments were interrupted a maximum of 20 min after the jumbo squid stopped moving. Nonetheless, recent data suggest that juvenile (R.R., unpublished) and adult jumbo squid (B.A.S., unpublished) can sustain severe hypoxia (1% O_2) for at least 6–12 h and therefore might use lethargy as a strategy to extend the residence time in the OMZ. It is noteworthy that our experiments were conducted with juveniles and therefore our results might not apply in adults, even though metabolic rates in juvenile and adult squid should not differ, as muscular squid display an unusual scaling relationship (tendency to isometry). This might be a consequence of: (1) tubular geometry (mantle diameter increases faster than thickness with growth), exchange surfaces (surface area$^{1/2}$:volume$^{1/3}$ increases with size) (O'Dor and Hoar, 2000) and cutaneous respiration (~60%) (Pörtner, 1994; Pörtner, 2002); (2) ontogenetic differences in locomotory expenditure, because the squid cost of transport may not decrease as much with size as that in other animals (e.g. mammals, birds and fish), where adults are more energy efficient; and (3) energetic requirements during all stages of the squid's short life cycle (namely, for growth and reproduction). High sustained production costs during all ages may result in a *B* value (scaling coefficient) that does not decrease with increasing body mass (Seibel, 2007; Rosa et al., 2009). However, it remains unclear whether adults will adjust their ventilatory mechanisms to low oxygen levels in a similar way to juveniles, especially as little is known about the potential of oxygen uptake *via* the skin under hypoxia.

LIST OF SYMBOLS AND ABBREVIATIONS

AMR	active metabolic rate
d_s	squid density
E_{O_2}	O_2 extraction efficiency
IMR	inactive metabolic rate
L	mantle length
L_c	cylinder length
M	weight mass (kg)
MC	mantle contractions
MR	metabolic rate
MR_{max}	maximum active metabolic rate
OMZ	oxygen minimum zone
r_C	contracted mantle radius
r_H	maximum (hyperinflated) mantle radius
RMR	routine metabolic rate
$\dot{V}$	flow rate
$\dot{V}_V$	ventilatory volume per minute
V_w	stroke volume
x	mantle thickness (mm)

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II Aerobic and anaerobic pathways in jumbo squid (*Dosidicus gigas*) under metabolic suppression in the oxygen minimum zones

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Aerobic and anaerobic pathways in jumbo squid (*Dosidicus gigas*) under metabolic suppression in the oxygen minimum zones

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A B S T R A C T

The Humboldt (jumbo) squid, *Dosidicus gigas*, is a large oceanic squid endemic off the Eastern Tropical Pacific that undertakes diel vertical migrations into mesopelagic oxygen minimum zones. Thereby, it encounters oxygen levels below its critical oxygen partial pressure. To better understand the metabolic mechanisms and the potential of metabolic suppression in these top predators, we exposed juvenile *D. gigas* to oxygen levels found in the OMZ (1% O_2, 1kPa, 10ºC) and investigated: i) oxygen consumption rates, ii) changes in energy reserve stores (phosphagen and nucleotide pool) including the adenylate energy charge, iii) accumulation of anaerobic end products (octopine, succinate), iv) aerobic and anaerobic energy production, and v) the individual ATP contribution of the energy resources quantified. Under severe hypoxia, *D. gigas* suppressed its metabolism by 45-60% ($p<0.05$) with an increasing trend according to exposure time. Anaerobic energy production increased from 6.3 ± 1.6 to 21.0 ± 5.5 µmol ATP g^{-1} wet mass h^{-1} ($p<0.05$), but it did not compensate the energy loss due to reduced oxygen consumption rates (70-80%; $p<0.05$). Concomitantly, the energy reserve stores were significantly depleted, namely phospho-L-arginine (25.2 ± 5.3 to 5.3 ± 0.7 µmol g^{-1} wet mass; $p<0.05$) and ATP (5.6 ± 0.5 to 1.3 ± 0.3 µmol g^{-1} wet mass; $p<0.05$). ATP degradation was accompanied by a simultaneous increase in ADP and AMP levels ($p<0.05$) that led to a significant drop in the adenylate energy charge from 0.9 ± 0.0 to 0.5 ± 0.0 ($p<0.05$). Phopsho-L-arginine breakdown generated a significant L-arginine build-up ($p<0.05$) that, in turn, was converted into the anaerobic end product octopine (3.8 ± 1.0 to 27.9 ± 6.9 µmol g^{-1} wet mass; $p<0.05$). The contribution of ATP production via succinate was minor (maximum 1.2%), but accumulated under severe hypoxia from 0.2 ± 0.0 to 0.7 ± 0.1 µmol g^{-1} wet mass ($p<0.05$). Thus, the present findings support the idea that metabolic suppression in juvenile *D. gigas* plays an important role in species' hypoxia tolerance within the OMZ environment. Moreover, we show that the contribution of anaerobic energy production is maintained with progressing hypoxia, switching from rapid energy reserve depletion during early hypoxia to fermentative pathways in late hypoxia.

1. Introduction

The jumbo or Humboldt squid *Dosidicus gigas* is the largest ommastrephid squid (up to 2.5 m total length and 50 kg total weight; Nesis, 1983) with a perennial geographical distribution centered in the Eastern Tropical Pacific (ETP). However, it has recently been expanding its range along the subtropical and temperate coasts of both North and South America (Keyl *et al.*, 2008; Zeidberg and Robison, 2007). *D. gigas* plays a critical ecological role both as prey (Clarke and Paliza, 2001; Abitia-Cardenas *et al.*, 2002; Ruiz-Cooley *et al.*, 2004) and predator (Markaida and Sosa-Nishizaki, 2003), but also has an important economical value since it constitutes the world's largest cephalopod fishery (Rodhouse *et al.*, 2006).

Besides horizontal movements, *D. gigas* is known to undertake diel vertical migrations into the mesopelagic realm, where it encounters zones of low oxygen during day time (Bazzino *et al.*, 2010; Gilly *et al.*, 2006; Rosa and Seibel, 2008). These oxygen minimum zones (OMZs) are hundreds of meters deep and thousands of kilometers wide and have apparently expanded to higher latitudes in the last 50 yr (Stramma *et al.*, 2008). Thus, vertical migrations into the OMZ bring *D. gigas* into an environment in which the dissolved oxygen level is only a few percent of the saturated value at the surface (Gilly *et al.*, 2006). In fact, jumbo squids spend 70% of their day time below their critical oxygen partial pressure (P_{crit}, ~ 1.7 kPa; Seibel, 2013; Trueblood and Seibel, 2013).

Low oxygen (O_2) levels are known to greatly limit the vertical distribution and ecology of many marine animals (i.e. swordfish, marlin and sharks; Brill, 1994, 1996; Lowe *et al.*, 2000; Wishner *et al.*, 1998, 2000 and 2008), but the jumbo squid thrive in such harsh environment by managing hypoxia via a highly temperature and pH dependent high-oxygen-affinity respiratory protein (Seibel, 2013) and a high potential to regulate its ventilatory mechanisms (Trübenbach *et al.*, 2013a). In this context, metabolic suppression has been discussed as further strategy in *D. gigas* to cope with O_2 shortage (Rosa and Seibel, 2008 and 2010; Trübenbach *et al.*, 2013a, b).

Aquatic animals normally respond to hypoxia by first attempting to maintain O_2 delivery, as the energy yield of oxidative phosphorylation is way more efficient than ATP production from fermentative pathways (Hochachka and Somero, 2002). Below the species specific P_{crit}, energy production cannot be maintained solely aerobically and the organisms try to: i) conserve energy expenditure, ii) reduce energy turnover, and finally iii) enhance energetic efficiency of those metabolic processes that remain and derive energy from anaerobic sources (Holton and Randall, 1967; Burggren and Randall, 1978; Van den Thillart and Smit, 1984; Wu and Woo, 1985; Dunn and Hochachka, 1986; Boutilier *et al.*, 1988; Chew and Ip, 1992; Randall *et al.*, 1992; Dalla Via *et al.*, 1994; Hochachka, 1997). It is worth noting that metabolic suppression of total (aerobic and anaerobic) metabolism is a prerequisite for the survival of prolonged bouts of O_2 limitation (Guppy and Withers, 1999; Hochachka and Somero, 2002; Bickler and Buck, 2007), especially in animals exposed to temporary O_2 deprivation. Its extent depends on: i) species metabolic demand, ii) phylogenetically available biochemical pathways, and iii) its capacity for O_2 transport (Seibel, 2011). In general, organisms suppress their metabolism by 50–95% and supplement the remaining energy demand using a combination of available O_2 and anaerobic metabolic pathways (Rosa and Seibel, 2008, 2010). The energy turnover downregulation is primarily achieved by shutting down expensive cellular processes, such as biosynthesis (Kwast and Hand, 1996), ion pumping (Buck and Hochachka, 1993), or the down regulation and/or modification of certain regulatory enzymes of anaerobic and aerobic pathways (Rahman and Storey, 1988; Storey, 1988; Dalla Via *et al.*, 1994; Hochachka, 1997). Thereby, ATP turnover rates and demands can be down regulated by more than an order of magnitude, and metabolic rates reduced by 5–20fold during hypoxia (Storey, 1996; Hochachka, 1997).

To better understand the metabolic plasticity and the potential of metabolic suppression in *D. gigas*, we exposed juvenile jumbo squids to O_2 levels typically found in the OMZ (1% O_2, 1kPa, 10ºC) to investigate the effect of severe hypoxia on: i) O_2 consumption rates, ii) depletion of energy reserve stores (phosphagen and nucleotide pool), iii) accumulation of anaerobic metabolites

(octopine, succinate), iv) adenylate energy charge (AEC), v) aerobic and anaerobic energy production, with the individual ATP contribution (of the different energy resources).

2. Materials and Methods

2.1. Specimen collection

Juvenile Humboldt squids, *D. gigas* (d'Orbigny, 1835), (4.1 – 29.7 g wet mass) were collected *via* dip net in the Gulf of California (27°N, 111°W; 28°N, 113°W), on the surface at night, in June 2011 (aboard the *RV New Horizon*, Scripps Institute, CA, USA) and were immediately transferred to 10°C seawater (environmental temperature at OMZ) aquaria on board the vessel.

2.2. Experimental procedure and oxygen consumption rates

Animals were placed in a flow-through respirometry set-up (270 ml volume, Loligo Systems, Tjele, Denmark; Rosa and Seibel, 2008, 2010), and allowed to acclimate for 8-12 h before starting measurements of O_2 consumption. Respirometers were immersed in a large thermostatted (Lauda, Lauda-Königshofen, Germany) water bath at 10ºC, an average temperature found at 250 m in the OMZ (Rosa and Seibel, 2010). Filtered (0.2 µm) and treated (50 mg l^{-1} streptomycin) seawater was pumped from a water-jacketed, gas-equilibration column through the respirometers at a constant flow rate (average 120 ml min^{-1}). The water in the column was bubbled continuously to maintain incoming water at high (21% O_2) or low PO_2 (certified gas mixture with 1% O_2). Oxygen concentrations were recorded at the entrance and the exit of each chamber with two Clarke-type O_2 electrodes connected to a 928 Oxygen Interface (Strathkelvin Instruments, North Lanarkshire, Scotland). The system was calibrated using air- and nitrogen-saturated seawater and checked for electrode drift and for microbial O_2 consumption before and after each trial. All experiments were carried out in darkness and at atmospheric pressure. Afterwards, specimens were immediately weighted on a motion-compensated precision shipboard balance system (Childress and Mickel, 1980). A total of 5 specimens were investigated for normoxia (21% O_2), early hypoxia (EH; 1% O_2; hypoxia exposure time < 160 min) and late hypoxia (LH; 1% O_2; hypoxia exposure time > 180 min) treatments according to Trübenbach *et al.*, 2013a. Oxygen consumption rates were quantified as the average rate for the entirety of each respiratory run and are given as µmol O_2 g^{-1} wet mass h^{-1}.

2.3. Phosphagen pool, octopine and succinate

The anaerobic metabolites (octopine and succinate) and the phosphagen pool [phospho-L-arginine (PLA) and L-arginine (L-Arg)] were quantified spectrophotometrically via conversion of NAD^+/NADH (at 339 nm; Shimadzu, UV-1800) according to the standard enzymatic procedures of Gäde (1985a, 1985b) and Beutler (1989). The method entailed the preparation of perchloric acid (3 M) extracts from frozen mantle tissues and determinations were made immediately after neutralization with $KHCO_3$ (2 M). Concentrations were quantified as µmol g^{-1} wet mass.

The octopine measurement is based on the oxidation of octopine to pyruvate and L-arginine by octopine dehydrogenase (ODH; Sigma-Aldrich, St. Louis, MA, USA) in the presence of NAD^+, whereas the increase in NADH is proportional to the amount of octopine (Gäde, 1985a).

PLA was quantified indirectly via L-Arg after its conversion via acid hydrolysis. Therefore 0.1 ml of perchloric extract was mixed with 0.1 ml of hydrochloric acid (HCl, 1M), incubated for 90 seconds at 100°C, immediately chilled on ice and neutralized with 0.1 ml sodium hydroxide solution (1 M). Then L-Arg is reductively condensed with pyruvate to octopine by the action of ODH (Sigma-Aldrich, St. Louis, MA, USA) and the decrease in NADH concentration is proportional to the amount of L-Arg (Gäde, 1985b).

Succinate was quantified via a succinic acid kit (Megazyme, Ireland) according to Beutler (1989). The measurement is based on the conversion of succinate to succinyl-CoA in the presence of ATP and succinyl-CoA synthetase with the concurrent formation of ADP and inorganic Pi. ADP reacts with phosphoenol-pyruvate (PEP) to form pyruvate and ATP in the presence of pyruvate kinase. The pyruvate produced is reduced to L-lactate by L-lactate dehydrogenase (LDH) in the presence of NADH by forming NAD^+. The amount of NAD^+ formed is stoichiometric with succinate and the decrease

in NADH adsorption used to determine succinate.

2.4. Nucleotide pool and adenylate energy charge (AEC)

The extraction of the nucleotides [adenosine 5'-triphosphate (ATP), adenosine 5'-diphosphate (ADP), adenosine 5' – monophosphate (AMP)] was adapted according to the procedure of Mendes *et al.* (2001). Frozen muscle tissue samples (0.5 g) were homogenized in 2.5 ml of 0.6 M perchloric acid, centrifuged (20,000 g, 10 min, 0°C) and supernatants neutralized with 1 M potassium hydroxide (KOH). After incubation at 0°C for 30 min, extracts were filtered (0.2 µm) to remove potassium perchlorate. Aliquots were blast frozen (2 h) in 3 ml vials at -80°C and stored until further analysis. Nucleotide concentrations were quantified by high performance liquid chromatographic (HPLC) at 254 nm using a Hewlett Packard 1050 HPLC system. Separations were performed via a Hewlett Packard LiChrosorb RP-18 column (10 mm, 200×4.6 mm) that operates isocratically (at 30°C and 1.6 ml min^{-1}) and a 0.1 M phosphate buffer (pH 6.9) as mobile phase. Nucleotide standards were obtained from Sigma-Aldrich (St. Louis, MA, USA). Nucleotide concentrations were calculated as µmol g^{-1} wet mass.

The adenylate energy charge was quantified according to Atkinson (1968) via following equation:

$$AEC = [(ATP+½ADP)/(ATP+ADP+AMP)], \quad (1)$$

where ATP, ADP and AMP (in µmol g^{-1} wet mass) represent the different nucleotide concentrations of the adenylate energy pool (see above).

2.5. Anaerobic and aerobic ATP production

Total metabolism consists of the energy produced *via* oxidative phosphorylation (estimated from the O_2 consumption rate) and that produced by anaerobic metabolic pathways (McDonald *et al.*, 1998). Aerobic production of energy, in ATP equivalents, is 6 ATP's per O_2 molecule consumed. Anaerobic production is accounted for by anaerobic energy equivalents (AEE) and measured by the increase in anaerobic end-products (octopine and succinate) plus the concentration changes in energy reserve stores (ATP and PLA) via following equation (modified after McDonald *et al.*, 1998):

$$AEE = 1.5*(\Delta octopine) + \Delta ATP + \Delta PLA + 2.5*(\Delta succinate), \quad (2)$$

whereby the term 2.5*(Δsuccinate) was included according to Grieshaber *et al.*, 1994, as anaerobic degradation of 1 mol of free amino acid (AA) L-aspartate results in 1 mol succinate by generating 2.5 mol ATP. Anaerobic and total (aerobic and anaerobic) energy production was quantified in µmol ATP g^{-1} wet mass h^{-1}. Metabolic suppression and the individual energy contribution of the different energy resources quantified were calculated in percentage (%).

2.6. Statistics

All data are expressed as means ± SD. Significant differences to hypoxia (1% O_2, early and late) were assessed using one-way ANOVA. Previously, normality and homogeneity of variances were verified by Kolmogorov–Smirnov and Bartlett tests, respectively. Moreover, percentage data (i.e. metabolic suppression, individual ATP contributions) were previously transformed by arc sin square root function. Subsequently, post-hoc tests (Tukey HSD) were performed. In data sets with inhomogeneous variances, Kruskal-Wallis tests were applied. All statistical analyses were performed for a significance level of 0.05, using Statistica 11.0 software (StatSoft Inc., Tulsa, USA).

3. Results

Oxygen consumption rates in juvenile jumbo squids decreased significantly from 14.9 ± 6.1 to 4.3 ± 1.2 µmol O_2 g^{-1} wet mass h^{-1} during early hypoxia (EH, 1% O_2, 1kPa) and then declined non-significantly to 2.5 ± 0.9 µmol O_2 g^{-1} wet mass h^{-1} at late hypoxia (LH, $p>0.05$, Fig. 1, Tab. 1). The overall reduction in oxygen consumption rates was 71 and 83%, respectively.

