UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES

Ocean and Earth Science

The effects of daily cyclic hypoxia on the ecophysiology of the Atlantic ditch shrimp, *Palaemon varians*

By

Luca Peruzza

Thesis for the degree of Doctor of Philosophy

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ABSTRACT

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THE EFFECTS OF DAILY CYCLIC HYPOXIA ON THE ECOPHYSIOLOGY OF THE ATLANTIC DITCH SHRIMP, *PALAEMON VARIANS*

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Oxygen partial pressure (pO_2) is not always constant in aquatic environments and can vary on different time scales, from hours to weeks. In many coastal environments, such as estuaries, lagoons or marshes, pO_2 levels can vary on a daily base, resulting in daily cyclic hypoxia. By monitoring temperature and pO_2 in the Lymington salt marshes (UK), I was able to quantify diel and seasonal pO_2 variability in this coastal habitat: diel oscillations in pO_2 were measured in winter, spring and summer, and the greatest pO_2 oscillations were recorded in summer, when pO_2 could fluctuate from ~42 kPa to ~ 1 kPa every 12 hours, causing diel cyclic hypoxia. Even if cyclic hypoxia is common in numerous coastal areas around the world and affects many species, this phenomenon is less studied in comparison to acute or chronic hypoxia. The aim of this thesis was to characterize the short-term effects and the long-term consequences of daily cyclic hypoxia on the physiology of an important decapod crustacean, *Palaemon varians*. This species was found in the Lymington salt marshes (UK) and, in the laboratory, was subjected to a cyclic hypoxic regime that mimicked conditions measured in the field during summer.

In the laboratory, a short 8-hour exposure to hypoxia (pO_2 < critical oxygen pressure, p_{crit}) induced behavioural and metabolic changes and suppressed feeding and ammoniacal excretion. Long-term exposure to diel cyclic hypoxia induced changes in the transcriptome of the animals, prompted an acceleration of the moult cycle (validated at transcriptional and phenotypic level) and eventually resulted in morphological changes to the gills, which increased lamellar surface area. Further, long-term exposure to cyclic hypoxia impaired animal's growth (in terms of body weight and length), reduced ammoniacal excretion and negatively influenced reproduction by reducing egg yolk content. Interestingly, long-term acclimation to cyclic hypoxia increased thermal tolerance and copper tolerance in comparison to control animals, probably as a consequence of the morphological changes to the gills induced by cyclic hypoxia.

Overall, results underline that a short hypoxic exposure repeated daily was able to induce in *P. varians* alterations at multiple levels of biological organisation. In particular, the observed long-term consequences (i.e. growth reduction, reduced ammoniacal excretion and impaired reproduction) might have important ecological implications for the species and for its ecosystem.

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Academic Thesis: Declaration Of Authorship

I, Luca Peruzza declare that this thesis and the work presented in it are my own and has been generated by me as the result of my own original research.

"The effects of daily cyclic hypoxia on the ecophysiology of the Atlantic ditch shrimp, *Palaemon varians*"

I confirm that:

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Peruzza L, Gerdol M, Oliphant A, Wilcockson D, Pallavicini A, Hawkins L, Thatje S, Hauton C. (in review) The consequences of daily cyclic hypoxia on the European grass shrimp *Palaemon varians*: from short-term responses to long-term effects. Functional Ecology

Signed:

Date:

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Chapter 1 Introduction

1.1 Physics of gas – fundamental principles:

The air that constitutes the atmosphere of our planet is composed by a mixture of gases with nitrogen, oxygen, argon and carbon dioxide being the most abundant. Each gas possesses a partial pressure, defined as the pressure that a gas in a mixture of gases would exert if it occupied alone the same volume as the mixture at the same temperature (Henrickson, 2005). For example, if in a closed vessel filled with air all gases are removed apart from one (e.g. oxygen), at the end of the process the pressure inside the vessel coincides with the partial pressure of that gas (e.g. oxygen). In a mixture of gases, by summing the partial pressure of each gas we obtain the total pressure of the mixture, as stated by Dalton's law of partial pressures (Henrickson, 2005). At sea level, where the atmospheric pressure is ~101 kPa, the partial pressure of oxygen (pO_2) is 20.9 kPa.

The partial pressure of a gas is a fundamental property in chemistry and biology as it governs how gases dissolve and diffuse into solutions. In fact, as stated in Henry's law, the amount of dissolved gas (e.g. oxygen) in a solution in terms of its concentration (C_{O2}) is proportional to its partial pressure (pO_2) in the gas phase above the solution. Hence, by increasing the pressure above the liquid, more molecules of oxygen will enter in the solution until pO_2 of the gas in the liquid equals pO_2 of the gas above it. For example, water pO_2 is ~21 kPa at sea level when the water is in equilibrium with the atmosphere. The concentration of a gas in a solution depends not only by the partial pressure of the gas but also: *i*) on the liquid (e.g. fresh water has higher solubility compared to salt water, (Henrickson, 2005)) and *ii*) on the temperature of the solution (an increase in temperature decreases the solubility of the gas). It is important to note that, while temperature does not influence pO_2, it accelerates the metabolism of ecthoterm animals, which in turn metabolize oxygen more quickly (Clarke & Fraser, 2004). From a physiochemical point of view C_{O2} and pO_2 describe different concepts and should not be used synonymously; in order to explain this difference, a simple experiment (i.e. mixing oil and water in a flask, from Massabuau and Abele (2012)) is reported in Figure 1.1. After bubbling air in a mixture of oil and water oxygen will be equally distributed in the system. When bubbling

stops, oil and water will separate. While an equal pO_2 can be measured in water and in oil, the difference in oxygen solubility between the liquids results in a very different C_{O2}.

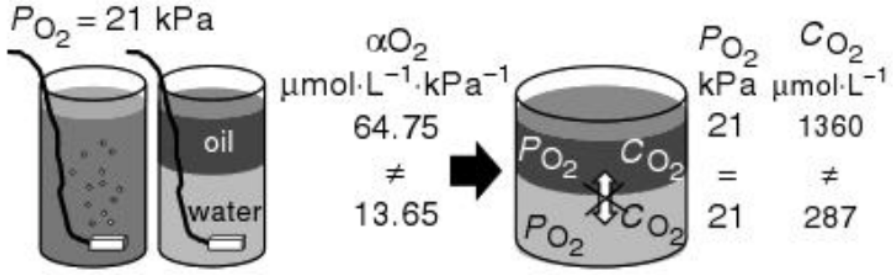

Figure 1.1: Distribution of oxygen between compartments having different O_2 solubility (αO_2). The distribution of O_2 between compartments is driven by the O_2 partial pressure (pO_2) gradient and not by the O_2 concentration (C_{O2}) gradient, in accordance with Henry's law. Taken from Massabuau and Abele, 2012.

As previously stated, partial pressure additionally governs the diffusivity of gases (i.e. their movements) across compartments (e.g. from water to blood), according to Fick's law:

$M_{O2} = A^* 1/E * k_{O2}^* \Delta pO_2$

Where M_{O2} is the oxygen flowing from one compartment to the other; A and E are respectively the surface area and the thickness of the membrane separating the compartments (e.g. the gill epithelium); kO_2 is the Krogh's constant of diffusion and ΔpO_2 is the difference of pO_2 between the two compartments (e.g. water and blood). Fick's law is extremely important as it governs gas exchanges in all living organisms and it is worth noting that the oxygen concentration does not appear in this law. As brilliantly expressed by Massabuau and Abele (2012), "the oxygen concentration *per se* is basically never a key limiting factor for respiration in an animal that lives in a large water body". This is extremely important as many authors used to refer to oxygen concentration when defining an hypoxic ecosystem instead of using oxygen partial pressure, generating a lot of confusion around the definition of hypoxia (as discussed later in this introduction).

1.2 Hypoxia in aquatic environments:

In the aquatic environment pO_2 is not always constant in time (Garcia et al., 2005; Helm, Bindoff, & Church, 2011) and not all aquatic ecosystems have the same pO_2. In Oxygen Minimum Zones (OMZs), due to respiration of organisms and slow water turnover (Naqvi et al., 2010), water pO_2 is consistently "low" (with pO_2 values down to ~0.5 kPa (Kiko et al., 2015)). In other regions, typically rivers, estuarine areas and shallow-water habitats, pO_2 can fluctuate on a seasonal basis. Examples of seasonal fluctuations can be found in the Chesapeake Bay in North America or in the Yangtze River in China, (Levin et al., 2009). This seasonal fluctuation is typically the result of upwelling or eutrophication events: after an initial algal bloom caused by these events, phytoplankton subsequently dies and it is decomposed by detritivorous species, which eventually consume the oxygen in the water (Levin et al., 2009; Hofmann et al., 2011). Additionally, pO_2 can fluctuate on a daily basis, increasing during the day due to an excess of O_2 photosynthetic production over O_2 respiration, and decreasing during the night as a result of continued O_2 respiration but no O_2 photosynthetic production (Guasch et al., 1998; Li & Brouwer, 2013a). Hence pO_2 fluctuations can be part of an organism's normal life experience (Breitburg, 2002): in fact, many taxa are able to tolerate, to some extent, pO_2 oscillations of moderate intensity e.g. (Riedel et al., 2014; Spicer, 2014), whereas other species are able to tolerate extreme oscillations. For example: the killifish *Fundulus grandis* can experience pO_2 levels varying from >21 kPa to ~0.5 kPa every 12 hours during the summer (Cheek et al., 2009). While pO_2 is an important factor in shaping the physiology of organisms, other factors (e.g. temperature) play an equally important role.

In the scientific community there is growing evidence that the temperature of the planet is increasing as a result of a directional climatic change, and that this change is occurring at an unprecedented rate in comparison to previous climate oscillations (Pachauri et al., 2014). The Intergovernmental Panel on Climate Change report (Pachauri et al., 2014) highlighted that, across the globe, the upper 75m of the water column has warmed by 0.11 ˚C per decade from 1971 to 2000. Models predict that warming of the oceans will continue throughout the 21st century (Fig. 1.2) and will be accompanied with a global mean sea level rise of some meters that might submerge many coastal habitats (e.g. marshes) and cities (Pachauri et al., 2014). In example, the reduction of salt marshes extent in Georgia (USA) is predicted between 20 and 45 % (Craft et al., 2009).

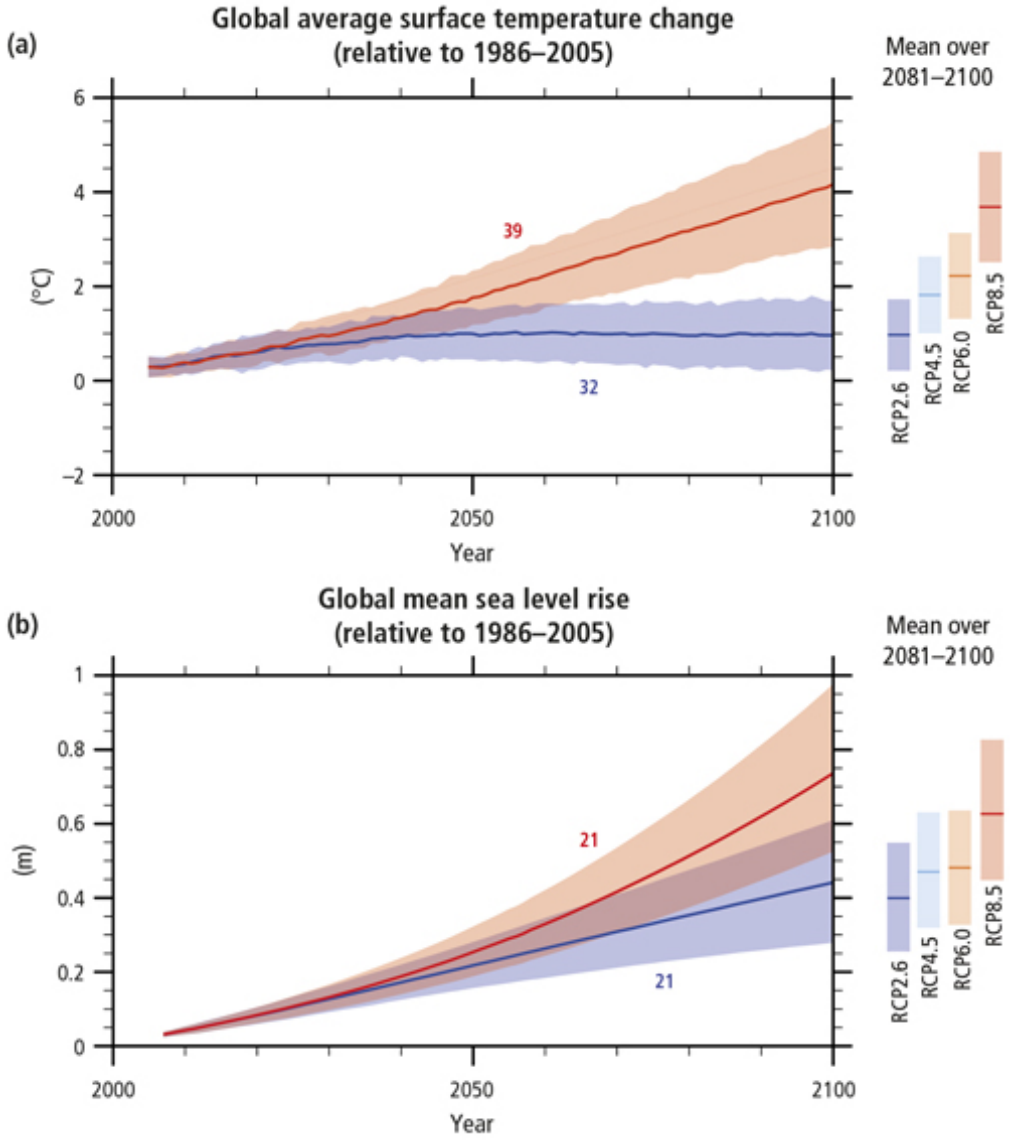

Figure 1.2: The IPCC multi-model simulated time series from 1950 to 2100 showing **A)** the projected change in global annual mean surface temperature and **B)** the projected mean sea level rise relative to 1986-2005 from four projected scenarios: Representative Concentration Pathways (RCP, patterns of greenhouse concentration trajectories projected by the year 2100) 2.6, 4.5, 6.0 and 8.5. The coloured numbers represent the number of models used to derive the multi-model mean.

As mentioned before, temperature inversely affects solubility of gases. Consequently, climate change is causing a decrease of oxygen solubility (Conley et al., 2009; Stramma et al., 2010), an increase in stratification of the water column and an increase of respiration rates at global scale (Wright, Konwar, & Hallam, 2012; Melzner et al., 2013). Since the 1980s it has been well-documented how vast coastal areas of the sea temporarily become hypoxic, resulting in mass mortality of organisms (Rosenberg, 1980; Stachowitsch, 1984; Boesch & Rabalais, 1991; Diaz & Rosenberg, 2008), with affected sites as large as 70 000 km^2 in marginal seas (Hilborn et al., 2003). Therefore, as the world warms, it is predicted

that hypoxic events may be exacerbated (Watson, Zinyowera, & Moss, 1996; Short & Neckles, 1999; Christensen et al., 2007; Whitney, Freeland, & Robert, 2007; Altieri & Gedan, 2015), becoming more frequent, more severe and persisting longer (Watson et al., 1996; Short & Neckles, 1999; Christensen et al., 2007).

