Angelica Amaduzzi (a60147)

EFFECTS OF HIGH CO_2 AND HYPOXIA ON THE EMBRYONIC DEVELOPMENT OF EUROPEAN CUTTLEFISH *(SEPIA OFFICINALIS)*

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Faculdade de Ciências e Tecnologia

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Doutor Juan Fuentes (CCMAR/UALG)

Doutor António Sykes (CCMAR)

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Declaração de autoria de trabalho

Declaro ser o autor deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

(Angelica Amaduzzi)

Abstract

The increasing atmospheric CO_2 concentrations due to anthropic influence is causing the acidification of the oceans, which often co-occurs with hypoxia events. These are becoming a growing concern for marine ecosystem and for aquaculture due to the potential consequences on early life stages of marine animals and in particular for invertebrates, who form their internal skeletons out of calcium carbonate. The European cuttlefish (*Sepia officinalis)* is an excellent model to assess the effects of climate change, since it possesses a calcium carbonate structure from early development that grows very fast with a short life cycle. The present thesis aims to determine the effects of two environmental stressors, high CO_2 environment and hypoxia events, in European cuttlefish embryogenesis, hatching rates and physiological characterisation through metabolic rates. Two separate experiments were performed. During the first, the cuttlefish eggs were kept in an indoor semi-open system with temperature maintained stable at 20°C and exposed to an acute event of hypoxia during the embryonic development (either day 10 or day 20). In the second experiment, cuttlefish eggs were kept in an outdoor open seawater system and exposed to high CO_2 or to high CO_2 plus an acute hypoxia event (either day 10 or day 20). The data obtained from the first experiment suggested that the embryos, kept at a temperature of 20°C can manage an acute hypoxia event without substantial consequences on their development. In the second outdoor experiment a much lower hatching rate was recorded, compared to the first experiment. This might be reconnected to the higher water temperature. Based on these data, it can be hypothesised that temperature could be one of the main key factors for cuttlefish eggs survival. Even so, it is not clear which is the threshold temperature for cuttlefish during the embryonic development.

Keywords: CO_2, Cuttlefish, Embryogenesis, Hypoxia, Metabolism, *Sepia officinalis*

Resumo

O aumento das concentrações atmosféricas de CO_2 devido à influência antrópica, tem vindo a causar a acidificação dos oceanos. Esse fenómeno geralmente ocorre, em conjunto com eventos de hipoxia, devido a temperaturas elevadas da água e escorrência de nutrientes da terra. Devido ao declínio global do oxigénio oceânico e ao aumento das atividades humanas, a acidificação e a hipoxia dos oceanos tornaram-se uma grande preocupação para a biodiversidade marinha e dois importantes fatores de stress ambientais para os ecossistemas marinhos. No entanto, a hipoxia e a diminuição do pH da água podem-se tornar problemas potenciais para as condições de aquacultura intensiva, devido aos custos associados à manutenção da saturação de oxigénio assim como, de possíveis consequências nos primeiros tempos de vida dos animais marinhos. Isso é particularmente importante em invertebrados, que formam seus esqueletos internos de carbonato de cálcio. O choco Europeu (*Sepia officinalis*) é um excelente modelo para avaliar os efeitos das alterações climáticas, nomeadamente no que diz respeito ao aumento de CO_2 e eventos de hipoxia nos oceanos, uma vez que esta espécie de cefalópodes possui uma estrutura de carbonato de cálcio desde o início do desenvolvimento, que cresce muito rapidamente com um ciclo de vida curto. Apesar disso, poucos estudos indicam até que ponto a combinação de ambos fatores de stress (aumento de CO_2 e hipoxia) provocam efeitos adaptativos neste animal, especialmente durante o desenvolvimento embrionário. A presente tese visa determinar os efeitos de dois fatores de stress ambientais, ambiente de elevado CO_2 e eventos de hipoxia, na embriogénese, taxas de eclosão e caracterização fisiológica através de taxas metabólicas de *S. officinalis*. Foram realizados dois testes em separado. Durante o primeiro, os ovos de choco (n = 2400) foram mantidos em sistema semiaberto com temperatura constante a 20°C. Os ovos foram expostos a um evento agudo de hipoxia durante o desenvolvimento embrionário (no dia 10 ou no dia 20) de forma a estudar como estes eventos podem afetar o desenvolvimento embrionário e a eclosão. Na segunda experiência, 1200 ovos de choco foram mantidos em sistema aberto. Os ovos foram divididos em quatro grupos: controle, expostos a CO_2 400ppm, e três grupos mantidos em 1600 ppm de CO_2, dois dos quais também foram expostos a um evento de hipoxia aguda (dia 10 ou dia 20). O objetivo foi analisar como diferentes tratamentos afetam a formação de ossos de choco, desenvolvimento embrionário e taxa de eclosão. Em ambas experiencias foram registrados e analisados estatisticamente dados de qualidade da água (salinidade, oxigénio, temperatura e pressão atmosférica), dados biológicos (peso húmido médio (MWW), taxa de eclosão, peso da progénie) e dados metabólicos (consumo de oxigénio). Os resultados da primeira experiência mostraram uma forte variabilidade do peso dos ovos ao longo do tempo relacionada ao desenvolvimento embrionário. A análise estatística do MWW mostrou que todos os efeitos considerados (tempo, eventos de hipoxia e tratamentos), foram estatisticamente diferentes. Apesar disso, o tempo (dias de amostragem) pode ser considerado o único fator relevante. Os animais mantidos a uma temperatura estável de 20°C começaram a eclodir a partir do dia 25, com um pico de eclosão por volta do dia 40. A taxa de eclosão foi em torno de 70- 75%. Uma taxa de malformação entre 1 e 3.5% foi observada na progénie. Os dados obtidos sugerem que os embriões, mantidos a uma temperatura de cerca de 20°C, podem gerir um evento de hipoxia aguda sem consequências substanciais no seu desenvolvimento. Os resultados da segunda experiência demostram uma variabilidade de MWW semelhante à observada na primeira experiência. Um déficit semelhante foi observado também nos registos dos dados da respirometria. A análise estatística do MWW mostrou que todos os efeitos considerados (tempo e tratamentos) foram estatisticamente diferentes. Apesar disso, o

tempo (dias de amostragem) pode ser considerado o único relevante. Os ovos começaram a eclodir por volta do dia 20 a uma temperatura média da água de 22.4°C. A taxa de eclosão foi em torno de 31.7% e nenhuma malformação foi registada. Como os ovos foram obtidos dos mesmos reprodutores e a qualidade da água foi a mesma, pode-se considerar que a diferença significativa na progénie pode estar relacionada com a temperatura. Os ovos foram mantidos ao ar livre em água que atingia frequentemente 27°C durante os dias mais quentes, não havendo portanto qualquer controlo da temperatura. Com base nestes dados, considera-se como hipótese de que a temperatura pode ser um dos principais fatores chave para a sobrevivência dos ovos de choco. Apesar disso, não foi evidente qual é a temperatura limite para o choco durante o desenvolvimento embrionário. Mais estudos devem ser realizados, considerando a incubação múltipla e testando a exposição a longo prazo para entender como as mudanças climáticas afetam esta fase do ciclo de vida do choco.

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$\dot{M}O_2$	rates of oxygen consumption
MWW	Mean wet weight
pCO_2	partial pressure of CO2
vs	versus

1. Introduction

1.1. Acidification of the oceans

The oceans cover seventy percent of the earth's surface. Their large volume and the ability of seawater to buffer carbon dioxide (CO_2) allows the oceans to absorb approximately half of all anthropogenic carbon CO_2 emissions to the atmosphere (Sabine *et al.*, 2004).

The CO2 produced by human activities penetrates the surface layers of the ocean and is transported by ocean currents to deeper waters. However, once atmospheric CO2 levels increase, the ability of the ocean to take it up decreases due to the reduced buffering ability of seawater as CO2 accumulates. Yet, the increase of anthropogenic CO2 emissions over time together with other climate-related variables, including a global temperature rise, have resulted in the elevation of the partial pressure of CO2 (pCO_2) and reduction of seawater pH, a phenomenon also known as ocean acidification (Caldeira & Wickett, 2003; Denman *et al.,* 2007).

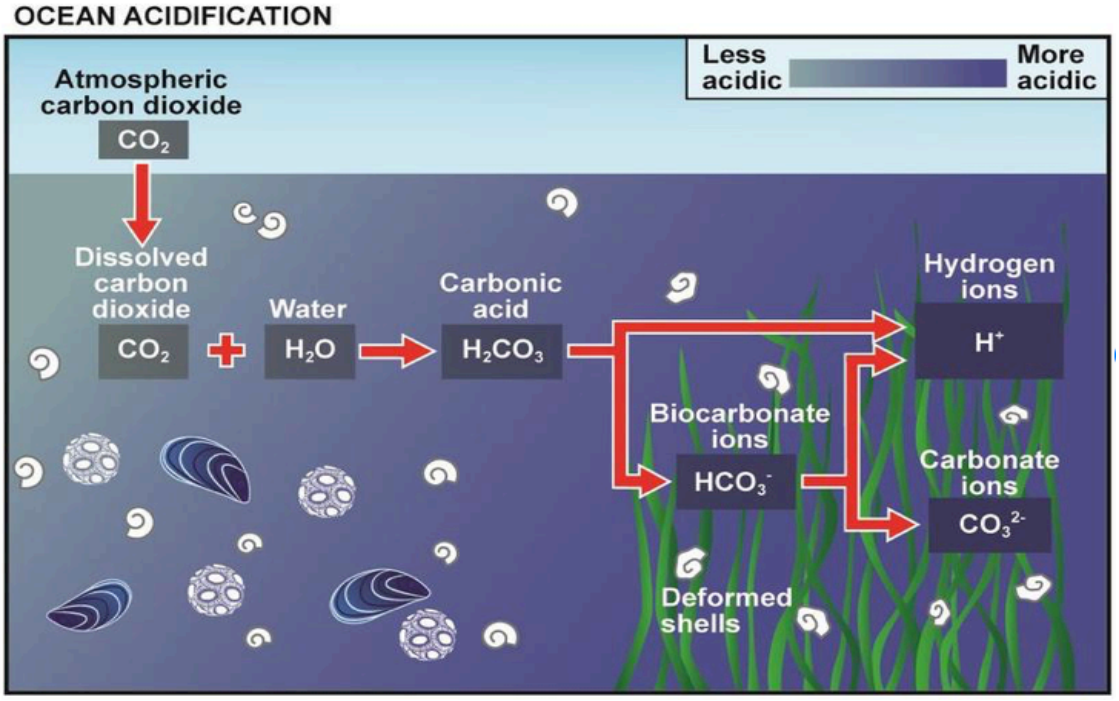


Figure 1.1 *Schematic diagram of ocean acidification. The reaction between dissolved carbon dioxide (CO_2) and water results in an increase in the concentration of hydrogen ions (H+); additional changes include an increase in bicarbonate ions (HCO_3), and a great decrease in carbonate ions (CO_3^2); carbonate ions CO_3^{2-} will modify the carbonate saturation state; these changes cause acidification (Image adapted from Birchenough et al., 2017).*

Ocean acidification causes a decrease of carbonate ions (CO_3^{2-}), thereby reducing the calcium carbonate ($CaCO_3$) saturation state in seawater (Kleypas *et al.,* 2006). Elevated

partial pCO_2 in seawater (also known as hypercapnia) could drive important alterations of the marine ecosystem by causing changes in the water carbonate chemistry (Orr *et al.*, 2005; Langdon & Atkinson, 2005; Kleypas *et al.,* 2006; Fabry & Seibel, 2008), as it has been detectable in upper ocean layers for several decades (Chen & Millero, 1979; Brewer *et al.,* 1997). Furthermore, hypercapnia can cause modifications and potential problems in marine animals, e.g., by provoking additional metabolic requirements in those exposed to these conditions (Pörtner, 2008; Blackford, 2010).

Hypercapnia particularly affects marine organisms that form shells or internal skeletons out of amorphous calcite (calcifiers), primarily aragonite or calcite, which are more soluble than other forms of carbonate (Zeebe & Wolf-Gladrow, 2001). Several studies have shown that the calcification rate of calcifiers, such as corals (Hoegh-Guldberg *et al.*, 2007), coccolithophores (Riebesell *et al.,* 2000; Zondervan *et al.,* 2001), foraminiferans (Spero *et al.,* 1997; Bijma *et al.,* 2002) and bivalves (Gazeau *et al.,* 2010; 2011; Thomsen *et al*., 2010) decrease with increasing pCO_2, even in seawater supersaturated with $CaCO_3$ (Gattuso *et al.*, 1998, Riebesell *et al.,* 2000, Bijma *et al.,* 2002, Kleypas *et al.,* 2006, Gazeau *et al.,* 2007; Fabry *et al*., 2008). Additionally, an increased pCO_2 may also have complex effects on the physiology, growth, and reproductive success of these marine organisms.

On the other hand, previous studies reported unchanged or increased calcification rates under high seawater pCO_2 in echinoderms (Wood *et al*., 2008; Gooding *et al.*, 2009; Ries *et al.,* 2009), decapod crustaceans (Ries *et al.,* 2009), juvenile cephalopods (Gutowska *et al.,* 2010a; 2010b) and teleost fish (Checkley *et al.,* 2009; Munday *et al.,* 2011).

In any case, the potential effects of this decline in pH on marine ecosystems and organisms are still poorly understood. Further studies should be done on the animals who possess complex life cycles, where it is often assumed that larval and juvenile stages may be the most vulnerable to environmental perturbations. (Pörtner, 2008; Pörtner & Farell, 2008).

1.2. Hypoxia in the sea

In the past few decades, concomitantly with the acidification of the oceans, there are reports of a global oceanic oxygen decline (Schmidtko *et al.,* 2017).

Low oxygen episodes occur naturally (Ekau *et al.,* 2010) and they are usually confined to well-defined areas or timescales (Whitney *et al.,* 2007; Diaz & Rosenberg, 2008). Many

estuaries and coastal zones experience long-term hypoxia events lasting weeks to months due to the density stratification of the water body (Grieshaber *et al.,* 1994).

Under these conditions animals do not experience an instantaneous and complete lack of oxygen, they will rather be exposed to gradually declining partial pressures of oxygen instead. Furthermore, the degree of hypoxia tolerance varies among animals, from terrestrial mammals that survive only minutes to hours without O_2 to hypoxia-tolerant fish, reptiles and invertebrates capable of withstanding anoxia for weeks to months (Grieshaber *et al*., 1994; Bickler & Buck, 2007).

Several studies have reported sublethal physiological stress followed by reduced growth and reproductive potential in the non-migrating organism that survived hypoxia events (Diaz & Rosenberg, 2008; Vaquer-Sunyer & Duarte, 2008). Moreover, early life stages are assumed to be more sensitive to oxygen stress than older life stages (Levin *et al*., 2009).

One of the main causes of hypoxic episodes is the excessive anthropogenic input of nutrients and organic matter into coastal ecosystems (Cloern, 2001; Diaz, 2001; Chan *et al*., 2008).

According to Diaz (2001), the growing density of human centers along the coasts and the increased presence over the years of hypoxic zones are important environmental stressors to marine species (Cosme & Hauschild, 2016; Townhill *et al.,* 2017) that could lead to losses in local marine biodiversity (Vaquer-Sunyer & Duarte, 2008). Moreover, extreme hypoxia can cause the formation of O_2- depleted dead zones devoid of higher marine life entirely (Vaquer-Sunyer & Duarte, 2008). In the future, low oxygen episodes are predicted to become more frequent, more severe, and to last longer (Diaz & Rosenberg, 2008).

To date, most of the research has been done on the latter stages of marine life cycles, and the combined effects of elevated CO_2 hypoxic events and temperature on the marine ecosystem are poorly known. Additionally, oxygen stress promoted by changes in climate conditions could represent a major bottleneck for species survival not only in the wild, but also for those produced in aquaculture (Rosa *et al*, 2013).

This thesis focuses on the effects of hypoxia and high levels of CO_2 on the embryonic development of the cephalopod *Sepia officinalis*. However, to explore and assess these effects, we must first understand the biological cycle and how it could be disturbed.

1.3. The European cuttlefish (*S. officinalis)*

Sepia officinalis belongs to the Class Cephalopoda, Order Sepiida, Family Sepiidae, and Genus *Sepia*, which includes around 100 species. This species can be found mostly in the Northeast Atlantic and the Mediterranean Sea, but also, from the North Sea to Northeast Africa (Jereb *et al.,* 2015). *S. officinalis* lives in coastal waters and on the continental shelf at depths lower than 150 m (Boletzky, 1983).

During the past 30 years, cephalopods have been an aquaculture candidate (Boucaud-Camou, 1989; Vaz-Pires *et al*. 2004; Sykes *et al.* 2006; Vidal *et al.,* 2014). Among the Genus *Sepia, S. officinalis* has been successfully reared in extensive aquaculture experiments at a medium scale in several EU countries such as Italy, France and Portugal (Sykes *et al.,* 2014). Cultured *S. officinalis* is used also as an animal model for biological and biomedical research (e.g., physiology, ecology, neuroscience, nutritional biochemistry, aging, molecular biology, or immunology) and for public exhibition in aquariums (Sykes *et al.,* 2014).

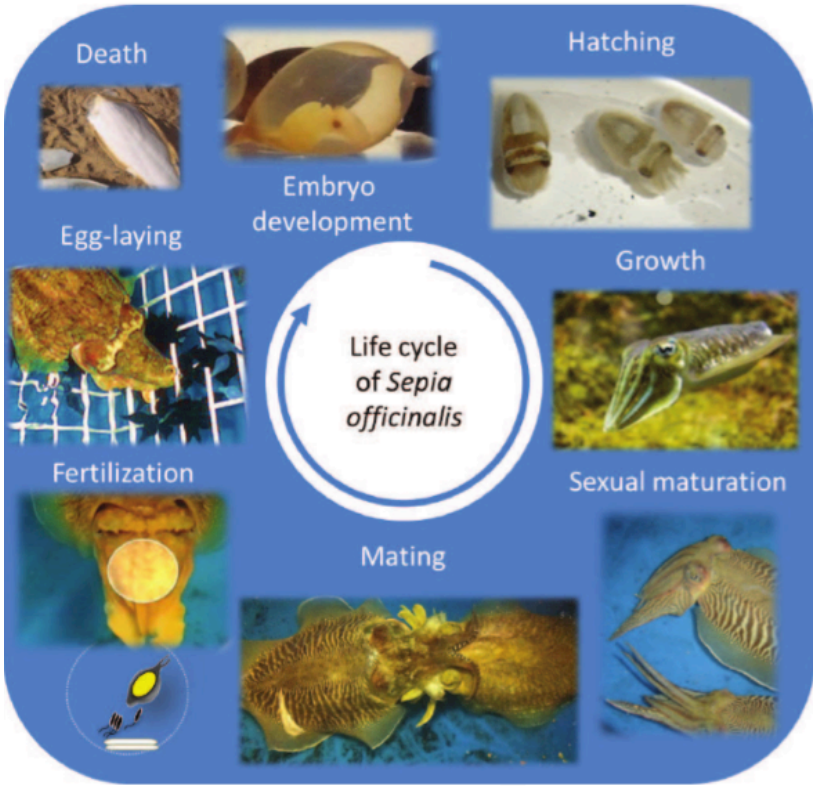


Figure 1.2 *Main successive stages of the life cycle of the cuttlefish S. officinalis. (Image adapted from Zatylny-Gaudin & Henry, 2018)*

The cycle of life of *S. officinalis* is relatively short, ranging from 6 months to 2 years (Guerra *et al.,* 2006). When sexual maturity is reached, cuttlefish display a very typical

behaviour of courtship, where males mature earlier than females. Females receive the spermatophores in the paired seminal receptacles, under the buccal mass, where they may remain and be used for as much as 2–5 months (Hanlon *et al*., 1999).