Concomitantly, there was a significant change in the composition of the nucleotide pool from normoxia to EH ($p<0.05$), but not from EH to LH ($p>0.05$; Fig. 2, Tab. 1). More specifically, ATP levels (Fig. 2A) significantly decreased from 5.6 ± 0.5 (normoxia) to 1.3 ± 0.3 µmol g^{-1} wet mass (LH; $p<0.05$), which was accompanied by a significant increase in ADP (0.8 ± 0.2 to

3.3 ± 0.4 µmol g^{-1} wet mass; p<0.05; Fig. 2B; Tab. 1) and AMP concentrations (0.1 ± 0.0 to 1.0 ± 0.2 µmol g^{-1} wet mass; p<0.05; Fig. 2C; Tab. 1). On the other hand, the adenylate energy charge (AEC; Fig. 2D, Tab. 1) declined significantly from 0.9 ± 0.0 at normoxia to 0.6 ± 0.0 at EH (p<0.05), and to 0.5 ± 0.0 at LH (p>0.05; Fig. 2D, Tab. 1).

Moreover, the phospho-L-arginine store was also significantly depleted in both hypoxia treatments, namely from 25.2 ± 5.3 (normoxia) to 8.4 ± 2.8 µmol g^{-1} wet mass at EH (p<0.05; Fig. 3A, Tab. 1). It further declined to 5.3 ± 0.7 µmol g^{-1} wet mass at LH (p>0.05). At the same time, the end product of PLA breakdown, L-arginine, significantly increased from 15.3 ± 4.5 (normoxia) to 33.3 ± 7.4 µmol g^{-1} wet mass (EH), but it decreased significantly to initial values at LH (13.7 ± 2.9 µmol g^{-1} wet mass; Fig. 3B, Tab. 1). The total phosphagen pool (PLA+L-Arg) was kept unchanged from normoxia to EH (p>0.05), but it decreased significantly from 40.5 ± 9.0 (EH) to 18.9 ± 3.5 µmol g^{-1} at LH (p<0.05; Fig. 2C; Tab. 1). Consequently, the ratio of PLA/(PLA+L-Arg) decreased significantly from 0.6 ± 0.1 at normoxia to 0.2 ± 0.0 at EH (p<0.05) and then to 0.3 ± 0.0 at LH (p<0.05; Fig. 3D, Tab. 1).

The anaerobic metabolite, octopine, was significantly accumulated during hypoxia exposure, ranging from 3.8 ± 1.0 at normoxia to 27.9 ± 6.9 µmol g^{-1} wet mass at LH (p<0.05; Fig. 4A; Tab. 1). Moreover, succinate levels also increased significantly from 0.2 ± 0.0 (normoxia) to 0.4 ± 0.1 µmol g^{-1} wet mass at EH (p<0.05), and to 0.7 ± 0.1 µmol g^{-1} wet mass at LH (p<0.05; Fig. 4B, Tab. 1).

The anaerobic ATP production, expressed as anaerobic energy equivalents (AEE), increased significantly throughout the hypoxia exposure, varying from 6.3 ± 1.6 (normoxia) to 21.4 ± 5.6 µmol ATP g^{-1} wet mass h^{-1} at LH (p<0.05; Fig. 5A, Tab. 1). In contrast, the total (aerobic and anaerobic) energy production declined significantly from 95.4 ± 35.3 (normoxia) to 46.6 ± 7.9 µmol ATP g^{-1} wet mass h^{-1} at EH (p<0.05), and then decreased non-significantly to 36.6 ± 3.7 µmol ATP g^{-1} wet mass h^{-1} at LH (p>0.05; Fig. 5B, Tab. 1). Consequently, a significant suppression of total metabolism (45% at EH and 58% at LH) was observed (p<0.05; Fig. 5C, Tab.1).

Last, aerobic energy production decreased significantly from 100% at normoxia to 55% at EH (p<0.05) and continued falling to 41% at LH (p>0.05; Fig. 5D, Tab. 1). The residual 45% of energy production was met anaerobically at EH, whereby 30% were achieved solely via energy reserve depletion (ATP: 6% and PLA: 24%; see Fig. 5D, Tab. 1). On the other hand, at LH, total anaerobic ATP production was 59% with the majority (36%) generated via fermentative pathways (octopine: 35%; succinate: 1%; Fig. 5D, Tab. 1).

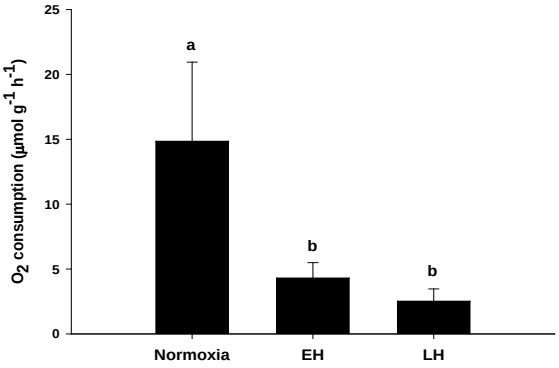

Fig. 1 Figure 1 – Oxygen consumption rates (in µmol g^{-1} wet mass h^{-1}) of juvenile *Dosidicus gigas* under normoxia (21% O_2), and early (EH) and late hypoxia (LH; 1% O_2). Values are expressed as means ± SD. Different lowercase letters indicate significant differences (p < 0.05, n = 5).

4. Discussion

4.1. Metabolic rates, energy reserve depletion and fermentative pathways

In the present study, oxygen consumption rates were significantly reduced in juvenile jumbo squids exposed to severe hypoxia (1% O_2) (EH: 71% and LH: 83%, respectively; Fig. 1), which corroborates our previous findings (65-85%; Rosa and Seibel, 2008, 2010; Trübenbach *et al.*, 2013a and b). The decline in O_2 consumption, besides a temperature-dependent response, has been linked to metabolic suppression (Rosa and Seibel, 2008 and 2010; Seibel, 2011). Jumbo squids spend ≥ 70% of their day time below their P_{crit} (~1.7kPa; Gilly *et al.*, 2006; Seibel, 2013; Trueblood and Seibel, 2013), the O_2 level where anaerobic energy production kicks in, and that is usually accompanied by the onset of metabolic suppression.

In well-oxygenated waters (and under routine conditions), the energy demand of marine animals is met aerobically via mitochondrial oxidative phosphorylation. However, under severe hypoxia (below P_{crit}), O_2 uptake is insufficient and has to be supplemented by anaerobic ATP production to maintain routine metabolic rates. Squids are known to store phospho-L-arginine in their muscles (Pörtner and Finke, 1998); this compound is found in high concentrations in the mantle and acts like phosphagen (i.e. as a reservoir of energy-rich phosphate). It maintains ATP at a constant level (Grieshaber *et al.*, 1994; Hochachka and McClelland, 1997) when the muscle is active and the ATP demand is higher than aerobic ATP provision by the mitochondria (Pörtner and Finke, 1998). Thereby, in a single reaction, the phosphate-bond energy is rapidly transferred (Grieshaber *et al.*, 1994) from the PLA store and then coupled to ADP. The ATP level in the muscle tissue of juvenile *D. gigas* could not be buffered via PLA breakdown resulting in a significant depletion of ATP and PLA reserve stores at EH, that was accompanied by a simultaneous increase in ADP and AMP (Fig. 2A, B and C, Fig. 3A, Tab. 1). These findings are in agreement with previous data in the coastal squid *Loliguncula brevis*, where PLA degradation could not prevent the breakdown of ATP during intense muscular activity (Finke *et al.*, 1996).

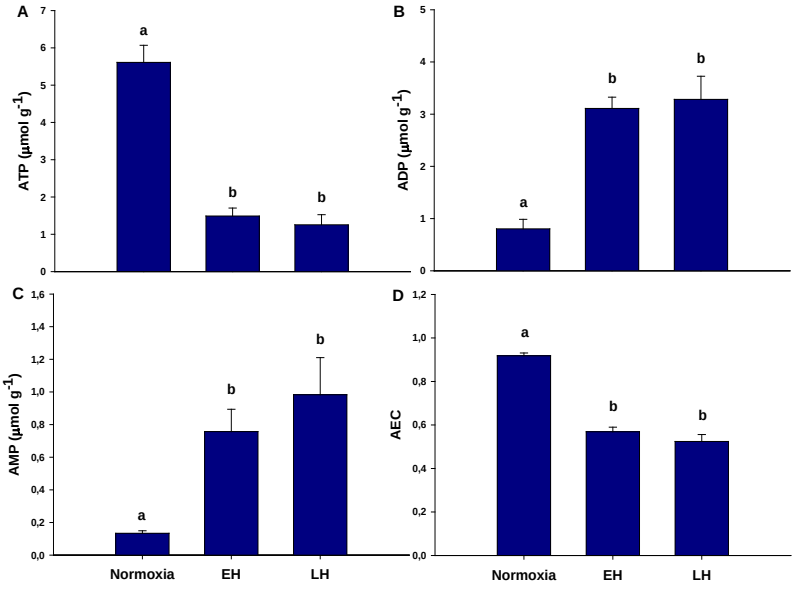

Fig. 2 Effect of hypoxia (1% O_2) on the nucleotide pool (in µmol g^{-1} wet mass) in muscle tissue of juvenile *Dosidicus gigas*: A) ATP, B) ADP, C) AMP, and D) the adenylate energy charge (AEC). Values are expressed as mean ± SD (n=5). Lowercase letters indicate significance between the oxygen treatments ($p < 0.05$).

Glycogen depletion also plays an important part in the anaerobic ATP production (Hochachka and Somero, 1973; Shulman et al., 2002). In cephalopods, pyruvate (end product of the Embden-Meyerhof-Parnas pathway) condenses with L-arginine and forms the opine, octopine (Grieshaber and Gäde, 1976). Thus, L-arginine is either produced by hydrolysis of PLA or is taken from the extracellular pool (Pörtner and Finke, 1998). However, the build-up of octopine is accompanied by proton (H^+) formation leading to marked intracellular acidosis (Pörtner *et al.*, 1991, 1993 and 1996) and acid-base disturbances under long-term hypoxia. In juvenile jumbo squids, PLA depletion

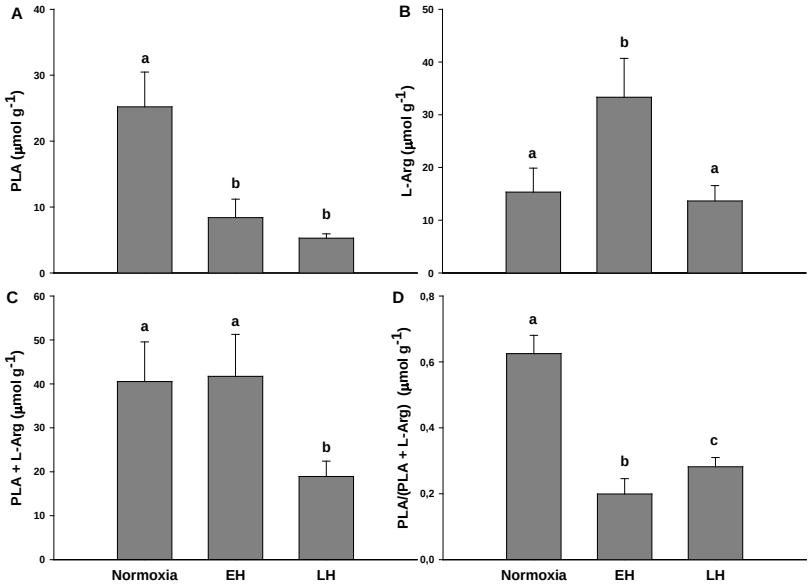

Fig. 3 Effect of hypoxia (1% O_2) on the phosphagen pool (in µmol g^{-1} wet mass) in muscle tissue of juvenile *Dosidicus gigas*: A) phosphor-L-arginine (PLA), B) L-arginine (L-Arg), C) phosphagen pool (PLA+L-Arg), and D) the ratio PLA/(PLA+L-Arg). Values are expressed as mean ± SD (n=6). Lowercase letters indicate significance between the oxygen treatments ($p < 0.05$).

in the muscle tissue resulted in a significant accumulation of L-arginine at EH, whereas the total phosphagen pool (PLA + L-Arg) remained unchanged (Fig. 3, Tab. 1). On the other hand, L-Arg and total phosphagen concentrations significantly decreased at LH with a concomitant (and significant) build-up of octopine (Fig. 3 and 4A, Tab. 1). This indicated that there was a switch from rapid energy reserve depletion under EH (via ATP and PLA) to fermentative processes under LH (Fig. 5D, Tab. 1). In hypoxia tolerant organisms, glycogen fermentation is coupled to the transamination of free amino acids (L-aspartate and L-glutamate) by generating succinate and ATP (via malate and fumarate synthesis; Grieshaber *et al.*, 1994). Additionally, during sustained severe hypoxia (i.e. under progressed acidosis), succinate can also be produced directly at the phosphoenol branching point, a pathway that elevates anaerobic ATP production (Grieshaber *et al.*, 1994). However, under low pO_2, the respiratory chain will become O_2 limited and succinate accumulates, indicating the onset of cytosolic and mitochondrial hypoxia (Grieshaber *et al.*, 1994; Finke *et al.*, 1996). Succinate levels significantly increased in EH and LH (Fig. 4B, Tab. 1), probably due to free amino acid (i.e. L-aspartate and L-glutamate) and/or protein degradation. Many mollusks have major carbohydrate reserves and glycogen stores as high as 5% of total body weight (i.e. bivalves; Giese, 1969), but in active mollusks like squids the glycogen storage potential is less than 0.4% of body weight (Rosa et al. 2005) and therefore a few minutes of activity can significantly deplete them (Storey and Storey, 1983). Squids, instead, developed the ability to maximally catabolize proteins (Hochachka *et al.*, 1973; Storey and Storey, 1978; O'Dor and Wells, 1987; Shulman *et al.*, 1984, 1993). Therefore, the high power outputs via protein degradation in squids may have been achieved by the evolution of enzymes that increase the ability to feed amino acids into the Krebs cycle (O'Dor and Webber, 1986). Moreover, in the vertical migrating squid *Sthenoteuthis oualaniensis*, a considerable amount of the protein substrates (up to 50%) was even used anaerobically under well-oxygenated conditions, a strategy that has been linked to 'overfeeding' and increased hypoxia tolerance (Shulman *et al.*, 2002).

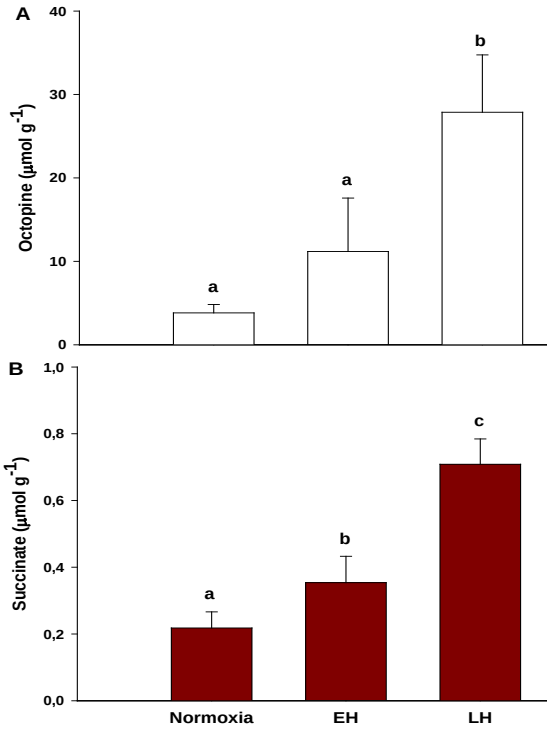

Fig. 4 Effect of hypoxia (1% O_2) on the production of anaerobic metabolites (in µmol g^{-1} wet mass) in muscle tissue of juvenile *Dosidicus gigas*: A) octopine and B) succinate. Values are given as means ± SD (n=5). Lowercase letters indicate significance between the oxygen treatments normoxia (21% O_2), early and late hypoxia (1% O_2) ($p < 0.05$).

In our previous study, we found out that there were significant changes in the locomotory activity of juvenile jumbo squids during the transition from EH to LH (Trübenbach *et al.*, 2013a). In fact, jumbo squids at EH still showed a high level of activity (cycling behavior) that disappeared under LH with the onset of lethargy. This behavioral strategy was assumed to be associated to energy reserve depletion and possible changes in squid's energy expenditure, since progressing hypoxia leads to intracellular acidosis and severe acid-base disturbances. Thus, *D. gigas* might spend more energy in essential processes like ion regulation instead of locomotion. The reduction in locomotory activity is a common strategy employed to safe energy under hypoxia. For instance, in the Atlantic cod fish (*Gadus morhua*) the motility was reduced by 60% when the dissolved O_2 concentration fell below 3 mg O_2 l^{-1} (Schurmann and Steffensen, 1994), whereas the eelpout *Zoarces viviparous* rested nearly motionless at the bottom when the O_2 level dropped below 2.9 mg O_2 l^{-1} (Fischer *et al.*, 1992) and the Norway lobster *Nephrops norvegicus* ceased its digging behavior when O_2 was reduced to 2.5 mg O_2 l^{-1} (Eriksson and

Baden, 1997). Our adenylate energy charge (AEC) data, an indicator of organism's health condition, supports the idea that under progressing hypoxia (LH), the jumbo squid is more stressed, since the values dropped significantly from initial 0.9 (for healthy, reproducing and growing organisms) to 0.6 at EH and further declined to 0.5 at LH (AEC: 0.5-0.7; stress indicator; Zaroogian and Johnson, 1989).