1.3 What are the current limitations in defining hypoxia at ecosystem level?

Probably the most common definition of hypoxia states that this condition occurs when oxygen concentration in the water falls below 2 mg L^{-1} (Diaz & Rosenberg, 1995; Wu, 2002; Brouwer et al., 2004; Vaquer-Sunyer & Duarte, 2008), at which point benthic fauna show aberrant behaviour (Diaz & Rosenberg, 2008). In spite of its usage, this definition has some limitations from a physiological and an ecological point of view, from the units of measurement used, to the application of one threshold value for many different species and habitats.

Traditionally, researchers have set thresholds to distinguish whether an ecosystem was hypoxic or not (e.g. ~4.8 kPa (~2 mg O_2 L^{-1}) for shallow-water habitats or ~1.2 kPa (~0.5 ml L^{-1}) (Helly & Levin, 2004; Vaquer-Sunyer & Duarte, 2008; Hofmann et al., 2011) in permanent OMZs). The choice of these thresholds is determined by an observed "abnormal condition" at any level of the ecosystem (i.e. an aberrant behaviour in benthic fauna (Diaz & Rosenberg, 2008; Hofmann et al., 2011) or a negative impact on biogeochemical cycles in marine ecosystems (Conley et al., 2009; Pena et al., 2010) - for a complete list see Hofmann et al. 2011). The problems of this method are: *i*) the choice of the "abnormal condition" appears to be subjective; *ii*) often the ecological conclusions seem to be the result of an inductive process, which means that a general conclusion (i.e. the threshold of hypoxia in the ecosystem) is derived from particular instances (i.e. studies of hypoxia tolerance on one/few species (Diaz & Rosenberg, 2008; Gooday et al., 2009)); *iii*) the potential arbitrary nature of the chosen threshold makes comparisons among studies very difficult because results from distinct studies may be derived from different assumptions of hypoxia.

Very frequently in the literature (Wu, 2002; Brouwer et al., 2004; Diaz & Rosenberg, 2008) authors have seemingly taken for granted the common thresholds of

hypoxia without testing their appropriateness to the studied species. The consequence of this approach is that one threshold value for hypoxia tends to be applied to many species and different ecosystems and sometimes to the whole water column (Hofmann et al., 2011). Recently, Vaquer-Sunyer and Duarte (2008) examined 872 published experiments, reporting oxygen thresholds for 206 different species, and found that the thresholds for hypoxia (in terms of lethal concentration and lethal time) differed broadly between (and also within) taxa, varying from 8.6 mg O_2 L^{-1} for the first larval stage of the crustacean *Cancer irroratus*, to persistent resistance to complete anoxia of the oyster *Crassostrea virginica* (Vaquer-Sunyer & Duarte, 2008). In particular, they reported that ~45% of the species had a lethal concentration higher than the common threshold value of 2 mg O_2 L^{-1}, broadly used in the literature as a threshold for hypoxia. These results clearly point out why the appropriateness of a threshold of hypoxia should be tested and not taken for granted and why a single value cannot be applied globally as a threshold to hypoxia.

Among the aquatic habitats, the importance of estuaries, marshes and lagoons is widely recognized for conservational and economic importance (Diaz & Solow, 1999; Aguzzi et al., 2005; Cattrijsse & Hampel, 2006; Altieri & Gedan, 2015). In fact, these habitats are typically rich in biodiversity and are important nursery areas for many exploited crustaceans and fish species (Cattrijsse & Hampel, 2006). Current management efforts to limit hypoxic episodes and conserve these ecosystems are mainly based on nutrient reduction plans to restrict oxygen fluctuations and prevent oxygen depletion (Steckbauer et al., 2011). For example, since 1990 Denmark has reduced nitrogen and phosphorous discharges by 50 and 80% respectively (Carstensen et al., 2006). To ensure that these management efforts are appropriately targeted, and subsequently assessed as being successful in limiting oxygen fluctuations, a robust scheme with which to establish when species experience hypoxia needs to be established. In the light of the above considerations, it is evident how the most used definition of hypoxia possesses some limitations and why a different definition of hypoxia should be preferred.

1.4 How is hypoxia identified at individual level?

The concept of hypoxia is primarily associated with respiratory physiology because it describes a condition linked with reduced gas exchanges due to low oxygen availability in the environment (Grieshaber et al., 1994) and, as explained before, pO_2 is the physical parameter that represents the actual driving force in gas exchanges in organisms in accordance with Fick's law (Massabuau & Abele, 2012). In fact, as explained by Childress (Childress & Seibel, 1998), hypoxic conditions occur when the animals are not able to maintain a gradient (in terms of ΔpO_2) to drive diffusion of oxygen from the exterior environment (e.g. $pO_{2\ water}$) eventually to their mitochondria (e.g. $pO_{2\ blood/haemolymph/body\ fluids/cytoplasm}$).

Traditionally, and in respiratory physiology, animals have been described as 'oxygen regulators' when they maintain oxygen consumption rates (MO_2) constant in response to decreases in pO_2 (e.g. some anchialine shrimp species (Havird et al., 2014), Fig. 1.3, left panel), and 'oxygen conformers' when they reduce MO_2 in a linear manner with decreasing pO_2 (i.e. cnidarians and some annelids, Figure 1.3 left panel) (Herreid, 1980; Richards, 2011). However, complete conformity or regulation is rarely found, and an intermediate response is typical (Figure 1.3, right panel) (Mueller & Seymour, 2011). The pO_2 value at which an oxy-regulator is no longer able to maintain constant MO_2 and shifts to oxy-conformity has been termed the "critical oxygen pressure" (p_{crit}) (Tang, 1933; Mueller & Seymour, 2011).

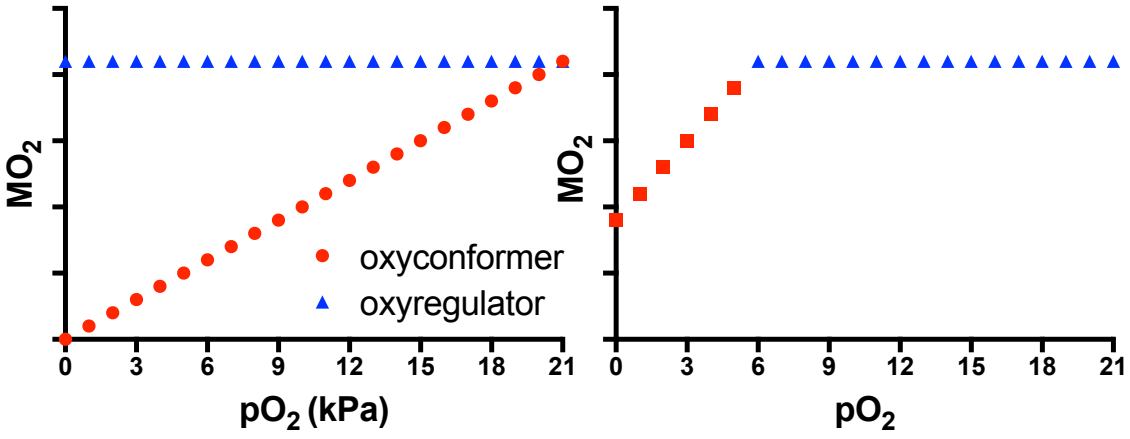

Figure 1.3: Schematic diagram showing the different physiological responses to a decline in pO_2. On the x-axis pO_2 is expressed in kPa, whereas the y-axis has arbitrary units. Left graph shows the response of a complete oxyregulator (e.g. some anchialine shrimp species) in blue triangles and the response of a complete

oxyconformer (e.g. cnidarians) in red circles. Most commonly an intermediate response is observed in aquatic ectotherms, as shown in the right panel. In this case animals behave like oxyregulators until a pO_2 threshold is reached. Below this pO_2 threshold they can be classified as oxyconformers.

The critical oxygen partial pressure represents an important physiological parameter because: *i*) important physiological changes take place when the body is exposed to this condition (see below); *ii*) all the physiological adaptations below this threshold are temporary solutions and survival of the animal in this condition is time dependent (Richards, 2011). For these reasons hypoxia was defined, in this thesis, as a stressful condition occurring when, in water, pO_2 reached the p_{crit} value of the species. Figure 1.4 explains some of these changes using as example an organism living in a shallow water environment. As stated by Fick's law, any gas exchange is primarily dependent on the establishment and maintenance of a partial pressure gradient (ΔpO_2) between the water (where pO_2 can vary from 21 to 0 kPa) and the body fluids, such as blood or haemolymph and eventually the mitochondria (Childress & Seibel, 1998) ($\Delta pO_2 = pO_{2\ water} - pO_{2\ blood/haemolymph/body\ fluids/cytoplasm}$).

When $pO_{2\ water}$ = 21 kPa (Fig. 1.4a): in this condition the supply of O_2 (as the amount of O_2 present in the blood and available for the cells) is maximal and therefore sufficient energy is allocated to the different energy-demanding functions to ensure maximum fitness (referred to as the individual reproductive success, Fig. 1.4a box A, B) (Sokolova et al., 2012). In this "optimum" state (Portner, 2001; Sokolova et al., 2012) individuals are able to complete their normal life cycle.

When 21 kPa > $pO_{2\ water}$ > p_{crit} (Fig. 1.4b): Partial pressure gradient – ΔpO_2 – is lowered (in comparison with the previous condition) in a direct way according to the value of $pO_{2\ water}$ (Fig. 1.4b) and two phases can be identified:

i) initially a decline in O_2 supply is observed (McMahon, Burggren, & Wilkens, 1974). The rate and duration of decline differs (i.e. blue dotted-lines, Fig. 1.4b) depending on the species, on the ontogenetic stage (e.g. larval, juvenile or adults), on the physiological condition of the individual (e.g. reproductive or non reproductive), and on the level of $pO_{2\ water}$ (Leiva et al., 2015). For example: at 18 kPa the decline will be less severe than the decline observed at 10 kPa);

ii) by resorting to so-called "aerobic adjustments" (Herreid, 1980; Wood & Shelton, 1980) (e.g. increase in gill ventilation (McMahon, 2001)) O_2 supply can be restored to normal (or almost normal) levels, according to the $pO_{2\ water}$ level experienced

(McMahon et al., 1974). In this stressful condition (also termed as "pejus" state (Portner & Farrell, 2008)) energy allocation is modified (Portner, 2001) (Fig. 1.4 box C, D), but animals can complete, even if with reduced efficiency (Coiro, Poucher, & Miller, 2000), their biological cycle because energy allocated to physiological processes is still sufficient.

When $pO_{2\ water}$ < individuals' p_{crit} (Fig. 1.4c): Oxygen movements between water and blood is strongly reduced (according to Fick's law). Therefore O_2 supply falls rapidly, the body resorts to "aerobic conformity adjustments" (Herreid, 1980) (i.e. anaerobic metabolism and metabolic suppression (Richards, 2011)) and enters into a hypometabolic state where the most expensive ATP-demanding processes (like protein synthesis or the maintenance of ion gradients) are extensively reduced (Richards, 2011). The consequence of this condition is that energy allocation is insufficient to sustain the various physiological processes (Richards, 2011) and, with time, all the processes apart from maintenance are shut down (Fig. 1.4 box E, F). In this condition when anaerobic substrates (used for anaerobic metabolism) are exhausted, eventually death will occur (Richards, 2011).

In summary, it is evident how the p_{crit} itself separates two key physiological conditions: above it, where the lowering in pO_2 can be indefinitely balanced by using aerobic adaptations and below it, where aerobic adaptations are insufficient and profound changes of the whole body physiology (that cannot be maintained indefinitely (Seibel, 2011)) take place.

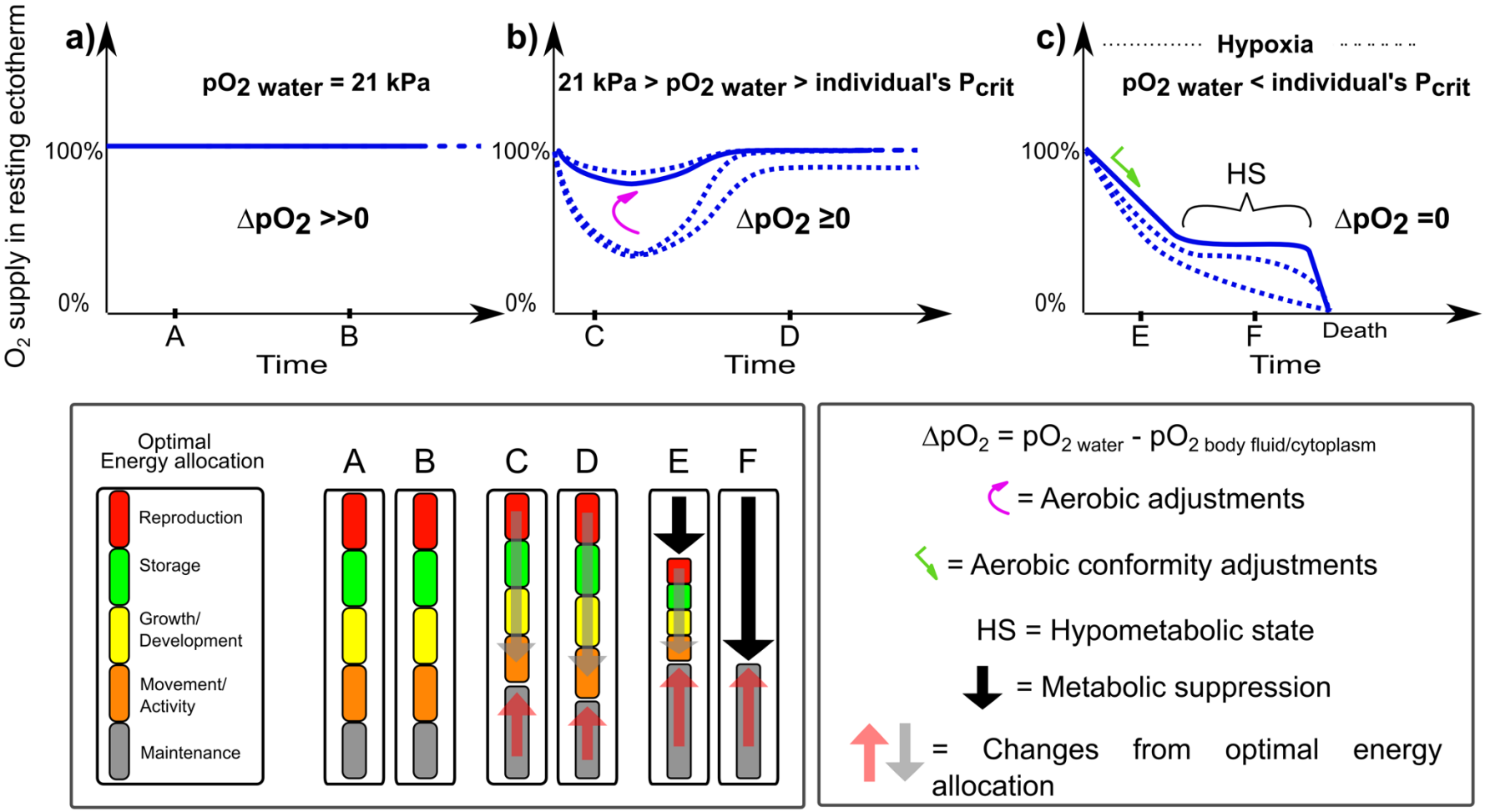

Figure 1.4 (legend overleaf)

Figure 1.4 (previous page): Schematic illustrating the importance of the critical oxygen level (p_{crit}) in defining hypoxia in a species. Physiological responses to three environmental scenarios at a given temperature, salinity, and hydrostatic pressure are considered: **a)** when oxygen partial pressure in the water, pO_2 water, is the highest value (21 kPa in the example); **b)** when 21 kPa>pO_2water >p_{crit}; and **c)** when pO_2 water < species' p_{crit}. Dotted blue lines indicate different declines in oxygen supply depending on the species, ontogenetic stage, physiological condition and water pO_2 level considered. Below the graph is reported the bioenergetic framework for assessing stress, as proposed by Sokolova et al. (2012) and adapted. The size of the boxes has been made equal for the sake of clarity and do not represent a quantitative measure of energy/resource allocation. Letters (A, B, C…) on the x-axis indicate different time points for which the bioenergetic framework is shown in the respective box. Changes in energy allocation are presented as qualitative changes.