After breeding repeatedly (Hanlon *et al.,* 1999), females will lay several hundred eggs over a period of a few days, in waters at depths below 30-40 m (Guerra, 2006) from late winter to early summer (Rodhouse, 1998), depending on the environmental factors.

In cold waters (e.g., English Channel), cuttlefish reproduce during the second year of life and for a short breeding period of 2 - 3 months, usually between the end of spring and the beginning of summer (Boucaud-Camou *et al.,* 1991; Önsoy & Salman, 2005). In contrast, in the Mediterranean, the majority of cuttlefish reproduce during the first year of life and over a longer period (Guerra & Castro, 1988).

This species reaches sexual maturity at very different sizes/weights (Sykes *et al*., 2006) and has an estimated potential fecundity of a maximum of 8000 eggs in nature (Capaz *et al.*, 2020). *S. officinalis* presents an intermittent spawning with no parental care of the eggs and the female dies shortly after spawning (Rocha *et al.,* 2001). The offspring hatch as isometric copies of adults with an average length of 6-9 mm (Melzner *et al.*, 2007) and a weight of 0.09-0.1 g (Sykes *et al.*, 2012; Capaz *et al.*, 2020).

Cuttlefish have an internal cuttlebone (Rodhouse, 1998), made of an aragonitic-organic composite, composed of a calcareous phragmocone containing several delimiting lamellae (septae), separated by small vertical pillars and walls forming chambers (Birchall &Thomas, 1983). These chambers contain gas and are used to regulate the animal's vertical position in the water column (Denton & Taylor, 1964). The cuttlebone is an associated buoyancy control mechanism (Denton & Gilpin-Brown, 1959) that is adjusted by moving liquid in or out of the shell chambers via an osmotic pump (Greenwald *et al*., 1980). The first chambers of the cuttlebone are synthesized during the embryonic phase within the egg case. The cuttlebone is produced rapidly, following the exponential growth rates observed in embryonic and juvenile cephalopods (Forsythe *et al*., 2002; Melzner *et al.,* 2005).

1.3.1. Embryonic development of *S. officinalis*

After the copula, female cuttlefish lay eggs on hydrodynamic locations attached to wild flora, fauna, or human-related structures (Boletzky, 1983). According to Sykes *et al.* (2014), fertilized eggs usually present a spirally coiled, black-stained envelope to protect

the embryos from predation (Dickel *et al.,* 2006) with a flask shape, a diameter ranging from 1.2 to 1.4 cm (Sykes *et al.,* 2006) and a thickness around 1.5 mm (Lemaire, 1971).

Embryonic development is temperature-dependent and may range from 25 days at 25 °C (Sykes *et al.,* 2006 and Figure 1.3b) to 80−90 days at 15 °C (Boletzky *et al.,* 2006 and Figure 1.3a). The development ends with hatchlings that resume a necto-benthic lifestyle (Hanlon & Messenger, 1996) and appear externally as isometric copies of adults (Melzner *et al.,* 2007).

Embryonic life can be divided into 30 stages (Lemaire, 1970). During the segmentation (from Stages 1 to 9) the telolecithal egg presents a meroblastic discoidal cleavage associating blastomeres in the central position and blastocones on its fringe (Zatylny-Gaudin & Henry, 2018). Afterwards, during epibolic gastrulation (stages 10–17), blastocones disappear under the ectoderm plate following the peripheral ring of blastula cells that will form the ring-shaped endo-mesoderm (Lemaire, 1971). At the end of gastrulation, the vitelline syncytium and extraembryonic ectoderm surround the yolk and internalize the vegetal pole to form the yolk sac (Lemaire, 1971; Zatylny-Gaudin & Henry, 2018). From the end of stage 17 organogenesis begins (Lemaire, 1970).

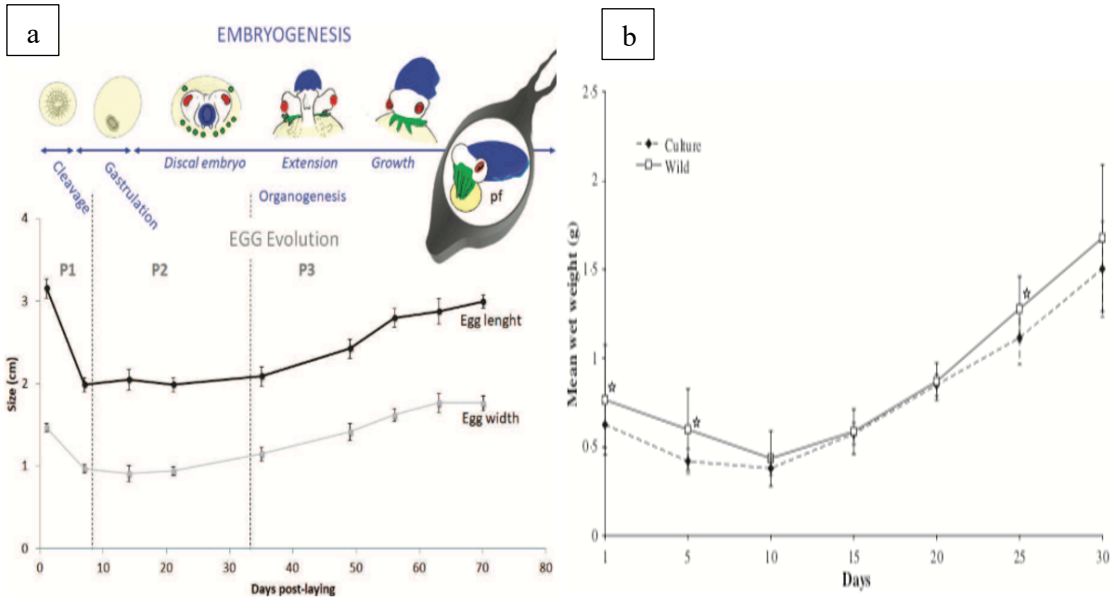


Figure 1.3a: *Evolution of S. officinalis egg size during embryogenesis at 16°C. Evolution phases of the egg case: P1, polymerization of the egg case; P2, stabilization of the egg case; P3, thinning and delamination of the egg case. Illustration of different stages of embryogenesis during cleavage, gastrulation and organogenesis. Yellow: vitellus, red: future eyes, blue: future mantle and shell, green: future arms; pf, perivitelline fluid. (Figure adapted from Zatylny-Gaudin & Henry, 2018).* ***Figure 1.3b:*** *Egg mean wet*

weight (MWW) trends over embryonic development at 19.5 ± 1.09 °C. Vertical bars represent standard deviation. Stars represent differences for P < 0.05. (Figure adapted from Sykes et el. 2009).

The cleavage period corresponds to the first phase (P1) of egg evolution (Zatylny-Gaudin & Henry, 2018). A few hours after laying, the egg cell is covered with lamina propria and surrounded by a thick gelatinous capsule (1.4 mm, ±0.6 mm). In contact with seawater, the capsules polymerize, thus reducing the volume of the egg by about 30% and its thickness by 50% (Zatylny-Gaudin & Henry, 2018).

During the embryonic development both mean wet weight (MWW) (Sykes *et al.*, 2009) and size (Zatylny-Gaudin & Henry, 2018) show a similar lowering tendency during the first 10 days (P1, Figure 1.3a and 1.3b) while expelling the water from the inside and consequent hardening of the egg, after which this was inverted until the end of the embryonic development (Sykes *et al.*, 2009).

According to Zatylny-Gaudin & Henry (2018), after 15 days of incubation and following polymerization, the capsule thickness is reduced, and the outer and inner layers can be distinguished (Figure 1.4a, section A and B). Polymerization of the capsule proteins helps tighten the layers of coiled outer and inner envelopes, highlighting an increasing melanin gradient from the inner layers to the outer layers. The egg is then tightly wrapped by a hardened, strong yet elastic capsule. These morphological characteristics of the capsule define the second phase of egg evolution (P2, Figure 1.3a) and correspond to gastrulation and the beginning of organogenesis (P3, Figure 1.3a) (Zatylny-Gaudin & Henry, 2018).

Thereafter, the embryo develops within the limits of a disk located at the animal pole, at the surface, or above the yolk mass, while the capsule size and thickness remain unchanged (Lemaire, 1971; Zatylny-Gaudin & Henry, 2018).

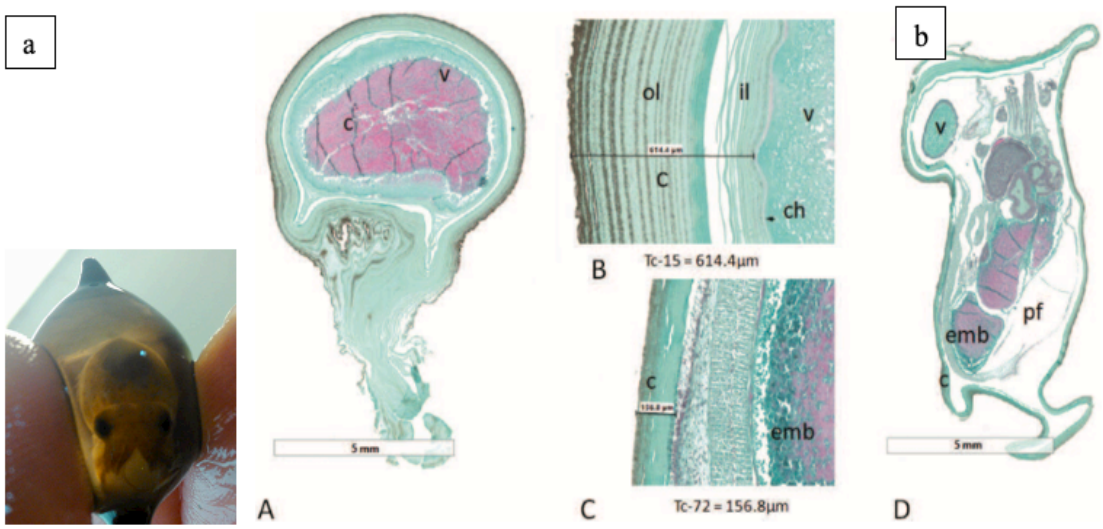


Figure 1.4a *shows a cuttlefish egg in the last phases of development (Stage 30) when the chorion becomes more transparent and it is possible to see the embryo (approx. mantle length 6 mm) completely formed inside the egg (Figure adapted from O'Brien et al., 2016).* ***Figure 1.4b*** *shows a longitudinal section of the egg after 15 days (A) and 72 days (D). ANG stained in Prenant-Gabe triple staining. Magnification of the egg case including capsule thickness after 15 days (B) and 72 days (C). C, capsule or egg case; ch, chorion; emb, embryo; il, inner layer; pf, perivitelline fluid; ol, outer layer; Ct, capsule thickness; v, vitellus (Figure adopted from Zatylny-Gaudin & Henry, 2018).*

Organogenesis marks the beginning of the last phase, after the closure of the yolk sac, which ends with hatching and corresponds to 2/3 of the development period (Lemaire, 1971).

This phase is characterized by a massive increase of water accumulation (about 1 ml) (Zatylny-Gaudin & Henry, 2018) between the vitelline and chorion chamber to maintain the osmolarity until hatching (Figure 1.4a, section D) (Paulij *et al.*, 1991; Boletzky, 2003). According to Sykes *et al*. (2009), during the last days of embryogenesis, the egg reaches a water content between 85% and 95%. The volume of this liquid is supposed to provide a buffer against mechanical injuries, changes in environment salinity (Pechenik, 1983), or acidity (Taylor, 1973) and stretch the capsule to its maximum size (De Leersnijder & Lemaire, 1972), making it more transparent (Figure 1.4b) (Boletzky, 1983; O'Brien *et al*., 2016). Moreover, the increase of perivitelline fluid (PVF) is involved in the availability of oxygen to the developing embryo because, together with the reduction in thickness of the chorion, it decreases the diffusion distance for oxygen, facilitating gas exchange (Lemaire, 1970; Wolf *et al.,* 1985). This process is necessary to support the increasing metabolic demands of the developing embryo (Cronin, 2000).

During the last phase, a few days before hatching, the embryo completes its growth and has assimilated much of the yolk reserves, enabling faster assimilation of energy

resources (Zatylny-Gaudin & Henry, 2018). The outer and inner capsule envelopes have now completely merged, and the outermost layers of the capsule including melanin appear to be delaminated (Zatylny-Gaudin & Henry, 2018).

At the time of hatching (stage 30 according to Lemaire *et al.*, 1970), the release of enzymes by the Hoyle organ located on the end of the dorsal mantle facilitates the emergence of the juvenile (Cyran *et al.,* 2013). Hatching is also facilitated by the thinning of the capsule (Figure 1.4a, section C) (Zatylny-Gaudin & Henry, 2018).

Although there is some information related to the composition, physiology, and metabolism in distinct phases of the embryonic development in cuttlefish (Lemaire 1970; Wolf *et al*., 1985; Boletzky; 1987; 1989; 2006; Cronin, 2000*;* Sykes e*t al.,* 2009) data concerning the possible consequences of ocean acidification and hypoxia events during this phase remains undocumented.

1.4. Embryonic respiration and aquaporins

One of the major differences between terrestrial and aquatic vertebrates is that the latter group has a potentially greater difficulty in maintaining osmotic homeostasis because they are surrounded by an external environment which is almost always in osmotic dis-equilibrium with their body fluids. In comparison to terrestrial animal species, aquatic organisms such as teleost fish and cephalopods may well possess a distinct set of mechanisms in order to adapt and survive the osmotic challenges posed by the seawater (hyper-osmotic) or freshwater (hypo-osmotic) environments (Sakamoto *et al.*, 2015).

Historically the transport of water across hydrophobic membranes was considered to occur via non-specific leakage. After the studies regarding the increased water permeation in certain cells or tissues, such as human erythrocytes and the urinary bladder of frogs, specific water channels were also thought to exist (Agre *et al.,* 2001; Agre, 2005).

Aquaporins, discovered by Peter Agre in 1992 (Agre *et al.*, 1993), are integral membrane proteins whose main role is to facilitate the transport of non-ionic compounds (Heymann & Engel, 1999) and water between cells (Agre, 2005).

Aquaporins have been documented in all kingdoms of life, the largest repertoire has been found in plants (Park *et al.*, 2010). A subclass of aquaporin (AQP) water channels, termed aquaglyceroporins, are also able to transport ammonia, CO_2, glycerol and perhaps urea and other small solutes (Hara-Chikuma & Verkman, 2006).

Aquaporins having been first observed in fish in 2000 (Cutler & Cramb, 2000). In cephalopods, aquaporins has been recently studied in *Sepia pharaonis*, where they seem to play a key role in the reception of signals derived from ionic and osmotic imbalances (Ren *at al.*, 2020). In another study with *Doryteuthis pealeii*, it is suggested the contribution of aquaporins-like proteins to the granulate structure of chromatophores, pigmentary organs that are responsible of the colour change of the animal (Williams, 2019). However, knowledge about aquaporins and their role in cephalopods, in particular *Sepia officinalis,* seems still limited.

The goal of the experiment is to identify if aquaporins are involved in the maintenance of the osmotic equilibrium between the eggs and the environment when these are introduced in an environment with a different osmotic pressure (distilled water).

1.5. Potential consequences of acidification of the oceans and hypoxia events on S. *officinalis*

Highly mobile and active marine organisms as cephalopods need an efficient and powerful ion regulatory apparatus to maintain constant blood pH despite fluctuations in seawater pCO_2. (Melzner *et al.,* 2009). This ability to regulate extracellular pH could be one explanation for the increased tolerance of some organisms to adapt to the acidification of the oceans (Melzner *et al*., 2009). Despite its efficiency, one of the side effects is that the processes may require a higher energetic cost of maintenance in acidified conditions (Hu *et al.,* 2011).

A second concern involves the potential effects in the formation of the cuttlebone due to increasing pCO_2 in marine water. According to Gutowska *et al.* (2008), the European cuttlefish *S. officinalis*, maintained calcification and growth in juveniles after 40 days of exposure to high pCO_2. In a second study, Gutowska *et al.* (2010b) reported a 20-50% increase of the $CaCO_3$ fraction in juvenile's cuttlebones along with a structural change of the calcified matrix in water with a high level of pCO_2 (~4000 µatm, pH_{NBS} ~7.23 and ~6000 µatm, pH_{NBS} ~7.10).

In the study of Dorey *et al*. (2013), it has been demonstrated how adverse abiotic conditions (high level of pCO_2 and high temperature of the water) in the PVF have implications regarding the calcification capacities of the embryo and the developmental conditions for the subsequent juvenile life. According to Dorey *et al.* (2013), the growth of both embryos and juveniles was unaffected by pH, whereas calcium incorporation into

the cuttlebone increased significantly with decreasing pH at 16° and 19°C. This phenomenon of hyper calcification was observed only in a limited number of animals, but it does not guarantee functional performance and calls for a better mechanistic understanding of the calcification processes (Dorey *et al.,* 2013).

It has been demonstrated that early cuttlefish life stages are more vulnerable towards hypercapnia than juveniles and adults (Sigwart *et al.,* 2016). For instance, juvenile performance can be impaired by stressful experiences such as food limitation, change in water movements, pollution, or UV- irradiation during larval or embryonic life (Pechenik, 2006).

Rosa *et al*. (2013) hypothesized that ocean acidification, warming and expanding hypoxia may act together as the main trigger for premature hatching and smaller post-hatching body sizes (Kamler, 2008) and, consequently, dictate negative effects on survival and development of posterior ontogenetic stages.

Despite this, the information concerning the direct impacts of stressor events, as the effects of high pCO_2 and hypoxia, on the embryogenic development biology, physiology and hatching rate are still scarce.