4.2. Metabolic suppression

The anaerobic ATP production (AEE) in *D. gigas* was significantly elevated under severe hypoxia, but the routine energy turnover (total ATP production) could not be maintained and was significantly reduced (Fig. 5A and B, Tab. 1). This indicated active metabolic suppression (EH: 45%; LH: 60%), and the differences between EH and LH (Fig. 5C) seems to be the result of a further (non-significant) reduction in the O_2 consumption rates at LH (Fig. 1) that might be explained by changes in locomotory and ventilatory performances (Trübenbach *et al.*, 2013a). In our previous

Table 1 Results of one-way ANOVA and Kruskal-Wallis tests evaluating the effects of early and late hypoxia (1% O_2) on oxygen consumption rates (µmol O_2 g^{-1} wet mass h^{-1}), octopine (µmol g^{-1} wet mass) and succinate concentrations (µmol g^{-1} wet mass), phosphagen and nucleotide pools (in µmol g^{-1} wet mass), anaerobic and total energy production (µmol ATP g^{-1} wet mass h^{-1}), metabolic suppression (in %) and individual energy contribution (in %) in juvenile *Dosidicus gigas*.

	df	F/H	p
Oxygen consumption			
RMR	2	12.0	0.003
Anaerobic metabolites			
Octopine	2	10.6	0.005
Succinate	2	67.2	0.000
Phophagen pool			
PLA+L-Arg	2	13.4	0.000
L-Arg	2	21.4	0.000
PLA	2	47.7	0.000
PLA/(PLA+L-Arg)	2	126.6	0.000
Nucleotide pool			
ATP	2	272.6	0.000
ADP	2	104.2	0.000
AMP	2	41.0	0.000
AEC	2	429.3	0.000
Energy production			
AEE	2	17.3	0.000
Total ATP	2	10.8	0.004
Metabolic suppression	2	8.8	0.004
Energy contribution			
Aerobic	2	103.4	0.000
Octopine	2	23.7	0.000
Succinate	2	75.7	0.000
ATP	2	317.8	0.000
PLA	2	227.3	0.000

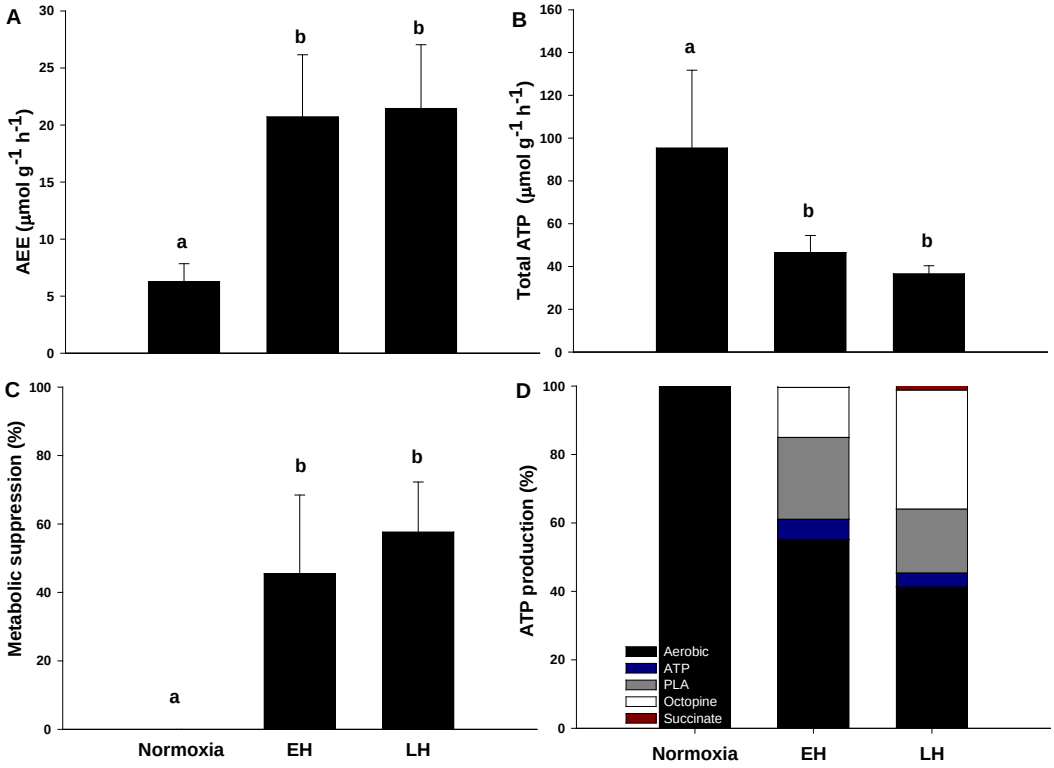

Fig. 5 Effect of hypoxia (1% O_2) on the energy turnover (in µmol ATP g^{-1} wet mass h^{-1}) in muscle tissue of juvenile *Dosidicus gigas*: A) anaerobic energy equivalents (AEE), B) total (aerobic and anaerobic) ATP production, C) metabolic suppression (in %), and D) the individual contribution of different energy resources (in %). Values are expressed as mean ± SD (n=5). Lowercase letters indicate significance between the oxygen treatments ($p < 0.05$).

study, jumbo squids maintained a high level of activity at EH by breathing more deeply (i.e. increased mantle contraction strength and reduced mantle contraction frequency) resulting in an elevated O_2 extraction efficiency of 60% (at inactive metabolic rate; without consideration of potential skin respiration), whereas *D. gigas* at LH was lethargic, probably as consequence of progressing acidosis, acid-base disturbances and energy reserve depletion (Trübenbach *et al.*, 2013a). In cephalopods, locomotory and respiratory activities are closely tied (Wells *et al.*, 1988) and therefore a reduction in locomotion is expected to impair O_2 uptake. Even though O_2 extraction efficiency was still 40% increased in lethargic jumbo squids (without consideration of potential skin respiration; Trübenbach *et al*., 2013a), probably by using the collar-flap system to uncouple ventilation from locomotion (Bone *et al.*, 1994), it still was 20% lower than in EH. The progressing hypoxia might lead to uncompensated acidosis in the blood of jumbo squids that, in turn, interferes with the O_2 uptake efficiency of their highly pH sensitive respiratory protein (Pörtner, 1994; Pörtner, 2002; Melzner *et al*., 2007). Thus, metabolic suppression is a physiological strategy that enable jumbo squid to survive prolonged periods of O_2 deprivation within the OMZ, but it's extend is restricted, since fermentable substrates are limitative and deleterious end-products (e.g. protons [H^+]) accumulate (Boutilier, 2001).

5. Conclusions

Juvenile jumbo squids are capable to undergo metabolic suppression, an important strategy in diel vertical migrating organisms of OMZs, to extend their residence time in food-rich and low-competitive hypoxic waters. Reduced aerobic energy capacity is partially compensated by an increase in anaerobic energy production. Moreover, we found a switch from rapid energy reserve depletion at EH to fermentative pathways at LH that seem to be reflected in jumbo squid's locomotory and ventilatory performance. It is worth noting that our anaerobic ATP calculations do not take into account energy production met by protein and/or amino acid degradation, a mechanism that seems to be important in terms of hypoxia tolerance, and thus the potential of metabolic suppression might be overestimated. In fact, *D. gigas* might use anaerobic protein degradation to improve its energy output, but further investigations are required.

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III Anaerobic muscle protein degradation in jumbo squids thriving in oxygen minimum zones?

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Short note, Marine biology, submitted

SHORT NOTE

Anaerobic muscle protein degradation in jumbo squids thriving in oxygen minimum zones?

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Abstract The jumbo squid, *Dosidicus gigas*, is an oceanic top predator in the Eastern Tropical Pacific that undergoes diel vertical migrations into mesopelagic oxygen minimum zones (OMZs). Besides glycogen breakdown, the pathways of the squid's metabolic (suppression) strategy are poorly understood. Here, juvenile *D. gigas* were exposed to oxygen levels found in the OMZ off Gulf of California (~1% O_2, ~ 10ºC at 250 m) with the aim to identify, via proteomic tools, eventual anaerobic protein degradation at such depths. Under hypoxia, total protein concentration decreased non-significantly from 79.2 ± 12.4 mg g^{-1} wet mass to 74.7 ± 11.7 mg g^{-1} wet mass ($p>0.05$). Yet, there was a significant decrease in heat shock protein (Hsp) 90 and α-actinin expression ($p<0.05$). The lower α-actinin expression at late hypoxia was probably related to decreased protection of the Hsp90 chaperon machinery resulting in increased ubiquitination ($p<0.05$) and subsequent degradation. Thus, the present findings indicate that *D. gigas* degrade, at least under progressing hypoxia, specific muscle proteins anaerobically. Moreover, the ubiquitin-proteasome system seems to play an important role in hypoxia tolerance, but further investigations are necessary to discover its full potential and pathways.

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Introduction

Jumbo or Humboldt squid *(Dosidicus gigas)* is a jet-propelled oceanic top predator endemic off the Eastern Pacific and one of its most pronounced features is the ability to perform diel vertical migrations into oxygen minimum zones (OMZs). Consequently, it spends > 70% of the day-time below its critical oxygen partial pressure (P_{crit}, ~1.6 kPa; Gilly et al. 2006; Seibel 2013). It is worth noting that active muscular squids were thought to be precluded from hypoxic areas (Pörtner 2002), as consequence of their physiological and anatomical restraints (Pörtner 1994, 2002; Melzner et al. 2007). Unexpectedly, *D. gigas* thrives in mesopelagic OMZs via metabolic suppression (Rosa and Seibel 2008, 2010; Trübenbach et al. 2013). Marine organisms exposed to hypoxia commonly suppress their metabolism by 50-95%, as energy reserve stores are limited and fermentative pathways less efficient, and supplement the remaining energy demand using a combination of available O_2 and anaerobic metabolic pathways (Seibel 2011). The main biochemical fuel under hypoxia is glycogen (i.e. gastropods, bivalves and sluggish fish; Shulman et al. 2002), but in squids, glycogen reserves are less than 0.4% of body weight (Rosa et al. 2005) and therefore a few minutes of activity can significantly deplete them (Storey and Storey 1983). Moreover, cephalopods evolved the ability to maximally feed on proteins (O'Dor and Wells 1986; Lamarre et al. 2012) and available evidence does indicate that squids, at least, under extreme conditions like at the end of their migrations show high levels of muscle proteolysis due to fasting and exercise (O'Dor et al. 1984; Shulman et al. 2002). Further, it has been shown that one of the most important mechanisms of adaptations, which provide

normal existence of planktonic crustaceans and fish in oxygen deficiency, may be anaerobic utilization of protein in energy metabolism. These data are supported by physiological research on whole organism (Schmidt-Nielsen 1975; Douglas et al. 1976; Shulman et al. 1993; Svetlichny et al. 1998), as well as subcellular and molecular studies (Hochachka et al. 1973). A similar phenomenon was noted for some predatory fish after hunting (Sukumaran and Kutty 1977). Under well-oxygenated conditions the nektonic squid *Sthenoteuthis oualaniensis* even used a considerable amount of the protein substrates (20 to 50%) anaerobically due to 'overfeeding', an adaptation that was linked to hypoxia tolerance (Shulman et al. 2002). This example of a peculiar functional hypoxia was connected, not with O_2 deficiency in the external environment, but with the necessity to utilize 'endogenous' O_2, which is formed during catabolism of reserve substances and probably from tissue destruction (Shulman et al. 1993).

Under food deprivation, the lipid reserves in cephalopods are called upon first, followed by an obligate dependence on the use of protein as a metabolic fuel (Boucher-Rodoni and Magold 1985; Houlihan et al. 1998; Grigoriou and Richardson 2009; Lamarre et al. 2012). In mammalian cell lines and especially in mammalian muscle during starvation, the ubiquitin-proteasome system (UPS) is the dominant route of protein degradation (Wing et al. 1995; Medina et al. 1995). This pathway was recently discovered in mantle tissue of starving *Sepia officinalis* and might fuel the metabolism with additional amino acids (Lamarre et al. 2012).

To identify the potential of anaerobic protein degradation in the muscle tissue of juvenile jumbo squids under severe hypoxia in the OMZs (1% O_2, 1 kPa), we performed: i) total protein concentration measurements, ii) protein expression profiles via 1D SDS-PAGE and western blot analysis, and iii) protein identification by MALDI-TOF/TOF.

Materials and methods

Specimen collection

Juvenile jumbo squids, *D. gigas* (d'Orbigny 1835), (2.3 – 29.7 g wet mass) were collected with a dip net in the Gulf of California (27°N, 111°W; 28°N, 113°W), on the surface at night, in June 2011 (aboard the *RV New Horizon,* Scripps Institute, CA, USA) and were immediately transferred to 10°C seawater (environmental temperature at OMZ) aquaria on board the vessel.

Experimental procedure

Animals were placed in a flow-through respirometry set up (270 ml volume, Loligo Systems, Tjele, Denmark) (Rosa and Seibel 2008; Rosa and Seibel 2010), and allowed to acclimate for 8–12 h. Respirometers were immersed in a large thermostatically controlled water bath (Lauda, Lauda-Königshofen, Germany) at 10°C, a temperature approximating that found at 250 m in the OMZ. Filtered (0.2 µm) and treated (50 mg l^{-1} streptomycin) seawater was pumped from a water-jacketed, gas-equilibration column through the respirometers at a constant flow rate (average 120 ml min^{-1}). The water in the column was bubbled continuously to maintain incoming water at high (21% O_2, 21 kPa,) or low PO_2 (certified gas mixture with 1% O_2, 1 kPa). All experiments were carried out in darkness and at atmospheric pressure. Afterwards, specimens were immediately weighed on a motion-compensated precision shipboard balance system (Childress and Mickel 1980) and muscle tissue samples frozen in liquid nitrogen. A total of 15 specimens were investigated, with 5 replicates for the normoxic treatment (21% O_2, 21 kPa) and 10 for the hypoxic one (1% O_2, 1 kPa). According to our previous study (Trübenbach et al. 2013) the hypoxic treatment was subdivided into early hypoxia (EH, < 160 min, N=5) and late hypoxia (LH, >180 min, N=5).

Protein extraction and concentration

Mantle tissue samples (0.2-0.7 g wet mass) of jumbo squids were homogenized (potter S type, B. Braun Biotech, IL, USA) in 1 ml RIPA buffer [(50 mM Tris-HCl pH 7.4; containing protease and phosphatase inhibitor (PMSP: phenylmethyl-sulfonyl fluoride)] and then centrifuged for 5 min at 1000 rpm (4°C; minispin, Eppendorf, Germany) to separate from debris. Supernatants were again centrifuged (30 min, 13,000 rpm, 4°C) and the final extracts stored at -80°C until further proceeded. It is worth noting that our protein extracts were performed at neutral pH (7.5) that mainly solves sarcomeric proteins, and therefore myofibrillar proteins like actin, paramyosin and myosin heavy chain (75-85% of total protein concentration; soluble at pH > 11 or < 3) might not be considered in our study.

Protein concentrations were determined immediately via the Bradford method using a microplate reader (Surprise, Tecan Group Ltd., Switzerland). A standard curve was generated with bovine serum albumin (BSA; Sigma-Aldrich, St. Louis, MO, USA) and deionized H_2O as blank (Bradford reagent Protein Assay Dye Reagent Concentrate, Bio-Rad laboratories

GmbH, München, Germany). The absorbance was quantified at 595 nm in a 96-well microplate and the protein concentrations were calculated in mg ml^{-1} and mg g^{-1} wet mass.

Protein profiles via 1D SDS-PAGE

For gel electrophoresis, 225 µg of each sample (N=5 per treatment) was treated with loading buffer (LB: 2% sodium dodecyl sulfate (SDS), 20% glycerol, 5% β-mercaptoethanol (β-ME; w/v/v) in 62.5 mM Tris–HCl pH 6.8) and denaturated by heat prior to loading (5 min at 95°C). Protein separation was performed via SDS-PAGE in 10% and 15% polyacrylamide separation/resolution gels (N=2, each) and 4% stacking gels. Electrophoresis was carried out with a standard vertical electrophoresis unit (18x16 cm, SE 600 series, Hoefer, MA, USA) at a constant current of 30 mA. During protein separation, the running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH≈ 9) was maintained at 10°C via a water bath (Lauda, Germany) to ensure high resolution of the gels. The separated proteins were visualized by staining overnight (ON) with Coomassie solution (2 g Coomassie r-250, 500 ml distilled water, 400 ml methanol, 100 ml acetic acid). Protein expression and profile patterns were analyzed with TotalLab Quant software (TotalLab Ltd., Newcastle upon Tyne, UK). Differences in protein expression (protein 2 and 3) were quantified as ratio by normalization to the constant expression of protein 1 (see Fig. 2A, B and C).

In gel protein analysis via MALDI-TOF/TOF

Bands of interest were manually excised from the gels using a disposable scalpel and thoroughly washed with deionized H_2O. Samples were sent in 1 ml deionized H_2O to IPATIMUP (Porto, Portugal) for protein identification by peptide mass finger print (PMF) following peptide sequencing/fragmentation (MALDI-TOF/TOF, PMF+MS/MS). Peptides of jumbo squid proteins were identified using the database of UniProt for mollusks (see Fig. 2D).

Western blots

Proteins (20 µg and 40 µg (Hsp90) per extract) were separated by SDS-PAGE (12% resolution/separation gels and 4% stacking gels) and transferred onto nitrocellulose membrane (Schleicher and Schüll, Dassel, Germany) by electroblotting. Membranes were blocked ON at 4°C in 20 ml blocking solution [BS, 1%, western blocking reagent, Roche; diluted in TBS (1x) (10 mM Tris-HCl, 150 mM NaCl, pH 7.5)].The primary antibodies used were raised against 1) α-actinin (H-300, Santa Cruz Biotechnology Inc., CA, USA), 2) Hsp90 (H-114, Santa Cruz Biotechnology Inc., CA, USA), and 3) ubiquitin (Sigma-Aldrich, MO, USA), diluted in 0.1% BS (1:500, 1:250 and 1:1000, respectively) and incubated ON at 4°C. Afterwards, membranes were washed 3x 15 min in TBS-T (1x) (10 mM Tris-HCl, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5) and subsequently stained with the secondary antibody solution for 3 h at 4°C (α-actinin and Hsp90: anti-rabbit IgG, Perking Elmer, MA, USA, dilution 1:2000 in 0.1% BS and ubiquitin: anti-mouse IgG: Sigma-Aldrich, MO, USA, dilution 1:4000 in 0.1% BS). Then membranes were washed 4x 10 min in TBS-T (1x) and activated with 1 ml of chemiluminescence solution (Luminata™ Classico, Western HRP substrate, Millipore, MA, USA) prior to detection (Image Quant, LAS-500, GE Health Care, Buckinghamshire, GB). Proteins were quantified using ImageJ software (Wayne Rasband, National Institute of Mental Health, Bethesda, MD, USA).

Statistics

Values are expressed as mean ± sd. One-way ANOVA's and Kruskal-Wallis tests were applied to indicate statistical differences. For all statistical analysis, STATISTICA (Tulsa, OK, USA) version 11.0 was used.