1.5 Physiological adaptations to low pO_2:

Given the fact that pO_2 can substantially change, aquatic species have evolved a series of adaptations to adjust their respiratory and circulatory systems to the different pO_2 levels, even if the ability to tolerate low water pO_2 values varies greatly among (and also between) taxa (Vaquer-Sunyer & Duarte, 2008). The ability to acquire O_2 from its environment is the key factor (Hughes, 1973; Richards, 2011) and there is a general consensus that tolerant species (i.e. species frequently exposed to low pO_2 values) have lower p_{crit} values compared with hypoxia sensitive species (Herreid, 1980; Childress & Seibel, 1998; Chapman & McKenzie, 2009; Mueller & Seymour, 2011; Seibel, 2011). Across metazoans the adaptations to live in habitats with reduced pO_2 (compared to saturation, pO_2 ~ 21kPa) are reasonably similar, even if their extent and degree vary greatly according to environmental characteristics, animal lifestyle and behaviour.

Since the p_{crit} threshold indicates a point of key physiological change in the species, beyond which MO_2 is lowered, adaptations that are set in motion before water pO_2 reaches p_{crit} are collectively called aerobic regulations (Herreid, 1980), in contrast to mechanisms adopted when water pO_2 is lower than p_{crit}, which are known as aerobic conformity (Herreid, 1980). The first set of aerobic regulations used by the organisms include behavioral mechanisms: in fact, mobile animals can respond to a lowered pO_2

simply by changing environment (Newell & Courtney, 1965; Gamble, 1971), by manifesting aquatic surface respiration (Chapman & McKenzie, 2009) and/or aerial emergence (common in burrowing crayfish like *Procambarus clarkii*, (Taylor, Butler, & Sherlock, 1973; Richards, 2011)). Additional aerobic regulations aim at highly effective removal of O_2 from water with circulatory and respiratory adaptations. In fact, the rate of blood perfusion can be increased by acting on heart beat rate or cardiac stroke volume (Wood & Shelton, 1980; Burleson & Silva, 2011; Motyka et al., 2017) or, as in some decapod crustaceans, by partitioning cardiac output in order to maximize blood flux through the gills (McMahon, 2001; Guadagnoli & Reiber, 2005). Additionally ventilation, gill perfusion and gill area can be increased (Herreid, 1980; Childress & Seibel, 1998; McMahon, 2001; Seibel, 2011), as many studies on crayfish of the genus *Orconectes* spp. have shown (e.g. McMahon (2001)), oxygen transport can be improved through increasing O_2-binding affinity (with different types of pigments or effectors (McMahon, 2001; Gorr et al., 2004; Gorr et al., 2010)) and the concentration of oxygen-binding pigments can be increased, as documented in *Daphnia magna* (Gorr et al., 2004). An additional interesting adaptation, widely reported in fish but also in reptiles and amphibians like the toad, *Bufo marinus,* (Morris, 2004; Bicego, Barros, & Branco, 2007; Gorr et al., 2010), consists in decreasing body temperature through the hypoxia-induced behavioural hypothermia, in which the animal seeks colder water in order to slow the metabolism and consequently the oxygen consumption.

When environmental pO_2 falls below the species' p_{crit}, then the animal's physiology adjusts MO_2 in relation to pO_2 decrease and these adjustments are grouped as aerobic conformity (Herreid, 1980). In this condition, due to insufficient oxygen provision to the cells, basal metabolism is suppressed and aerobic ATP production is diminished. In order to compensate for the reduced ATP production, ATP needs of the body are lowered via metabolic suppression, which is realised mainly by suppressing the most expensive cellular processes, such as protein turnover and maintenance of transmembrane ion gradients (Richards, 2011) (e.g. the jumbo squid, *Dosidicus gigas*, is able to suppress by 40% its ATP demand (Seibel et al., 2014)). The decrease in ATP production can be partially (heterometabolic response) or totally (homeometabolic response) compensated by anaerobic metabolism (Herreid, 1980). In any case, anaerobic metabolism is a temporary solution because it has main limitations in its use, such as the requirement for abundant available energetic substrates, like glycogen, (to cope with the low ATP yield), the disposal of metabolic wastes (usually organic acids)

and the need to recycle reduced electron carriers (Richards, 2011; Seibel, 2011). Organisms resorting to anaerobic metabolism typically show, when normoxic conditions return, "oxygen debt," which is a physiological process used to deal with metabolic waste and regenerate ATP stores (Herreid, 1980). Therefore the severity and duration of hypoxia, the quantity of energetic substrates, the strategy to deal with metabolic wastes and the type of anaerobic response are the factors that determine the tolerance time of the animals under anaerobic conditions.

At a molecular level, cells can detect pO_2 and its variation mainly, though not exclusively, because of particular proteins called Hypoxia-Inducible Factors (HIFs, (Wu, 2002; Gorr et al., 2010)) that were reported for the first time in the 1990s (Wang & Semenza, 1993). The presence of HIFs is ubiquitous in the animal kingdom, from nematodes to fish and humans (Nikinmaa & Rees, 2005; Gorr, Gassmann, & Wappner, 2006; Brouwer et al., 2007; de la Vega et al., 2007; Hampton-Smith & Peet, 2009; Hardy et al., 2012; Liu, Shin, & Cheung, 2014). At cellular level, they act as oxygen sensors, capable of transducing from minutes to hours of a marked shortage in oxygen supply by binding target gene sequences called Hypoxia-Response Elements (HREs) like haemoglobin in the crustacean *Daphnia magna* (Gorr et al., 2004). The principal cytological/molecular responses induced by HIF in fish are illustrated in Figure 1.5 but new findings are continuously discovered. Recently, the regulatory function of HIFs in recruiting certain genes during moulting and infection in crustaceans has also been demonstrated (Terwilliger et al., 2008; Gorr et al., 2010), underlining their importance in the physiology of animals. Another important response at cellular level has been called "preparation for oxidative stress" and consists in an increase in the antioxidant defences (among them Selenium-dependent Glutathione Peroxidase, Se-GPX (Gorr et al., 2010)) after exposure to hypoxia (Brouwer et al., 2007; Massabuau & Abele, 2012), as has been reported in hypoxic tolerant species, but not in sensitive ones (Gorr et al., 2010). Increased antioxidant defences are an important mechanisms in order to protect cellular components and biomolecules from the oxidative effect of Reactive Oxygen Species (ROS), which tend to form when tissues are exposed to normoxic conditions after a period in hypoxia.

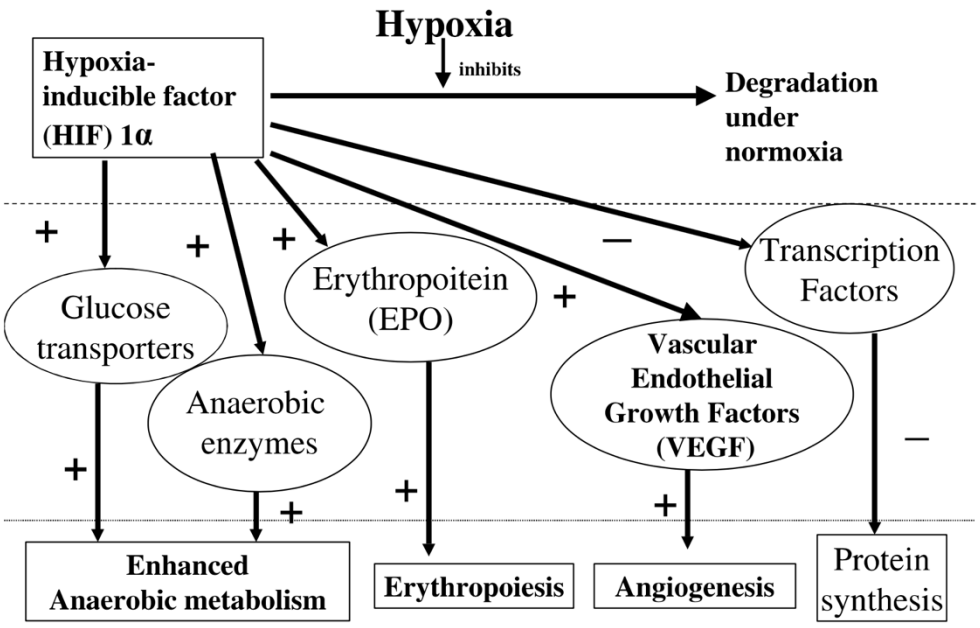

Figure 1.5: Principal molecular responses to hypoxia and related gene regulation. From Wu, 2002.

1.6 Chronic hypoxia:

In the past, a great part of the literature has focussed the attention on the effects of chronic hypoxia (meaning a constant exposure to low pO_2 values ranging from days to weeks (Greenberg & Ar, 1996; Pichavant et al., 2001; Brouwer et al., 2007; Landry et al., 2007), and has elucidated the majority of the mechanisms involved in the physiological response to chronic hypoxia (Section 1.5) (Herreid, 1980; Wu, 2002; Gorr et al., 2010). Chronic hypoxia is able to cause major changes in species composition, species diversity and species richness (Wu, 2002; Gunderson, Armstrong, & Stillman, 2015), with the permanent or periodic removal of sensitive species from habitats (Fig. 1.6, from (Wu, 1982)). The recovery time of communities following chronic hypoxia varies depending on the severity and duration of hypoxia, with examples from the continental shelf of New Jersey – USA (Falkowski, Hopkins, & Walsh, 1980), from the Kattegat – Sweden (Rosenberg, 1980), from Pomeranian Bay – southern Baltic Sea (Powilleit & Kube, 1999) and from the Adriatic Sea (Stachowitsch, 1991), all indicating that several years can be necessary for the recovery of benthic communities (e.g. only

36 % of benthic biomass was recorded three years after an hypoxia-induced benthic defaunation occurred in the Adriatic Sea, (Stachowitsch, 1991)).

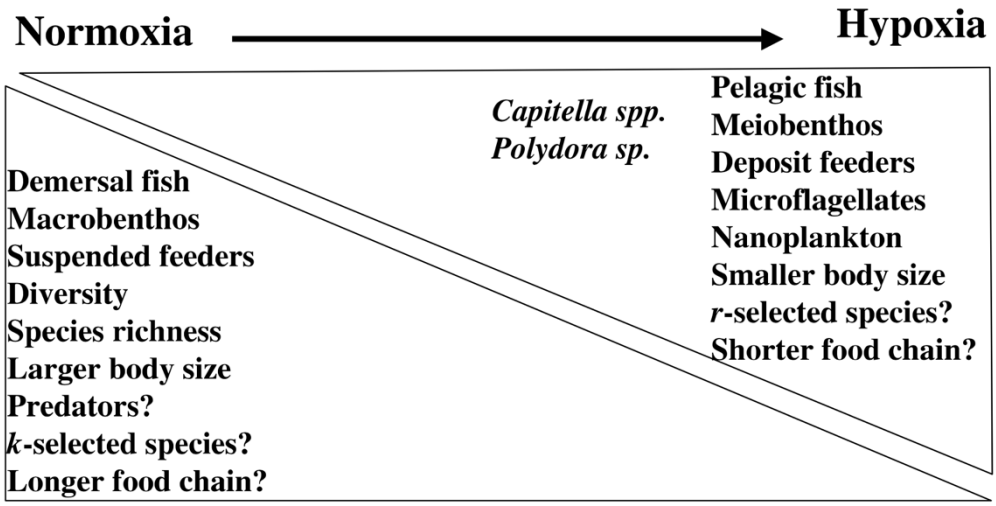

Figure 1.6: Diagram showing changes in community structure and function expected to occur along a hypoxic gradient. Possible changes are marked with a question mark. The polychaetes from the genus *Capitella* and genus *Polydora*. Modified from Wu, 2002.

1.6.1 Consequences of chronic hypoxia on whole organism's physiology:

Aquatic organisms have evolved a series of physiological mechanisms (discussed in detail in section 1.4) in order to live in environments where pO_2 is not always constant but changes through time. These adaptations have an energetic cost that is necessarily subtracted from other physiological activities: in fact, as explained by Sokolova et al. (2012), energy allocation can be diverted away from physiological processes (i.e. growth or reproduction) when species are subjected to stress conditions. The degree of impairment of the different physiological processes is species specific and depends on the magnitude and duration of the stressor (Sokolova et al., 2012). Chronic exposure at 3.8 kPa for one month reduces specific growth by ~50% and the gonado-somatic index by ~30% in the killifish *Fundulus grandis*, significantly delaying the onset of spawning (Landry et al., 2007). 42-days exposure to ~10 kPa significantly reduces growth in the turbot *Scophthalmus maximus* and the bass *Dicentrarcus labrax* (Pichavant et al., 2001). Similarly, growth reductions have been shown in echinoderms, mollusks, annelids, crustaceans and other fish in response to chronic hypoxia (Wu, 2002). Interestingly,

Pichavant et al. (2001) noted a decrease in food intake in juvenile turbot *Scophthalmus maximus* and sea bass *Dicentrarchus labrax* exposed to hypoxia (7.5 – 10.6 kPa, at 22 ˚C) and argued that this reduced food intake "might be an indirect mechanism by which prolonged hypoxia reduces growth" by reducing the oxygen demanding process of digestion. Similar observations have been reported in juveniles of the shrimp *Fenneropenaeus chinensis* kept for 10 days at 5 kPa (Wei et al., 2008), and for other decapod crustaceans, molluscs and annelids (Wu, 2002).

While chronic hypoxia induces a reduction in growth across many different taxa, the effects on reproduction and development are more equivocal. Brouwer et al. (2007) reported that females of grass shrimp, *Palaemonetes pugio*, exposed to chronic hypoxia (~ 3.8 kPa at 27 ˚C) had a higher fecundity than females kept in normoxia, whereas a reduction in the number of egg capsules was found in the gastropod *Nassarius festivus* kept at ~3.9 kPa (28 ˚C) for 7 weeks (Cheung et al., 2008). Further, a retarded gonad development with a reduced reproductive output was reported for carp *Cyprinus carpio* chronically exposed to ~2.5 kPa (Wu, 2002). While Oppen-Berntsen, Bogsnes, and Walther (1990) reported that natural hatching in mature salmon eggs is induced by hypoxia, Diez and Davenport (1990) showed that embryos of dogfish *Scyliorhinus canicula* cannot complete development when exposed to 20% oxygen saturation. In a similar way, detrimental effects on development (mainly a delayed development) were found in: oyster (*Crassostrea virginica*) larvae kept for more than 24 hours at ~1 kPa (at 24 ˚C) (Baker & Mann, 1994); mussel (*Mytilus edulis*) embryos kept more than 60 hours at pO_2 between 1 and 3.5 kPa (Wu, 2002); and grass shrimp (*P. pugio*) embryos kept at ~3.8 kPa (Brouwer et al., 2007).