2. Justification of the thesis topic

The increasing atmospheric CO_2 concentrations due to anthropic influence is causing the acidification of the oceans. This phenomenon often co-occurs with hypoxia events due to elevated water temperatures and accelerated nutrient delivery from land.

Due to a global oceanic oxygen decline and an increase in human activities, ocean acidification and hypoxia are becoming a growing concern for marine biodiversity and two important environmental stressors to marine ecosystems. Nonetheless, hypoxia and decreasing pH of the water could become potential problems for intensive aquaculture conditions because of the costs associated with keeping oxygen saturation at normal levels and the potential consequences on early life stages of marine animals. This is particularly important in invertebrates who form their internal skeletons out of calcium carbonate.

The European cuttlefish (*S. officinalis)* is an excellent model to assess the effects of climate change, namely regarding the increase of CO_2 and hypoxia events in the oceans, since this cephalopod species possess a calcium carbonate structure from early

development that grows very fast with a short life cycle. This accelerated development has triggered the interest in its introduction as a new species for aquaculture diversification. Despite this, few studies have investigated the extent to which the combination of both stressors, increases of CO_2 and hypoxia, elicits adaptative effects in this animal, especially during embryonic development.

3. Objectives of the work

The present thesis aims to determine the effects of two environmental stressors, high CO_2 environment and hypoxia events, in European cuttlefish (*S. officinalis*) embryogenesis (duration in days according to Sykes *et al.,* 2009 and stages of development as reported by Lemaire *et al.*, 1970), hatching rates (Sykes *et al.,* 2014) and physiological characterisation through metabolic rates (Rosewarne *et al.,* 2016).

Two separate experiments were performed. During the first, the cuttlefish eggs were exposed to an acute event of hypoxia during the embryonic development (either day 10, when the egg expels the water from the inside and become harder; or day 20) in order to study how acute hypoxia events can affect cuttlefish embryonic development and hatching rate.

In the second experiment, the eggs were maintained at 1600 ppm of CO_2, two groups were also exposed to an acute hypoxia event (either day 10 or day 20), in order to study how the different treatments affect the formation of the cuttlebone, the embryonic development and the hatching rate.

4. Materials and Methods

4.1. Collection of the eggs

Eggs were obtained from multiple generation captive-bred populations not only to reduce the impact on the environment, but also to have precise information regarding the age of the eggs and to have a batch homogeneous as much as possible.

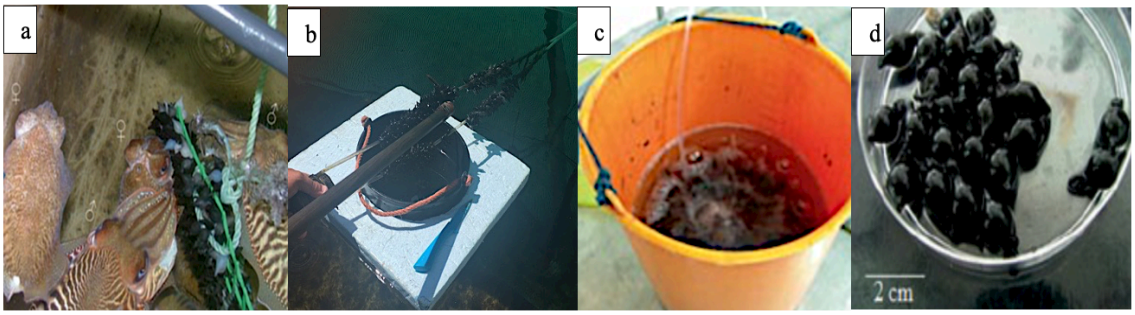


Figure 4.1 *shows the principal steps necessary to obtain the eggs for the experimental phase. The eggs lay by the females are attached to the net and airlifts* ***(a)*** *are removed with a knife and* ***(b)*** *and collected in a bucket with strong aeration* ***(c)****. After, the eggs are selected by colour and shape* ***(d)*** *and the not suitable are discarded. (Photos a, c, d, from Sykes et al. 2014; photo b by A. Amaduzzi).*

After copulation, the eggs are attached by the females to ropes, nets and airlines (Figure 4.1a). These are removed using a small knife to cut the egg lace connected to the plastic (Figure 4.1b), this prevents the damage of the chorion. Freshly laid eggs are characterized by a soft and gelatinous texture that makes it necessary to handle them carefully to avoid causing any harm.

After being removed from the breeding tank, the eggs were collected in a bucket with strong aeration (Figure 4.1c) that makes them move gently before being individualized from the spawn and selected by colour and shape. Oval black eggs were considered viable (Figure 4.1d), while eggs of any other shape and/or colour were considered non-viable and discarded (Sykes *et al.,* 2014). In both experiments, eggs were randomized divided into treatments.

4.2. Hypoxia Experiment

Three replicate 2.5L tanks were set for four different DO_2 conditions (100% DO_2; 75% DO_2; 50% DO_2 and 25% DO_2) respectively for group Day 10 and Day 20, for a total of 24 tanks (Figure 4.2). These were settled in an indoor semi-open system with temperature maintained stable at 20°C using titanium heaters. Each tank hosted 100 individualized normal cuttlefish freshly laid eggs (Sykes *et al.,* 2014).

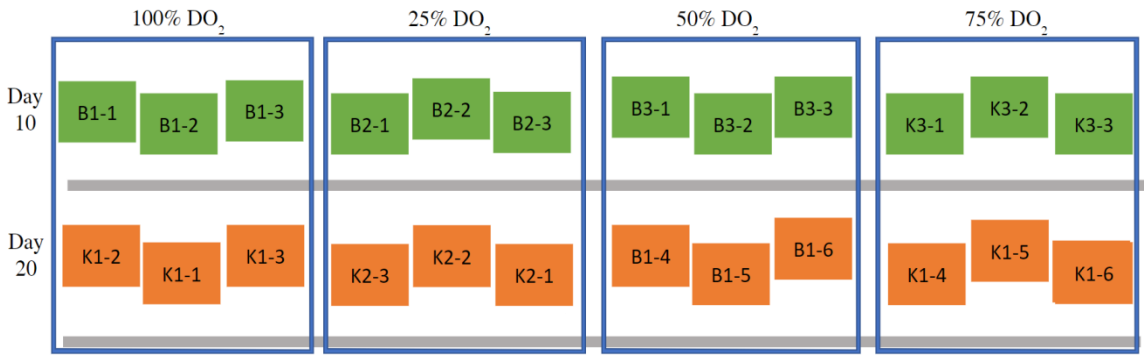


Figure 4.2 *shows the organisation of the 24 tanks on 2 shelves, the first one (green colour) where there were the eggs used during the Hypoxia event at Day 10 arranged in triplicate per treatment. On the second shelf (orange colour) were organized the eggs used during the Hypoxia event on day 20. The blue lines show the triplicates divided by treatment. Every tank contained 100 eggs for a total of 2400 eggs.*

Every tank contained a rectangular air stone, at the middle, and it was covered to avoid excess light intensity (intensity of 100 lux). Water entered the tank from the top and exited through the water level filter (1mm mesh). The eggs remained in the system for 45 days of normal development. The photoperiod resembled natural geographical conditions in spring (14 hD : 10 hN).

4.2.1. Seawater quality

Temperature, salinity, and DO_2 were measured daily at 9:30 am, together with pH and atmosphere pressure. Both temperature and DO_2 were measured with a VWR DO220 probe, while salinity was measured with a VWR EC300 salinity meter. In addition, a MaximDallas temperature chip (Thermochron – DS1921G; MAXIM, Sunnyvale, CA, USA) recorded data every 15-30 minutes. Salinity was measured every day with a WTR Cond3310 salinity meter.

Temperature was maintained stable at 20ºC by using a heating and cooling system. pH was measured in the collecting filtration tank and in the temperature regulation tank every day manually by using a VWR PH20 pH meter.

4.2.2. Biological Data

A representative batch of eggs was sampled at day 1 to estimate the average MWW of the sample size (around 18% of the embryos). Every 5 days, the eggs MWW (g) was measured to check the development. On the days of the hypoxia events (days 10 and 20), the MWW was taken just before the start of the experimental trial. Data regarding the number of hatchlings per treatment, the weight of the animals and the incidence of

malformation were recorded daily. Photos of the malformations were taken every time new hatchlings were found using a camera inserted in a stereomicroscope and connected to a laptop. To take the pictures animals were put in a Petri dish with a 4 mm sheet of squared paper under and filled with water from their tank.

4.2.3. Metabolic Data

Every 10 days, (day 10 and day 20 of the experiment) a given hypoxia level (100%, 75%, 50% and 25% DO_2 saturation) was applied to all the eggs of each replicate for 2 h. The water with the specific DO_2 was prepared in isolated tanks of 1 L, after the eggs from each replicate were collected and moved to a specific tank. To keep the level of hypoxia stable, the 1L tanks were covered with bubble wrap and a dark plastic cover to protect the eggs from the light (Figure 4.3b and 4.3c). The aim was to determine rates of oxygen consumption ($\dot{M}O_2$).

Three eggs, after being randomly selected, were inserted in the respirometer system where they recovered by being subject to full DO_2 saturation, meanwhile, data was recorded every second for 50 minutes. The recovery procedure was applied also to the other eggs of the same replicate in a separated isolated tank meanwhile the respirometry data was being collected. Background $\dot{M}O_2$ (resulting from consumption of bacteria and electrode) was also assessed with no animal (n=2) in the chamber for respectively two hours and overnight (approximately six hours).

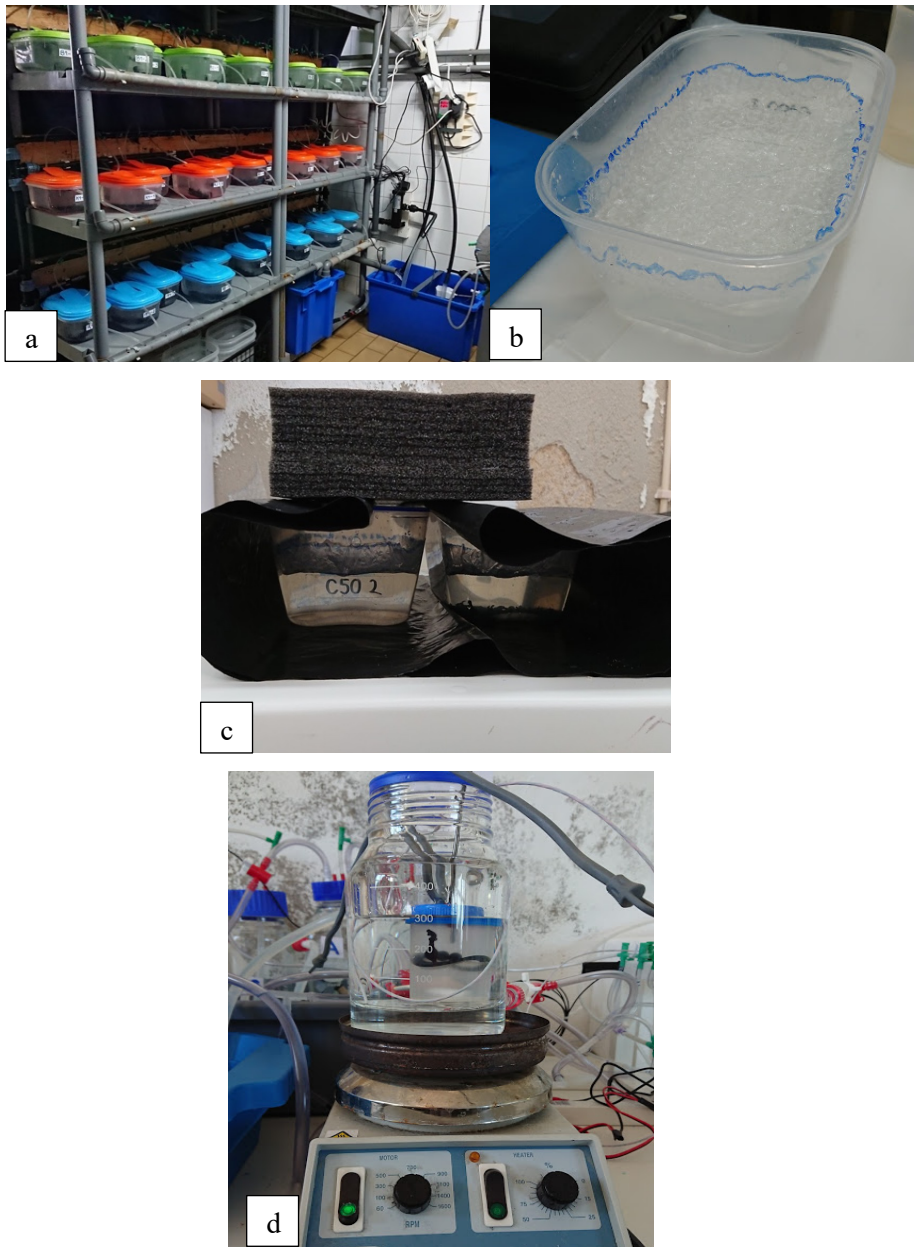


Figure 4.3 *shows the main steps of the hypoxia event procedure: at day 10 the eggs of the first row (greet top) were exposed to the different levels of hypoxia, at day 20 the eggs in the second row received the same treatments (**a**). Meanwhile, the water with the level of hypoxia was prepared and maintained stable by using bubble wrap to prevent the gas exchange (**b**). The eggs were exposed to a determined hypoxia event for 2 hours, meanwhile, they were covered with a dark cover (**c**). After the stressor event, 3 randomly selected eggs were inserted into the respirometry system and data was collected for 50 minutes (**d**).*

Out of the total time recorded during the respirometry, only the data from minute 15 to minute 30 (in total 15 minutes) was used for the linear regression. This was done to prevent potential bias as, for example, potential bubbles of air left in the chamber.

Bacterial respirometry was taken into consideration. Vessels containing marine water only were kept and measured to see the bacteria oxygen consumption (if any) and the results were subtracted to the actual values obtained during the recording of the three egg

respirometry. To obtain the final oxygen consumption of a single egg the results obtained were divided by three.

4.2.3.1. Respirometry system

Respiration analyses were conducted in an close respirometry system (Lefevre *et al.,* 2015) made of: a) one cylinder type chamber, where the three eggs were set (BPA free plastic; 3.1 x 3.2 cm) together with the oxygen probe collecting data; b) one cylinder type container (VWR 500 ml) with water maintained at 20 ºC thanks to c) an external recirculating pump (Brushless DC Pump QR30E DC 12V, 4.2W), which was regulated to achieve a given flow connected to d) a refrigeration machine manually settled at 20ºC.

Hypoxia levels of DO_2 were achieved by gassing seawater with pure nitrogen (N_2) using a ceramic stone in a closed 7 L reservoir.

The temperature inside the chamber was constantly checked by using a compact FireStingO_2 PC-controlled (USB) fiber-optic temperature meter (Figure 4.4).

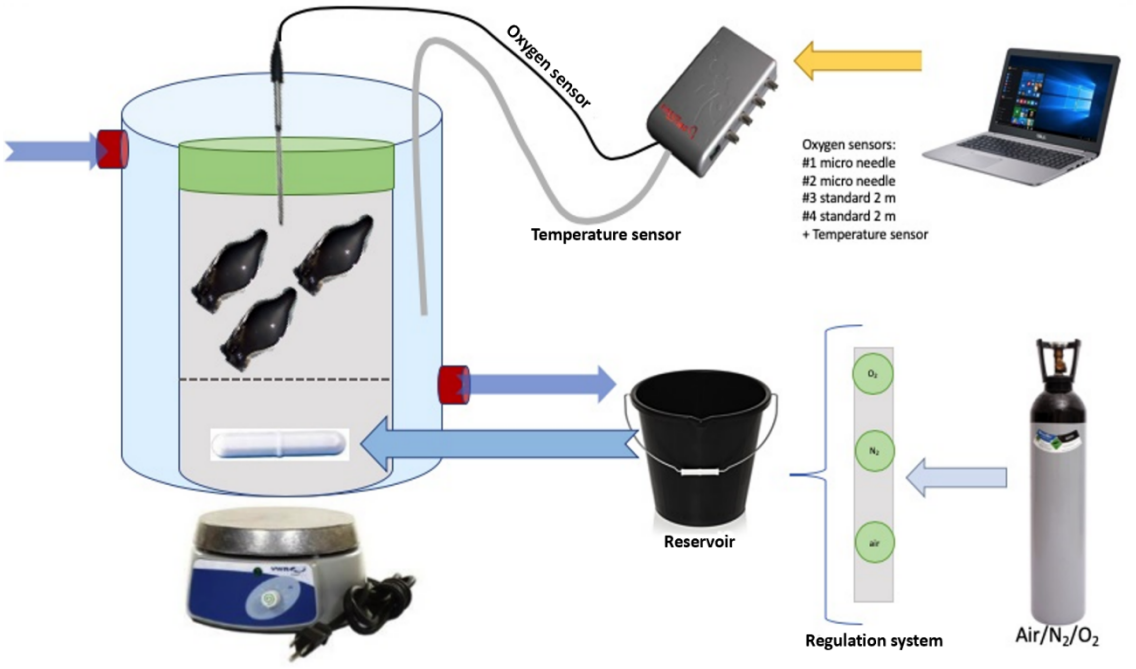


Figure 4.4 *shows how the respirometry system was organized: the computer was connected to two FireStingO_2 PC-controlled (USB) fiber-optic oxygen meter sensors which were inserted respectively inside the eggs chamber (inner one with green top) and on the temperature cylinder (external one in light blue). Oxygen levels were constantly monitored and regulated through a system of valves, directly connected to a N_2 or O_2 tanks or air pump. The chamber and the cylinder were isolated from each other, this allowed us to refill the chamber with gassed water with O_2 after the hypoxia event. To maintain a stable temperature, the water was passing through the external cylinder and the refrigerator/heating system was manually settled at 20 °C.*

The system had a total volume of 294 mL. While in recirculation and flushing, seawater entered and exited on opposite sides of the chamber at a flow of 60 L h-1 (3V) and 82 L h-1 (4.5V), respectively at a temperature of 20.0 ± 0.08°C. Dissolved oxygen saturation data was collected with a robust oxygen probe (3 mm diameter) connected to a compact FireStingO_2 PC-controlled (USB) fiber-optic oxygen meter inserted in an isolated chamber with the eggs and the gassed water (Figure 5.4). The vessel was then places into the system to keep the temperature stable. Pyro Workbench software was used to calibrate the fiber-optic oxygen meter and collect the data. At the end of the experiment, the data was transferred to Microsoft Excel 2016.

After the procedure, the eggs were removed, weighed, and then allowed to recover in normal rearing conditions until the end of the experiment.