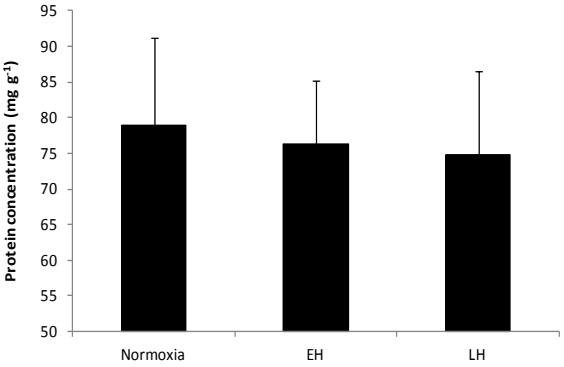
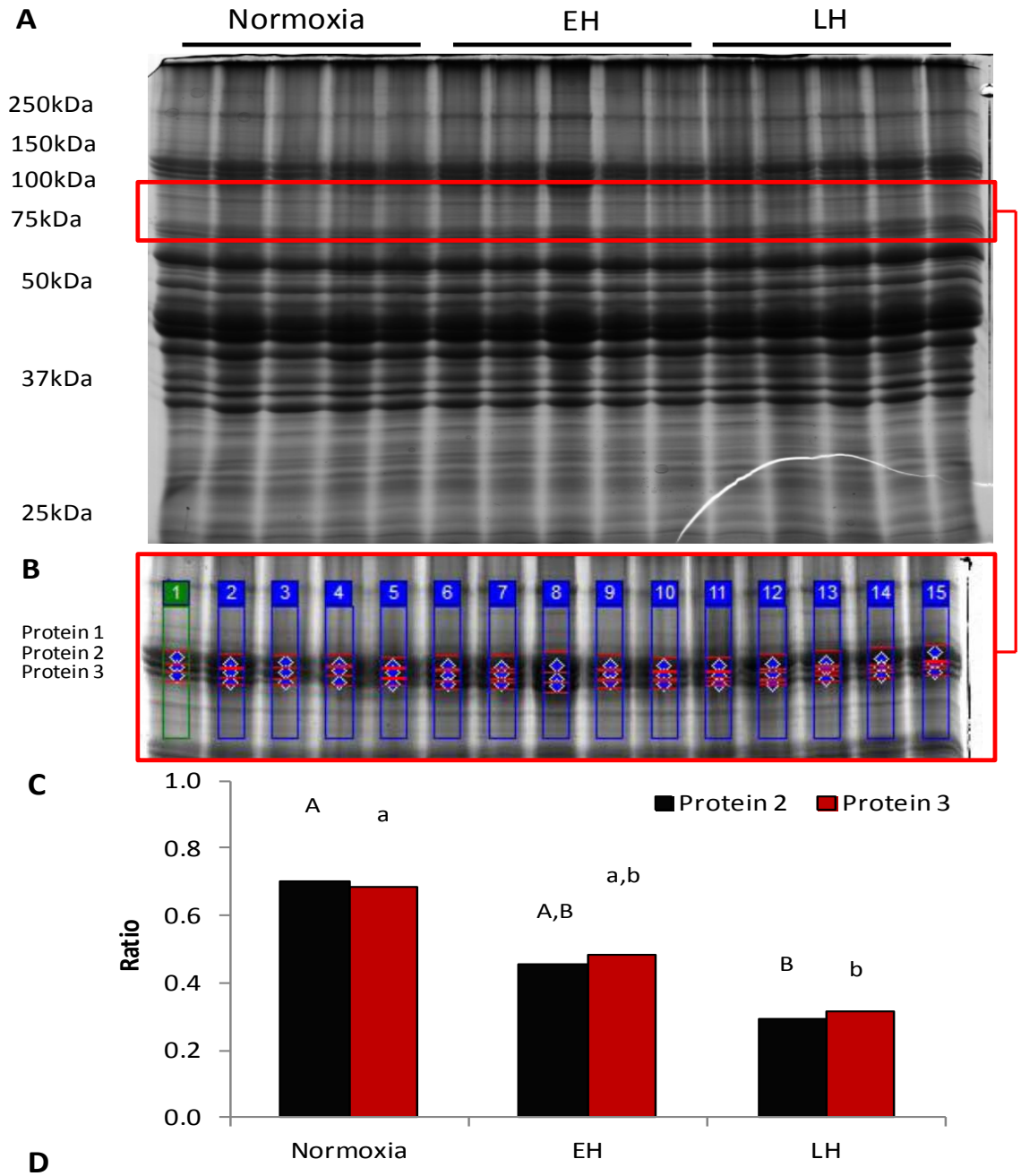


Fig. 1 Effects of hypoxia (1% O_2, 1 kPa) on the total protein concentration (in mg g^{-1} wet mass, N=5) in muscle tissue of juvenile *D. gigas*.

Results

The total protein concentration in the muscle tissue of juvenile *D. gigas* did not vary significantly with hypoxia exposure (Fig. 1; F=717.2, P=0.812), a finding that was also supported by the overall low variation in the protein expression profiles (10 and 15% 1D SDS- PAGE gels; see Fig. 2A). Yet, SDS-PAGE (S) and western blot (W) analysis revealed a significant reduction in the protein expression of α-actinin(~ 100 kDa) (S: H=12.02, P=0.003, Fig. 2; W: F=5.89, p=0.038, Fig. 3A) and Hsp90 (~ 90 kDa) (S: H=10.14, P=0.006,

Fig. 2; W: F=19.34, P=0.002, Fig. 3B) at late hypoxia (LH), which was also accompanied by a significant increase in the ubiquitination level of target proteins in the size of ~ 100 kDa (W: H=7.20, p=0.027, see red arrow in Fig. 3C) indicating the activation of the UPS.



D

Protein Number	Protein Identification	Acession Code	Protein Score	Protein Score C. I. %	Total Ion Score	Total Ion C. I. %	Peptides Matched
1	Phosphorylase	tr|Q596I0|Q596I0_CRAGI	81	99.9	69	100	7
2	α-actinin	tr|Q8ITH0|Q8ITH0_BIOGL	205	100	150	100	16
3	Heat shock protein 90	tr|F6KYK3|F6KYK3_MYTCH	104	100	82	100	4

Fig. 2 Effects of hypoxia (1% O_2) on the protein expression in the muscle tissue of juvenile *Dosidicus gigas*: A) SDS-PAGE protein profile (10% separation polyacrylamide gel), B) magnified area of the 10% SDS-PAGE showing the analyzed proteins 1, 2 and 3, C) statistical analysis of the differently expressed proteins 2 and 3 presented as ratio to the constantly expressed protein 1; letters indicate statistical differences ($p<0.05$, N=5, capital letters: protein 2; lower case letters: protein 3), and D) table of protein identification by MALDI-TOF/TOF analysis (peptides were analyzed in the database of UniProt for mollusks).

Discussion

Metabolic suppression of total metabolism is a prerequisite for the survival of prolonged bouts of O_2 limitation (Guppy and Withers 1999; Hochachka and Somero 2002; Bickler and Buck 2007), and our recent studies revealed that juvenile jumbo squids suppress their metabolism up to 60% when exposed to severe hypoxia (1% O_2, 1 kPa; ~3h; Rosa and Seibel 2008, 2010; Trübenbach et al. 2013). Thereby the remaining energy demand is supplemented using a combination of anaerobic metabolic pathways (Trübenbach et al. to be published elsewhere) and elevated O_2 uptake (Trübenbach et al. 2013). Commonly, metabolic suppression is accompanied by a decrease in protein synthesis (i.e. 50% in crucian carp: Smith et al. 1996; 70 to >90% in freshwater turtles: Land et al. 1993; Bailey and Driedzic 1996; Fraser et al. 2001), as it is one of the major energy consuming processes (18–26% of cellular energy expenditure; Hawkins 1991). As such, the downregulation of protein turnover is one of the major contributing factors to the depression in ATP turnover and metabolic suppression at the whole animal level (Guppy et al., 1994). It has to be coordinated with a blockage of protein decomposition in order to maintain structural integrity of the organism to make an instantaneous recovery possible when O_2 becomes available again (Grieshaber et al. 1994). In the present study, the total protein concentration in the muscle tissue of juvenile *D. gigas* did not vary significantly with hypoxia indicating that the majority of proteins were maintained under cessation of protein synthesis. Nonetheless, SDS-PAGE and western blot analysis revealed a significant reduction in the expression of Hsp90 at LH. Hsp90, despite being a heat-shock protein, is one of the most abundant proteins in unstressed cells (1–2% of cytosolic protein), where it plays a housekeeping function by controlling the activity, turnover, and trafficking of a variety of proteins (Pratt and Toft 2003). It has been demonstrated that Hsps play an important role in stress protection (Buchner 1999; Caplan 1999) and that hypoxia induces multiple vascular growth factors that upregulate Hsp90 expression (Gerber et al. 1997; Almgren and Olson 1999).

Yet, several studies reported that the expression of Hsps differs not only between tissues of the same species, but also between different species and stimuli (Dietz 1994; Currie et al. 2000; Deane and Woo 2003; Feidantsis et al. 2009, 2012). For example, fish white muscle exposed to extended fasting (*Dicentrarchus labrax*, Antonopoulou et al. 2013; *Salvelinus alpines;* Vijayan et al. 2006) and heat stress (*Acipenser transmontanus*,

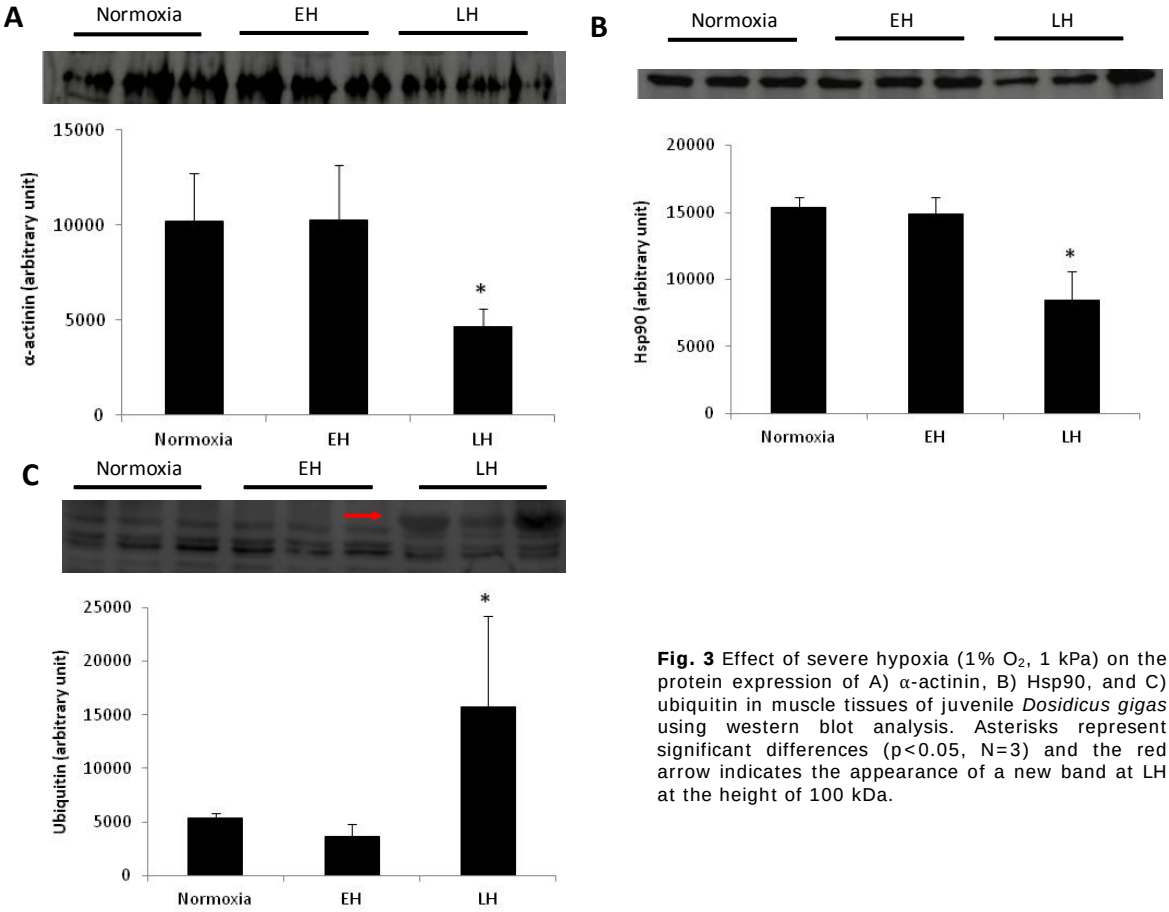


Fig. 3 Effect of severe hypoxia (1% O_2, 1 kPa) on the protein expression of A) α-actinin, B) Hsp90, and C) ubiquitin in muscle tissues of juvenile *Dosidicus gigas* using western blot analysis. Asterisks represent significant differences ($p<0.05$, N=3) and the red arrow indicates the appearance of a new band at LH at the height of 100 kDa.

Han et al. 2012) revealed lower Hsp90 levels linked to metabolic disruption of adaptive responses. If protein synthesis is decreased by hypoxia, less Hsp will be needed for various aspects of protein metabolism such as repairing or translocation of newly synthesized protein within or between cells (Hendrick and Hartl 1993), which might explain the decrease in Hsp90 in muscle tissue of *D. gigas* under metabolic suppression. Moreover, Hsp90 interacts with several regulatory co-factors in a sequential manner to assemble a functional chaperone machinery (Smith 1993; Pratt and Toft 2003) that folds specific proteins or even directs misfolded proteins to the cellular degradation machine for destruction (i.e. via UPS; Höhfeld et al. 2001).

Most of the Hsp90-regulated/stabilized co-proteins that have been discovered to date are involved in signal transduction like the hypoxia inducible factor 1α (HIF-1α; Minet et al. 1999; Isaac et al. 2002; Falson et al. 2005), which, after dimerization with the β-unit (ARNT) in the nucleus, activates specific target genes under hypoxia promoting angeogenesis, red blood cell maturation, glucose uptake and glycolysis (Bruick 2013). Although the von Hippel Lindau (VHL) gene product, the ubiquitin ligase responsible for regulating HIF-1α protein levels, efficiently targets HIF-1α for rapid proteasome-dependent degradation under normoxia, HIF-1α is resistant to the destabilizing effects of VHL under hypoxia (Huang et al, 1998; Kallio et al. 1999; Issac et al. 2002). Further, the HIF-1α/Hsp90 complexes dissociate under hypoxia (i.e. when HIF-1α is activated; Minet et al. 1999) and thus Hsp90 might hold HIF-1α in a repressed state and/or chaperoning its folding and the "activable" confirmation of HIF-1α under normoxic conditions (Whitelaw et al. 1993; McGuire et al. 1994; Antonsson et al. 1995). Thus, jumbo squid under LH might downregulate the protein expression of Hsp90 to increase the amount of stabilized HIF-1α, but it is still unclear whether the decrease in Hsp90 observed was due to active downregulation or due to degradation (i.e. via UPS).

In the present study, the expression of α-actinin in jumbo squid muscle was also significantly affected by LH. α-actinin is a cytoskeletal actin-binding protein and is localized at the Z-disk and analogous dense bodies, where it forms a lattice-like structure and stabilizes the muscle contractile apparatus in striated, cardiac and smooth muscle cells (Squire 1997; Otey and Carpen 2004; Sjöblom et al. 2008). Besides binding to actin filaments, α-actinin associates with a number of cytoskeletal and signaling molecules, cytoplasmic domains of transmembrane receptors and ion channels, rendering its important structural and regulatory roles in cytoskeleton organization and muscle contraction (Sjöblom et al. 2008). As α-actinin has been discovered as co-protein of Hsp90 (Falson et al. 2005), we may argue that the lower α-actinin expression at LH (Fig. 2 and 3A) was probably related to decreased protection of the Hsp90 chaperon machinery resulting in increased ubiquitination (Fig. 3C) and subsequent degradation. Moreover, the degradation of α-actinin might favor the inefficiency of jet-propulsion and might explain the lethargic behavior observed at LH in our previous study (Trübenbach et al. 2013).

The present findings indicate that juvenile *D. gigas*, at least under progressing hypoxia, might degrade specific muscle proteins anaerobically to extend its hypoxia tolerance. Moreover, the ubiquitin-proteasome system seems to play in important role, but further investigations are necessary to discover its potential and pathways. Such understanding may provide valuable information about how this species is responding to the vertical and horizontal expansion of the OMZs triggered by global climate change (Stramma et al. 2008).

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IV Hypoxia tolerance and antioxidant defense system of juvenile jumbo squids in oxygen minimum zones

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Hypoxia tolerance and antioxidant defense system of juvenile jumbo squids in oxygen minimum zones

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ABSTRACT

Jumbo squid (*Dosidicus gigas*) is a large oceanic squid endemic off the Eastern Tropical Pacific that undertakes diel vertical migrations into mesopelagic oxygen minimum zones. One of the expected physiological effects of such migration is the generation of reactive oxygen species (ROS) at the surface, promoted by the transition between hypoxia and reoxygenation states. The aim of this study was to investigate the energy expenditure rates and the antioxidant stress strategies of juvenile *D. gigas* under normoxia and hypoxia, namely by quantifying oxygen consumption rates, antioxidant enzyme activities [including superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST)], heat shock protein expression (Hsp70/Hsc70), and lipid peroxidation [malondialdehyde (MDA) levels]. A high significant decrease (68%) in squid's metabolic rates was observed during hypoxia ($p < 0.05$). This process of metabolic suppression was followed by a significant increase in Hsp70/Hsc70 expression ($p < 0.05$), which may be interpreted as a strategy to prevent post-hypoxic oxidative damage during the squid's night upwards migration to the surface ocean. On the other hand, in normoxia, the higher SOD and CAT activities seemed to be a strategy to cope with the reoxygenation process, and may constitute an integrated stress response at shallower depths. GST activity and MDA concentrations did not change significantly from normoxia to hypoxia ($p > 0.05$), with the latter indicating no enhancement of lipid peroxidation (i.e. cellular damage) at the warmer and normoxic surface waters. The understanding of such physiological strategies that are linked to oxygen deprivation and reoxygenation phases may provide valuable information about how this species is quickly responding to the impacts of environmental stressors coupled with global climate change.