1.7 Daily cyclic hypoxia in shallow water and coastal habitats:

In contrast to chronic hypoxia, cyclic hypoxia is characterized by periodic fluctuations in pO_2 from normoxia to hypoxia and back to normoxia, with a cycle that typically lasts 24 hours (Coiro et al., 2000; Brown-Peterson et al., 2011). As previously stated, pO_2 fluctuations are expected to increase in severity and the predicted decline in oxygen content will be more pronounced in all aquatic habitats within 30 km off the coast (Gilbert et al., 2010). This zone comprises habitats that have very high biodiversity and are economically important (Altieri & Gedan, 2015). Among them, estuary, marshes and lagoons can be characterized by a strong, diel variability in the main environmental parameters (i.e. temperature and pO_2 Bulger (1984); Cheek et al. (2009); Oliphant (2013)), which could make the species that live in these habitats more vulnerable to further climate changes. In fact, as explained by Tomanek (2010), organisms living in highly variable environments will likely experience a greater impact from hypoxia and climate change, because their physiological response to stress: "is already maximised and will be difficult to modify in order to retool for a rapidly changing world," (Tomanek, 2010). Therefore, there is a pressing need to quantify the magnitude of the impact from cyclic hypoxia on coastal organisms. In spite of our knowledge in relation to chronic hypoxia (dating back to the mid 50s), only in recent years researches have focussed on the effects of diel cyclic hypoxia on coastal organisms, recognising that also a short hypoxic exposure repeated each day might have a significant impact on the physiology of organisms (Brown-Peterson et al., 2011; Remen et al., 2012). In fact, even a short hypoxic exposure might trigger an energetic debt, in terms of energy expenditure (Seibel, 2011), that must be paid in order to re-establish the normal homeostasis of the body. Studies that reproduce "natural" cyclic hypoxic conditions are relevant for a vast number of species from estuaries, lagoons and marshes where this phenomenon is currently experienced; however, the incidence of such studies is still rare. This is partly because pO_2 oscillations are more difficult to create in the laboratory and partly because they require long-term exposures. Moreover species from these habitats are generally adapted to hypoxia (Cochran & Burnett, 1996) and hence, on the short term (i.e. days), changes from cyclic hypoxia are subtle and only become more apparent in the long-term (i.e. from weeks to months).

In order to study cyclic hypoxic conditions a few *in situ* studies have been performed (Stierhoff, Targett, & Power, 2009; Brown-Peterson et al., 2011; Li & Brouwer, 2013a). Unfortunately, as brilliantly expressed by Spicer in his review (Spicer, 2014), the weakness of *in situ* experiments is that researchers cannot draw causal conclusions due to the presence of multiple factors, with some of them being extraneous (Spicer, 2014). By contrast, researchers have tried to estimate the impact of cyclic hypoxia on some physiological variables (i.e. growth), by understanding the impact of chronic hypoxia on the same variables (Coiro et al., 2000; Stierhoff, Targett, & Miller, 2006), but have often reported that discrepancies occur between the estimated and the observed magnitude of the studied effects (Coiro et al., 2000). Currently, only few experiments have assessed the impact from daily cyclic hypoxia on some physiological aspects of a limited number of coastal species (Stierhoff et al., 2006; Brown-Peterson et al., 2008; Cheek et al., 2009; Stierhoff et al., 2009; Brown-Peterson et al., 2011; Remen et al., 2012; Li & Brouwer, 2013a; Davidson, Targett, & Grecay, 2016) (Table 1.1). This thesis will contribute to a better understanding of how cyclic hypoxia is able to affect the physiology of a coastal organisms and how this can be reflected in its habitat.

Table 1.1: Comparison of some studies assessing the effects of cyclic hypoxia on physiological parameters of marine ectotherms.

Study	Species	Common name	Cyclic hypoxic regime	Effects
Stierhoff et al., 2006	*Pseudopleuronectes americanus*	Summer flounder	4.9 - 27 kPa, 20 ˚C	~50 % reduction in specific growth rate
	P. dentatus	Winter flounder	4.9 - 27 kPa, 25 ˚C	~35 % reduction in specific growth rate
Brown-Peterson et al., 2008	*Palaemonetes pugio*	Grass shrimp	4.2 - 22 Kpa, 27 ˚C 7.5 h d^{-1} in hypoxia	Females produced only 2 broods (compared to 3 in controls)
				~50% reduction in relative fecundity
Cheek et al., 2009	*Fundulus grandis*	Killifish	moderate cycle: 6.5 kPa for 1.5h d^{-1}:	~50% reduction in male GSI and 11-ketotestosterone (11KT)
				~50% reduction in female GSI
			severe cycle: 2.4 kPa for 3.4h d^{-1}:	~40% reduction in testosterone and 50% reduction 11KT
				35% reduction in female GSI and 50% reduction estradiol
Stierhoff et al., 2009	*P. americanus*	Summer flounder	Field study	Correlation between mean pO_2 levels and RNA:DNA content
Li et al., 2013	*P. pugio*	Grass shrimp	Field study comparing normoxic and cyclic hypoxic site	Animals from normoxic site were ~10% longer and ~22% heavier than shrimps from cyclic hypoxic site.
				Downregulation of *Vitellogenin*
Brown-Peterson et al., 2011	*P. pugio*	Grass shrimp	Field study comparing 2 bay systems:	
			- WC: 2.3 - 21 kPa, with 2.9h d-1 below 5kPa	WC: reduction in fecundity (34%) and % gravid females (60%)
			- MC: 4.1 - 21 kPa, with 0.7h d-1 below 5 kPa	MC: reduction in % gravid females (40%)
Davidson et al., 2016	*Paralichtys dentatus*	Flounder	moderate cycle: 7.4 - 21 kPa	No effect on growth
			severe cycle: 204 - 21 kPa	~30% growth reduction

1.8 Study organism:

The Atlantic ditch shrimp *Palaemon varians*, formerly know as *Palaemonetes varians* (Leach, 1814), is a brackish water species in the family Palaemonidae, within the order Decapoda (http://www.marinespecies.org/aphia.php?p=taxdetails&id=587704). Palaemonid shrimps have a global distribution in coastal waters, from 48˚ S to 71˚ N (Figure 1.7A). The sub-family Palaemoninae includes species with adaptations to low salinity habitats and have putatively colonized freshwater habitats from brackish environments (Ashelby et al., 2012).

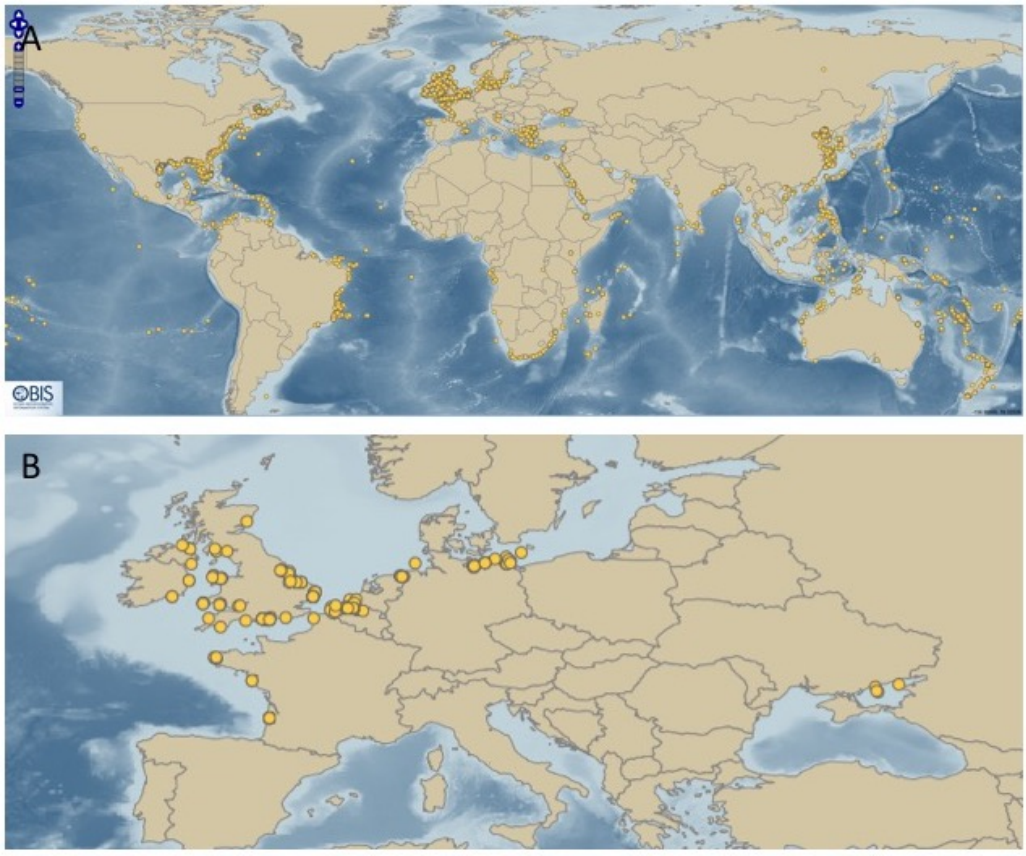

Figure 1.7: The global distribution of **A)** the family Palaemonidae and **B)** Palaemon varians, each dot represents a population. Taken from the freely-available OBIS database: www.iobis.org (OBIS 2015, accessed on 20th September 2017).

1.8.1 Habitat and ecology:

P. varians, specifically, inhabits "inland coastal ponds, characterized by stagnant, highly turbid water (Aguzzi et al., 2005)", with broad variations in salinity, temperature and pO_2 (Welsh, 1975; Healy, 1997; Aguzzi et al., 2005). It is commonly distributed along the coastlines of Western Europe and down the North East Atlantic coast as far Morocco, also being found in areas of the Mediterranean Sea (Figure 1.7B, (Dolmen, 1997; Dolmen, Hindley, & Kleiven, 2004; Aguzzi et al., 2005)). In the UK, *P. varians* is ubiquitously found along the coastline in many salt marsh habitats commonly associated with estuaries (Figure 1.7B).

The studied population originates from the marshlands adjacent to Eight Acre Pond on the outskirts of Lymington in southern Hampshire (UK, 50°44'19.8" N and 50°44'22.2" W). These marshes are part of the "Lymington-Keyhaven nature reserve" since 1973 covering nearly 200 Ha and, within the New Forest they are part of the New Forest National Park (a designated Ramsar site number UK11047). The Reserve is of international importance for the large numbers of breeding, feeding and roosting birds that it supports; black-headed gulls along with little and sandwich terns breed in significant numbers, along with waders such as oystercatcher, ringed plover, redshank and raptors such as barn owls (information from http://www.hiwwt.org.uk/reserves/lymington-and-keyhaven-marshes/). Marshes are key coastal habitat of conservation importance along many temperate coastal fringes (Dolmen et al., 2004) for their important roles as nursery habitats for estuarine fish, crustacean species and other nekton (Mathieson et al., 2000; Cattrijsse & Hampel, 2006).

The salt marshes of Lymington are enclosed within seawalls that are connected to the western arm of Solent. The area is comprised by a series of drainage ditches found next to the main channels. The ditch is around 100 m long, 1-3 m wide and 0.5-1 m deep. The habitat is characterized by lentic waters, with a small tidal influence but with large fluctuations in environmental parameters (Oliphant, 2013). Gauze, brambles, grasses, and other scrub vegetation over-hang the edges of the ditch and, during summer months, extensive weed grows within the ditch providing protection for invertebrate fauna. Predatory birds, such as white egrets and grey herons, and predatory fishes were noted in this ditch (personal observations). Shrimp were most often caught in high densities adjacent to the bank underneath over-hanging vegetation. Within the Lymington salt marshes an annual variability in temperature, ranging from ~0 ˚C to 25 ˚C, and salinity, ranging from ~1 to ~ 43 psu, was observed from 2011 (Oliphant, 2013). Given *P. varians*'

ability to tolerate large variations in environmental conditions, has led to it being used as a model organism where its physiological responses to temperature (Ravaux et al., 2012; Oliphant, Hauton, & Thatje, 2013; New et al., 2014), pressure (Oliphant et al., 2011; Morris, 2015; Morris et al., 2015), metal toxicity (Brown, Thatje, & Hauton, 2017) and pO_2 (Nielsen & Hagerman, 1998) have been studied.

Similarly to the congeneric grass shrimp *Palaemonetes pugio*, commonly found in salt marshes in USA (Welsh, 1975), *P. varians* is a benthic detritivore that contributes to the mechanical breakdown of refractory organic matter and, simultaneously, it is a primary and secondary consumer (Escaravage & Castel, 1990b; Aguzzi et al., 2005). Its ecological role is therefore pivotal within the marsh ecosystem because its detritivorous activity makes detrital energy available at a variety of trophic levels and raises the efficiency of transfer to the food web (Welsh, 1975).

In the wild, *P. varians*' life cycle is of 2 years duration (Jefferies, 1964), with one reproductive season per year, lasting from March to September (Jefferies, 1964). As all decapod crustaceans, *P. varians* possesses gills (branchiae, Fig. 1.8), which are the primary sites of respiration (Felgenhauer, 1992) even if they additionally play a physiological role also in ion regulation and excretion (Gilles & Péqueux, 1985). Gills are located on each side of the body and are located inside a gill chamber, a structure delimited by the carapace on the outer side and the thoracic wall on the inner side. The gill chambers open on all sides, except on the dorsal side, allowing water to reach the respiratory surfaces where gas exchange is performed. The spatial architecture, and hence morphology, of *P. varians*' gills is called phyllobranch and consists in a main branchial axis from which flat paired lamellar branches (called lamellae or plates) extend (Felgenhauer, 1992). Each lamella has a leaf-like shape, is covered with cuticle on both sides and is vascularized; as a result of this anatomical structure, gas exchange is are made possible because the haemolymph is forced to flow through the lamella in close contact with water inside the gill chamber (Felgenhauer, 1992).