4.3. CO_2 Experiment

Three replicate tanks were established for four different CO_2 condition: normal (CO_2 400ppm); high CO_2 (CO_2 1600ppm); high CO_2 with hypoxia event (50% DO_2) at day 10 of embryonic development and high CO_2 with hypoxia event (50% DO_2) at day 20 of embryonic development at Ramalhete Marine Station (Figure 4.5).

The experiment was performed in an outdoor open seawater system at a temperature around 22 °C using the conditions, setup and feedback systems for CO_2 control previously described by Gregorio *et al.* (2019). The eggs remained in the system for a period of up to eight weeks during embryonic development. (Lemaire *et al.,* 1970; Sykes *et al.,* 2009; 2014). Every 100L tank housed 100 normal cuttlefish freshly laid eggs, selected by shape and colour (Sykes *et al.,* 2014).

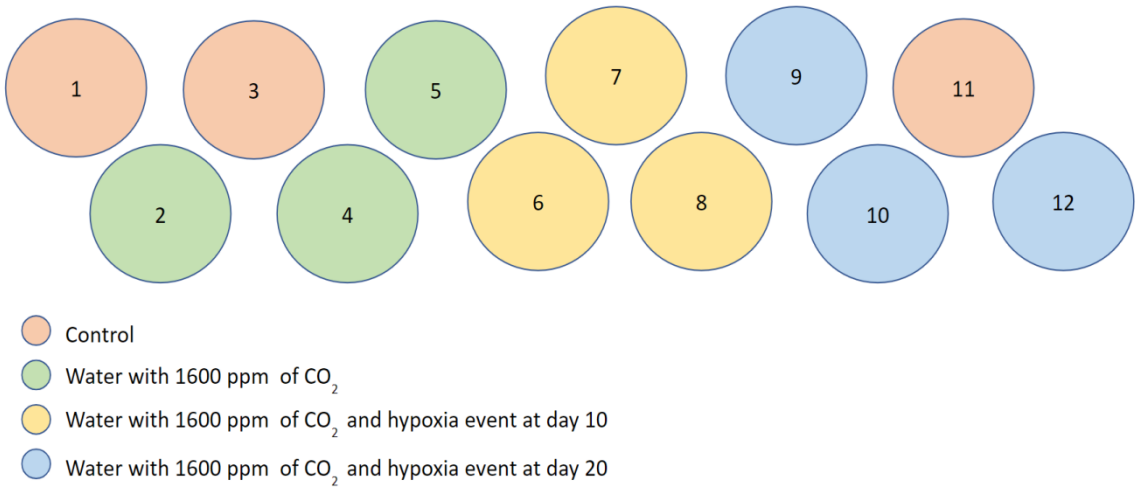


Figure 4.5 *shows the tanks setting in Ramalehete, where the control (red tanks) was maintained with marine water at 400 ppm of CO_2. The other tanks were maintained stable at 1600 ppm of CO_2 with events of hypoxia at day 10 (yellow tanks) and day 20 (blue tanks). Every tank contained 100 eggs for a total of 1200 eggs.*

Each tank was round-shaped and contained one airlift and one stone that made them move gently in a circular motion, preventing them from stacking on the bottom (Sykes *et al.,* 2006). The tanks were covered to avoid excess light intensity (mean intensity of 100 lux). Seawater entered the tank through the bottom and existed through the middle filter (1 cm mesh). The water flow was checked regularly to guarantee the same conditions in all tanks. The photoperiod was set to resemble natural geographical conditions in spring (14 hD : 10 hN).

4.3.1. Water quality

Oxygen and temperature were measured manually every day at 9:30 am using a multi-sensor probe VWR DO220, together with pH and atmosphere pressure. iBottom temperature chips were inserted in the tanks to monitor the temperature hourly. Salinity was recorded every day with a WTR Cond3310 salinity meter. pH was measured daily in all the tanks using a VWR PH20 pH meter manually calibrated through pH 4, 7 and 10 buffers (accuracy of ±0.02, pH ±1 digit).

4.3.2. Biological Data

Every 5 days, the eggs MWW (g) was measured to check the development over the weeks. The MWW was taken just before the start of the experimental trial on the days of the hypoxia events (days 10 and 20). Data regarding the number of hatchlings per treatment, the weight of the animals and the incidence of malformations.

Photos of the new hatchlings were taken when malformations were observed. The new-born cuttlefish bodies were checked every time there were new-borns and photos were collected if malformations were observed.

4.3.2.1. Aquaporins

Every 10 days (D10 and D20) three eggs were taken from each replicate tank and put on a petri dish with distillate water (enough to cover them) for 15 minutes. Every 10 seconds a photo was taken with a GoPro 3 Hero black digital camera positioned on a tripod. The camera was maintained in the same position throughout the experiment in order to maintain the same prospection and allow the program Adobe Lightroom to compare precisely if there was a change in volume or/and shape of the egg.

Both the programs were analysing the profile of the objects in the pictures and underling the differences by overlapping the last image to the first. If differences were found the difference in area of pixel was coloured in red.

4.3.3. Metabolic Data

At day 10 and 20 of the study, respectively, a given hypoxia level (50% DO_2 saturation) was applied for 2 h to the groups of eggs at high CO_2 with hypoxia event at day 10 or day 20 of embryonic development.

Afterwards, three randomly selected eggs from every treatment group, were inserted in the respirometer system where they were subject to full DO_2 saturation. Data of each

group was recorded every second for 50 minutes, aiming to determine rates of oxygen consumption ($\dot{M}O_2$). The equipment, setting and methodology used for the trial was the same described above in the hypoxia experiment (see Section 4.2.3).

4.4. Statistical analysis

IBM SPSS software was used for the statistical analysis. MWW of the eggs at day 5 was tested for both normal distribution and equality of variances using the Shapiro-Wilk test and the Levene's test (Zar, 1999), respectively. The significant level was set at 0.05. MWW data recorded at day 5 were subject to a one-way ANOVA with Bonferroni *post hoc* test (Zar, 1999). Data analysis was done considering the division of the experiments into three stages: biological data, hatching rate and respirometry.

4.4.1. Hypoxia experiment

At the end of the experiment, Levene's test (Zar, 1999), if the null hypothesis was rejected, a lower level of significancy would be used (*P*-value = 0.01) for the analysis of the variance (ANOVA). A three-way ANOVA was performed, considering the MWW as the dependent variable and considering three factors: i) time (sampling days); ii) hypoxia events at day 10 and day 20 and iii) treatments, followed by a Bonferroni *post hoc* test (Zar, 1999). The results were graphically represented to better interpret the significancy of the interaction.

4.4.1.1. Hatchlings

A Levene's test (Zar, 1999) was performed on the hatchling data. In case of the rejection of the null hypothesis a lower level of significancy would have been used for the ANOVA (*P*-value = 0.01). A three-ANOVA test was used to test the null hypothesis of equal means for a variable of interest, the hatching rate, considering all possible combinations of the three grouping factors, that are i) time (sampling day), ii) treatments and iii) day of the hypoxia event (at day 10 and day 20). Afterward, multiple comparisons were performed using Bonferroni *post hoc* test (Zar, 1999). The results were graphically represented to better interpret the significancy of the interaction.

4.4.1.2. Respirometry

A linear regression was performed on the data collected during the respirometry phase of the four treatments (100%, 25%, 50% and 75% DO_2) during the hypoxia event on day 10 and day 20 of embryonic development. In the linear regression, the amount of oxygen

consumed (expressed in mg/L**)** was used as the dependent variable and the time in hours (h) as the independent variable.

4.4.2. CO_2 experiment

At the end of the experiment, a Levene's test (Zar, 1999) was performed, if the null hypothesis was refused, a lower level of significancy would have been used (*P*-value = 0.01).

A two-way ANOVA was performed with data obtained, considering the MWW as the dependent variable and considering two factors: i) time (sampling days) and ii) treatments. Afterward, Bonferroni *post hoc* test (Zar, 1999) was used to determine multiple comparisons. The results were graphically represented to better interpret the significancy of the interaction.

4.4.2.1. Hatchlings

A Levene's test (Zar, 1999) was performed on the hatchling data obtained, if the null hypothesis was rejected, a lower level of significancy would be used (*P*-value = 0.01) for the ANOVA. A two-way ANOVA was performed, considering the MWW as the dependent variable and considering two factors: are i) time (sampling day) and ii) treatments, followed by a Bonferroni *post hoc* test (Zar, 1999). The results were graphically represented to better interpret the significancy of the interaction.

4.4.2.2. Respirometry

A linear regression was performed on the data collected during the respirometry phase of the four treatments, control (CO_2 400ppm), high CO_2 (CO_2 1600ppm), high CO_2 with hypoxia event (50% DO_2) at day 10 of embryonic development and high CO_2 with hypoxia event (50% DO_2) at day 20 of embryonic development. In the linear regression, the amount of oxygen consumed (expressed in mg/L**)** was used as the dependent variable and the time in hours (h) as the independent variable.

4.5. Ethical statement

All the procedures were approved by CCMAR Animal Welfare Committee (ORBEA CCMAR-CBMR) and Direcção-Geral de Alimentação e Veterinária (DGAV) of the Portuguese Government, according to National (Decreto-Lei 113/2013) and EU

legislation (Directive 2010/63/EU) on the protection of animals used for scientific purposes. Procedures were only applied to live animals by authorized users.

5. Results

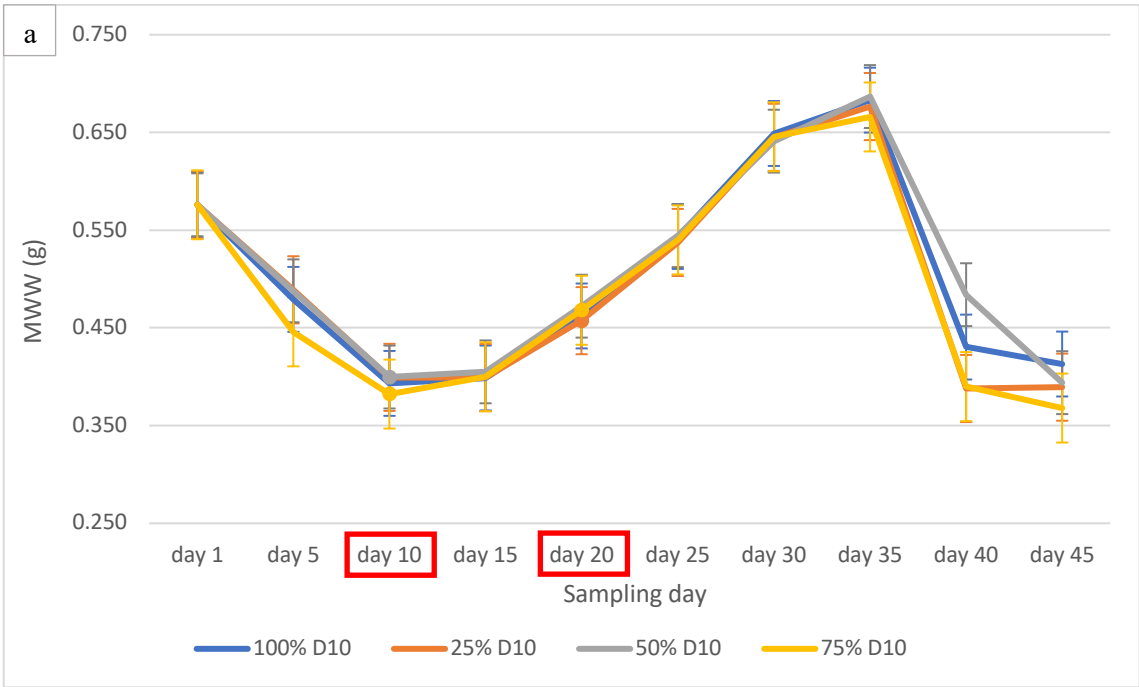
5.1. Hypoxia experiment

5.1.1. Water quality

Temperature of the water was maintained stable during the experiment at 19.59 ºC ± 0.4 with an 8.15± 0.03 pH and a mbar of 1016 ± 2. DO_2 was 101.9%±1.41 and the salinity was 36.66 ‰ ± 0.32.

5.1.2. Biological data

The average MWW of the representative sample of eggs at day 1 was 0.576 g. During embryonic development, the weight of the eggs showed a significant variation, as it is possible to observe in Figures 5.1a and 5.1b. Starting from day 5, the mass of the eggs decreased, passing from approximately 0.450 g to 0.380 g at day 10. After, the average weight in all the treatments started to increase, reaching the maximum around day 30-35 (around 0.654 g).



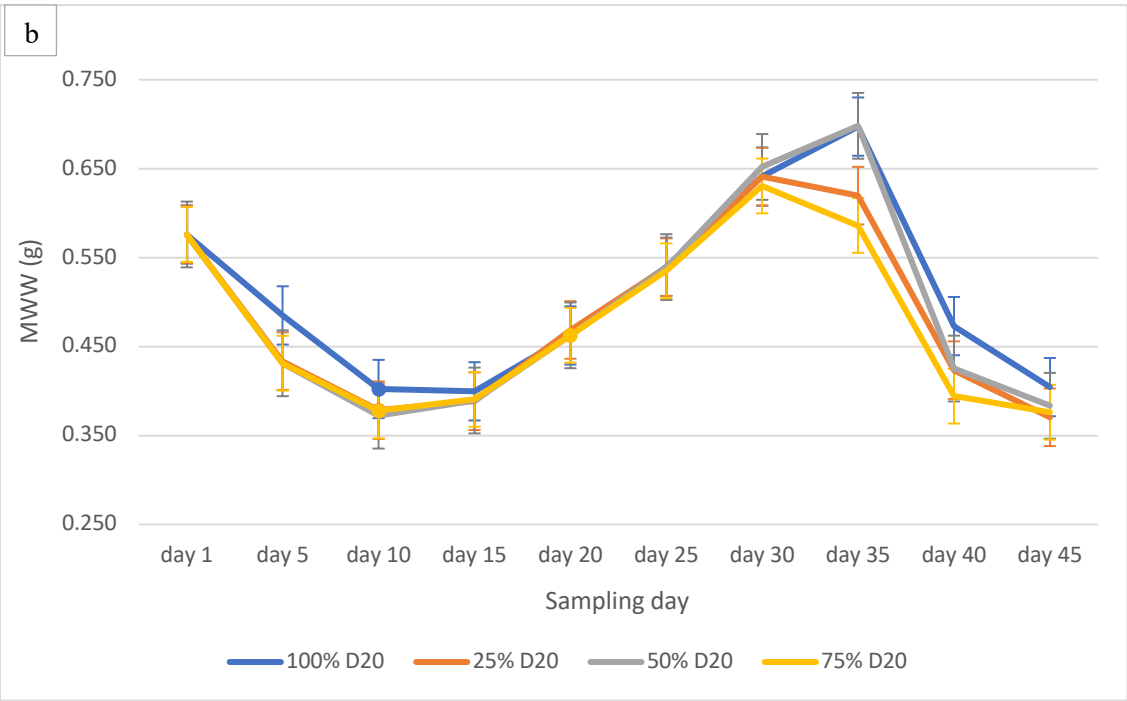


Figure 5.1a *and* ***5.1b*** *shows the MWW variation over time of the treatments (hypoxia event at day 10 (D10) on the top and hypoxia event at day 20 (D20) below). Results are reported ad average± SEM of n=2400 eggs. The red icons indicate the day of the hypoxia event. As it is possible to observe the average weight decrease from 0.450 g to less than 0.400 g around day 40.*

At day 30, control at day 20 (100% D20) and 50% DO_2 level at day 20 (50% D20) were the treatments with the highest presence of eggs heavier than 0.500 g, which was considered the moment after which the eggs could hatch. Passed this day the eggs exceeding 0.500 g were considered late hatching (approximately 20% of the eggs left at day 40) and the other aborts.

Levene's test on the MWW at day 5 showed no statistical difference between treatments. The normality tests noted a significant difference, through graphic inspection, no strong violation of the normality was observed and the sample was considered normally distributed.

The one-way ANOVA highlighted significant differences between the MWW of the eggs at day 5, meaning that at least two replicates of the treatments were dissimilar. Bonferroni *post hoc* test showed that treatments 25% DO_2 and 50% DO_2 appeared significantly different from treatments 75% DO_2 and 100% DO_2, respectively. Concluding that the weight of the eggs at day 5 was not homogeneous across the four treatments.

Levene's test performed at the end of the experiment on the MWW data collected showed significantly differences. A lower level of significance was chosen to perform the ANOVA. The *P*-value was set at 0.01.

The three-way ANOVA results showed that all the effects, both principal and both of interaction, resulted significantly different with a *P*-value of 0.01, with the only exception of the factor hypoxia event at day 10 and day 20 (*P*-value = 0.018). The partial eta-squared, which measures the power of the effect considered, of the factor 'day' resulted to be the only remarkable effect on the dependent variable (partial eta-square= 0.303). All the other effects, despite being significant, do not appear relevant (partial eta-squared ≤0.004).

Multiple comparisons were performed using Bonferroni *post hoc* test showed significant differences in the MWW of the eggs over time, between treatments and between hypoxia events.

A remarkable variability in weight was observed, related to the development day (sampling day of the MWW), as already described above. The weight on day 5 appears significantly different compared to all the other sampling days, with the only exception of days 20 and 40. Day 10 and 15 do not appear significantly different between each other and with day 45. Starting from day 25 until day 35, the weight of the eggs appears to be always significantly different in all treatments.