1. Introduction

Jumbo squid, *Dosidicus gigas*, is a large pelagic squid (can reach 2 m of total length and 50 kg of weight) with a geographical distribution centered in the Eastern Tropical Pacific (ETP). Yet, its historical range has been recently expanding along the subtropical and temperate coasts of both North and South America (Keyl et al., 2008; Zeidberg and Robison, 2007). Besides the poleward movements, *D. gigas* is known to undertake diel vertical migrations into intermediate depths where it encounters zones of low oxygen (Bazzino et al., 2010; Gilly et al., 2006; Rosa and Seibel, 2008). These oxygen minimum zones (OMZs) are hundreds of meters deep and thousands of kilometers wide and have apparently expanded to higher latitudes in the last 50 yr (Stramma et al., 2008). For instance, the stable OMZ of the Gulf of California typically extends from ~250 to 800 m (southern part; $\leq 10^{\circ}C$, $\leq 20\ \mu M\ O_2 = \leq\ 2\%\ O_2$, see Fig. 1), but can be as shallow as 60 m (at the mouth of the Gulf; Fiedler and Talley, 2006), and results from microbial metabolism of sinking organic material generated by high surface productivity (Alvarez-Borrego and Lara-Lara, 1991; Roden, 1964). Thus, vertical migrations into the OMZ bring *D. gigas* into an environment in which the dissolved oxygen level is only a few percent of the saturated value at the surface (Gilly et al., 2006). Low oxygen levels are known to greatly limit the vertical distribution and ecology (i.e. predation, food competition) of many marine animals (Brill, 1994, 1996; Lowe et al., 2000; Wishner et al., 1998, 2000, 2008), but the jumbo squid thrive in such harsh environment by managing hypoxia via metabolic suppression (Rosa and Seibel, 2008, 2010; Trübenbach et al., in press).

One of the expected effects of the OMZ environment on the physiology of *D. gigas* is the generation of reactive oxygen species (ROS) at the surface, promoted by the transition between hypoxia and reoxygenation states, as upward migrations increase oxygen

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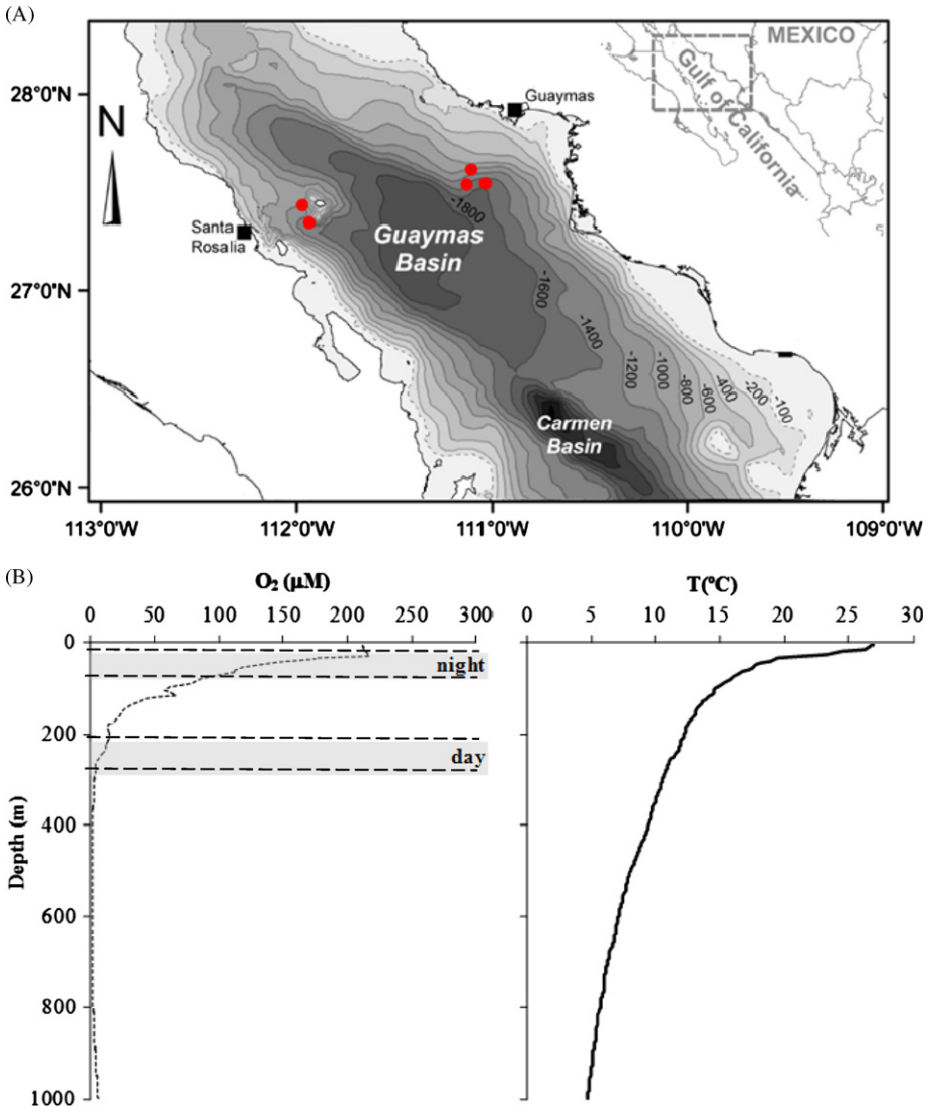

Fig. 1. Study area in the Gulf of California, Mexico (between Santa Rosalia and Guaymas). (A) Sampling locations are represented with red circles. (B) Oxygen levels and temperature conditions along a depth gradient. Measurements were obtained with a CTD profiler and oxygen sensor from R/V New Horizon at one of the stations located in the Guaymas basin. Grey bars (within long-dashed lines) represent the main depth range occupied by *D. gigas* during daytime (around 250 m) and night time (around 70 m) periods (obtained by tagging data, Gilly et al., 2006). (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

consumption with each cell generating about 0.1% ROS per molecule oxygen consumed (Fridovich, 2004).

In aerobic organisms, oxidative stress occurs when reactive oxygen species (ROS) cause damage that cannot be balanced by the organism's antioxidant defense system. ROS are molecules derived from oxygen, such as the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH.), with the latter one being the most reactive and destructive one (Pannunzio and Storey, 1998). ROS are mostly formed in mitochondria during O_2 reduction in the electron transport chain and can damage biological macromolecules (i.e. lipids, proteins and DNA (for review see Halliwell, 2006; Halliwell and Gutteridge, 2006), leading to functional alterations in cells and tissues. The most frequent cellular injury caused by non-neutralized ROS is called "peroxidation", which is the reaction of ROS with organism's lipids, especially membrane-associated ones (Lesser, 2012). The lipid peroxidation process is usually determined via malondialdehyde (MDA), one of the terminal products of the peroxidative breakdown of lipids (Pannunzio and Storey, 1998; Uchiyama and Mihara, 1978).

Many molecules, like reduced glutathione (GSH), vitamins, heat shock proteins and antioxidant enzymes, can eliminate or change the molecular configuration of ROS and by that protect organisms from cellular damage. An efficient antioxidant defense system is characterized by: (i) superoxide dismutase (SOD), which transforms O_2^- to H_2O_2, (ii) catalase (CAT), which converts H_2O_2 into H_2O and O_2 independently of any substrate and thereby inhibits its accumulation in cells and tissues, (iii) glutathione reductase (GR), which furnishes cells with the antioxidant glutathione (GSH, i.e. by reacting with O_2^- and OH.) and further supplies the enzyme activity of glutathione peroxidase (GPx) and glutathione-S-transferase (GST), (iv) GPx, which also eliminates H_2O_2 using GSH as substrate, and (v) GST, which, in association with GSH, transforms xenobiotics into other conjugates as part of a detoxification route (Lesser, 2006).

Besides changes in O_2 level, the jumbo squid also experience significant thermal habitat shifts during the night upwards migration to the surface ocean (Rosa and Seibel, 2008, 2010). One may argue that the squid's cellular heat shock response may include the up-regulation of genes encoding heat shock proteins

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(Hsps) as part of the cell's internal repair mechanism. The cytosolic heat shock proteins Hsp70 and Hsc70 are highly conserved members of the Hsp70 family (Ciavarra et al., 1994; Morimoto et al., 1990) and are found in diverse organisms (from bacteria to mammals) functioning as molecular chaperones and preventing protein unfolding among other important roles (Iwama et al., 1998, 1999). In addition to heat shock, the expression of Hsp70 has also been reported to be induced under various stress conditions such as pathogen infection, amino acid analog (Mosser et al., 1988; Williams and Morimoto, 1990), irradiation (i.e. UV, Yamashita et al., 2010), exposure to pollutants (i.e. heavy metal ions; Boone and Vijayan, 2002; Mosser et al., 1988; Yamuna et al., 2000), hypoxic condition (Cheng et al., 2003) and osmotic stress (Spees et al., 2002). Thereby, Hsp70 can be elevated several hundred times compared to non-stress conditions (Chuang et al., 2007). Hsc70, in turn, is constitutively expressed in normal cells and moderately induced (only several folds) under normal growth conditions (Ali et al., 1996; Chuang et al., 2007).

It is expected that ROS formation is enhanced with temperature increase (at shallower depths) as a consequence of squid's higher oxygen consumption rates, which will also trigger the antioxidant defense system and heat shock response. Although ROS formation under elevated temperature is well documented, it is still controversial under hypoxic conditions. High oxygen tensions lead to elevated ROS formation, but as a result from changes in mitochondrial redox state at low oxygen supply, ROS could also increase, as electron transport in the lower part of the respiratory chain is reduced (Brand, 2000; Hochachka and Lutz, 2001; Schumacker, 2003). However, several studies have shown that, during oxygen-restricted periods, some aquatic organisms (e.g. marine worms, gastrotrichs and turbellarians: Lesser, 2006; crustaceans: Desai and Prakash, 2009; Romero et al., 2007; fishes: Cooper et al., 2002; Hermes-Lima and Zenteno-Savín, 2002) activate antioxidant enzymes and heat shock proteins (Cheng et al., 2003; Teixeira et al., in press) as a biological tool to minimize post-hypoxic oxidative damage resultant from the reoxygenation.

Thus, the aim of this study was to analyze, for the first time, the link between hypoxia tolerance and antioxidant defense system of juvenile *D. gigas*, by exposing squids to oxygen levels found in the OMZ (1% O_2=10 μM O_2, 10 °C; Fig. 1) and quantifying: (i) the oxygen consumption rates, (ii) heat shock protein (Hsp70/Hsc70) response, (iii) lipid peroxidation (MDA level) and (iv) antioxidant enzyme activities (SOD, CAT and GST).

2. Materials and methods

2.1. Samples

Juvenile jumbo squids, ranging from 5.4 to 13.5 g wet weight, were collected in the Gulf of California (Santa Rosalia, 27°N, 112°W, and Guaymas, 27°N, 111°W; Fig. 1) at the surface during the night (with a hand-held dip net) aboard the R.V. New Horizon (Scripps Institute, California). After capture, specimens were immediately transferred to 10 °C seawater (average temperature around 250 m in the OMZ, Fig. 1) aquaria on board the vessel, where they were maintained up to 12 h before placement in a respiratory chamber. It is worth noting that all experiments were conducted with juvenile stages and it is not known if both juveniles and adult jumbo squids display similar diel vertical migration behavior (i.e. if they encounter the same minimum oxygen levels during descent). Nonetheless, we know that both stages show similar tolerance to low oxygen levels and suppress their metabolism to exact the same extent (Seibel, Trübenbach and Rosa, unpublished data). Moreover, the mass-specific metabolic rates in squids scale almost isometrically (Seibel, 2007; Rosa et al., 2009) and therefore shouldn't change much between juveniles and adults. This may be a consequence of the: (i) tubular geometry (mantle diameter increases faster than thickness with growth), exchange surfaces (surface area$^{1/2}$:volume$^{1/3}$ increases with size) and cutaneous respiration (~50%); (ii) smaller ontogenetic differences on locomotory expenditure, because squid's cost of transport may not decrease as much with size as in other animals; (iii) high energetic requirements during all stages of the squid's short-life cycle (for more discussion see Seibel et al., 2007; Rosa et al., 2009).

2.2. Hypoxia exposure and oxygen consumption rates (OCR)

Animals were placed in a flow-through respirometry set-up (Loligo Systems, Denmark; Rosa and Seibel, 2008, 2010), and allowed to acclimate for 12 h before starting measurements of oxygen consumption rates (routine metabolic rates). Respirometers were immersed in a large thermostatted (Lauda) water bath at 10 °C. To avoid bacterial contaminations, the seawater has been filtered (0.2 μm) and treated (50 mg L^{-1} streptomycin) before it was pumped at a constant flow rate (average 120 mL min^{-1}) from a water-jacketed, gas-equilibration column through the respirometers. The water in the column was bubbled continuously to maintain incoming water at high (normoxia, 21% O_2=210 μM O_2=100% O_2 saturation in seawater) or low oxygen partial pressure (PO_2) (hypoxia, certified gas mixture with 1% O_2=10 μM O_2=4.8% O_2 saturation in seawater). Oxygen concentrations were recorded at the entrance and at the exit of each chamber with two Clarke-type O_2 electrodes connected to a 928 Oxygen Interface (Strathkelvin Instruments, Scotland). The system was calibrated using air- and nitrogen-saturated seawater. Further, before and after each trial, the experimental setup was checked for electrode drift and microbial oxygen consumption. All experiments were carried out in darkness (stress avoidance) and at atmospheric pressure. Afterwards, specimens were immediately weighted on a motion-compensated precision shipboard balance system (Childress and Mickel, 1980) and oxygen consumption calculated according to their wet weight (ww) as μmol O_2 g^{-1} ww h^{-1}. A total of 30 specimens were investigated in terms of oxygen consumption rates, with 18 replicates for the normoxic treatment (21% O_2) and 12 for the hypoxic one (1% O_2). After the respiratory runs, a small portion of the mantle was immediately frozen in liquid nitrogen for further biochemical analysis (six specimens per treatment).

2.3. Analyses

2.3.1. Preparation of tissue extracts

Tissue homogenates (n=6 for normoxia and n=6 hypoxia) were prepared by using 150 mg of frozen mantle from each squid. All samples were homogenized (Ultra-Turrax, Ika, Staufen, Germany) in 500 μL phosphate buffer saline (PBS) solution (pH 7.3; 0.14 M NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4). All homogenates were then centrifuged (20 min at 14 000 × g at 4 °C) and Hsp70/Hsc70 production, lipid peroxidation and antioxidant enzyme activities (SOD, CAT and GST) were quantified in the supernatant fraction (according to Rosa et al., 2012). All enzymatic assays were tested with commercial enzymes obtained from Sigma (St. Louis, USA).

2.3.2. Heat shock response (HSR)

The heat shock response (HSR) was assessed from heat shock protein (Hsp70/Hsc70) production via enzyme-linked immunoabsorbent assay (ELISA) based on a protocol from Njemini et al. (2005).

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The homogenate supernatant (10 μL) was diluted in 250 μL PBS from which 50 μL was added in a 96 well microplates (Nunc-Roskilde, Denmark). After incubation (overnight, 4 °C) the microplates were washed (3 ×) with 0.05% PBS-Tween-20 (Sigma-Aldrich, USA) and the reaction stopped by adding 100 μL of blocking solution (per well; 1% bovine serum albumin (BSA), Sigma-Aldrich, USA). After incubation (2 h, room temperature (RT)) and washing step (PBS-Tween-20, see above), the primary antibody (anti-Hsp70/Hsc70, Acris USA), which detects 72 and 73 kDa proteins corresponding to the molecular mass of inducible Hsp70 and Hsc70, was added (50 μL of 5 μg mL^{-1} solution per well) and then incubated for 90 min at 37 °C. After removal of the non-linked primary antibody (washing step with PBS-Tween-20), the secondary antibody (anti-mouse IgC, Fab specific, alkaline phosphatase conjugate, Sigma-Aldrich, USA) was added (50 μL of 1 μg mL^{-1} solution), and further incubated for 90 min at 37 °C. Then microplates were washed again and 100 μL of substrate (SIGMA *FAST*™ *p*-nitrophenyl phosphate tablets, Sigma-Aldrich, USA) was added to each well and incubated 10–30 min at RT. The reaction was stopped with 50 μL NaOH solution (3 N; each well) and the absorbance determined in a 96 well microplate reader (BIO-RAD, Benchmark, USA) at a wavelength of 405 nm. The amount of Hsp70/Hsc70 was calculated from a standard curve based on serial dilutions (0–2000 ng mL^{-1}) of purified Hsp70 active protein (Acris, USA) and given as μg (Hsp70/Hsc70) g^{-1} ww.

2.3.3. Lipid peroxidation (MDA concentration)

Lipid peroxidation was obtained via the terminal product malondialdehyde (MDA) that accumulates due to oxidative cellular damage. The thiobarbituric acid reactive substances assay (TBARS assay; Uchiyama and Mihara, 1978) was used, in which thiobarbituric acid reacts with MDA to yield a fluorescent product that was detected spectrophotometrically at 532 nm. Homogenates were treated with 8.1% dodecyl sulfate sodium, 20% trichloroacetic acid (pH 3.5), thiobarbituric acid, mixture of n-butanol and pyridine (15:1, v/v) (Sigma-Aldrich, Germany) (Correia et al., 2003). To quantify the lipid peroxidation, MDA concentrations were calculated with the computer program Microplate Manager 4.0 (BIO-RAD, USA) based on an eight-point calibration curve (0–0.3 μM TBARS) using MDA bis-(dimethyl acetal) (Merck, Germany). The results were expressed as pmol g^{-1} ww.

2.3.4. Superoxide dismutase (SOD)

SOD activity was determined spectrophotometrically (BIO-RAD, Benchmark, USA) based on Sun et al. (1988) at 550 nm (25 °C). The assay contained 50 mM potassium phosphate buffer (pH 7.8), 3 mM ethylenediaminetetraacetic acid (EDTA), 3 mM xantine solution, 0.75 mM nitroblue tetrazolium (NBT), 100 mU xanthine oxidase solution (XOD) and 1 U μL^{-1} SOD enzyme solution. SOD from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard. The results of this enzymatic assay are given in units (U) g^{-1} ww, where one unit of SOD is defined as the amount of sample causing 50% inhibition of NBT reduction.