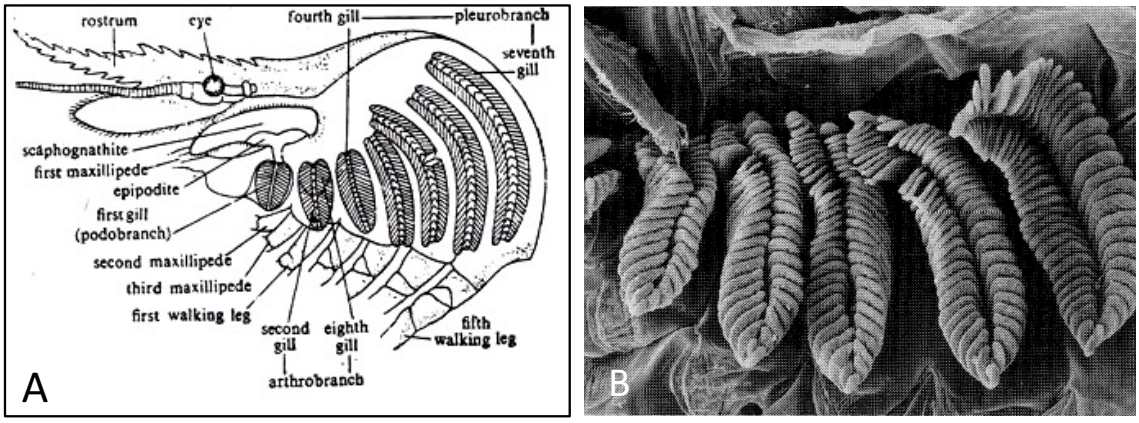

Figure 1.8: **A)** Schematic illustrating the gills and their collocation in the cephalothorax of a Palaemonid shrimp. **B)** Ultrastructure of decapod gills. Lateral view of phyllobranch gills of *Palaemonetes kadiakensis*. SEM x30. Modified from Felgenhauer, 1992.

1.8.2 *Palaemon varians*' physiological adaptations to decreasing pO_2:

As suggested by Taylor and Spicer (1987), differences between species in their tolerance to environmental parameters are likely dependent on the different habitats in which the species are found (Taylor & Spicer, 1987), with more tolerant species living in high variable environment such as rock pools or marshes. *P. varians,* commonly found in salt marshes, has a similar hypoxia tolerance (deducted by comparing the p_{crit} of the two species) to *Palaemon elegans*, commonly found in rock pools in the upper regions of the littoral zone (Morris & Taylor, 1985; Taylor & Spicer, 1987). Both *P. varians* and *P. elegans* possess a greater hypoxia tolerance than the congenerics *Palaemon adspersus*, *Palaemon serratus* and *Palaemon longirostris*, the former two commonly found in sub-tidal seagrass beds (Taylor & Spicer, 1989; Nielsen & Hagerman, 1998) and the latter in estuary habitats (Taylor, 1990).

In spite of the different p_{crit}, *P. varians* and all four congeneric species are able to maintain a considerable degree of respiratory independence by preserving approximately constant MO_2 rates over a wide range of pO_2 values (Taylor, 1990). The respiratory independence is granted by a number of respiratory responses; in fact, when facing a decline in water pO_2 all species are able to increase the time spent in actively ventilating the gills (by reducing the number of ventilatory pauses commonly found in normoxia) and are able increase scaphognathite beating rate, thus increasing overall ventilation volume (Taylor, 1990).

In *P. elegans* and *P. adspersus* a marked decrease in heart beat rate below p_{crit} is observed (Taylor & Spicer, 1989; Taylor, 1990), whereas in *P. varians* heart rate is maintained independent of pO_2 values (Hagerman & Uglow, 1984). This allows *P. varians* to maintain haemolymph circulation in which is present a particularly modified haemocyanin, whose characteristics are sufficient for the animals to maintain MO_2 at very low ambient pO_2 (Hagerman & Uglow, 1984). Although all five species have similar metabolic rates (Nielsen & Hagerman, 1998), *P. varians* has a higher haemocyanin concentration and a higher haemocyanin oxygen affinity in comparison to *P. adspersus* (Nielsen & Hagerman, 1998) and a higher total oxygen carrying capacity in comparison to *P. adspersus*, *P. serratus*, *P. longirostris* and *P. elegans* (Taylor & Spicer, 1989; Nielsen & Hagerman, 1998). This means that *P. varians* is able to extract more oxygen from the water at lower pO_2 values and carry more oxygen in its haemolymph in comparison to the other species. Finally, *P. varians* has an arterial blood oxygen tension (P_aO_2, a measure of physically dissolved O_2 in the haemolymph and not bound to the haemocyanin) of only 1.3 – 4 kPa, compared to P_aO_2 of 9.3 – 12 kPa for *P. adspersus* (Hagerman & Uglow, 1984).

The majority of our knowledge in relation to *P. varians*' adaptations to cope with low pO_2 comes from acute or chronic hypoxic exposure studies but we currently do not know which physiological responses are triggered when the animals are exposed to cyclic hypoxia and what are the long-term consequences of such exposure on physiological processes like growth or reproduction.

1.9 Thesis aims and objectives:

The Atlantic ditch shrimp, *Palaemon varians*, plays a fundamental role within its ecosystem and might be particularly vulnerable to future cyclic hypoxic events, which are expected to increase in severity and frequency. By mimicking natural diel oscillations in pO_2 currently experienced in the field and by using molecular techniques, alongside other measures of physiology, the thesis aims to understand in detail the consequences of prolonged daily realistic cyclic hypoxia on different aspects of the physiology of this coastal species. By determining the current degree of impairment on the physiology, inferences can be made on the ecological repercussions on the salt marsh habitat deriving from changes to the species at any biological or ecological level. Further, given the wide

tolerance of the species to the main physiochemical parameters, inferences can be made as to the possibility for *P. varians* to colonize other habitats which might develop favourable conditions for the life of the species.

The thesis has four main objectives:

1. Characterize natural diel oscillations in pO_2 during the summer within the Lymington salt marshes, where *P. varians* is regularly collected, and set up in the laboratory an experimental system in order to replicate the same fluctuations and perform the experimental work (Chapter 2).
2. Identify and characterize, by using transcriptomics, differential gene expression profiling and histology, the physiological mechanisms that allow *P. varians* to adapt to diel cyclic hypoxia conditions (Chapter 3).
3. Characterize how the energetic impact of prolonged daily cyclic hypoxia (up to 40 days) can impair growth, feeding, reproduction and larval development of *P. varians* (Chapter 4).
4. Investigate how the thermal tolerance and survival of acute copper exposure are changed, following a daily cyclic hypoxic exposure of 28 days, by means of observing behavioural responses, determining survival rates and integrating these data with differential gene expression (Chapter 5).

Chapter 2 Diel fluctuations in temperature and pO_2 within the Lymington salt marshes (UK) and effects of a short-term hypoxic exposure on *Palaemon varians* in the laboratory

Summary:

Coastal environments such as salt marshes can be characterized by a substantial variability in physiochemical parameters and the magnitude of variation can be markedly greater than adjacent habitats. In order to quantify some environmental variability, one sampling site within the Lymington salt marshes (UK) was monitored over time. Temperature was continuously measured from October 2013 to January 2016; oxygen partial pressure (pO_2) was measured for one week in August 2016, in February 2017 and in May 2017. Results showed that temperature and pO_2 were highly variable both on a seasonal and daily basis. Seasonal variation in temperature spanned over 20 ˚C whereas diurnal variation varied from 2 ˚C in February to 6 ˚C in August. Diel fluctuations in pO_2 spanned from 15 kPa in February to almost 42 kPa in May and August. Further, diurnal variations in both temperature and pO_2 were compared with data available from three monitoring coastal sites in UK and confirmed the greater environmental variability (particularly in pO_2) present in Lymington. Overall result suggested that animals in the marsh might experience cyclic hypoxia on a daily basis. In order to study the effects of hypoxia in the laboratory, initially from an acute exposure and subsequently from a long-term cyclic exposure, one of the species living in the marshes, the shrimp *Palaemon varians*, was chosen as a model for its important role in the ecosystem. In the laboratory the critical oxygen pressure (p_{crit}) of *P. varians* was calculated at 22 ˚C, a temperature frequently measured in the marsh during summer and thus chosen as a reference temperature, and the effects of a short hypoxic exposure (8-hours) on oxygen consumption rates (MO_2) and lactate accumulation were assessed. At 22 ˚C, *P. varians* showed a p_{crit} of 4.55 ±0.66 kPa. During 8-h hypoxic exposure, an increase in tissue lactate was observed in hypoxic animals compared to normoxic animals after 2, 4 and 6-hour, whereas an increase

in MO_2 was only detected after 6-hours. Data presented underline how p_{crit} is able to trigger, in the short term, whole-body physiological stress responses at sub-lethal level, associated with the commencement of anaerobic metabolism.

2.1 Introduction:

Aquatic environments can be characterized by a substantial variability in the main physiochemical parameters (i.e. temperature, salinity, pH and pO_2) (Richards, 2011). These parameters can fluctuate on different time scales (i.e. from hours to weeks, as explained in Section 1.2) and the magnitude of these oscillations can be really extreme. For example, for an estuary habitat in Florida Bulger (1984) reported a temperature change of 8 ˚C in as little as 30 minutes. As described in Chapter 1, oxygen partial pressure (pO_2) is not constant in aquatic environments and, particularly in salt marshes, it can fluctuate on a daily basis (Guasch et al., 1998; Li & Brouwer, 2013a). In fact, research on salt marshes in New Jersey (Smith & Able, 2003), South Carolina (Cochran & Burnett, 1996) and Alabama (Cheek et al., 2009) have shown the considerable environmental variability of these habitats, with water pO_2 levels oscillating from supersaturated conditions (> 21 kPa) during the day, to hypoxic conditions (~ 1 kPa) during night-time. Unfortunately in European marshes, "the knowledge of the aquatic component has largely been ignored" (Cattrijsse & Hampel, 2006), resulting in a limited understanding of the variation of the main environmental parameters.

Across the coast of United Kingdom, numerous salt marshes can be found (Dolmen et al., 2004). Among them, the Lymington salt marshes are an important biodiversity hotspot for fish, birds and mammals (as explained in Section 1.8.1). In an attempt to quantify the environmental variability within the Lymington salt marshes, Oliphant (2013) monitored the fluctuations in temperature and salinity and reported a great variability in these parameters but did not measure variations in pO_2. In the first part of this Chapter fluctuations in pO_2 were be measured on a daily basis in August 2016, February 2017 and May 2017 to provide a better understanding of the physiochemical parameters that shape the physiology of organisms living in the Lymington marshes.

In the second part of the Chapter, focus was moved on some physiological mechanisms that are triggered when animals are facing a short hypoxic exposure. As

shown in Section 1.4, the majority of animals shows two different responses when facing a decline in water pO_2: initially they can be classified as oxy-regulators and then as oxy-conformers. The transition between regulation and conformity has been called the critical oxygen pressure – p_{crit} (Tang, 1933; Mueller & Seymour, 2011) and is associated with whole body changes to animal's physiology, starting from molecular (Wu, 2002) and up to behavioural level (Wannamaker & Rice, 2000). This condition is extremely important because the costs of basal metabolism cannot be sustained solely with aerobic metabolisms, thus anaerobic metabolism is triggered (Childress & Seibel, 1998; Seibel, 2011) and other physiological processes (e.g. growth and reproduction) are suppressed (Sokolova et al., 2012). Survival in this condition is time limited and depends on the amount of anaerobic substrates available (Richards, 2011), as explained in Section 1.4.

To understand some of the changes deriving from a short-term exposure to hypoxia, one of the species living in the marshes, the shrimp *Palaemon varians*, was chosen as a model for its role in the ecosystem. In fact, export of organic material is largely absent in European marshes (Cattrijsse & Hampel, 2006) and decaying marsh flora are processed by detritivorous species, such as *P. varians* (Aguzzi et al., 2005), which plays a fundamental role in transfer of nutrients and energy in the ecosystem (Welsh, 1975; Aguzzi et al., 2005).

In the laboratory, after determining *P. varians*' critical oxygen level at 22 ˚C (a reference temperature for summer conditions typically measured in Lymington marshes) metabolic changes (namely lactate accumulation and increase in oxygen consumption rates) were quantified in animals exposed to hypoxic conditions ($pO_2 < p_{crit}$) for up to 8 hours.

2.2 Specific chapter hypothesis:

1. Lymington salt marshes are characterized by a considerable environmental variability in temperature and oxygen on a seasonal and diel scale.
2. During exposure to hypoxia for a short term (8-h), the shrimp *Palaemon varians* will increase oxygen consumption rate (MO_2) and lactate content in the muscle.

2.3 Material and Methods:

2.3.1 Environmental variability:

The species used for all this work, the Atlantic ditch shrimp *Palaemon varians*, originates from the marshlands adjacent to Eight Acre Pond on the outskirts of Lymington in southern Hampshire (UK, 50°44'19.8" N and 50°44'22.2" W). The salt marshes are enclosed within seawalls that are connected to the western arm of Solent. The area is comprised by a series of drainage ditches found next to the main channels. The ditch is around 100 m long, 1-3 m wide and 0.5-1 m deep. The habitat is characterized by lentic waters, with a small tidal influence but with large fluctuations in environmental parameters (Oliphant, 2013).

Environmental conditions (i.e. temperature and oxygen partial pressure pO_2) at the collection site in Lymington, UK (Fig. 2.1) were monitored. While temperature data could be measured continuously for over two years (from October 2013 to January 2016), pO_2 data could only be collected in three discrete weeks throughout the year to describe some of the variability that can occur in different seasons within the ditch. A temperature data logger (nke instrumentation S2T600 Temperature data logger, Hennebont, France) was deployed at one site along the ditch (black arrow, Fig. 2.1). The logger was fixed to a metal stake and was positioned adjacent to the bottom of the channel, at a height that shrimp would frequent (approximately 5 – 10 cm from the bottom of the channel). Temperature data was continuously logged from October 2013 to January 2016 every 30 minutes and periodically downloaded to a laptop.