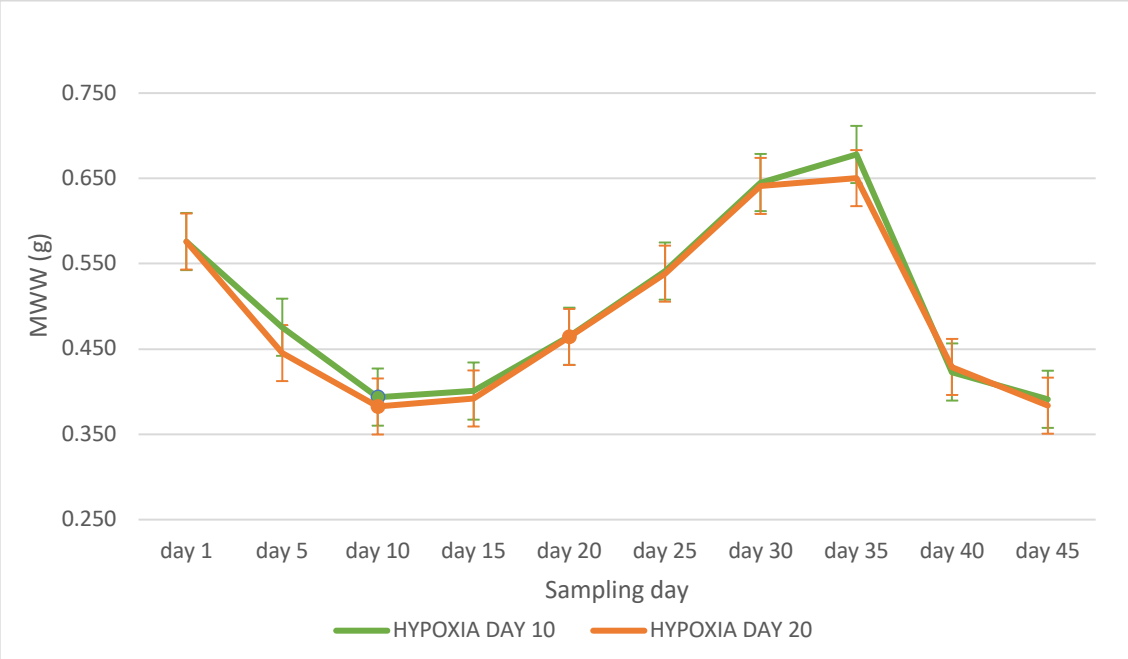


Figure 5.2 *shows the MWW(g) variation of the two hypoxia events over time. Results are reported ad average± SEM of n=2400 eggs. The red icons indicate the day of the hypoxia event.*

Statistical differences were found in the interaction between day and hypoxia events, represented graphically in Figure 5.2. Results showed that eggs under hypoxia at day 20 had significant lower mass both at day 5 and 35, when instead at day 40 it increased,

becoming higher than the average MWW of hypoxia event at day 10. On day 45 the average MWW of both the hypoxia events result aligned.

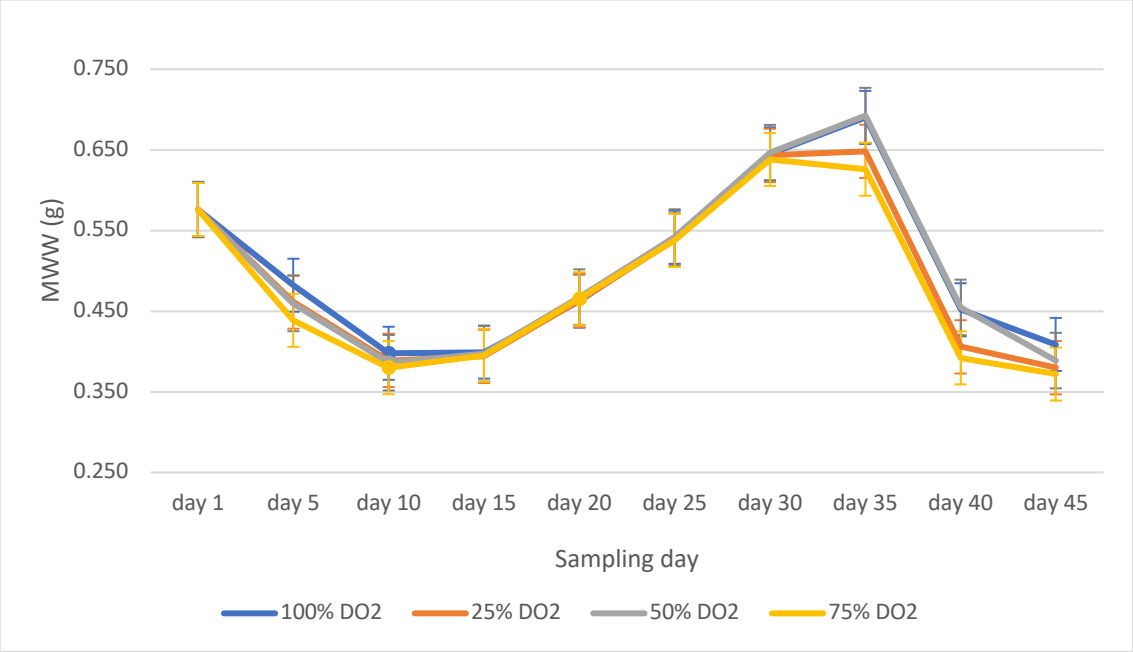


Figure 5.3 *shows the variation in MWW(g) per treatment over time. Results are reported as average± SEM of n=2400 eggs. The red icons indicate the day of the hypoxia event.*

Considering instead the treatments, the interaction between day and treatments seems to be mainly related to the increase in MWW at day 35, especially in treatments with oxygen at 50%DO_2 and the control (100% DO_2). This difference appears to be gradually fading at days 40 and 45.

5.1.3. Hatchlings

At a stable temperature around 19.6 ºC, the eggs started to hatch from day 25 of the experiment, where the highest hatching rate was observed in the treatments 25% and 50% DO_2 (about 5% of the eggs). As it is possible to observe in the graphs below, treatments 25% and 75% DO_2 day 20 recorded a higher hatching rate already at day 35 compared to the other treatments, followed by another similar quantity of animals born at day 40 (Figure 6.4). In all the other treatments, instead, a well-defined peak in the hatching rate was observed at day 40.

On day 45, around 7% of the eggs out of the total ones left per tank hatched (around 30 eggs). That day, treatments 50% DO_2 day 10 and the 100%DO_2 day 20 recorded the major number of hatchlings (around 14.4% vs. 5% in the other treatments).

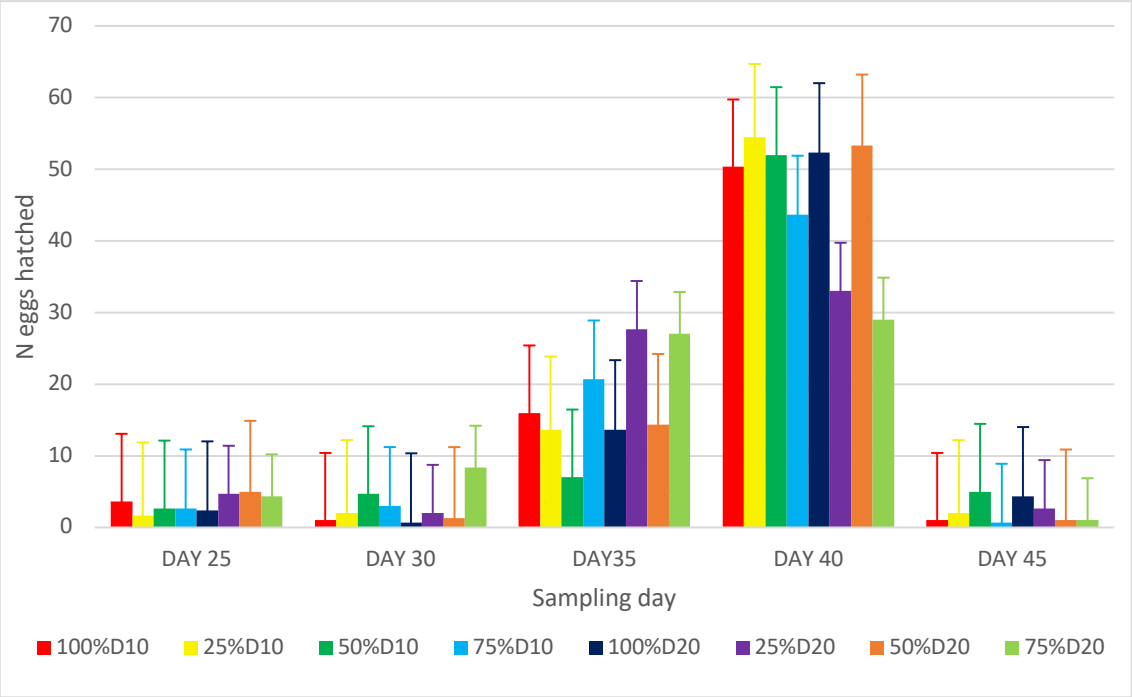


Figure 5.4 *shows the number of eggs hatched per day, starting from day 25 until the last day of the experiment (day 45). Results are reported as average±SEM. As it is possible to observe, the highest number of hatchlings were found on day 40. The animals found in between the sampling day were counted as hatched during the following sampling day (e.g., the cuttlefish hatched between day 31 and day 34 were counted all together at day 35).*

At the end of the experiment, the hatching rate in all the treatments was around 70%, the lowest percentage was observed in treatment 75% DO_2 Day 20 (69.7%), the highest in treatment 50% DO_2 Day 20 (75%). Data was analysed to evaluate the possible presence or absence of main effects due to individual factors (time, treatment and hypoxia event), also evaluates the significance of their interaction.

Levene's test performed at the end of the experiment on the hatching rate data showed significant differences. Consequently, a lower level of significance was chosen for performing the ANOVA. The *P*-value was set at 0.01.

The three-way ANOVA, followed by Bonferroni post hoc test, showed that all the effects, both principal and both of interaction, were significantly difference. The partial eta-squared, which measures the power of the effect considered, of the factor 'day' resulted to be the only remarkable effect on the dependent variable (partial eta-squared = 0.500). All the other effects, despite being significant, do not appear relevant (partial eta-squared ≤0.01).

A remarkable variability was observed in the number of hatchlings related to the factor day. The hatchlings were found starting from day 25, which is the reason why no

significant difference was observed from day 5 until day 20. From day 25 all the treatments results are significantly different.

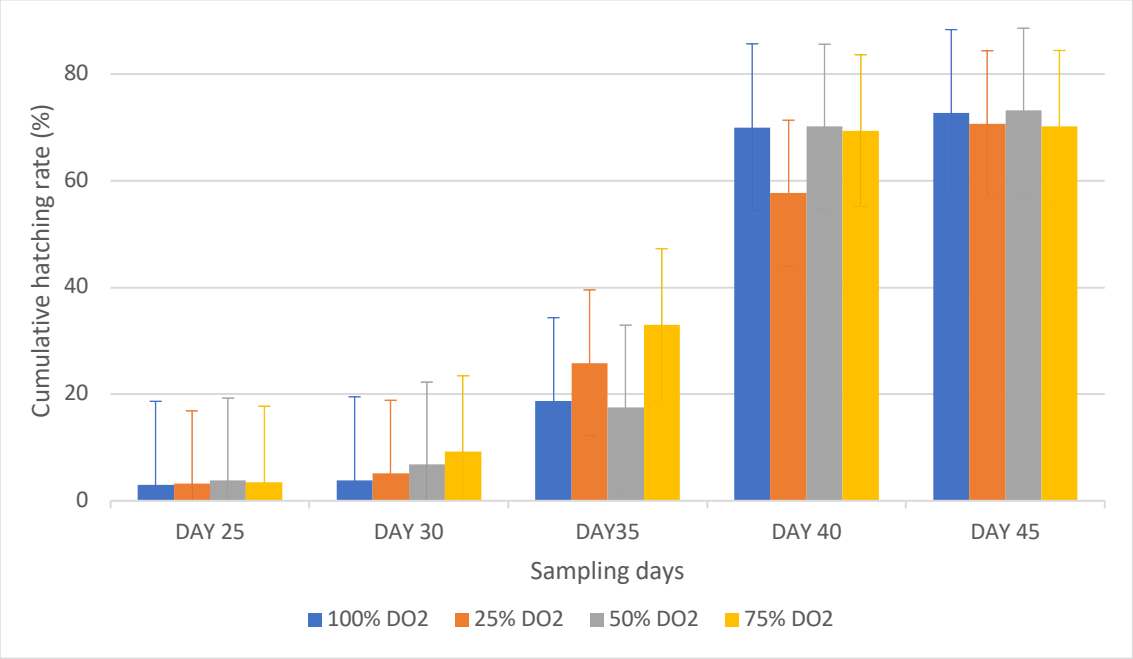


Figure 5.5 *shows the average cumulative hatching rate plus SEM over time (from day 25 until day 45) for the four experimental treatments (100% DO_2, 25% DO_2, 50% DO_2, 75% DO_2).*

Considering only the factor treatments over the sampling days, represented graphically in Figure 5.5, no significant effect was observed in the hatching rate, with the only exception of treatment 75% DO_2. The significance of the interaction between day and treatment, although also very modest, could be found in the fact that the percentage of hatching at day 35 seemed to be considerably higher in groups 25% and 75% DO_2 than in groups 50% and 100% DO_2. However, this difference seemed to reset to zero at day 45 as the remaining eggs were abort, which weight was similar to the one recorded at day 10 and 15 of development. The percentage of aborts at the end of the trial was around 27% for control and 50%DO_2, 29% for 25% DO_2 and 30% in treatment 75% DO_2, respectively.

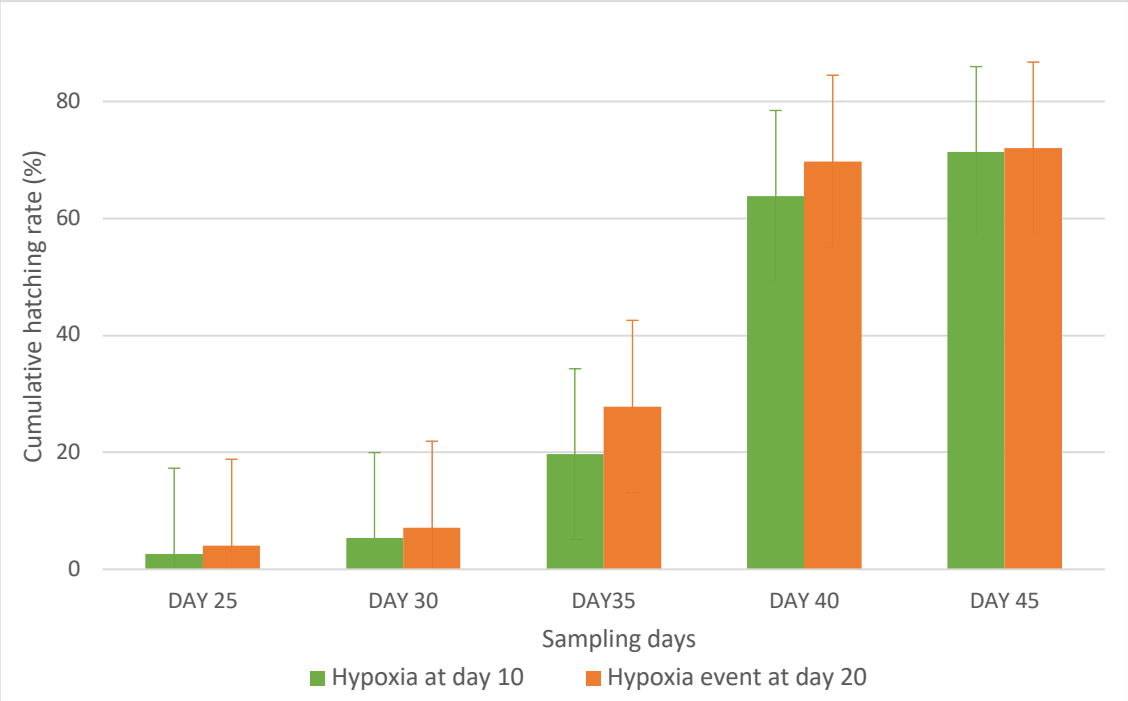


Figure 5.6 *shows the average cumulative hatching rate plus SEM over time of the two hypoxia events groups (from day 25 to 45), showing that the Hypoxia event at day 10 results to have a hatching rate lower than the group which was exposed to hypoxia on day 20.*

The significance of the interaction between day and hypoxia event, albeit of a very modest entity, could be found in the fact that the hatching percentage of hypoxia event at day 20 seems to be higher at days 35 and 40 and then aligns with the data from hypoxia event at day 10 on day 45. No significant differences were found in hatchling weight, which had an average of 0.092 g.

Table 5.1 *shows the initial and final MWW of the eggs, hatching rate, malformation rate and the mean weight ± standard deviation for each treatment (Day 10 100%, 25%, 50%, 75% DO_2 and Day 20 100%, 25%, 50%, 75% DO_2).*

	D10 100% DO_2	***D10 25% DO_2***	***D10 50% DO_2***	***D10 75% DO_2***	***D20 100% DO_2***	***D20 25% DO_2***	***D20 50% DO_2***	***D20 75% DO_2***
MWW at day 5 (g)	0.479 ±0.11	0.489 ±0.11	0.488 ±0.12	0.446 ±0.10	0.485 ±0.11	0.434 ±0.09	0.431 ±0.09	0.431 ±0.10
MWW at day 45 (g)	0.413 ±0.19	0.389 ±0.13	0.394 ±0.14	0.368 ±0.13	0.404 ±0.18	0.370 ±0.12	0.383 ±0.13	0.376 ±0.15
Hatchling weight (g)	0.092 ±0.04	0.94 ±0.05	0.090 ±0.01	0.091 ±0.01	0.088 ±0.01	0.092 ±0.04	0.094 ±0.04	0.092 ±0.04
Hatching Rate (%)	72 ±3.0	71.3 ±11.93	71.3 ±5.13	70.7 ±1.15	73.3 ±1.53	70 ±5.29	75 ±1.0	69.7 ±4.04
Malformation Rate (%)	1.67 ±1.15	1.33 ±0.58	2.43 ±1.62	2.33 ±1.51	1.67 ±1.15	1.00 ±1.00	3.50 ±2.12	2.67 ±2.08

The malformation rate was between 1 and 3.5 %, the highest presence of malformation was noted in the treatments 50% and 75% DO_2 of day 20, 3.5 ± 2.12 and 2.67 ± 2.08 (Table 5.1). In both the control groups (100% DO_2 day 10 and 100% DO_2 day 20) was observed a malformation rate of 1.7%. The lowest rate was observed in both the treatment with the highest level of hypoxia (day 10 and 20 with 25% of DO_2).

The physical abnormalities were localized principally in the eyes, mantle coloration and chromophore and in a few cases on the tissues of the animals.

The most common malformation involved the mantle formation, which could have involved a small area of it, Figure 5.7 image C, or the total surface of the mantle. In some severe cases, the mantle formation resulted incomplete, and the cuttlebone was visible (Figure 5.7 image I).

All the animals with mantle abnormalities seemed unable to control the contraction and relaxation of the muscles around individual chromatophores, which allow them to camouflage. Furthermore, the animals did not show any response to light stimulus.