2.3.5. Catalase (CAT)

Catalase activity was determined according to Aebi (1984). The reaction contained 50 mM potassium phosphate buffer (pH 7.0), 12.1 mM H_2O_2 (as substrate) and was started by the addition of the sample. The consumption of H_2O_2 (extinction coefficient 0.04 mM cm^{-1}) was monitored using a spectrophotometer (Helios, Unicam, UK) at 240 nm. The absorbance was measured each 15 s for 180 s at 25 °C. Standard catalase activity was obtained using a bovine catalase solution (1523.6 U mL^{-1}; Sigma-Aldrich, Germany). The results are given as mU g^{-1} ww.

2.3.6. Glutathione-S-transferase (GST)

The determination of GST activity was performed according to the procedure described by Habig et al. (1974) and optimized for 96 well microplates. The GST enzyme activity was determined spectrophotometrically at 340 nm, every minute for 6 min using a microplate reader (BIO-RAD, Benchmark, USA). Thereby the increase in absorbance is directly proportional to the GST activity. The assay contained 200 mM L-glutathione reduced, PBS and 100 mM CDNB (1-chloro-2,4-dinitrobenzene) solution. Equine liver GST (Sigma-Aldrich, Germany) was used as standard. The results are expressed as U g^{-1} ww.

2.4. Statistics

Pearson's correlation coefficients were used to get an indication of the relationships among all the variables measured (oxygen consumption rates, heat shock protein response, lipid peroxidation and antioxidant enzyme activities). Subsequently, to test the effect of hypoxia in such physiological/biochemical variables, t-student and Mann–Whitney tests were performed. Values are expressed as mean ± SE. For all statistical analysis software STATISTICA (Tulsa, USA) version 10.0 was used.

3. Results

The exposure to hypoxic conditions, similar to those found in the OMZ of the Gulf of California led to a significant decrease (~ 68%) in the oxygen consumption rates (OCR) of *D. gigas* (Fig. 2). OCR decreased from 12.4 ± 0.8 μmol O_2 g^{-1} ww h^{-1} to 3.9 ± 0.5 μmol O_2 g^{-1} ww h^{-1} (Mann–Whitney, $p \ll 0.001$, Table 3). Hypoxia also had a differential effect in the activity of the several antioxidant enzymes, MDA accumulation and heat shock response.

Heat shock expression, namely Hsp70/Hsc70 levels, increased significantly from 22.7 ± 1.9 μg g^{-1} ww in normoxia to 40.7 ± 5.1 μg g^{-1} ww in hypoxia (Mann–Whitney, $p = 0.01$; Fig. 3 and Table 3). Lipid peroxidation did not change significantly between treatments, showing slightly elevated values under normoxic conditions (t-student, $p > 0.05$, Fig. 4, Table 3).

The antioxidant enzyme activities (SOD, CAT and GST) are presented in Fig. 5. SOD activity decreased significantly (Fig. 5A) from normoxia (38.5 ± 2.6 U g^{-1} ww) to hypoxia (20.8 ± 1.5 U g^{-1} ww; t-student, $p < 0.001$; Table 3). CAT activity also

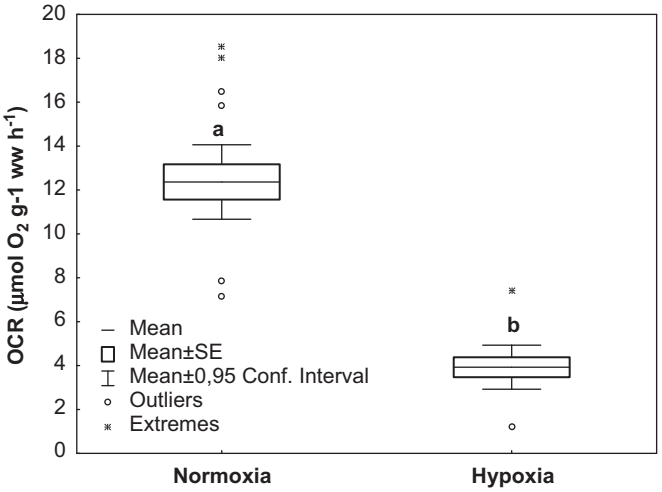

Fig. 2. Effect of hypoxia (1% O_2; 10 °C) on the oxygen consumption rate (OCR; in μmol O_2 g^{-1} ww h^{-1}) of juvenile jumbo squid, *D. gigas* (normoxia: $n = 18$; hypoxia: $n = 12$). Letters are indicating significant differences ($p < 0.05$).

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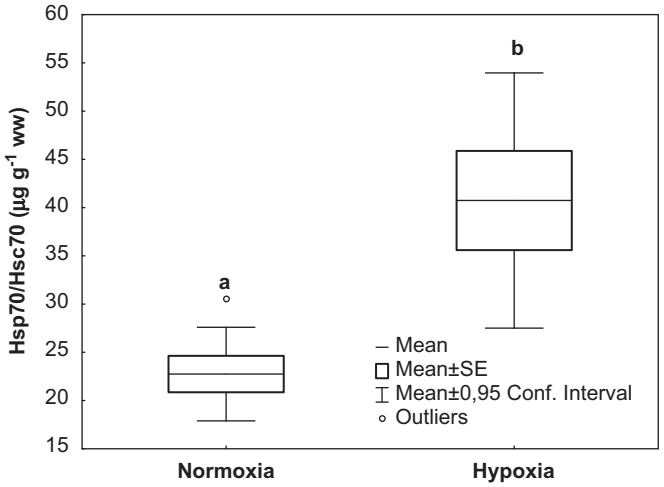

Fig. 3. Effect of hypoxia (1% O_2; 10 °C) on the heat shock response (Hsp70/Hsc70 concentrations, in µg g^{-1} ww) in the mantle tissue of juvenile jumbo squid *D. gigas* (normoxia: $n=6$; hypoxia: $n=6$). Letters are indicating significant differences ($p<0.05$).

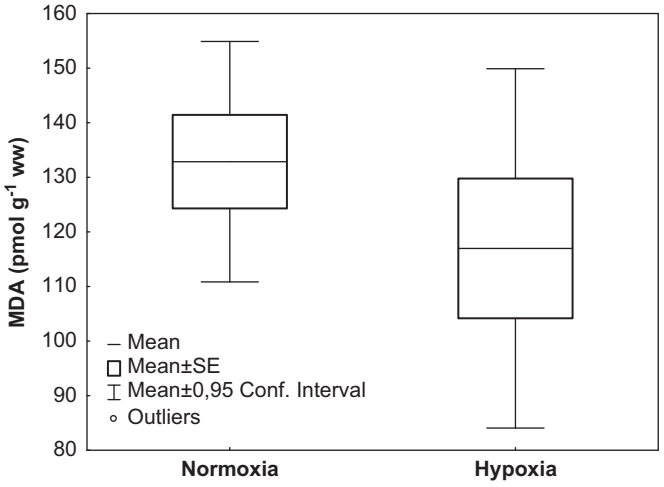

Fig. 4. Effect of hypoxia (1% O_2; 10 °C) on lipid peroxidation (MDA concentrations, in pmol g^{-1} ww) in the mantle tissue of juvenile jumbo squid *D. gigas* (normoxia: $n=6$; hypoxia: $n=6$). Letters are indicating significant differences ($p<0.05$).

followed the same trend, declining significantly from 413.3 ± 68.1 mU g^{-1} ww to 73.0 ± 15.7 mU g^{-1} ww in hypoxia (Mann–Withney, $p<0.01$; Fig. 5B and Table 3). In contrast, GST activity did not vary significantly between treatments (normoxia: 1.14 ± 0.03 U g^{-1} ww; hypoxia: 1.18 ± 0.08 U g^{-1} ww; t-student, $p>0.6$; Fig. 5C and Table 3).

As Table 1 demonstrates, there was a highly significant positive correlation between OCR and both SOD ($r=0.87$, $p<<0.001$) and CAT activities ($r=0.84$, $p=0.001$), and a highly significant negative correlation between OCR and Hsp70/Hsc70 levels ($r=-0.66$, $p<0.05$). It is also worth noting that there was a significant negative relationship between Hsp response and MDA concentration ($r=-0.58$, $p<0.05$).

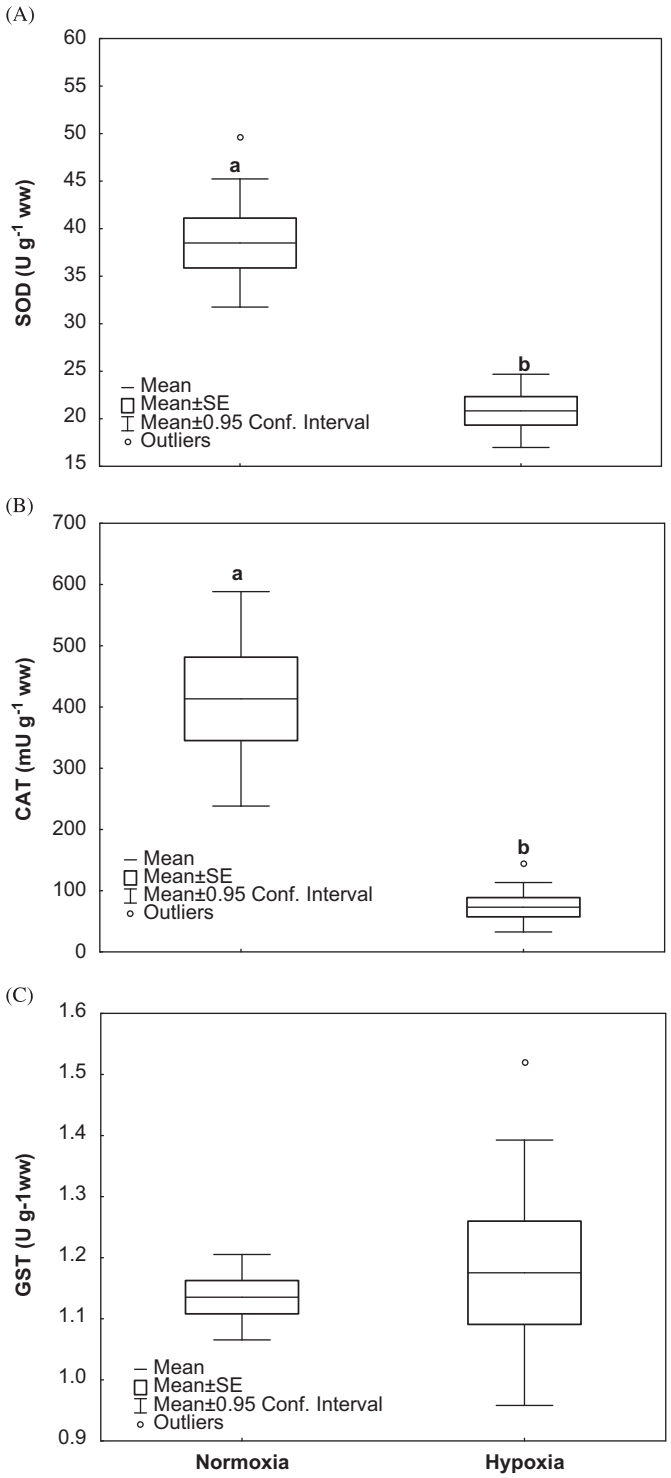

Fig. 5. Effect of hypoxia (1% O_2, 10 °C) on antioxidant enzyme activities in the mantle tissue of juvenile jumbo squid *D. gigas* (normoxia: $n=6$; hypoxia: $n=6$). (A) superoxide-dismutase (SOD; in U g^{-1} ww), (B) catalase (CAT; in mU g^{-1} ww), and (C) glutathione-S-transferase (GST, in U g^{-1} ww). Letters indicate significant differences ($p<0.05$).

4. Discussion

4.1. Metabolic suppression under hypoxia

D. gigas is an oceanic top predator that undergoes diurnal vertical migrations into pronounced OMZs (Gilly et al., 2006). At the warmer and normoxic surface waters (during night time), *D. gigas* displays one of the highest metabolic rates of any animal on the oceans (Rosa and Seibel, 2008). Yet, during the day, the jumbo squid actively avoids the surface thermal habitat and descends into the OMZ, a migratory behavior that greatly decreases its high energy demand. An important feature of the OMZ in the Guaymas

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Table 1
Pearson correlation coefficients between oxygen consumption rates (OCR), heat shock protein (Hsp70/Hsc70) response, antioxidant enzyme activities, namely catalase (CAT), superoxide-dismutase (SOD) and glutathione-S-transferase (GST) and lipid peroxidation (malondialdehyde (MDA) concentrations) in the mantle tissue of the jumbo squid *D. gigas*.

	OCR	Hsp70/Hsc70	CAT	SOD	GST	MDA
OCR	1.00					
Hsp70/Hsc70	−0.6*	1.00				
MDA	0.16	−0.58*	1.00			
SOD	0.87*	−0.70*	0.21	1.00		
CAT	0.84*	−0.54	0.15	0.62*	1.00	
GST	−0.13	−0.13	−0.01	0.04	−0.17	1.00

* Indicates statistical significance at the 5% level of significance.

Basin (Fig. 1) is that its upper boundary tends to correspond roughly to the daytime location of the acoustic deep-scattering layer (DSL), which is largely composed of mesopelagic micronekton, particularly myctophids that constitute the diet of *D. gigas* (Markaida et al., 2008). Thus, one may argue that the diel migratory movements of this squid might also reflect the vertical positioning of the DSL.

It is already known that to cope with those prolonged periods of O_2 restriction in the OMZ (maximum durations of 400/800 min below 300 m (~ 1% O_2, 1 kPa/200 m ~2% O_2, 2 kPa); Gilly et al., 2006), the jumbo squid suppresses its metabolism about 70–80% (at 1% O_2; juveniles: Rosa and Seibel, 2008, 2010; Trübenbach et al., in press; adults). Our present work corroborated these previous findings, as OCR's were greatly reduced in juvenile jumbo squids (average 68%, Fig. 2). On the other hand, most active fish predators are excluded from the OMZ (Prince and Goodyear, 2006), as their potential to tolerate hypoxic conditions is much lower (< 150 μM O_2; Brill, 1994, 1996; ~45 μM O_2, big-eye tuna, Lowe et al., 2000). Therefore, lower predation pressure and resource (food) competition at deeper depths might also favor jumbo squid's migratory behavior.

4.2. Heat shock response (HSR) during transition from hypoxia to normoxia

In the present study, Hsp70/Hsc70 concentrations were significantly increased in the muscle of juvenile jumbo squid under hypoxia (1% O_2, 2–4 h, Fig. 3), and we argue that this up-regulation constitute a preparation for the reoxygenation phase, i.e. the transition from hypoxic to normoxic conditions during squid's upward migration. During such phase, as oxygen is reintroduced, the electrons intensively "leak" from the electron transport chain and generate a burst of oxyradicals (Lushchak and Bagnyukova, 2006). Additionally, the increase in ROS formation during reoxygenation must be accelerated by the simultaneous rise of temperature during the squid's vertical ascent to the surface ocean.

Metabolic suppression is usually characterized by the shut-down of energetically demanding cellular activities (Guppy and Withers, 1999; Hermes-Lima and Zenteno-Savín, 2002; Hochachka and Somero, 2002; Seibel, 2011; Storey and Storey, 2004). Although Hsp synthesis is energetically costly (Tomanek, 2010; Tomanek and Somero, 1999), such up-regulation may be crucial for squid's cellular fitness because heat shock proteins are known to play an important role in repairing, refolding, and eliminating damaged or denatured proteins due to ROS formation (see De Oliveira et al., 2005). In fact, in other aquatic organisms, it has also been shown that after suppression of ATP-demand and ATP supply pathways leading to inhibition of protein synthesis, there is a "rescue" of protein synthesis mediated by preferential expression of certain genes in hypoxia tolerant cells (Hochachka et al., 1996, 1997).

An elevated HSP response during hypoxia has also been previously described in barnacles and oysters (on molecular level; see Table 2). In the latter, the increase in the Hsp70 mRNA pool was suggested to impact the signal transduction regulation and therefore might play an important role in early transcriptional regulation in some tissues, as their cells are ready to react very quickly to any stress situation (David et al., 2005). Further, hypoxia-dependent induction of chaperones (Benjamine et al., 1992) might favor the stability (or life-time) of individual proteins (Hochachka and Lutz, 2001), necessary to survive such conditions.

4.3. Relationship between antioxidant enzyme capacity, metabolic rates and cellular damage

SOD and CAT activity levels in mantle tissue of juvenile *D. gigas* were similar to those found by Zielinski and Pörtner (2000) in *Loligo vulgaris* (SOD: 88.0 ± 25.1 U g^{-1} ww, CAT: 0.0 ± 0.0 U g^{-1} ww) and *Sepia officinalis* (SOD: 157.2 ± 38.9 U g^{-1} ww, CAT: 20.6 ± 25.3 U g^{-1} ww), and confirm the low antioxidant capacity in cephalopods. These authors argued that the low antioxidant enzyme capacity in cephalopods is negatively correlated to their (high) metabolic rates, and that might explain their relative short life-span. In fact, other species with much lower metabolic rates, like the intertidal mussel *Mytilus edulis* and the worm *Arenicola marina*, display much higher SOD and CAT enzyme activities (*M. edulis* – SOD: 615 ± 52 U g^{-1} ww, CAT: 5870 ± 1060 U g^{-1} ww, Gamble et al., 1995; *A. marina* – SOD: 860 ± 423 U g^{-1} ww, CAT: 1210 ± 360 U g^{-1} ww; Buchner et al., 1996) than cephalopod species. Also, the white muscle tissue (low mitochondria density) of freshwater fishes like goby (*Perccottus glenii*, Lushchak and Bagnyukova, 2007) and carp (*Cyprinus carpio*, Lushchak et al., 2005a) shows much higher activity (i.e. SOD: ~225–750 U g^{-1} ww, CAT: ~1200–3750 U g^{-1} ww) than those found in cephalopods. The cephalopod mantle is composed of a thin inner and outer mitochondria-rich layer and a massive middle mitochondria-poor layer (similar to white muscle in fishes; Gosline and DeMont, 1985), and that might explain the lower antioxidant capacity. Yet, some investigations also point out a clear correlation between ROS formation and metabolic rate (Ku et al., 1993). If this holds true for the highly aerobic jumbo squid, a higher ROS formation and oxidative damage are expected at normoxia. Yet, MDA concentrations in the squid's muscle (indicative of cellular damage) did not significantly change between normoxic and hypoxic conditions, demonstrating an efficient antioxidant defense system.