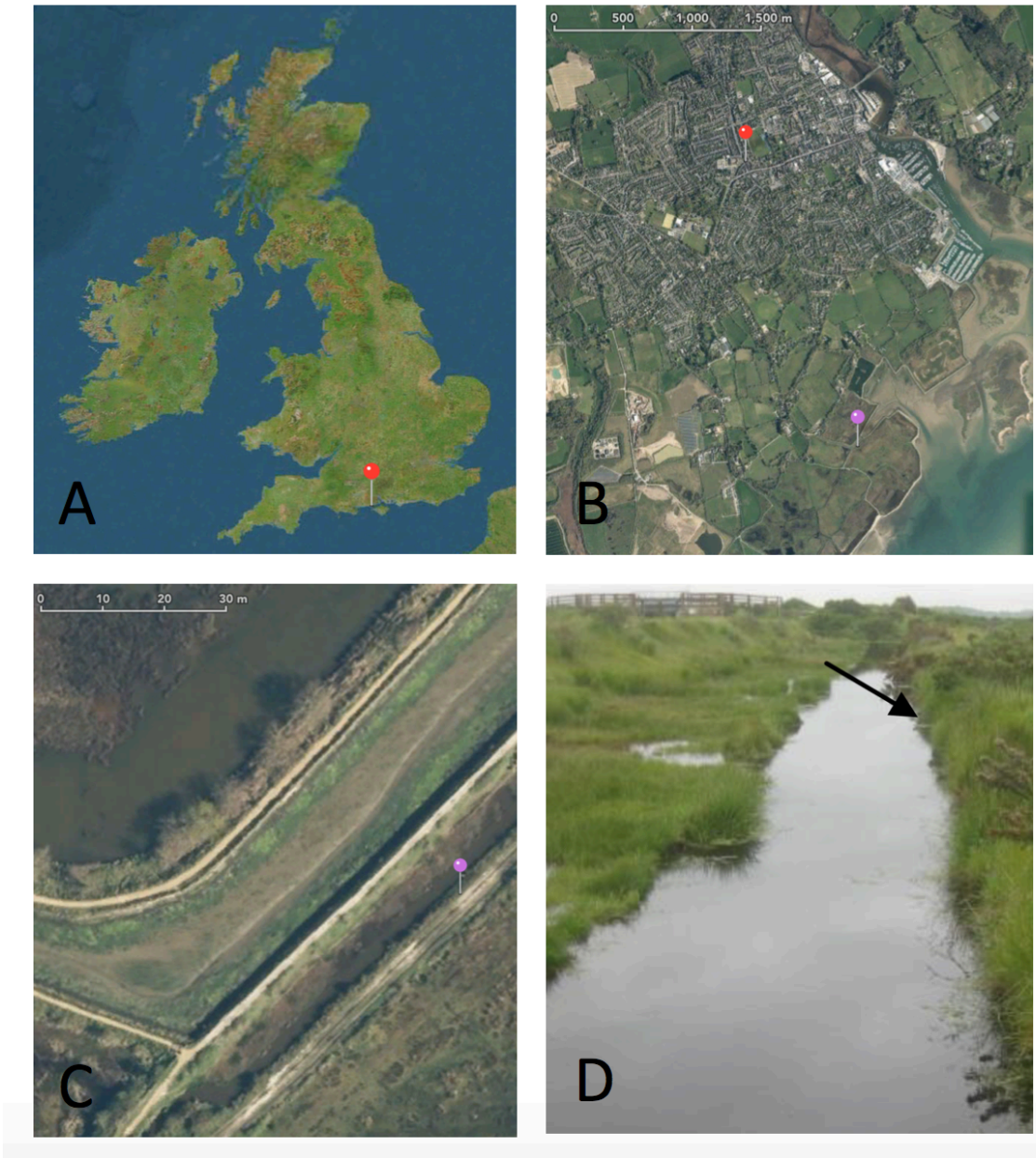

Figure 2.1: **A)** Geographic localisation of the town of Lymington, UK, indicated by the red pin. **B)** Localisation of the city of Lymington (red pin) and the sampling site within the Lymington salt marshes (purple pin, scale bar at top left corner). **C)** Higher magnification of the monitoring and sampling site. **D)** Picture of the sampled channel indicating the exact monitoring location (black arrow). Pictures A-C are taken from Maps (https://www.apple.com/ios/maps/), version 2.0. Picture D is taken from Oliphant, 2013.

Water pO_2 inside the channel was measured by building a custom oxygen-logger, consisting of an Anderaa Oxygen Optode model 3835 (Xylem, USA) fitted with an internal temperature sensor and connected with a cable to an r-p-r space logger S10 (Richard Paul Russell Ltd, Southampton, UK) and a 12V battery. Invaluable help and assistance in assembling and configuring the oxygen-logger was received by Mr Jon Campbell, Miss Urška Martinčič, Dr Alexander Beaton and Dr Susan Hartman (who kindly provided the

Oxygen Optode) from the National Oceanography Centre, Southampton. Logger and battery were housed in a waterproof box. In order to measure daily pO_2 variation in winter, spring and summer, one sampling week per season was randomly selected to perform the deployment and collect environmental data. For the entire duration of the sampling week, the oxygen optode measured water temperature (˚C) and air saturation (%) every 10 minutes. The optode was fixed to a metal stake and placed in the centre of the channel (approximately 50 cm away from the temperature logger nke) and adjacent to the bottom of the channel (approximately 5 – 10 cm from the bottom of the channel).

2.3.2 Laboratory determination of *Palaemon varians*' p_{crit}:

In order to calculate p_{crit}, an experimental chamber with a total sea water volume of 55mL was placed inside a water bath at a constant temperature of 22 ºC. The chamber was fitted with a stir bar to ensure proper mixing of the water inside the chamber when this was sealed. A Fibox 3 optical oxygen meter (PreSens Precision Sensing GmbH, Germany) equipped with an O_2 sensor spot was used to monitor pO_2 in the chamber. The sensor spot was fixed on the inner surface of the chamber and pO_2 could therefore be continuously measured in a non-invasive and non-destructive manner from outside, through the wall of the vessel. The instrument was calibrated daily with 100% O_2 saturated seawater and 0% O_2 saturated seawater (Oliphant et al., 2011).

Firstly, 10 shrimps collected from the monitored site in Lymington were acclimated at 22 ˚C (+1˚C/day) for at least two weeks prior to the experiments. Then, each animal was left to acclimate overnight inside the experimental chamber in order to eliminate the stress associated with manual handling of the animal. During acclimation water was gently pumped through one opening from the water bath inside the chamber. At the beginning of the experiment the circulation was isolated, the chamber was sealed and pO_2 was measured every 30 seconds. Simultaneously, with the beginning of each experiment a replicate chamber containing no animal was sealed and the pO_2 level was measured at the end of the experiment in order to quantify microbial respiration. The experiment ended: *i)* when pO_2 reached 0 kPa or *ii)* when pO_2 was near 0 kPa but was no longer decreasing. At the end of each experiment the animal was carefully blotted on paper and its wet weight was taken with an analytical balance (Denver Instrument si-234 Colorado - USA, weight ± 0.0001g).

Each mean pO_2 value (calculated over an interval time of three minutes) was converted to oxygen concentration (μmol L^{-1}) under the temperature and salinity conditions

used in the experiment according to Benson and Krause (1984). Oxgen consumption rate MO_2 (μmol O_2 g^{-1} min^{-1}, Ern et al. (2013)) was calculated from the measured pO_2 values according to the relation (Oliphant & Thatje, 2014):

$MO_2 = [([O_2]_{initial} - [O_2]_n) / t_n] * V / WW$

Where: $[O_2]$ is the oxygen concentration in the water (in μmol/l); t_n is the time (min); V is the volume of the chamber (L); WW is the wet body mass of the animal (g).

MO_2 was plotted against pO_2 and fitted with a curve using Prism 6 (GraphPad Software, San Diego, CA) after comparing different mathematical models: linear, quadratic, logarithmic and one-phase association. Among all the models, the one that gave the best fit in terms of R^2 values (Mueller & Seymour, 2011) was chosen. P_{crit} was then calculated according to Mueller et al (2011), by finding the highest vertical distance between the fitting curve and the straight line (representing hypothetical perfect conformity, see Chapter 1 Section 1.4) passing through the curve values at the initial and final pO_2 values recorded during the experiments. P_{crit} was determined individually for every animal used and then the mean of the population was calculated (n=10). In order to obtain a value that was representative of the entire population, animals of different size, sex and physiological condition (i.e. intermoult and ovigerous females) were used.

2.3.3 Design and creation of the experimental system:

For the purpose of investigating the effects of short-term hypoxic exposure (this Chapter) and the effects of prolonged cyclic hypoxic exposures (Chapters 3, 4 and 5), a flow-through aquarium was designed, optimised and constructed (Fig. 2.2A). Since long-term maintenance up to 40-days was necessary, the flow-through system was required to house a large number of animals in optimal conditions: e.g. creating an appropriate circulation to keep well oxygenated the water in all the parts of the system during normoxic periods and preventing accumulation of metabolic wastes, deoxygenating the water in a homogeneous way (in the hypoxic tanks only) to prevent the formation of anoxic zones in the aquaria. Further, the system needed to automatically produce cyclic fluctuations of the oxygen in the water at desired pO_2 level (≤ species' p_{crit}) and for a set amount of time (7/8-hours every night). Finally three independent experimental replicates at the level of the studied factor (hypoxia) were required (Riebesell U., 2010).

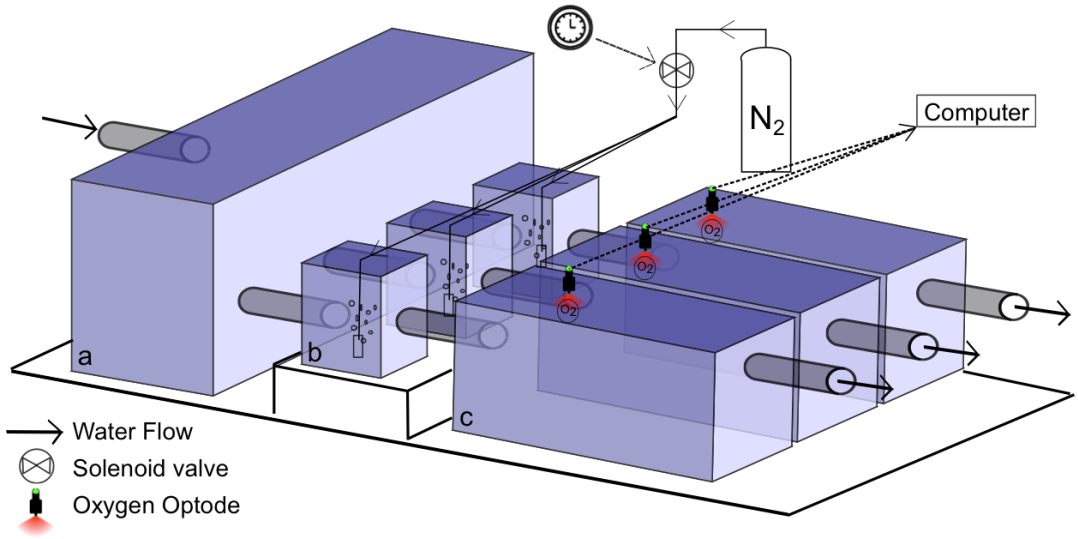

Figure 2.2: Schematic of the flow-through aquarium system. **a)** Holding tank (~40 L). Water coming from the sump (not shown in the picture, ~40 L) is warmed here and subsequently distributed to b. **b)** Bubbling tanks (1.5 L/each). In each tank Nitrogen (N_2) is bubbled independently, in order to replicate the factor "hypoxia" three times. **c)** Animal tanks (10 L/each). Each tank continuously receives water from one bubbling tank. Excess water goes back in the sump.

The flow-through system was closed, meaning that water coming out from the experimental tanks (Fig. 2.2 c) was filtered and oxygenated in one 120 L sump (not drawn in Fig. 2.2) and from the sump it was pumped again into the holding tank (Fig. 2.2 a). Two twin systems following the scheme in Fig. 2.2 were constructed, one to study hypoxic conditions and another as control. Table 2.1 shows the main physiochemical parameters measured in the two systems. During all the experimental work presented in this and following Chapters water pO_2 levels were continuously monitored using a Microx TX3 oxygen meter (PreSens Precision Sensing GmbH, Germany) equipped with an O_2 sensor and connected to a PC, used to log the data.

Table 2.1: Main physiochemical parameters measured in the two systems. Nitrite, Nitrate and Ammonia were measured using the commercial kits Nitrate/Nitrite marine test kit, Red Sea and Ammonia marine test kit, Red Sea (https://www.redseafish.com/). Temperature, salinity and pH are presented as Mean ± SD.

	Normoxic system	**Hypoxic system**
Temperature (˚C)	22.30 ±0.70	22.11 ± 0.67
Salinity (PSU)	33.4 ±0.9	33.1±1.1
pH	8.06±0.08	8.15±0.08
Nitrite (ppm)	< 0.05	< 0.05
Nitrate (ppm)	< 10	< 10
Ammonia (ppm)	< 0.2	< 0.2
Water flow rate (mL/min/tank)	100 mL	100 mL

2.3.4 Short-term (8-hour) hypoxic exposure:

Short-term hypoxic exposure was intended to quantify the effects of one single hypoxic exposure (water $pO_2 \leq$ species' p_{crit}) on whole body oxygen consumption – MO_2 – and lactate accumulation in muscle tissue. By using the aquarium system described above (Fig. 2.2), hypoxia was created by bubbling nitrogen (N_2) in the water (see Supplementary Table 1 – Appendix A for mean pO_2 value recorded). During the experiment, stocking density was 15 animals per tank. Sampling occurred every two hours from the start of the experiment (time 0) to the end (time 8). Each time, sampled animals were split between MO_2 measurements (n=4 per treatment per time point) and lactate measurements (animals flash frozen in liquid nitrogen and stored at -80 ˚C, n=4 per treatment per time point).

In order to quantify MO_2, sampled animals from both treatments were immediately placed in a 55 ml centrifuge tube filled with 100% aerated seawater at 22 ˚C. The tube was carefully closed, making sure that no air bubble was trapped inside it (Oliphant et al., 2011), and stored in a 22 ˚C water bath (Oliphant et al., 2011). After 30 minutes, pO_2 inside the tube was measured (final pO_2) using a temperature-adjusted oxygen meter and micro-optode (Microx TX 3, PreSens) previously calibrated with 100% O_2 saturated seawater and 0% O_2 saturated seawater (Oliphant et al., 2011). Control tubes containing no animals were processed similarly to quantify the effects of microbial respiration. Then, the final pO_2 difference between control and experimental tubes was calculated and MO_2

values were obtained as previously described (section 2.3.2). At the end of each experiment the animal was carefully blotted on paper and its wet weight was taken with an analytical microbalance (as above).

A Lactate Assay Kit (Mak064, Sigma-Aldrich, Hants, UK) was used to determine lactate content in the muscle tissue. The abdomen of animals stored at -80 ˚C was weighed and used for the analysis. Each sample was weighed and homogenized in four volumes (w/v) of Lactate Assay Buffer and centrifuged at 13000 *g* for 10 min at 4 ˚C. Then 6.25 µl of the sample were used for the analysis following the manufacturer's protocol.

Statistical analysis was performed using R version 3.1.2 (Team, 2014). Normality of the data and homogeneity of variances were assessed using Shapiro-Wilk test and Bartlett's test, respectively; two-way ANOVA and *post-hoc* TukeyHSD were used to test the effects of treatment and time on the response variables (MO_2 and lactate). Significance was accepted at p-value < 0.05.

2.4 Results:

2.4.1 Environmental variability:

During the entire monitoring period, seasonal fluctuations in temperature were observed (Fig. 2.3). Lowest average temperatures were recorded in January and February, with values of ~6 ˚C, whereas highest average temperatures were recorded in July, with values of ~23 ˚C. Diel standard deviations (grey bars, Fig. 2.3), tended to be smaller during winter and increased in spring and summer (as a consequence of greater thermal variation within the 24-hour in these seasons). In the summer (from June to September) at night time (from 2000 to 0800 BST), water temperatures ranging from 12 to 24 ˚C were recorded (Fig. 2.4) and temperatures ≥ 22 ˚C were frequently measured (~15% of the time).