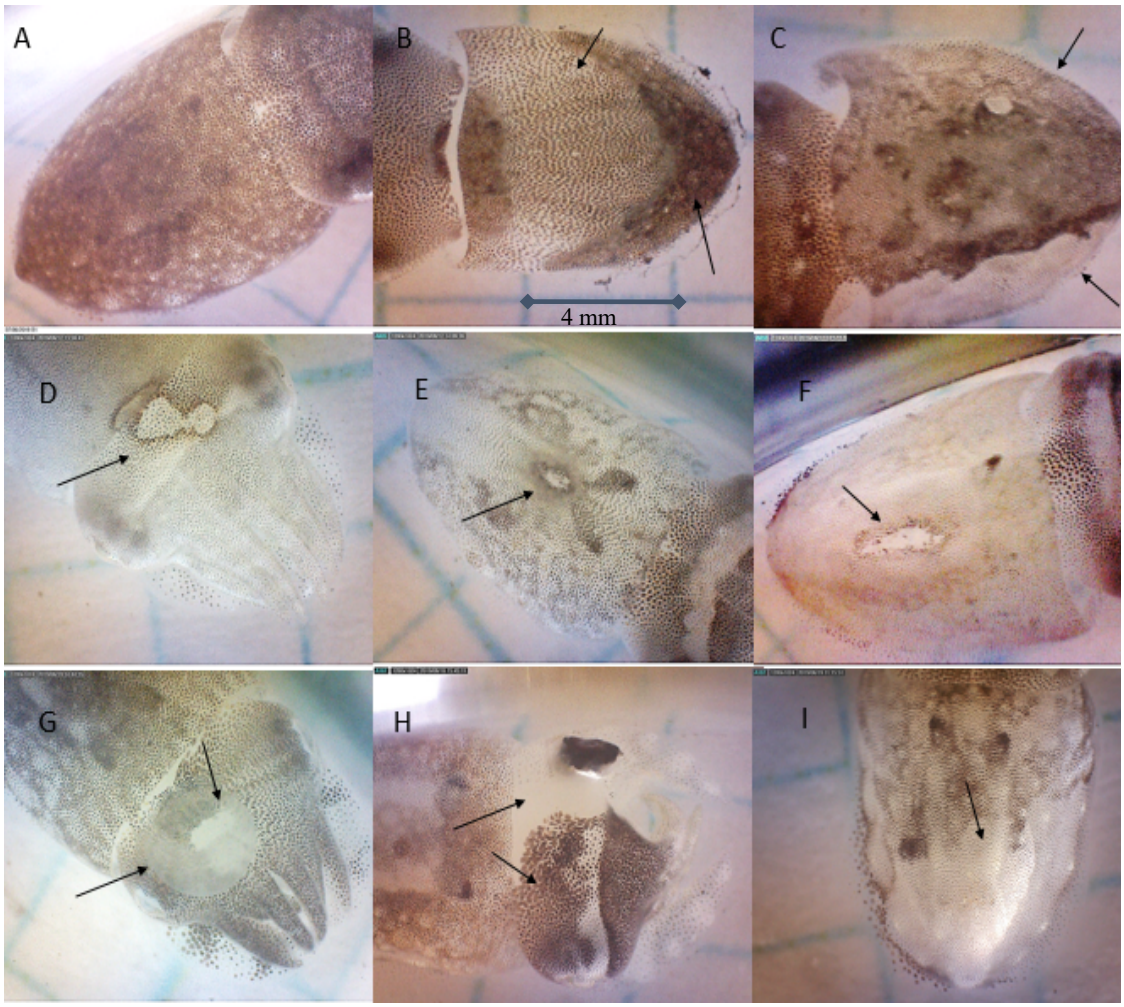


Figure 5.7 *shows some of the malformations observed during the experiment. A healthy cuttlefish should present a homogeneous colored and shaped mantle and a response to light and dark stimulus as in photo A. The absence of chromatophores was observed in small areas, as in photo H, where the two arrows point to the part without chromatophores (which results* in *white) and the part with them (in* a *darker color). Lack of chromatophores* was *observed also in located areas, as it is possible to see in* Figures *D E and F where the area without chromatophores* appears *as a wound with ragged edges. In more severe cases the absence of chromatophores was diffuse in all the body resulted in a "spotted" mantle, as* in *photo B, or partial transparency, like in picture I, where it was possible to see the cuttlebone. In some* cases, *a thickening of the superficial tissues was observed, as in Figure G. (Photo made by Angelica Amaduzzi).*

This kind of mantle malformations alone was mostly observed around day 25. From day 30 cases of eye malformations started to appear more often, alone or combined with mantle abnormalities. The highest incidence of malformations to the mantle was in the 50% DO_2 day 10 condition.

In a few cases, some sort of bubbles appeared in the tissues of the animals. In the worst case, the animal was not able to keep the correct position in the water and was corkscrew swimming (Figure 5.8 image B). Eye malformations were the most frequents, together with mantle chromatophores abnormalities.

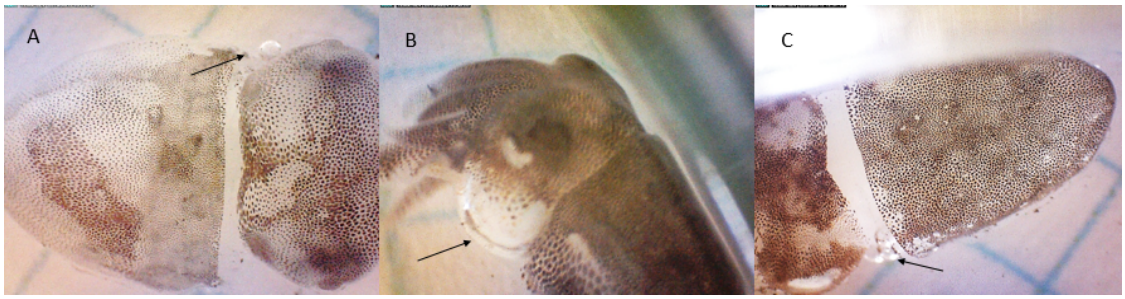


Figure 5.8 *shows malformations found during the hypoxia experiment. Some individuals were found with bubbles in the tissues. In the most severe cases, as in Figure B, the animals were unable to maintain the correct position in the water column and swim properly. (Photos taken by Angelica Amaduzzi).*

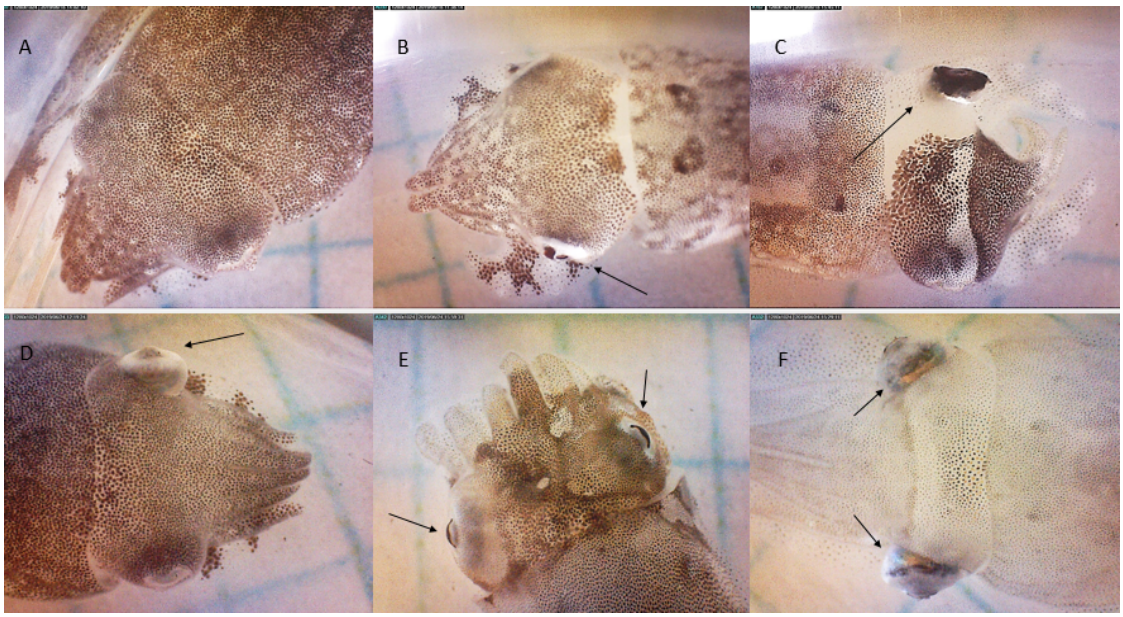


Figure 5.9 *shows the photos of the eye malformations observed during the hypoxia experiments. The animal presented in Figure A is a normal cuttlefish (control). Figures B and E show hatchlings with an intense dark-colored eye with a partial or total absence of the eyelid. Figures D and F show the eye of the cuttlefish covered by an extra layer of membranes which results in a bulge aspect. Figure C shows a dark area in proximity of the left eye, the eye results flat and without eyelid.*

The abnormalities observed involved the structure and dimension of the eyes. In some cases, the eyes appeared devoid of the typical W-shaped pupil (Figure 6.9 image E) and with a thickening of the membranes forming the eyes (Figure 6.9 images D and F) other animals, ophthalmic manifestation was observed, as alteration of the tissue coloration (Figure 6.9 images C and F) and absence of reaction to light stimulus, making the animals totally or partially blind. The highest rate of eye malformations was observed in tanks 75% DO_2 day 10.

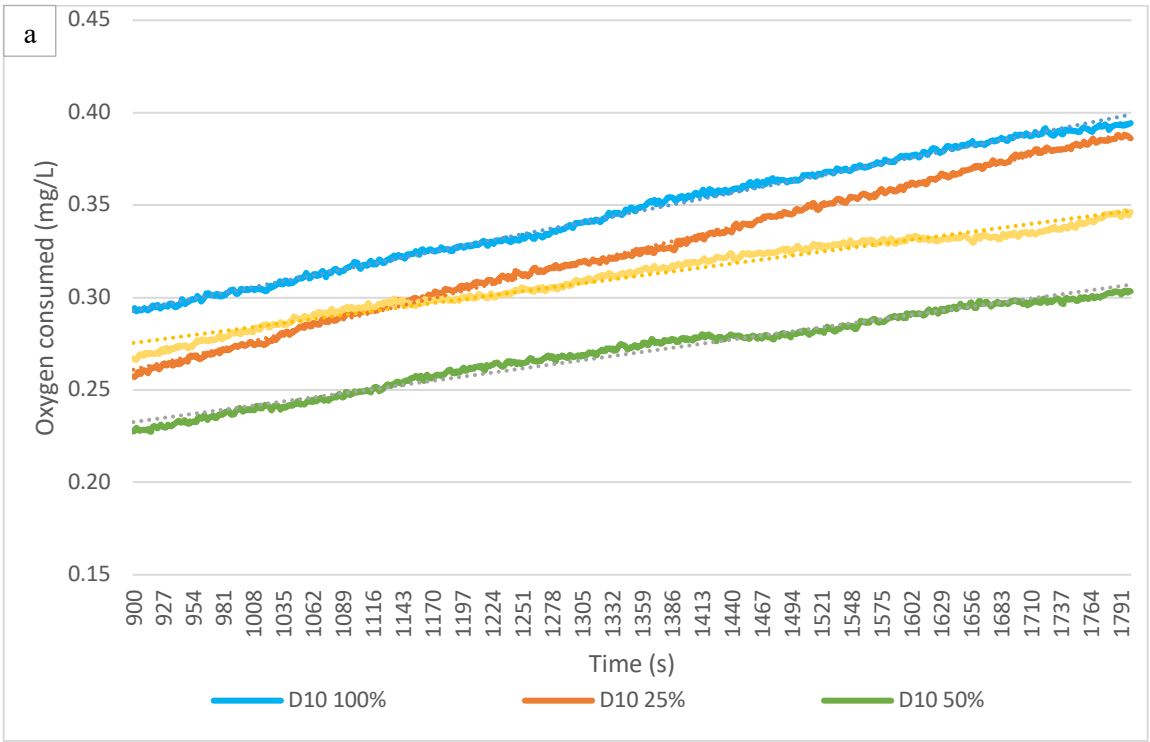
5.1.4. Respirometry data

The linear regressions, using as dependent variable mg/L oxygen consumed and as independent variable time (15 minutes). For each treatment, the coefficient was calculated (Table 5.2).

Table 5.2 *shows the results of the linear regression performed with SPSS.*

	D10 100%	**D10 25%**	**D10 50%**	**D10 75%**	**D20 100%**	**D20 25%**	**D20 50%**	**D20 75%**
Constant	,187***	,132***	,158***	,204***	,145***	,172***	,123***	,102***
Coefficient of time (h)	,042***	,051***	,030***	,029***	,042***	,055***	,036***	,029***

The results showed that the value of mg/L oxygen consumed by the eggs at time zero (the constant) appears different for each treatment. The treatment with the lowest value is group D20 75% DO_2 (0.102), instead D10 75% DO_2 showed the highest constant, exactly the double (0.204).



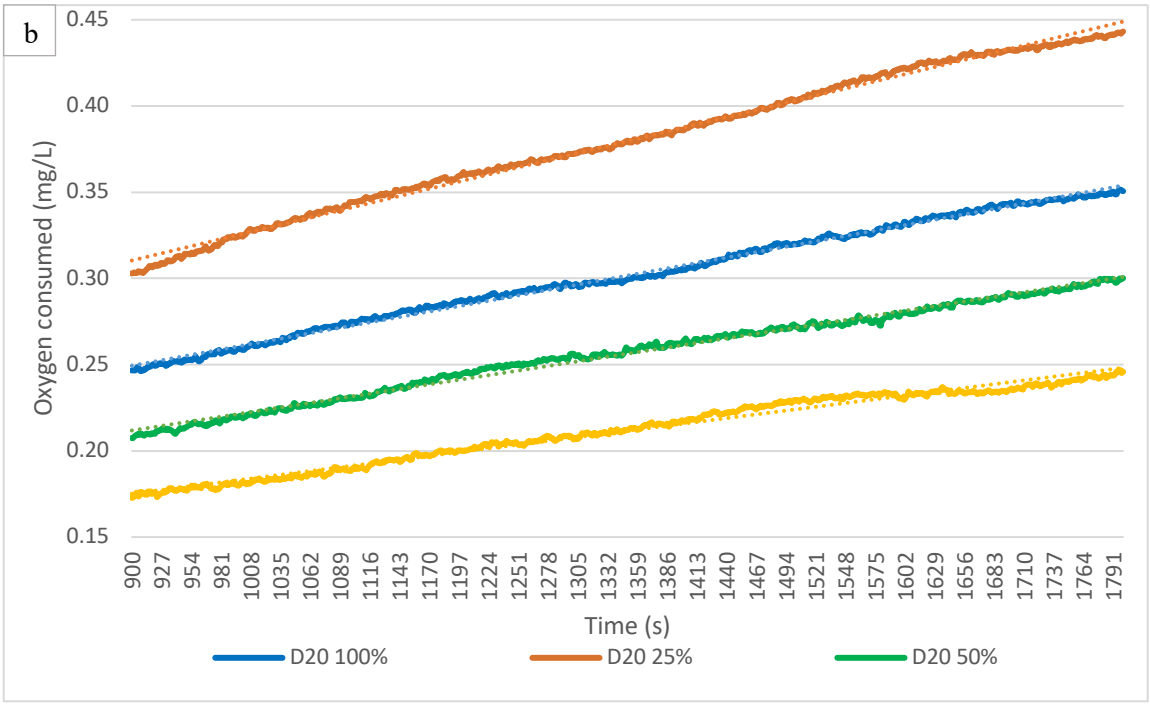


Figure 5.10a *and* ***5.10b*** *show the consumption of oxygen (mg/L) over time (15 minutes – from second 900 until 1800) per treatment of the eggs exposed to hypoxia at day 10 (Figure 5.10a) and day 20 (Figure 5.10b) of development, respectively. As it is possible to observe, the treatment D20 75% DO_2 results to be the one with the lowest value at time zero (900 s). D20 25% results instead, the treatment with the highest consumption rate at time zero, next to the control group D10 100% DO_2.*

The coefficients of time indicate the rate of mg/L oxygen consumed by the eggs per hour: all the eggs inserted in the respirometry chamber showed oxygen consumption. The highest increment in consumption of oxygen over time (0.055) was observed in group D20 25% DO_2. On the other hand, the lowest increment over time (0.029) was recorded for treatment D10 75% DO_2 and D20 75% DO_2.

5.2. CO_2 experiment

5.2.1. Water quality

Tanks were kept outdoor during the month of July with an average of 101.43% ± 1.21 DO_2, 36.68 ‰ ± 0.29 salinity and 1016.06 ± 2.15 mbar. Due to the outdoor setting, a strong fluctuation was observed in the water temperatures, which recorded an average temperature of 22.2°C ± 1.9, with a minimum of 18 °C and a maximum of 27°C on the hottest days.

Water pH in the tanks showed an average of 8.2± 0.04 in the Control, 7.8±0.05 in the High CO_2, 7.8±0.04 in the High CO_2 plus hypoxia at day 10 and 7.84±0.07 in the High CO_2 plus hypoxia at day 20.

5.2.2. Biological data

The average MWW of the representative sample of eggs at day 1 was 0.744 g. During embryonic development, the weight of the eggs showed a significant variation, as it is possible to observe in Figure 5.11. Starting from day 5, the mass of the eggs decreased, passing from approximately 0.446 g to 0.380 g at day 10. After, the average weight in all the treatments started to increase, reaching the maximum around day 20-25 (approximately 0.516 g).

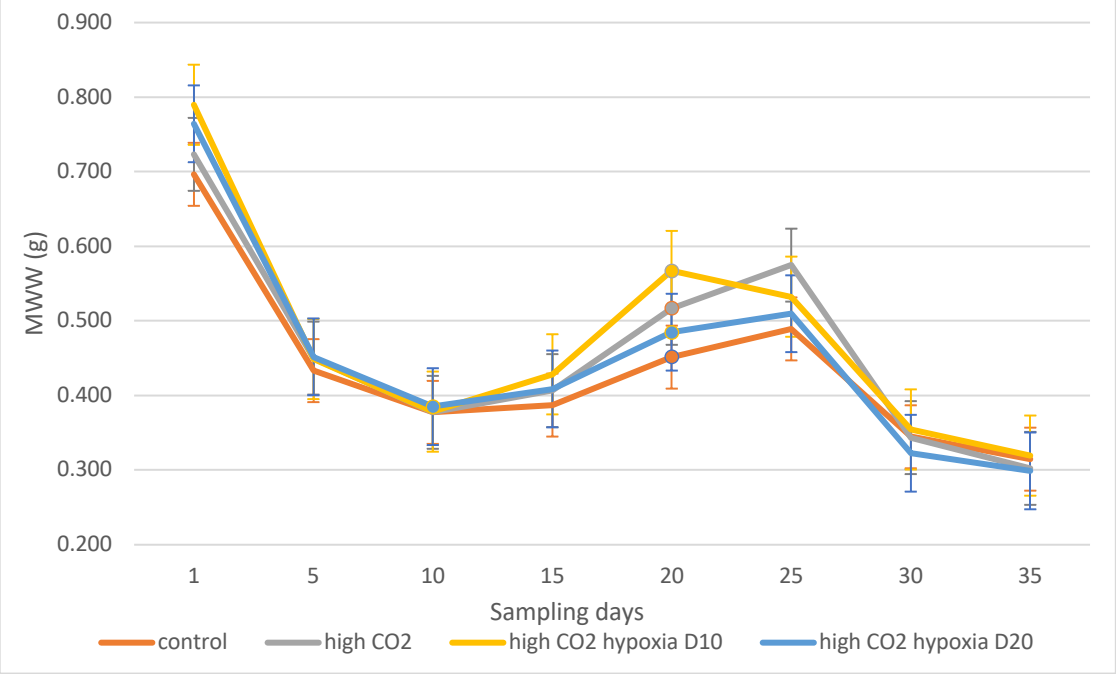


Figure 5.11 *represents the MWW variation during the embryogenic development of the cuttlefish eggs used during the CO_2 experiment. It is possible to see how the weight decrease from day 1 to day10, followed by*

a gradual increase as described previously in the hypoxia experiment. Results are reported ad average± SEM of n=1200 eggs. The red icons indicate the day of the hypoxia event.