4.4. Antioxidant enzymatic activity during transition from hypoxia to normoxia

Antioxidant enzymes are known to be intrinsically linked and dependent upon the activity of one another, and therefore, one would expect to see correlative changes in their activity (Cooper et al., 2002). In juvenile jumbo squid, both SOD and CAT activities were significantly correlated and reduced in hypoxia, a pattern also observed in other marine species from similar (OMZ) and different habitats (e.g. intertidal, subtidal; see Table 2). In normoxia, the enhanced SOD and CAT activities in the squid's muscle seemed to be a strategy to cope with the reoxygenation process and constitute an integrated stress response at shallower depths.

While the antioxidant defense strategies of OMZ (part-time or full) residents are poorly investigated (see Tremblay et al., 2010, 2012), those from intertidal species are much better understood. The latter are known to be highly efficient as they are exposed to

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Table 2
Review of the effects of hypoxia/anoxia in the antioxidant defense strategies of aquatic organisms. Letters represent following: H=hypoxia, A=anoxia, Air=air exposure, DVM=diurnal vertical migrations, d=days, h=hours, and ^{r}=recovery phase. Arrows in brackets indicate non-significant changes, 0=values not detectable, –=not measured in the cited study, and *=anoxia exposure (8 h).

Group/Species	Habitat	Exposure time	Sample	CAT	SOD	GST	MDA	Hsp70	References
Corals									
Veretillum cynomorium	Intertidal	Air (0.5–2.5 h)	Complete colony	↑	=	↑	=/↑ ^{r}	↑	Teixeira et al. (in press)
Mollusks									
Dosidicus gigas	OMZ	H (2–4 h)	Muscle	↓	↓	=	(↓)	↑	Present study
Littorina littorea	Intertidal	H (6 d)	Hepatopancreas	↓	↓	↓	=	–	Pannunzio and Storey (1998)
			Muscle (foot)	↑	↓	(↓)	=	–	
Crassostrea gigas	Intertidal	H (7–10 d/24 d)	Digestive gland	–	–	–	–	↓/↑	David et al. (2005)
Astarte borealis	Sublitoral	H (2 d/10 d)	Gills	(↑)/↓	(↑)/(↑)	–	–	–	Abele-Oeschger and Oeschger (1995)
Perna perna	Intertidal	Air (4 h/≥18 h)	Digestive gland	=/=	↑/(↓)	–/↑	–/↑	–	Almeida et al. (2005)/Almeida and Bainy (2006)
		Air (≥ 18 h)	Gills	=	(↓)	(↓)	↑	–	
Mytilus edulis	Intertidal	Air (6.5 h)	Digestive gland	–	↓	–	–	–	Letendre et al. (2008)
			Gills	–	=	–	–	–	
Crustaceans									
Balanus amphitrite	Intertidal	H (24 h)/A (24 h)	Muscle	↑/↑	↑/↑	–	–	↑/–	Desai and Prakash (2009)/Cheng et al. (2003)
Paralomis granulosa	Coastal	Air (6 h)	Gills	↑	↓	↑	(↓)	–	Romero et al. (2007)
			Muscle and hepatopancreas	↑	↑	↓	↑	–	
Chasmagnathus granulata	Estuarine/ Intertidal	A (8 h)	Gills	↑	↓	↑	=/↑ ^{r}	–	De Oliveira et al. (2005)
Nyctiphanes simplex	Coastal/above OMZ	DVM (warm season)	Complete animal	=	(↓)	=	(↑)	–	Tremblay et al. (2012)
Nematoscelis difficilis	OMZ		Complete animal	(↑)	(↑)	(↑)	(↑)	–	
Euphausia eximia	OMZ		Complete animal	(↓)	↓	(↓)	(↓)	–	
Polychaeta									
Heteromastus filiformis	Intertidal	H (6 h)/A (6 h)	Complete animal	↓/↑	↓/↓	–	–	–	Abele et al. (1998)
Arenicola marina	Intertidal	H (5 h/48 h)	Chloragog	↓/↓	=/=	–	–	–	Abele-Oeschger and Oeschger (1995)
Nereis diversicolor	Intertidal	A (6 h)	Complete animal (mature)	(↑)	(↑)	–	–	–	Abele-Oeschger et al. (1994)
Fish									
Carassius auratus	Freshwater	H (3 h/12 h)	Liver	=/(↑)	↑ *	↓/↑	↓/↓	–	Lushchak et al. (2001)/Lushchak et al. (2005b)
			Muscle	=/(↓)	–	↑/(↑)	↓/↓	–	
Perccottus glenii	Freshwater	H(2 h/10 h)	Liver	↓/↓	↑/↑	↑/=	=/↓	–	Lushchak and Bagnyukova (2007)
			Muscle	=/=	=/(↓)	↑/=	=/=	–	
Cyprinus carpio	Freshwater	H (5 h)	Liver	=	↓	–	↑	–	Lushchak et al. (2005a)
			Muscle	=	↑	–	↓	–	
Leiostomus xanthurus	Estuarine/ Coastal	H (12 h)	Gills	0	↑	–	–	–	Cooper et al. (2002)
			Liver	(↓)	=	–	–	–	
			Muscle	0	↑	–	–	–	

Table 3
Results of t-student and Mann–Whitney tests evaluating the effects of hypoxia (1% O_2) on oxygen consumption rates (OCR, $\mu mol\ O_2\ g^{-1}\ ww\ h^{-1}$), heat shock response (Hsp70/Hsc70 production, in $\mu g\ g^{-1}$ ww), lipid peroxidation (MDA concentration, in $pmol\ g^{-1}$ ww) and antioxidant enzyme activities (SOD, GST (in $U\ g^{-1}$ ww) and CAT (in $mU\ g^{-1}$ ww))in jumbo squid *D. gigas*.

	t/*U* value	*p*
Metabolic suppression		
OCR	1.00	0.000
Heat shock response		
Hsp70/Hsc70	1.50	0.010
Lipid peroxidation		
MDA	1.03	0.327
Antioxidant activity		
SOD	5.84	0.000
CAT	0.00	0.005
GST	−0.45	0.662

an extremely more variable environment (i.e. heat, irradiation, osmotic and hypoxic stress). Contrarily to the jumbo squid, some hypoxia-tolerant intertidal species are known to induce antioxidant production during hypoxia as a preparatory adaptation to protect against ROS production during reoxygenation (Abele and Pantarulo, 2004;Hermes-Lima and Zenteno-Savín, 2002; Pannunzio and Storey, 1998; Teixeira et al., in press). This increase in antioxidant enzyme activity during hypoxia is designated by "*preparation for oxidative stress*" and considered a key phenomenon in the protection against post-hypoxic free radical damage (Freire et al., 2011; Hermes-Lima and Zenteno-Savín, 2002).

Like the present study, there are many others that show that some antioxidant enzymes only increase during reoxygenation/recovery phase itself to minimize cellular damage (i.e. *Littorina littorea*: GST in hepatopancreas, CAT and SOD in muscle, Pannunzio and Storey, 1998; *Carassius auratus*: GST in white muscle tissue, Lushchak et al., 2001; *P. glenii*: GST in liver,

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Lushchak and Bagnyukova, 2007; see Table 2) leading to the conclusion that antioxidant defense systems are highly species (and even tissue) specific.

5. Conclusions

The present findings show that *D. gigas* may possess two distinct oxidative-stress related responses during its diel vertical migration. First, with the expected increase of ROS generation at the warmer (and normoxic) surface waters during night-time (as a consequence of the higher energy expenditure rates), SOD and CAT activities are enhanced. Second, while in the deep hypoxic conditions, there is a preparatory up-regulation of heat shock proteins to avoid cellular stress during the reoxygenation phase at the surface ocean. Thus, *D. gigas* seems to present a diversified group of physiological strategies (''metabolic suppression'' and ''Hsp preparation for oxidative stress'') to deal with oxygen deprivation and reoxygenation phases, and such understanding may provide valuable information about how this species is quickly responding to the impacts of environmental stressors coupled with global climate change (Rosa and Seibel, 2008; Zeidberg and Robison, 2007).

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3 General discussion and conclusions

The present dissertation addressed the physiological and biochemical mechanisms that enable the jumbo (or Humboldt) squid (*Dosidicus gigas*) to cope with prolonged dives into OMZs during daytime. This is a quite relevant research topic because (1) OMZs are vertically and horizontally expanding due to global warming with a steady decrease in O_2 level (Stramma *et al.*, 2008), (2) cephalopods are thought to be excluded from hypoxic areas due to anatomical and physical constraints (Pörtner, 2002), and (3) this squid species is target of the world's largest invertebrate fishing industry (14% of total squid catch; Rodhouse *et al.*, 2006). To determine the cost and benefits of such diel vertical migrations, I investigated the adaptive strategies in juvenile jumbo squids off the Gulf of California (also known as Sea of Cortez) with a focus on ventilation, locomotion, metabolism and antioxidant defense.

3.1. Biochemical and physiological adaptations in *Dosidicus gigas*

Based on the experimental results shown in the previous section, I can now address the research hypotheses formulated in the introduction:

a) Can D. gigas *regulate its ventilatory mechanisms to increase the O_2 uptake capacity?*

Despite locomotion and ventilation in cephalopods are closely tied via jet-propulsion (Wells and O'Dor, 1991), *D. gigas* shows an unexpected high capacity to regulate its ventilatory mechanisms. At normoxia, *D. gigas* is capable to regulate mantle contraction frequency and strength simultaneously according to activity (publication 1, Fig. 7B, D and 8), whereas other squids like *I. illecebrosus* and *L. opalescens* do not vary their ventilation *via* frequency with vigorous jetting, but *via* powerful contractions of their circular fibres (Webber and O'Dor, 1985; Wells *et al.*, 1988). At EH (< 160 min, 1 kPa O_2), jumbo squids breathe "deeply", with more powerful contractions and an enlarged inflation period (reduced mantle contraction frequency). Such deep-breathing behavior together with a reduction in the relaxed mantle diameter, favors diffusion pathways over gills and skin resulting in an elevated O_2 extraction efficiency (EO_2) of maximum 82% (33.6% with consideration of 60% potential skin respiration; $p<0.05$; publication 1, Fig. 7, 9 and 10). This finding supports the

high regulatory capacity of ventilation in *D. gigas*, as pelagic muscular squids that routinely depend on jet propulsion, are characterized by a low EO_2 (5–10%; maximum 25%; Wells *et al.*, 1988; Pörtner, 1994). Contrarily, at LH (> 180 min, 1 kPa O_2), all ventilatory processes are significantly reduced, except for EO_2 (45%/20% with and without 60% potential skin respiration). The elevation in EO_2 seems to be achieved by (1) using the collar flap system (uncoupling of ventilatory and locomotory mechanisms), as it is known for nautilus, resting sepia and to some extend for squids (Bone *et al.*, 1994) and (2) a further reduction in the relaxed mantle diameter ($p<0.05$; publication 1, Fig. 7, 9 and 10) to promote diffusion.

b) Does D. gigas *use metabolic suppression as strategy to survive prolonged descents into severe hypoxia?*

Even though *D. gigas* shows increased O_2 uptake potential (i.e. EO_2 values between 45-82% at hypoxia, see above) in the range of nektonbenthic cephalopods (50-90% in cuttlefish: Melzner *et al.*, 2006, 2007; 45-75% in octopus: Wells and Wells, 1985; Wells, 1990) and possesses a highly pH and temperature dependent high O_2-affinity respiratory protein (Seibel, 2013), under severe hypoxia (below P_{crit}), the energy demand cannot be met solely aerobically and has to be supplemented by anaerobic pathways (Grieshaber *et al.*, 1994; Childress and Seibel, 1998; Seibel, 2011). In fact, O_2 consumption rates in *D. gigas* are 70-80% reduced and cannot be compensated by the activation of anaerobic energy pathways (70% increased anaerobic energy equivalent (AEE) production) resulting in metabolic suppression (maximum 60% at LH; $p<0.05$; publication 2, Fig. 1 and 5A and C). At EH, anaerobic energy production (~ 45% of total metabolic performance) is primarily achieved via rapid energy reserve depletion (ATP and phosphor-L-arginine (PLA); $p<0.05$; publication 2, Fig. 2, 3 and 5D), means that the ATP level in muscle tissue of juvenile *D. gigas* cannot be buffered via PLA breakdown, as it was observed for *L. brevis* during intense muscular activity (Finke *et al.*, 1996). At EH, PLA depletion in *D. gigas* is accompanied by the accumulation of its breakdown product L-arginin that, in turn, condenses with the end product of glycolysis (pyruvate) to form octopine (Grieshaber and Gäde, 1976), the major anaerobic energy source at LH ($p<0.05$; publication 2, Fig. 4 and 5D). Thereby, the anaerobic energy contribution to total ATP production increases from 45% to 60% at LH due to a further decline in O_2 consumption probably as result of reduced EO_2. The continous decrease in O_2 consumption/uptake at LH cannot be balanced by an increase in AEE concentration (similar values at EH and LH; $p>0.05$; publication 2, Fig. 5A) leading to a further increase in metabolic suppression due to progressing hypoxia (from 45 to 60%; $p>0.05$; publication 2, Fig. 5C).

c) Does D. gigas *use anaerobic (muscle) protein degradation as mechanism to increase the energy output and extend hypoxia exposure time?*

One of the major contributing factors to the depression in ATP turnover is the downregulation of protein turnover (Guppy *et al.*, 1994) that has to be coordinated with a blockage of protein decomposition in order to maintain structural integrity of the organism to make an instantaneous recovery possible when O_2 becomes available again (Grieshaber *et al.* 1994). Total protein concentrations in the muscle tissue of juvenile *D. gigas* do not vary significantly with hypoxia ($p>0.05$; publication 3, Fig. 1) indicating that the majority of proteins are conserved under cessation of protein synthesis.

Glycogen storage potential in active muscular squids is less than 0.4% of body weight (Rosa *et al.*, 2005) and, under well-oxygenated conditions, they primarily feed on proteins (O'Dor and Webber, 1986; Lamarre *et al.* 2012), some even anaerobically, as it has been observed for *S. oualaniensis* (Shulman *et al.,* 2002). Even though total protein concentrations in muscle tissue of *D. gigas* only slightly decrease at severe hypoxia (1 kPa O_2), the study also indicates that specific muscle proteins (i.e. Hsp90 and α-actinin; $p<0.05$; publication 3, Fig. 2 and 3A and B) might be degraded anaerobically, at least under progressing hypoxia (> 180 min). Thereby, the lower α-actinin expression at LH seems to be related to a decreased protection via the Hsp90 chaperon machinery. This is resulting in increased ubiquitination ($p<0.05$; publication 3, Fig. 3C) and subsequent degradation. Therefore, the ubiquitin-proteasome system seems to play an important role in hypoxia tolerance, but further investigations are necessary to discover its full potential and pathways.

d) Does D. gigas *reduce its level of activity as strategy to conserve energy under severe hypoxia although locomotion and respiration in cephalopods are closely tied?*

The reduction in locomotory activity (i.e. reduced activity frequencies, lowered speed, lethargy; Fischer *et al.*, 1992; Schurmann and Steffensen, 1994; Eriksson and Baden, 1997; Rosa and Seibel, 2008, 2010) is a common strategy employed to safe energy under hypoxia especially under metabolic suppression (Childress and Seibel, 1998; Wu, 2002; Seibel, 2011), as fermentable substrates are limitative and simultaneously deleterious end products (e.g. H^+, organic compounds) accumulate (Boutilier, 2001).

D. gigas shows a distinct periodicity in the rate of O_2 consumption (activity) in the confines of a respiration chamber at normoxia (cycle length 20-60 min; see publication 1, Fig. 1A, 6A), and acoustic data revealed short-term cyclic vertical movements (~ 15-90 min) during day and night that has been interpreted as active forages by Gilly and colleagues (2006; Fig. 11). As the frequency and duration of brief vertical movements are similar to the

periodicity reported here, I argue that the respiratory cycles at normoxia may reflect the capacity to perform migratory forays. At EH, despite a similar diving behavior was observed via acoustic data during day time (Gilly *et al.*, 2006), the respiratory cycles observed (within the chambers) are shorter in length but increase in frequency accompanied by: (1) an enhacement in the number of escape-jets and (2) a faster switch between swimming modes ($p<0.05$; publication 1, Fig. 1B, 5 and 6). These findings demonstrate a switch towards a more efficient and energy saving locomotory performance that enables *D. gigas* to maintain a high degree of activity at EH. Moreover, the increased cycling between aerobic and anaerobic swimming phases over short time intervals may reduce the costs of transport and permit a long-term use of anaerobic resources. This strategy on the long-term might imply a maximized use of ambient O_2 and increase hypoxia tolerance, as it has been hypothesized for *L. brevis* (Finke *et al.*, 1996). Further, the deep-breathing behavior does not only enable *D. gigas* to increase EO_2, but also to pass the same amount of water through the mantle cavity per period of time, i.e. a stable ventilatory volume per min (see publication 1, Fig. 10B), which also explains the high degree of activity observed under EH and jumbo squid's ability to perform similar forages as seen under normoxia (Gilly *et al.*, 2006). Furthermore, *D. gigas* displays an enhanced usage of its lateral fins under progressive hypoxia, with a peak at the transition from early to late hypoxia. This also may contribute to minimize the costs of transport, as lateral fins can push a large mass of water especially at low velocities (O'Dor *et al.*, 1988; Wells and O'Dor, 1991).