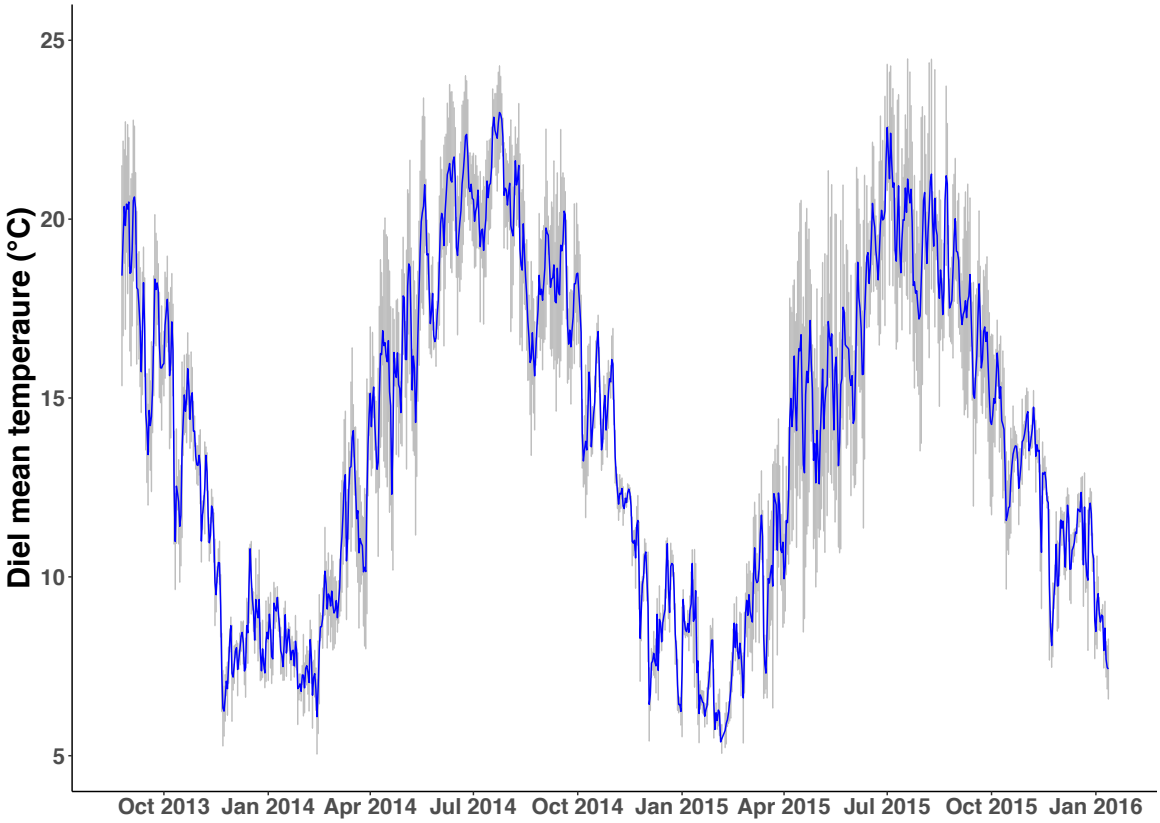

Figure 2.3: Diel mean water temperature (blue) ± SD (grey bars) measured with temperature logger nke in the sampling site within the Lymington salt marshes, approximately 5 – 10 cm from the bottom of the channel.

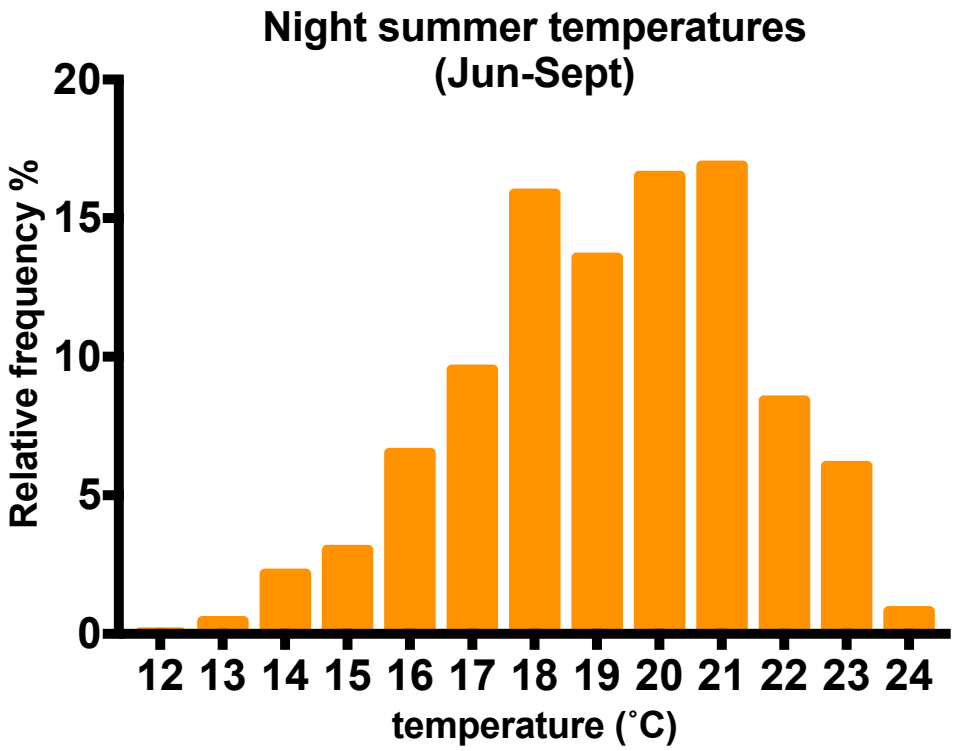

Figure 2.4: Frequency histogram of temperatures recorded in Lymington salt marsh between 2000 hrs and 0800 hrs (BST) in summer 2013 (June – September) and 2014 (June – September).

During 2016 and 2017, oxygen and temperature measurements were taken over one-week period in winter (February 16th – 23rd 2017), spring (May 14th – 22nd 2017) and summer (August 4th – 11th 2016), Figure 2.5. Measurements were taken approximately 5-10 cm from the bottom of the channel. A picture of the same section of the channel in the different seasons is also shown to illustrate the different vegetation content throughout the year (Fig. 2.5). Water pO_2 oscillated on a daily base throughout all the sampling weeks, increasing during the day (arguably due to photosynthetic production of O_2 in accordance to Smith et al., (2003)) and decreasing during the night (due to respiration). Diel pO_2 oscillations were less pronounced during winter, ranging from ~21 kPa (~ 100% air saturation) to ~5 kPa (~30% air saturation); diel fluctuations became very marked in spring and summer, where pO_2 could vary from ~42 kPa (~200% air saturation) to ~3.5 kPa and back to ~42 kPa within one day. In May, the amplitude of the oscillations seemed "irregular" (with peaks in pO_2 that changed considerably every day), in contrast to August where peaks in pO_2 ranged from ~36 kPa (~170% air saturation) to ~42 kPa and a ~39 kPa difference between adjacent peaks and troughs were detected every 12-hours.

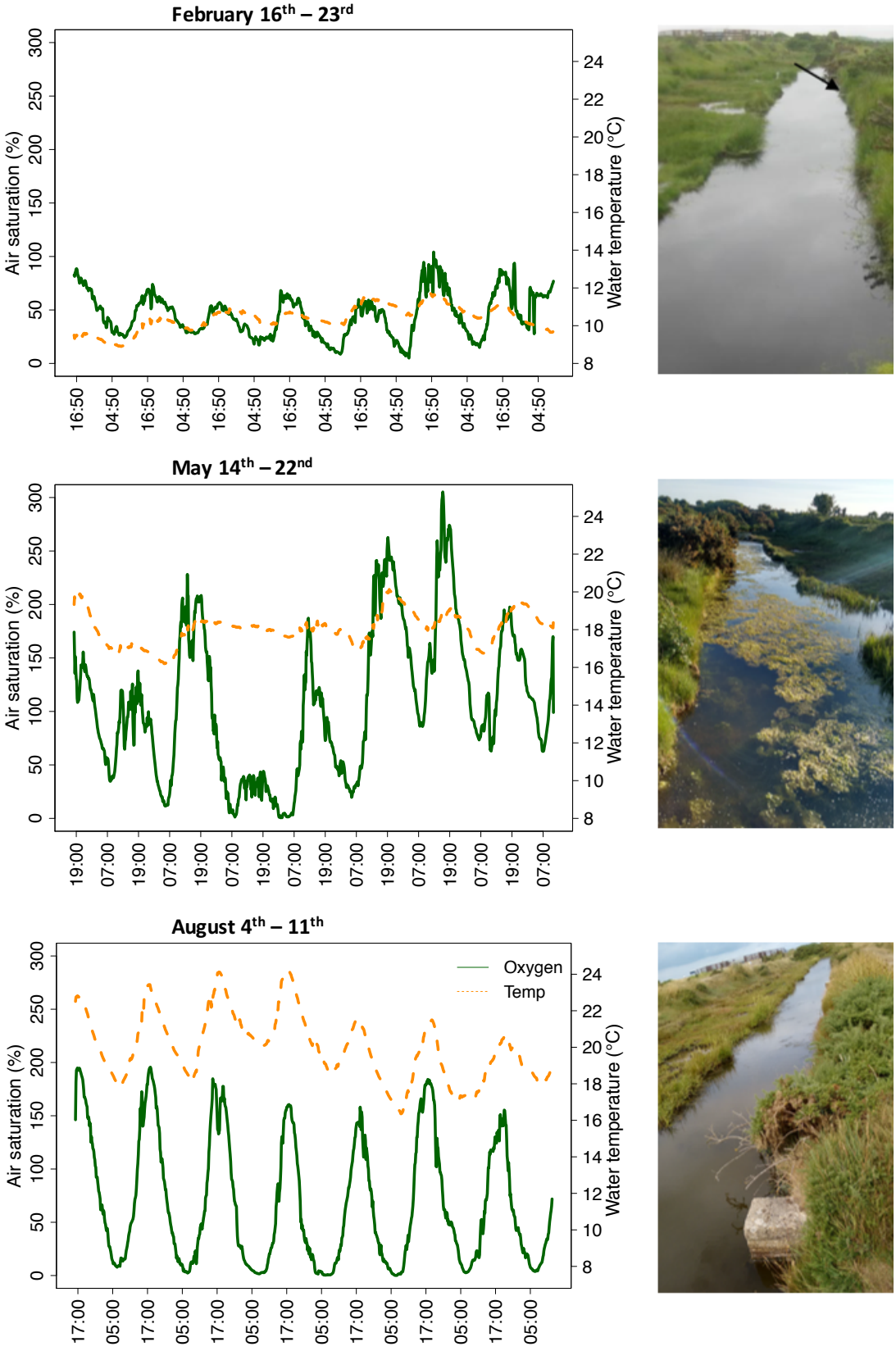

Figure 2.5: Oxygen and temperature measured within one channel from the Lymington salt marsh during one week in February (upper graph), May (middle graph) and August (lower graph). Photographs of the channel, taken in February (upper), May (middle) and August (lower), illustrate the differences in within the channel during time. Black arrow indicates the location of the logger. Upper picture is taken from Oliphant (2013).

2.4.2 Laboratory determination of *Palaemon varians*' p_{crit} and short-term (8-hour) hypoxic exposure:

The first experiment was designed to determine the critical oxygen level (p_{crit}) of the studied population. As p_{crit} is dependent on temperature (Herreid, 1980), the experimental temperature for all the experiments required constraining. A temperature frequently recorded during the summer was thought to be appropriate for the purpose of the research, since warm summer temperature accelerates metabolism of ectotherms, increasing their oxygen consumption and making the development of tissue hypoxia more likely (Cochran & Burnett, 1996). For those reasons, the experimental temperature was set to 22 ˚C, since water temperatures ≥ 22 ˚C were frequently measured (~15% of the time, Fig. 2.4) within the Lymington salt marsh.

An example of MO_2-pO_2 graph (used to determine p_{crit}), its best log-fitting curve, and the oxyconformer line is plotted in Figure 2.6. With the vertical distance method, the difference between the two curves was calculated for each pO_2 and the greatest difference was identified as the p_{crit} (in the example, the greatest difference corresponded to a p_{crit} of 3.5 kPa, Fig. 2.6). Within the studied population a mean p_{crit} of 4.55 ±0.63 kPa (n=10) was determined at 22 ˚C, Table 2.2.

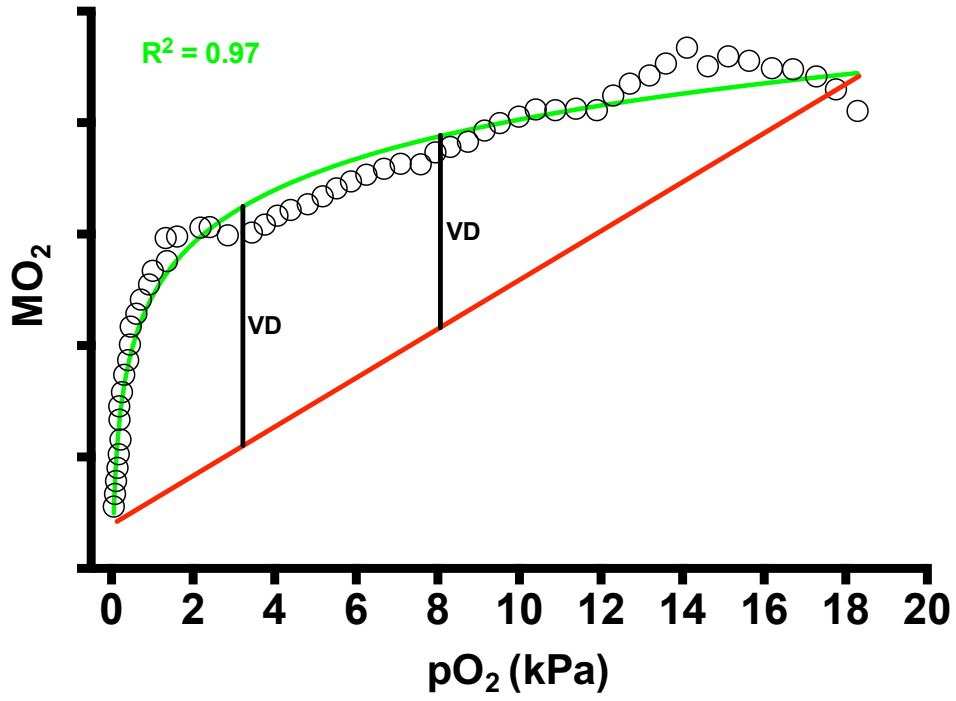

Figure 2.6: PO_2-MO_2 graph (open circles) of one experimental animal. Log best-fitting curve (R^2 = 0.966) in green. Hypothetical perfect conformer response in red. VD= vertical distance between the logarithmic curve

and the straight line. The highest value of VD corresponds, on the x-axis, to the p_{crit} value (in this example p_{crit} = 3.5 kPa).

Table 2.2: P_{crit} calculated for 10 experimental animals at 22°C. R^2 represents the goodness-of-fit for the logarithm model fitting MO_2-pO_2 data (Fig. 2.6). Wet weight of each animal is reported.

Animal	**P_{crit} (kPa)**	**R^2**	**Sex**	**Wet Weight (mg)**
1	5.5	0.95	-	282.2
2	3.5	0.96	Ovigerous Female	266.1
3	4.0	0.86	Female	289.4
4	4.5	0.82	Male	229.5
5	4.5	0.8	Male	116.7
6	4.5	0.79	Female	304.2
7	3.5	0.79	Male	160.8
8	4.5	0.65	Male	110.4
9	4.5	0.68	Male	227.8
10	5.0	0.61	Ovigerous Female	371.6

The subsequent experiment was designed to determine the effects of a single eight-hour exposure to hypoxia on animals' respiration rate and lactate content in the muscle (Fig. 2.7). Lactate and MO_2 data were normally distributed (Shapiro-test, p-val > 0.05) and homoscedastic (lactate: Bartlett's K-squared = 5.50, df = 9, p-val = 0.78; MO_2: Bartlett's K-squared = 11.53, df = 9, p-val = 0.24). Overall, MO_2 differed between treatments (p-value= 0.05, Table 2.3A) and Tukey's multiple comparisons revealed that MO_2 was statistically higher in hypoxic animals after 6-hours (Fig. 2.7A). When analysing lactate content, the interaction between time and treatment was found to be statistically significant (p-value < 0.0001, Table 2.3B). Tukey's multiple comparisons revealed that lactate in muscle increased significantly in hypoxic animals compared to normoxic animals after 2, 4 and 6-hours from the beginning of the experiment (Fig. 2.7B).