On day 20, the treatment of high CO_2 with Hypoxia event at day 10 had the highest incidence of eggs heavier than 0.500 g, which was considered the moment after which the eggs could hatch. After this period the eggs exceeding 0.500 g were considered late hatching (around 3% of the eggs left at day 35) and the other aborts.

Levene's test on the MWW at day 5 showed no statistical difference between treatments. The normality tests noted a significant difference, but through graphic inspection, no strong violation of the normality was observed, and sample was considered normally distributed.

The ANOVA did not highlight significant differences between the treatments on day 5. This meant that the MWW of the eggs at day 5 was homogeneous. The Levene's test performed on all the MWW data showed significant differences and a lower level of significance was chosen in order to perform the ANOVA. The *P*-value was set at 0.01.

The two-way ANOVA, followed by Bonferroni *post hoc* test, showed that all the effects, both principal and both of interaction. The partial eta-squared of the factor 'day' resulted to be the only remarkable effect on the dependent variable (partial eta-square= 0.242). All the other effects, despite being significant, do not appear relevant (partial eta-squared $\leq$0.007).

A remarkable variability in weight was observed, related to the development day (sampling day of the MWW). The weight on days 1 and 5 appears significantly different compared to all the other sampling days. Weight between day 20 and 25 does not appear significantly different, as well as between day 30 and day 35.

5.2.3. Hatchlings

The experiment was held in July, outdoor, following the natural variation of temperature. The first hatchlings were observed on day 20 of the experiment, in tanks with high CO_2 and high CO_2 plus hypoxia event at day 10.

As it is possible to observe in the graphs below, control and high CO_2 groups recorded a higher hatching rate already at day 30 compared to the other treatment.

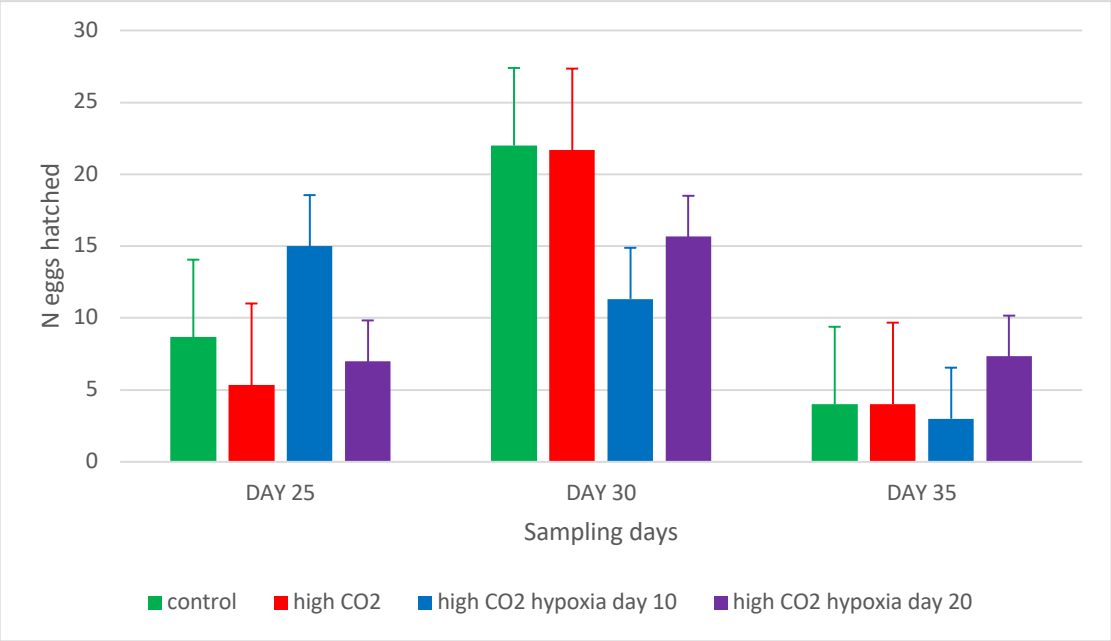


Figure 5.12 *shows the number of eggs hatched per day, starting from day 20 until the last day of the experiment (day 35). Results are reported ad average± SEM. As it is possible to observe, the highest number of hatchlings were found on day 30. The animals found in between the sampling day were counted as hatched during the following sampling day (e.g., the cuttlefish hatched between day 21 and day 24 were counted all together at day 25). Sampling day 20 is not shown in the graph as only three eggs hatched (two eggs for high CO_2 treatment and one high CO_2 plus hypoxia at day 10 treatment).*

All the other treatments, except for high CO_2 plus high hypoxia at day 10, recorded a peak of hatchling at day 30 of the experiment. At day 35, treatment high CO_2 plus hypoxia at day 20 recorded the highest number of eggs hatched (about 7 eggs). At the end of the experiment the hatching rate was 31.7%, the lowest percentage was observed in treatment high CO_2 plus hypoxia at day 10 (29.7%), the highest in treatment in the control group (34.7%).

Data were analysed to evaluate the possible presence or absence of main effects due to individual factors (time and treatment), and their interaction.

The Levene's test performed on all the hatchling data showed significantly differences, a lower level of significant was chosen to perform the ANOVA. The *P*-value was set at 0.01. The two-way ANOVA, followed by Bonferroni *post hoc* test, showed that all the effects were statistically different, with the only exception of the treatment (P-value = 0.066). The partial eta-squared, which measures the power of the effect considered, of the factor 'day' resulted to be the only remarkable effect on the dependent variable (partial eta-squared = 0.198). All the other effects, despite being significant, do not appear relevant (partial eta-squared $\leq$0.004).

The results showed a remarkable variability in the number of hatchlings related to the day, as already described above. The hatchlings were found starting from day 20, which is the reason why no significant difference was observed from day 5 until day 20. From day 25 all the treatments result significantly different.

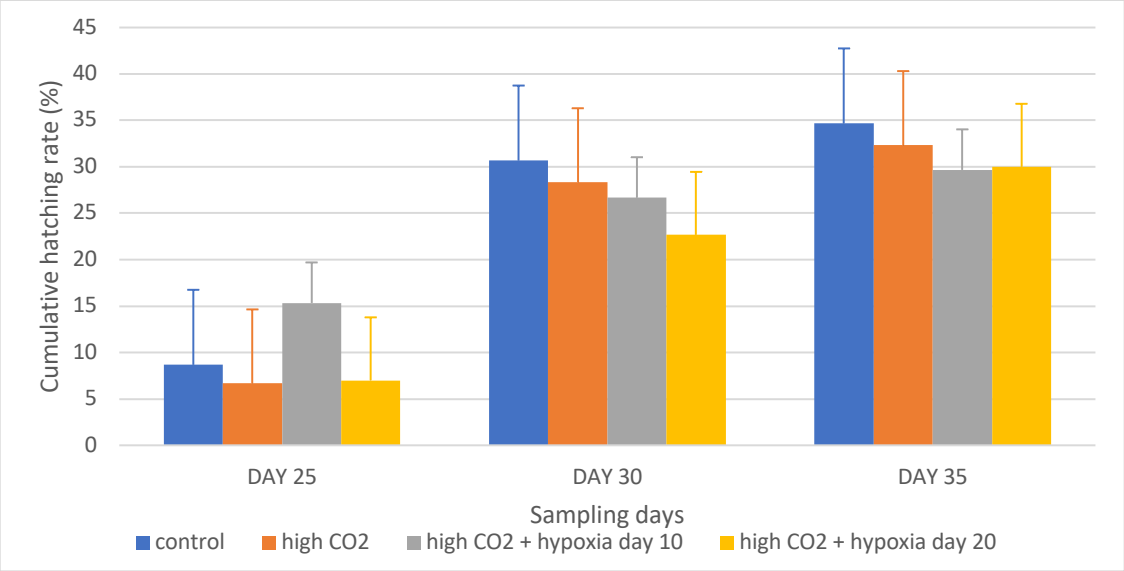


Figure 5.13 *shows the average cumulative hatching rate plus SEM over time (from day 25 until day 35) for the four experimental treatments (control, high CO_2, high CO_2 plus hypoxia at day 10 and high CO_2 plus hypoxia at day 20). Sampling day 20 is not shown in the graph as only 3 eggs hatched (two eggs for high CO_2 treatment and one high CO_2 plus hypoxia at day 10 treatment).*

Considering the treatments, represented graphically in Figure 5.13, no significant effect was observed in the hatching rate. The significance of the interaction between day and treatment, although also very modest, could be found in the fact that the percentage of hatching at day 25 seemed to be considerably higher in groups high CO_2 plus hypoxia at day 10, however, this difference seemed to decrease in the following days.

No significant differences were found between the weight of the hatchlings, which resulted around 0.083g. No malformations were observed during the experiment.

Table 5.3 *shows the MWW of the eggs plus standard deviation at day 5 and 35 and the hatching rate. No malformation was recorded during the experiment.*

	Control	High CO_2	High CO_2 + 50% DO_2 D10	High CO_2 + 50% DO_2 D20
MWW at day 5 (g)	0.433 ±0.13	0.450 ±0.12	0.449 ±0.14	0.452 ±0.15
MWW at day 35 (g)	0.315 ±0.12	0.302 ±0.11	0.318 ±0.13	0.299 ±0.10
Hatchling weight (g)	0.082 ±0.014	0.084 ±0.013	0.082 ±0.011	0.084 ±0.013
Hatching rate (%)	34.7 ±6.66	32.3 ±3.79	29.7 ±3.22	30 ±2.65

Eggs with a weight over 0.500 g (< 5% of the total amount left) at day 35 were taken from the tanks and opened with a scalpel to observe if they were aborts or late hatchlings.

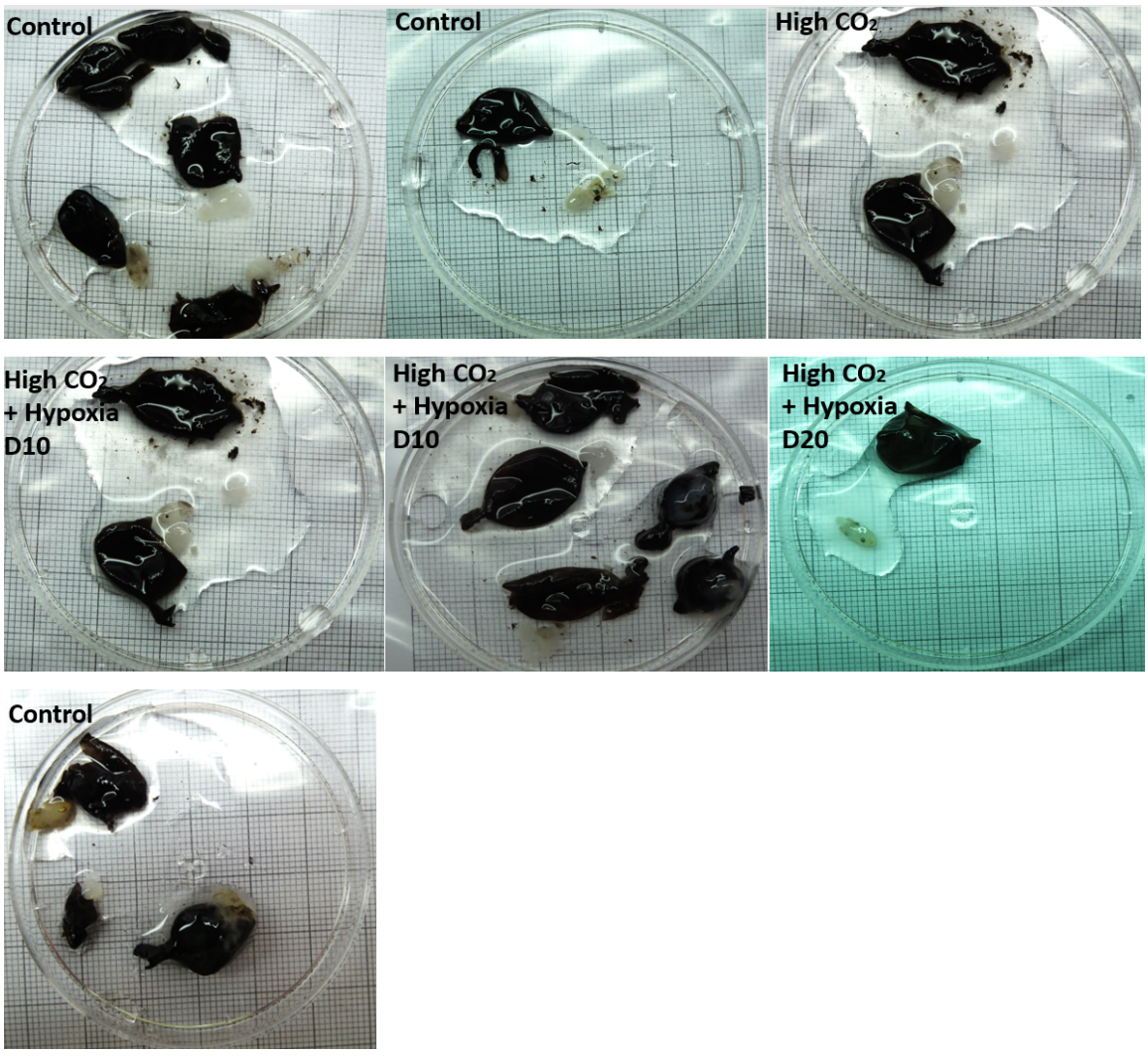


Figure 5.14 *shows the cuttlefish aborts found inside the eggs with a weight over 0.500 g. Aborts were found in the control tanks (1,3,11) as well as in hypoxia and CO_2 at day 10 (tank 7) and day 20 (9 and 11).*

As it is possible to observe in Figure 5.14, some of the animals, as tanks 1 and 11 were alive but their body was not completely developed. In other cases, the animals were missing fundamental parts of the body, demonstrating that the development process stopped during the second phase of development (tank 3 and 9). In the most severe case, as high CO_2 treatment (tank 5) and high CO_2 plus hypoxia at day 10 (tank 7), the content of the eggs was a mass of non-developed tissues or aborted at early stages of development. The treatment with the higher amount of abort was High CO_2 plus hypoxia at day 10.

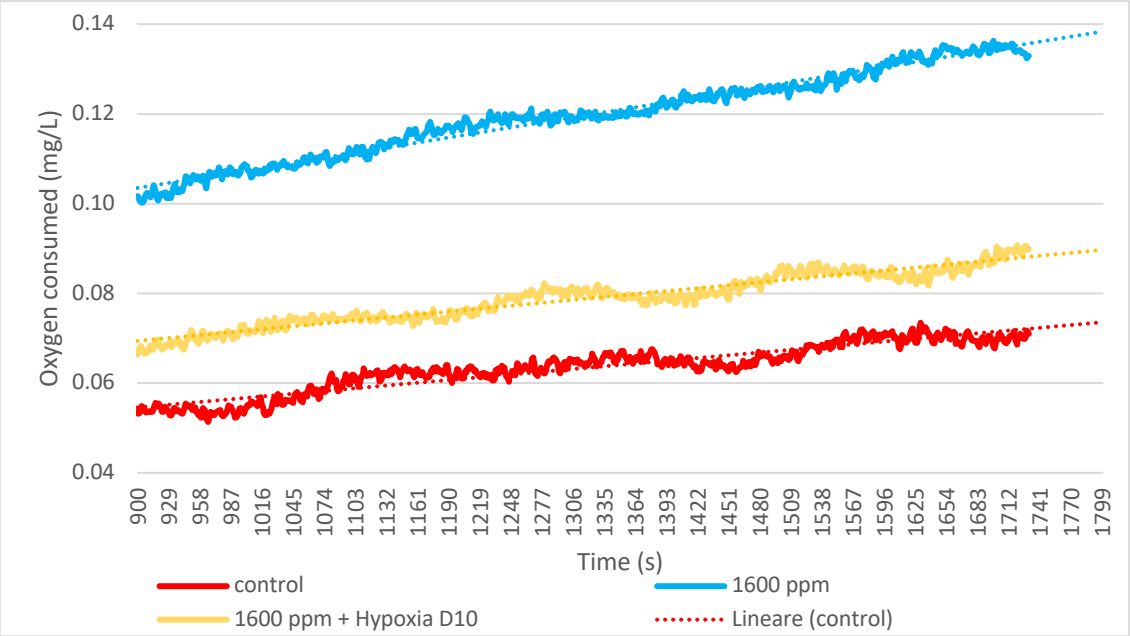
5.2.4. Respirometry data

Two linear regressions were performed, using as dependent variable mg/L oxygen consumed and as independent variable time (15 minutes). The first one took into consideration the data recorded after the hypoxia event on day 10 and the second one the data after the hypoxia event on day 20, in addition also data from high CO_2 plus hypoxia at day 10 were collected. Control and high CO_2 respirometry data were also recorded on both occasions. For each treatment, the coefficient was calculated (Table 6.8).

Table 5.4 *shows the results of the linear regressions performed with SPSS on day 10 and day 20 of the experiment.*

	Control	**High CO2**	**High CO2 + hypoxia D10**	**Control**	**High CO2**	**High CO2 + hypoxia D10**	**High CO2 + hypoxia D20**
Constant	,036***	,069***	,048***	,123***	,117***	,134***	,130***
Coefficient of time (h)	,008***	,014***	,008***	,025***	,029***	,023***	,025***

The results showed that the value of mg/L oxygen consumed by the eggs at time zero (the constant) is different according to the groups. The lowest value (0.036) is for the Control Group while the highest (0.069) is for the High CO_2 Group D10. The coefficients of time, indicating instead how much the Y increases for each additional hour, showed that the highest hourly increase (0.014) is for the high CO_2 group D10 while the lowest hourly increase (0.008) is for the Control and high CO_2 + hypoxia D10 groups.