However, the majority of extended dives at 300 m (~ 1 kPa O_2), revealed via acoustic tagging experiments, peaked between 140-240 min (Gilly *et al.,* 2006), which is in close agreement to the onset of late hypoxia (~ 170 ± 10 min) in my first study, where jumbo squids get lethargic. As consequence, no respiratory cycles are visible (publication 1, Fig. 1C). The lethargic behavior is probably connected to the switch from energy reserve depletion (at EH) to fermentative pathways (at LH), as octopine formation under progressing hypoxia is accompanied by intracellular acidosis and severe acid-base disturbances (Hochachka and Mommsen, 1983; Grieshaber et al, 1994). Therefore, *D. gigas* may spend more energy in essential processes like ion regulation instead of locomotion. Another reason for such a lethargic behavior might be the specific degradation of the structural protein α-actinin, which may impair the efficiency of jet-propulsion (publication 4, Fig. 2 and 3A). Unfortunately, the experiments have been interrupted maximum 20 min after the jumbo squids stopped moving. Anyhow, recent data suggest that juvenile (Rosa unpublished) and adult jumbo squids (Seibel unpublished) can sustain severe hypoxia (1 kPa O_2) for at least 6-12 hours and may use lethargy as a strategy to conserve energy and

slow down the accumulation of toxic end products and intracellular acidosis to extend residence time in OMZs.

e) Does D. gigas *possess an efficient antioxidant defense mechanism to prevent cellular damage during the reoxygenation phase while ascending?*

During the reoxygenation phase, for example in ascending jumbo squids, electrons intensively "leak" from the electron transport chain when O_2 is reintroduced and generate a burst of oxyradicals (ROS) (Lushchak and Bagnyukova, 2006). Hypoxia tolerant intertidal species are known to induce antioxidant enzyme activity (i.e. SOD, CAT, and GST) during hypoxia as a preparatory adaptation to protect against ROS production during reoxygenation (Pannunzio and Storey, 1998; Hermes-Lima and Zenteno-Savín, 2002; Abele and Pantarulo, 2004; Teixeira *et al.*, 2013).

In general, antioxidant enzyme activities in squids are low and negatively correlated to their high metabolic demands (i.e. *L. vulgaris* and *S. officinalis*), which might explain their relative short life-span (Zielinski and Pörtner, 2000). However, the antioxidant enzyme activities in muscle tissue of juvenile *D. gigas* were in close agreement with those found in *L. vulgaris* and *S. officinalis* (Zielinski and Pörtner, 2000). Yet, some investigations also point out a clear correlation between ROS formation and metabolic rate (Ku *et al.*, 1993). If this holds true for highly aerobic jumbo squids, a higher ROS formation and oxidative damage is expected at normoxia. Yet, MDA concentrations (indicative of cellular damage) in jumbo squid's muscle tissue do not significantly change between normoxic and hypoxic conditions ($p<0.05$; publication 4, Fig. 4), demonstrating an efficient antioxidant defense system. Moreover, SOD and CAT activities are enhanced under well-oxygenated conditions ($p<0.05$; publication 4, Fig. 5A and B) that seems to constitute an integrated stress response at shallower depths by buffering increased ROS formation, and, in addition, might even be a strategy to cope with the reoxygenation/recovery process. In a variety of other marine organisms some antioxidant enzymes increased during reoxygenation/recovery phase itself to minimize cellular damage, and their comparison revealed that antioxidant defense is highly species (and even tissue) specific (Pannunzio and Storey, 1998; Lushchak *et al.*, 2001; Lushchak and Bagnyukova, 2007). It is also worth noting that the increase in ROS formation during reoxygenation must be accelerated by the simultaneous rise of temperature during squid's vertical ascents to the surface ocean, but has not been investigated within my thesis. Therefore, further investigations should be conducted to round off the topic of reoxygenation and oxidative stress in *D. gigas*.

Moreover, Hsp70 concentration in muscle tissue of juvenile jumbo squids is significantly increased under severe hypoxia (1 kPa O_2) ($p<0.05$; publication 4, Fig. 3), which may constitute a preparation for the reoxygenation phase during squid's upward migration. Although protein synthesis, especially under metabolic suppression, is energetically costly (Tomanek and Somero, 1999; Tomanek, 2010), such upregulation may be crucial for squid's cellular fitness because Hsps are known to play an important role in repairing, refolding, and eliminating damaged or denatured proteins due to ROS formation (Oliveira *et al.*, 2005). In fact, it has been shown that after suppression of ATP-demand and ATP supply pathways accompanied by an inhibition of protein synthesis, there is a ''rescue'' of protein synthesis mediated by preferential expression of certain genes in hypoxia tolerant cells (Hochachka *et al.*, 1996, 1997). Such an elevated HSP response during hypoxia has been previously described in barnacles and oysters (on molecular level), and even might play an important role in early transcriptional regulation in some tissues, as their cells are ready to react very quickly to any stress situation (David *et al.*, 2005). Therefore, hypoxia-dependent induction of chaperones (Benjamine *et al.*, 1992) might favor the stability (or life-time) of individual proteins (Hochachka and Lutz, 2001), necessary to survive hypoxic conditions. However, Hsp70 concentrations are increased in muscle tissue of juvenile *D. gigas*, but, in contrast, Hsp90 expression declines indicating different functions (Höhfeld *et al.*, 2001; Falsone *et al.*, 2005) and confirms the specific "rescue" of proteins and/or genes under hypoxia. Moreover, Hsp70 has been discovered to assemble the Hsp90 machinery and therefore further investigations have to be conducted.

3.2. Ecological aspects and consequences

3.2.1. Biodiversity and community structure

Low O_2 concentrations are shaping the communities of fish and other aquatic animals (e.g. Maes *et al.*, 1998; Rosenberger and Chapman, 1999) by influencing biodiversity and species richness (Pihl, 1994; Diaz and Rosenberg, 1995). The expansion of hypoxic areas forces species to adapt quickly or to undertake avoidance movements (Renaud, 1986; Pihl *et al.*, 1991; Keister *et al.*, 2000) and, might favor the appearance of hypoxia tolerant species from neighboring habitats (Stevens *et al.*, 2006). In other words, hypoxia alters species community composition by eliminating sensitive organisms (or animal groups) and encourages the proliferation of a few tolerant ones (Dauer, 1993). Moreover, in expanding OMZs, there is a transition from a diverse midwater fauna to an ecosystem dominated by diel migrant biota that must return to surface waters at night. In the event of such a transition, the ecology of the region will be fundamentally altered (Seibel, 2011) and

biodiversity significantly reduced. Additionally, k-selected species might be replaced by r-strategists, as their potential to adapt to environmental changes is limited (Wu, 2002). Further, smaller specimens might out-compete larger ones, as their body-mass oxygen-consumption ratios are more advantageous (Burleson *et al.*, 2001), which will impact the age composition of stocks and communities, which may change recruitment abilities as well as predation pressure on food organisms. Hence, trophic interactions will change and carbon fluxes will follow different pathways (Ekau *et al.*, 2010). Therefore, complex food chain structures are expected to be replaced by simple ones, as it has been described in the "Fishing down the food web" concept by Pauly *et al.* (1998) that was accompanied by a reduction of the trophic level and an increase in the importance of small-sized species and specimen (Ekau *et al.*, 2010). Such changes in the community structure will definitely have consequences for the remaining species in the ecosystem. For instance, if other predator species occur, prey species may have to adapt to their different hunting behavior (Ekau *et al.*, 2010). Moreover, the introduction of "new" species (i.e. jellyfish) might not be usable for mankind with deleterious impacts on the fishing industry (Ekau *et al.*, 2010).

One of the expected winners of climate change and its induced extension of oceanic dead zones is gelatinous zooplankton, whereas other components of the classical marine food web like copepods and fish seem to be more negatively affected (Ekau *et al.*, 2010). Shoji *et al.* (2005) for example reported a reduced food uptake potential of juvenile Spanish mackerel on red sea bream larvae with decreasing O_2 level, while the consumption of fish larvae by moon jellyfish increased and hence contributed to a shift in the composition of the community. Jellyfishes, in general, show a high potential to rapidly adapt to disturbed environmental conditions (Richardson *et al.*, 2009), which makes them predestined to take a dominant role in ecosystems with declining O_2 conditions. Their opportunistic feeding behavior together with the reduced ability of many prey organisms to escape under low O_2 conditions may allow them to out-compete other organisms and control population levels of prey (Kodama *et al.*, 2006; Dutil *et al.*, 2007).

Surprisingly, squids that were thought to be excluded from hypoxic areas due to anatomical and physical constraints (Pörtner, 2002) also seem to benefit from expanding hypoxia. An increasing abundance of some squid species has been observed and total squid catches have tripled during the last 30 years (Morales- Bojorquez *et al.*, 2001; FAO, 2010), both suggesting an increasing role in pelagic food webs of neritic and oceanic ecosystems. Squids are typical r-strategists with an enormous growth potential (especially *D. gigas*; Mejía-Rebollo *et al.*, 2008), and seem to be more tolerant against low O_2 than many other pelagic organisms, although their inefficient jet-propulsion requires more energy than such as

undulatory swimming in fish (Pörtner and Zielinski, 1998). Contrarily to the hypothesized shift towards smaller-sized animals in hypoxic communities, the "deep hypoxic" living *S. oualaniensis* essentially increased its growth rate and biomass compared to the "shallow normoxic" living specimens (Bizikov, 1995; Shulman *et al.*, 2002) demonstrating its high potential and success to adapt to such extreme conditions. This finding was explained by an elevation in the energy efficiency of food consumption, decreased energy expenditures on aerobic respiration and locomotion and probably due to an increased eating performance, as food competition within the OMZ is lowered (Shulman *et al.,* 2002). Moreover, this squid seems to have the potential to degrade proteins anaerobically, even under normoxic conditions, a feature that supports its high hypoxia tolerance (Shulman *et al.*, 2002). However, the biomass of jumbo squids also increased over the past years, which has been linked to an extension of their distribution area in the Eastern Pacific Ocean (Gilly, 2005). In fact, jumbo squid catches have increased from 15,000 t in 1990 to 818 000 t in 2006 (FAO, 2010) and their distribution area extended from 30° N in 1980 to 60° N in 2005/2006 (Gilly, 2005). Expansion of the OMZ is expected to elevate the refuge of vertical migrating top predators, such as *D. gigas*, from highly mobile predators such as mako sharks and thus supports an overall increase of their biomass (Vetter *et al.*, 2008). Moreover, the extending OMZ serves as an increasing refuge space for hypoxia tolerant mesopelagic fish species and benefits a rapid biomass increase in such top predators, as it has been observed for jumbo squid off Chile and California (Field *et al.*, 2007; Field, 2008). Argúelles *et al.* (2008), in addition, argued that increased food availability might even lead to a larger size-at-maturity in *D. gigas* and thus enhances its reproductive potential and competitiveness.

3.2.2. Synergistic effects

It is important to note that many of the ecological responses related to hypoxia are confounded by other factors. For example, it is well known that hypoxia caused by organic pollution is often associated with elevated levels of ammonia, hydrogen sulphide and particulate organic matter (Wu, 2002). Even more important, global warming, caused by anthropogenic greenhouse gas formation, is known to induce the expansion of hypoxia and impact ocean acidification (Oschlies *et al.*, 2008; Rosa and Seibel, 2008; Stramma *et al.*, 2008; Rabalais *et al.*, 2009). As these three climate-related factors are closely correlated, their synergistic effects are expected to influence physiological processes (Childress and Seibel, 1998; Somero, 2005; Tewksbury *et al.*, 2008; Pörtner, 2012) and may drive, at the community level, profound changes to trophic interactions (Edwards and Richardson, 2004; Ekau *et al.,* 2010), diversity, and biogeography (Perry *et al.,* 2005; Somero, 2005).

Mesopelagic, diel migrating animals influence the respiratory carbon flux, as they consume carbon near the surface and then transport it to depth, where it is excreted as CO_2 (Angel, 1989; Longhurst *et al.*, 1990). This process is responsible for a large fraction of the carbon exported from surface waters in some regions (Ducklow *et al.*, 2001). The OMZ influences this flux by altering the biomass, vertical distribution and physiology of resident organisms. In particular, if most migrators are suppressing metabolism at depth, then the amount of CO_2 released is similarly suppressed, thus decreasing the efficiency of the "biological carbon pump" (Seibel, 2011) and even might aggravate ocean acidification in surface waters. Moreover, as fishes seem to play an important role in global carbon cycle by balancing the production and dissolution processes of carbonate (Jennings and Wilson, 2009), the decline of fish stocks due to advancing hypoxia might enhance PCO_2 in upper water layers and thus, make organisms more vulnerable to hypoxia, resulting in a self-sustaining negative loop (Ekau *et al.*, 2010). In addition, the decrease in biodiversity in hypoxic areas will reduce ecosystem resilience and resistance and may decrease its function in nutrient cycling processes (Ekau *et al.*, 2010).

Thus, the synergistic impact of these three climate-related factors hypoxia, global warming and ocean acidification will compress the habitable night-time depth range of vertically migrating species (1) from below as result of OMZ shoaling, and (2) from above due to unfavorable high temperatures and decreasing pH (Fig. 4.1), as surface waters impose O_2 stress due to elevated O_2 demand at high temperatures and constrained O_2 transport due to ocean acidification (Rosa and Seibel, 2008) and high temperature (Ekau *et al.*, 2010). However, the vertical distribution and environmental tolerance is species-specific and therefore habitat compression will affect each species differently (Seibel, 2011).

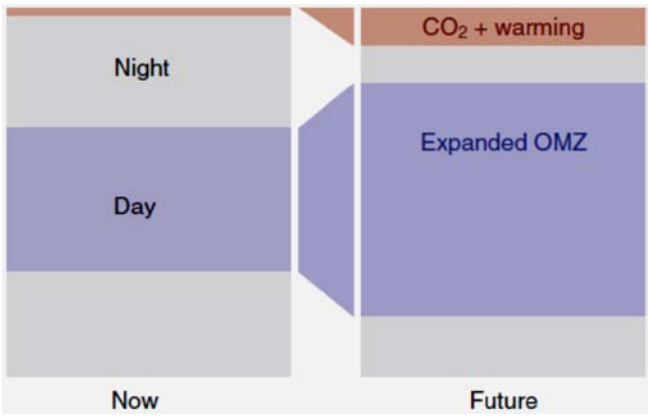

Figure 4.1 - Schematic illustrating compression of the night-time habitat range (gray) for vertically migrating species from the present (left) to a future ocean impacted by global warming, ocean acidification and expanding oxygen minimum zones (OMZs). Warm surface waters (red) are stressful to some species, and expansion of this zone will restrict the depth to which organisms can retreat to burn off an oxygen debt accrued during night-time forays into OMZs (blue) (after Seibel, 2011).

Jumbo squids seem to benefit from expanding hypoxia (i.e. increased refuge and food availability; Field *et al.*, 2007; Field, 2008; Vetter *et al.*, 2008), but their high sensitivity towards elevated temperatures (Q_{10} value of 7 between 20 and 25°C; Rosa and Seibel, 2008)

and PCO_2 may have severe consequences under progressing synergistic climate change conditions. In fact, by the end of the 21st century, the ETP is expected to warm, between 2° and 4°C because of weakening tropical circulations (Meehl *et al.*, 2007), and seawater pH will decline by as much as 0.3-0.4 unit. Warmer temperatures will elevate metabolism and require enhanced performance from a constrained O_2 transport system that will be impaired, especially at high temperatures, by ocean acidification (Pörtner, 2002). Thus warming and acidification may cause ventilatory and circulatory stress and/or cause acid-base imbalance that restricts the squid's aerobic scope and impairs swimming activity (Rosa and Seibel, 2008). Together these variables may reduce the squid's ability to respond to external stimuli, leaving it more vulnerable to its main predators (Rosa and Seibel, 2008). Moreover, if the OMZ continues to expand vertically in the ETP (Stramma *et al.*, 2008), *D. gigas* will have to retreat to even shallower waters at night to repay any accumulated O_2 debt and to hunt. Thus, ocean acidification and warming may create a ceiling that precludes these squids from entering near-surface waters, while the expanding hypoxic zone may elevate the floor below which they cannot penetrate during their night-time recovery from hypoxia (Rosa and Seibel, 2008). This hypothesized habitat compression (Prince and Goodyear, 2006) may alter the squid's behavioral and feeding ecology with cascading effects on growth and reproduction. Jumbo squids are an important commercial fishery throughout the ETP and are important components in the diets of birds, fishes, and mammals there (Davis *et al.*, 2007). However, it is known that O_2 transport in epipelagic squids is limited under restricted environmental conditions to meet very high rates of O_2 demand (Rosa and Seibel, 2008), and therefore *D. gigas* might suffer from progressing synergistic climate-related impacts.

3.3. Future prospective

To achieve a deeper understanding of the ecological processes and forecast the consequences of climate change, we need a better understanding of the physiological, biochemical and molecular adaptation mechanisms and potentials on the species level. "Rescue" of protein synthesis and degradation are key mechanisms under metabolic suppression, and my previous results demonstrate that *D. gigas*, at least under progressing hypoxia (> 3h, 1 kPa O_2) may use anaerobic (muscle) protein degradation (i.e. Hsp90, α- actinin) to increase hypoxia tolerance, but, in addition, activates specific proteins (i.e. Hsp70) for protection. Therefore, in the near future I will perform a proteome screening between normoxia, early and late hypoxia treatments to reveal key proteins and pathways (i.e. the UPS) that are involved in hypoxia tolerance. Moreover, I expect to find a time-

dependent transition in the proteasome between early and late hypoxia, as I already observed in the ventilatory and metabolic performance during my thesis. Further I would like to verify/supplement the proteomic data with RNA sequencing, which additionally might help to separate between protein degradation and active downregulation.

3.4. References

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4 Eidesstattliche Erklärung

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Erklärung gem. § 9 der Promotionsordnung:

Ich erkläre hiermit,

(i) dass die Abhandlung - abgesehen von der Beratung durch die Betreuerin oder den Betreuer - nach Inhalt und Form die eigene Arbeit ist,

(ii) dass diese Arbeit vor dem jetzigen Promotionsverfahren keinem anderen
Promotionsverfahren unterzogen wurde und

(iii) dass die Arbeit unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft entstanden ist.

(gez. Katja Trübenbach)