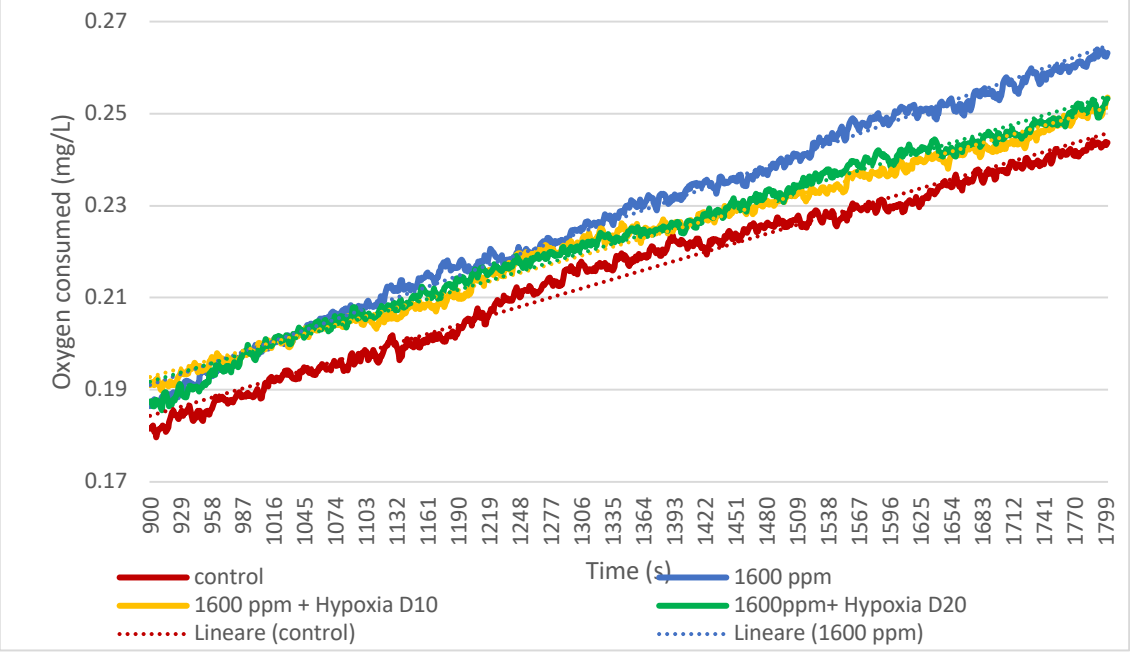


Figure 5.15a and 5.15b *show the consumption of oxygen (mg/L) over time (15 minutes – from second 900 until 1800) per treatment at day 10 and day 20, respectively.*

The results recorded at day 20 showed that the value of mg/L oxygen consumed by the eggs at time zero (the constant) is quite similar according to the groups. The coefficients of time indicate the rate of mg/L oxygen consumed by the eggs per hour: all the eggs inserted in the respirometry chamber showed a growing trend.

The lowest value (0.117) is for the high CO_2 group while the highest (0.134) is for the high CO_2 + hypoxiaD10 group. The coefficients of Time, in this case, highlighted that

the highest hourly increase (0.029) is for the high CO_2 group while the lowest hourly increase (0.023) is obtained for the high CO_2 + hypoxia D10 group.

5.2.5. Aquaporins trial

Every 10 days 3 eggs from every treatment were taken and put in a petri dish with distillate water for 15 minutes. The event was recorded with a camera set up on a tripod (Figure 5.17), and photos were taken every 15 seconds in order to record the possible changes in the volume of the eggs.

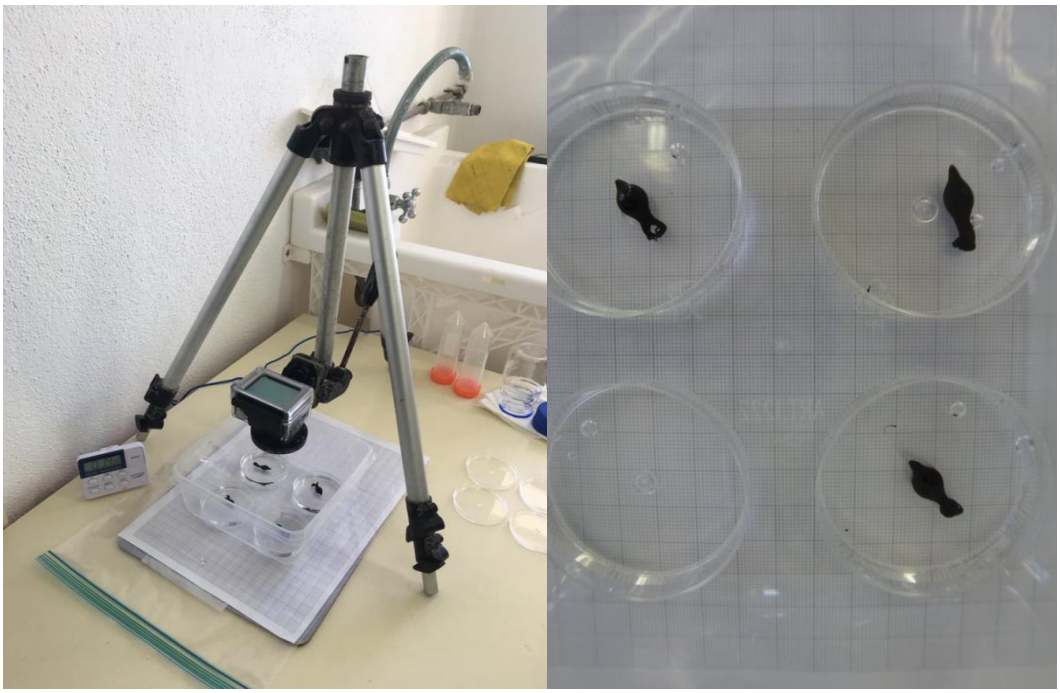


Figure 5.16 *shows the setting used to obtain the photo for the aquaporins trial. The camera (GoPro) was installed on the top of a tripod, which was rotated to capture photos of the egg during the 15 minutes of sampling. The eggs were inserted in Petri dishes, one per plate, and submerged with distilled water.*

Both the programs were used on the same images and in some cases the photos were also analysed manually due to the reflection of the water, which was giving false-positive results. No significant changes were observed on days 10 and 20. Most of the eggs, after being exposed to distilled water for 15 minutes, hatched and the animals had a weight in line with the ones observed in the tanks. No malformations were observed after the test.

6. Discussion

6.1. Hypoxia experiment

MWW of the eggs in the hypoxia experiment showed a trend similar to the ones observed in the studies of Sykes *et al*. (2009) and Zatylny-Gaudin & Henry (2018). It can be assumed that the decrease in weight around day 10 was caused by the expulsion of the water from the inside and consequent hardening of the egg, after which this was inverted until the end of the embryonic development (Sykes *et al.*, 2009).

From day 15 an increase in weight over time was observed, together with an increment of the percentage of eggs with a weight over 0.500 g. This phase, as described by Zatylny-Gaudin & Henry (2018), was characterized by a massive increase of water accumulation between the vitelline and chorion chamber to maintain the osmolarity until hatching (Paulij *et al.* 1991; Boletzky, 2003).

Statistical differences in MWW were found on day 5, before the hypoxia events. This can be reconnected to the intrinsic variability of the weight of the eggs. As it was described in Boletzky (1983) the dimension of the eggs, and consequentially the weight, is affected by the size/dimension of the female who laid them, as large females lay larger eggs.

At a stable temperature of 20°C eggs started to hatch at day 25 of the experiment in all the treatments, the highest number of hatchlings was observed between days 35 and 40 of the experiment. These development times were shorter in comparison to Sykes *et al.* (2006), where it was demonstrated that cuttlefish embryos need from 25 days at 25 °C days to complete their development.

This reduction in development time was already observed in previous studies where temperature significantly decreased during the embryonic period, from 48–49 days at 18.8ºC to 32–34 days at 22.8ºC (Rosa *et al.,* 2013). In the present experiment, the early hatching can be reconnected to maintaining the same temperature overnight, without simulating the temperature variation normally present in nature. This could have increased the development rate and caused the early hatching of the eggs.

A similar temperature-related pattern can also be observed in many heterotherms animals, as Atlantic salmon (*Salmo salar*), in which the approach to predict growth and development is based on degree-days (DD) (Amoroso *et al.*, 2016).

The same method was used in the study of Uriarte *et al.* (2012), where the effect of temperature on embryonic development under controlled conditions was studied in *Octopus mimus,* together with the influence of temperature on the time taken to reach stage XX using physiological time (DD) (Campbell *et al.*, 1974).

In the study of Uriarte *et al.* (2012), the time taken to reach stage XV (third part of the organogenesis) at a stable temperature of 21 °C was 24, 58 and 75% shorter than observed in eggs maintained at 18, 15 and 12 °C, respectively. It was demonstrated the presence of an inverse relationship between environmental temperature and the duration of embryonic development. Embryos kept at 18 and 21 °C grew faster and required less degree-days than those at 12 and 15 °C.

In the future the use of degree-days could be considered appropriate for predicting the physiological time of development and hatching phase of cuttlefish. Despite the potential, no optimum DD has been identified yet for European cuttlefish, nor a possible temperature threshold, as the available information in literature appears scarce.

Average hatching rate was around 71.7%, similar results were obtained in Capaz *et al.* (2020), where the average rate was 74.4% and in the study of Sykes *et al.* (2012) (average: 62%). Exposure to acute hypoxia events did not result in a relevant difference in terms of average animal weight (0.092 g), which resulted in line with the values recorded by Sykes *et al.* (2012) (0.099 g) and slightly heavier than in the study of Capaz *et al.* (2020) (0.080 g). Despite this, malformations were observed especially on the mantle, even if in a minor percentage of the new-borns (below 5%). Similar anomalies were observed in the embryos and hatchlings of the squid *Loligo vulgaris* (Rosa *et al.* 2012), where a temperature of 19 °C significantly affected the embryonic development, causing underdeveloped mantle, complete body deformities and eye dimorphism.

The cuttlefish mantle is composed of chromatophores, organs that are present in the skin of many cephalopods and are used for camouflage or signaling (Messenger, 2001). If the functionality of these organs is compromised, animals might not be able to blend in the environment nor communicate and their survival might be at risk.

Progressively decreasing oxygen levels have been hypothesized to eventually trigger hatching once critical values are reached inside the egg in both vertebrates and invertebrates (cephalopods: DeWachter *et al.,* 1988; Cronin & Seymour 2000).

Nevertheless, cuttlefish embryos did not show relevant differences after exposure to the acute event of hypoxia.

Respirometry analysis performed at 20 °C showed that the embryo inserted in the respirometry chamber showed a growing trend in oxygen consumption, with the highest trend in the most severe hypoxia event (25%DO_2) at day 20. Despite the stressor factor, eggs seemed to recover, and no statistical differences were observed in the hatching rate of the animals.

Although the theories see the embryonic phase to be more sensitive to oxygen stress than older life stages (Levin *et al.,* 2009), the results of the present trial suggest that cuttlefish embryos kept at a stable temperature of 20°C can manage an acute hypoxia event without substantial consequences to their development.

Similar results have been obtained in studies with juveniles and adults *S. officinalis* (Storey & Storey 1979), *Lolliguncula brevis* (Zielinski *et al.,* 2000) and *Dosidicus gigas* (Rosa & Seibel, 2008), where animals were exposed to short periods of strong hypoxia (Grieshaber *et al.,* 1994). Despite the observation that oxygen fluctuations seem to be a common trigger for metabolic depression in several species of different animal groups including bivalves and gastropods (Michaelidis *et al*., 2005; Pörtner *et al.*, 2005), results lead to think that cephalopods could withstand it.

Studies in cuttlefish (Gutowska *et al.*, 2010a; 2010b) demonstrated that *S. officinalis* has the potential to cope also with long-term exposure to the applied levels of hypoxia, together with other stressor factors as hypercapnia and elevated temperatures, but negative effects of the population level might be foreseen (Pörtner *et al.*, 2005; Pörtner, 2010; Melzner *et al.*, 2012). To have a heightened understanding of the phenomena and its potential effects on this marine species, further studies should be done, also addressing the potential effects of long-term exposure to a different level of hypoxia during embryonic development.

6.2. CO_2 Experiment

The variation of MWW showed a similar tendency, starting from day 10, already described in Sykes *et al*. (2009) and Zatylny-Gaudin & Henry (2018). It can be assumed that the decrease in weight was caused by the expulsion of the water from the inside and consequent hardening of the egg, after which this was inverted until the end of the embryonic development (Sykes *et al.,* 2009).

Despite this, a different trend can be observed between the two experiments: in the hypoxia experiment, the heaviest weight was recorded during the 40th day of the experiment, while in the CO_2 experiment it occurred during the first day of the trial (0.744 ± 0.058 g). During the expected highest peak of weight, corresponding to the moment when the egg mass increased due to the incorporation of water between the vitelline and chorion chamber, a lower weight (around 17%) was recorded in the CO_2 trial in comparison with the one with hypoxia.

A similar shortfall could be observed in the respirometry analysis trend where, even if eggs of the CO_2 experiment kept a growing trend in oxygen consumption, the slope and values recorded were considerably inferior to the ones from the hypoxia experiment. This might be connected to the hypothesis that one or more eggs were not alive while in the respirometry chamber, especially the first recording on day 10.

Despite the results obtained, eggs seemed to recover, and no statistical differences were observed in hatching rate between treatments and in average animal's weight (0.083 g), which results in line with the values obtained in Capaz *et al.* (2020) (0.080 g).

The first hatchlings were observed on day 20 of the experiment in tanks with high CO_2 and high CO_2 plus hypoxia at day 10 conditions at an average temperature of 22.4 ºC. In line with the results obtained from previous studies (Uriarte *et al.*, 2012), the development time significantly decreased due to the higher temperature.

As in the study performed by Rosa *et al.* (2013), pH did not elicit any significant change. Similar results were achieved in Lacoue-Labarthe *et al.* (2009) where, even if pH showed a strong effect on the egg weight, no significant impact was observed on the weight of hatchlings at the end of development.

Comparing the two experiments performed it can be noted that the hatching rate was visibly inferior in all the treatments of the CO_2 experiment (average hatching rate: 31.7% in the second experiment *vs* 71.7% in the first experiment), including the control. Because the eggs were obtained from the same breeders and water was provided from the same system, it can be hypothesized that the significant difference in hatchlings might be reconnected to the temperature. Tanks of the CO_2 trial were kept outdoor space of Ramalhete station where the water temperatures frequently reached 27°C during the hottest part of the day.

Based on the data collected, together with the absence of significant differences in hatching rate and weight of the hatchlings, it can be hypothesized that temperature might be one of the main key factors which can unfavourable the conditions inside the egg, more than environmental pH.

This hypothesis is also corroborated by previous studies which suggest that near-future CO_2 might not elicit major impacts on embryonic development (Dorey *et al.,* 2013), survival rates (Dorey *et al*., 2013), or body size (Lacoue-Labarthe *et al*., 2009; Hu *et al.,* 2011; Dorey *et al.,* 2013; Rosa *et al.,* 2013; Sigwart *et al.,* 2016) of *S. officinalis*.

Dorey *et al.* (2013), also investigated the effects of seawater pH and temperature on the PVF, showing that eggs swelling increased, together with oxygen consumption, in response to acidification or warming, but that this effect seemed to weaken once temperature increases, suggesting that eggs plasticity could be limited. Also, the difference of pCO_2 between the exterior and the interior of the egg diminished under warmer treatments; PVF pH was significantly lower at 16 °C than at 19 °C.

Despite it is not clear which is the threshold temperature for cuttlefish during the first phases of the life cycle, multiple trials have been performed to single it out it in the adult specimen. Richard (1971) suggested 30°C as the possible upper survival threshold for adult animals of *S. officinalis*, while Pascual (1978) reported only lower growth at 30°C compared to 22°C. Nevertheless, the effect of high temperature on embryonic development has been studied in other cephalopod species. In the squid *Loligo vulgaris reynaudii* (Oosthuizen *et al.,* 2002), an optimum range between 12 °C and ∼17 °C was determined for embryonic development. Outside the optimal temperature range abnormal ontogenesis occurred. In Rosa *et al.* (2013), warming *per se* caused a notable metabolic depression in the pre-hatching stage. These studies showed that early development stages were more sensitive to variable temperature regimes than later development stages.

Although this species has shown to be quite resilient to future climate changes, these studies showed that temperature might play a major role in determining the life span of *S. officinalis* (Richard, 1971; Pascual, 1978; Forsythe *et al*., 1994; Domingues *et al*., 2001a; 2001b; 2002; 2004). Further studies should be done to individuate a possible upper-temperature threshold in order to predict how this species will fare in the future.

7. Conclusion

S. officinalis is an animal that had raised a commercial interest in the past few years due to its fast growth rate and the possibility to raise it in captivity at high densities (Boletzky, 1983; Wells, 1994). Potentially cuttlefish can be considered a new aquaculture source of food (Sykes *et al.,* 2006). Concomitantly, in the past few decades, growing concerns were raised on the increase of hypoxia events in the coastal water, together with rising of the water temperature, which is expected to increase between 1.5°C and 5.8°C by 2100 based on the different projections (Meehl *et al.,* 2007), and the increase of carbon dioxide released in the marine environment. It is hypothesized that phenomena might decrease the pH of the surface water between 0.14 and 0.5 units, depending on the estimated scenario (Meehl *et al.,* 2007).

These events might harm the life cycle of many organisms and cause major losses in biodiversity. Between the species investigated *S. officinalis* seems to withstand acute and long-term exposure to a combination of factors related to climate change. The present experiments provided evidence that the early phase of life of *S. officinalis* can cope with an acute event of hypoxia and hypercapnia but appeared to be more sensitive than adults and juveniles to higher water temperature, as already described by Schipp *et al.* (1979).

This might trigger the survival of the species, especially in the warmest months of the year, when the temperature raises in the coastal water, where female cuttlefish usually lay eggs (Boletzky, 1983), are higher.

Despite the results obtained, further studies should be done to understand how climate change might affect this phase of the cuttlefish life cycle.

A more realistic incubation scenario could be set by replicating the presented experiments with eggs incubated in parallel under multiple increased temperatures and under conditions of hypoxia, hypercapnia and hypoxia plus hypercapnia.

This trial would help to understand better the relation between the environment and the interior of the egg and what kind changes can be elicited in terms of pCO_2 and PVF pH, define the thermal window for cuttlefish embryonic development and to comprehend further the influence of each stress factor considered, thus giving a clearer picture of the possible future changes in the species survival and geographical range.

For future trials, long-term exposure should be tested, e.g., reproductive and multi-generation studies, as it is expected that most of the consequences of climate change will be either long-lasting or persistent and realistic critical limits to these changes can be observed only if an organism has enough time to either reach a new steady state or perish (Pörtner *et al.*, 2005).

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