Table 2.3: Two-way ANOVA table carried out on **A.** MO_2 and **B.** lactate accumulation.

A. MO_2 - ANOVA Table					
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.14	4.00	0.03	F (4, 30) = 2.003	P=0.12
Time	0.04	4.00	0.01	F (4, 30) = 0.5621	P=0.70
Treatment	0.07	1.00	0.07	F (1, 30) = 4.161	P=0.05
Residual	0.51	30.00	0.02		

B. Lactate - ANOVA Table					
ANOVA table	**SS**	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	64.07	4.00	16.02	F (4, 30) = 3.149	P=0.03
Time	24.57	4.00	6.14	F (4, 30) = 1.207	P=0.33
Treatment	197.50	1.00	197.50	F (1, 30) = 38.83	P<0.0001
Residual	152.60	30.00	5.09		

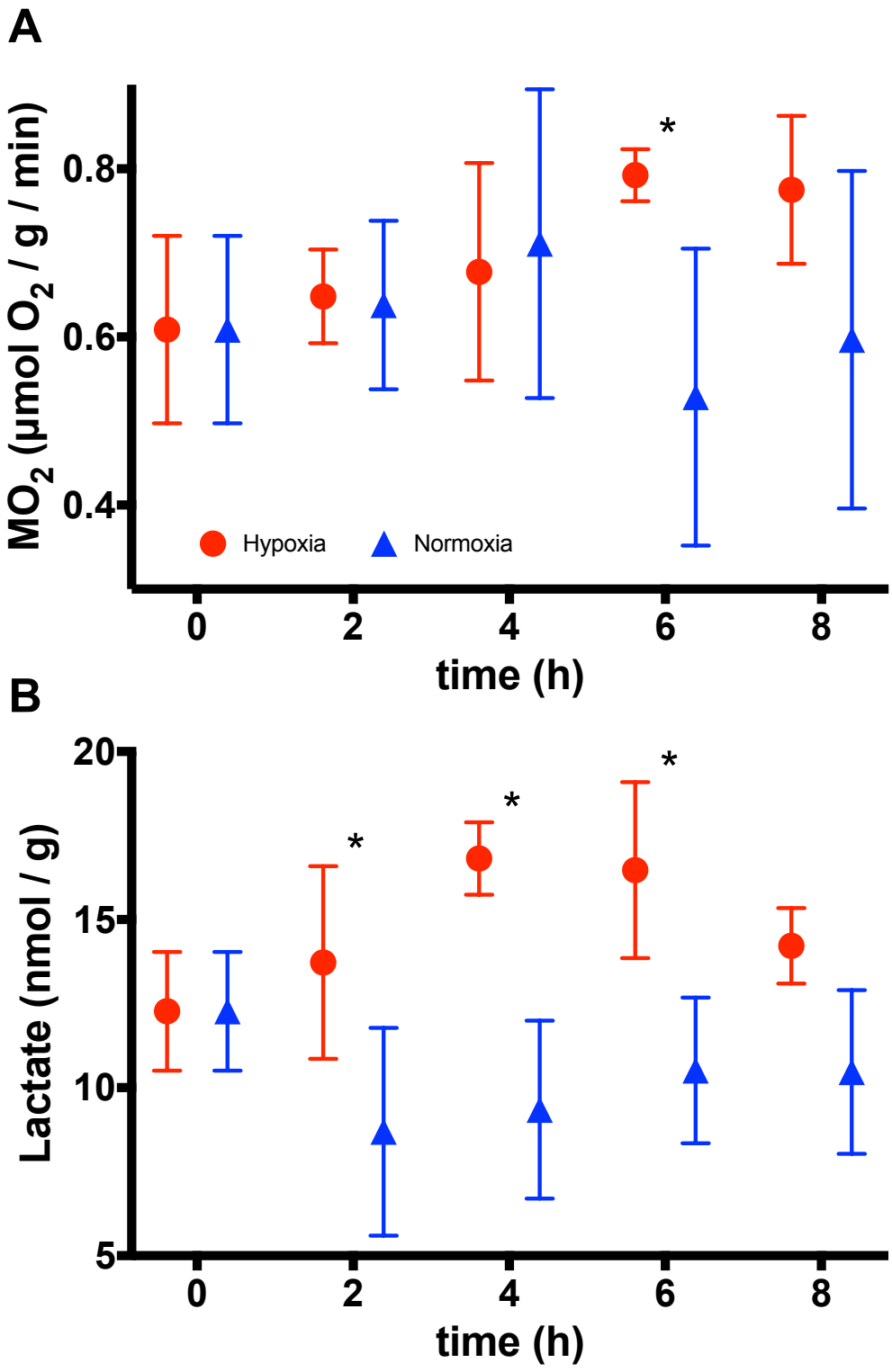

Figure 2.7: Effects of short term hypoxia (up to 8-hours) on **A)** MO_2 (using wet weight) and **B)** lactate content in muscle (mean ±SD). * indicates statistical difference between treatments (p-value <0.05) at the same time point.

2.5 Discussion:

2.5.1 Environmental variability:

All habitats on our planet are characterized by some degree of variability in their physiochemical parameters, but in some, among the others, the magnitude of variability is particularly intense (e.g. the inter-tidal environment (Helmuth, 1999; Somero, 2002)). Estuaries and salt marshes are a typical example where high variability in temperature, pO_2 and salinity is found (Marsden, 1976; Cheek et al., 2009; Oliphant, 2013). Previously, Oliphant (2013) quantified some of the variability in temperature and salinity that characterizes the Lymington salt marshes (UK) but did not include oxygen. This work continued the previous work but also moved forward and characterized some of the diel and seasonal variability in pO_2, providing a better understanding on the major environmental factors (and their magnitude of variability) that shape the biology of marsh species.

As I will demonstrate, the Lymington salt marshes are characterized by a larger and more marked thermal and pO_2 diel range, in comparison to other nearby shallow water habitats. Overall, annual thermal excursion ranged from ~5 to ~ 22 ˚C. Daily average temperatures from ~2 ˚C in winter to ~24 ˚C in summer were instead recorded by Oliphant (2013) in the same site in Lymington. However, it may be argued that this difference in thermal ranges was due to inter-annual variability, as some annual variability in temperature (~ ±1 ˚C) was already reported by Oliphant (2013). Overall, thermal range in Lymington was larger than the range measured in the nearby site of Calshot in the Solent (a strait that separates the Isle of Wight from the mainland of England), where the annual thermal range was reported as 6 to 18 ˚C (Hirst, Sheader, & Williams, 1999). In spite of the proximity of the two habitats, it is possible that the difference in thermal range was due to the fact that Lymington's marshes are characterized by stagnant waters and channels with a reduced depth (typically less than 1 m), which are more easily warmed and cooled by atmospheric agents (e.g. sun, wind, as reported by Oliphant (2013) and Marsden (1976)) than the nearby site of Calshot (where water is mixed by tides). Interestingly in the Lymington marshes, diel fluctuations in water temperature were lowest in winter, then increased during spring and summer and finally decreased again in Autumn, as suggested by the different length of the grey bars in Figure 2.3. In a similar fashion, rapid changes in pO_2 (within 12 hours) were found in all sampled weeks, with maximum fluctuations of ~42

kPa (~200% air saturation), consecutively observed over three days in August (Fig. 2.5). A similar magnitude of diel variability in pO_2 can be observed in other salt marshes, in agreement with Smith and Able (2003), who reported a diel variation of ~33 kPa in a salt marsh in New Jersey (from ~42 kPa at 1100 hrs to ~9 kPa at 2300 hrs in July 1990), with Cochran and Burnett (1996), who reported a diel variation of ~10.5 kPa in a salt marsh in South Carolina (from 18 kPa at 1800 hrs to ~7 kPa at 0700 hrs in August 1994) and with Cheek et al. (2009), who reported a diel variation of ~ 20 kPa in the marsh of Weeks Bay, Alabama (from 21.5 kPa at 1200 hrs to 1.3 kPa at 0000 hrs in July 2004).

Diel variability in temperature and pO_2 found in Lymington was probably due to a combination of different factors. In fact, it could be argued that, while the thick algal coverage present in the channel (particularly during spring, Fig. 2.5) was responsible for intense O_2 production during the day, on the other hand, the abundance of organisms (animals and plants) was responsible for significant O_2 consumption during the night in a similar way to what reported by Smith et al., (2003), Brown-Peterson et al., (2011) and Cheek et al., (2009). In fact, as proposed by Smith et al., (2003), it is likely that photosynthesis of benthic algae, phytoplankton and submerged aquatic vegetation rapidly increased pO_2 during the day, ultimately resulting in supersaturated conditions (Fig. 2.5). After nightfall oxygen production ceased but oxygen consumption continued (Christian, 1981). Since oxygen consumption is directly dependent on temperature (i.e. warmer temperatures increase oxygen consumption rates, (Ern et al., 2016)), it could be argued that, during warm summer nights, elevated oxygen consumption rates, coupled with reduced streamflow and reduced depth of the channel, resulted in the development of hypoxic conditions, in accordance with Cochran and Burnett (1996).

Throughout the year, the magnitude of diel oscillation in temperature and pO_2 differed between the seasons, being lower in winter and higher in spring and summer (Fig. 2.5). The difference in pO_2 patterns measured in May and August, which resulted in intense supersaturated conditions in May (up to ~ 63 kPa, ~300% air saturation, Fig. 2.5), might depend on the algal coverage that developed in the channel during spring (responsible for intense O_2 production) and was drastically reduced by mid-July (as can be seen by comparing pictures in Figure 2.5). On the other hand, during winter, diel pO_2 fluctuations were greatly reduced (only ~15 kPa) , probably due to low temperatures ($\leq$12 ˚C), which greatly reduced metabolism (and hence oxygen consumption) of ectotherms (Gillooly et al., 2001; Ern et al., 2016).

The environmental variability in temperature and pO_2 found in salt marshes is peculiar of these habitats, in contrast to the environmental variability that can be observed

in adjacent shallow water habitats. As an example, to show the different magnitude of variability, temperature and pO_2 time-series data from 4th to 11th August 2016 were plotted in Figure 2.8 with data obtained from Lymington salt marsh, Penarth Marina in Wales (51º 26' 47" N, 3º 10' 37" W, data from https://stormcentral.waterlog.com/SiteDetails.php?a=98&site=301), and two shallow water sites nearby Lymington: Bramble Bank (50° 47' 24" N, 1° 17' 9" W, data from http://www.bramblemet.co.uk/) and Southampton water (50° 52' 15" N 1° 22' 22" W, data from https://stormcentral.waterlog.com). By comparing the trend in temperature and pO_2 between Lymington and the other habitats, the different amount of variability between them could be easily appreciated. In fact, within the sampling period, maximum temperature fluctuations during a daily cycle were ~ 6 ˚C in Lymington whereas maximum fluctuations of ~3 ˚C were recorded in Bramble Bank, Penarth Marina and Southampton waters (Fig. 2.8A); in a similar way, diel pO_2 variation was only ~2 kPa (~ 10% air saturation) in Penarth Marina and Southampton waters whereas it was ~40 kPa (~200% air saturation) in Lymington (Fig. 2.8B). In conclusion, these data underline the different amount of environmental variability (particularly in pO_2) between the salt marsh habitat of Lymington and nearby coastal habitats.

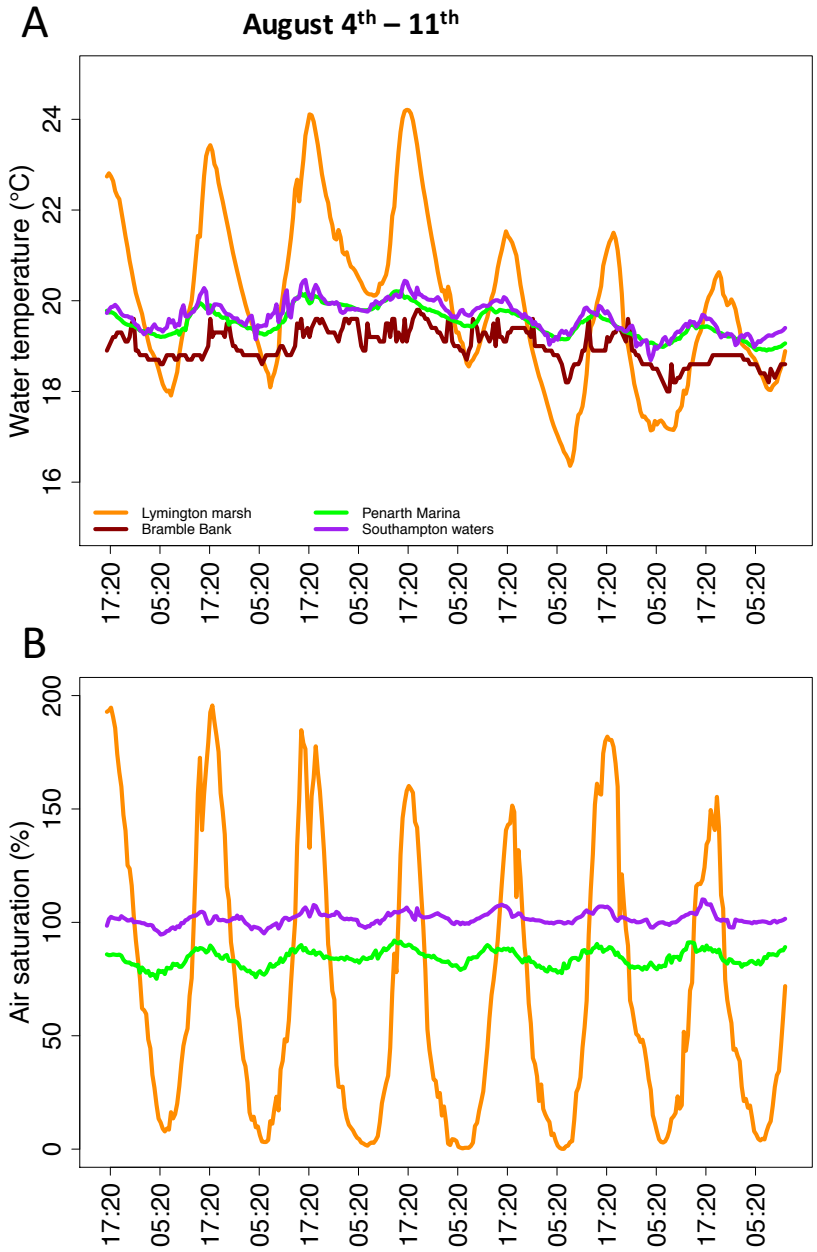

Figure 2.8: Temperature **A)** and pO_2 **B)** time-series data from 4th to 11th August 2016 for Lymington salt marsh, for Bramble Bank (50° 47' 24" N, 1° 17' 9" W, data from http://www.bramblemet.co.uk/) and for Penarth Marina (51º 26' 47" N, 3º 10' 37" W, data from https://stormcentral.waterlog.com/SiteDetails.php?a=98&site=301) and Southampton water (50° 52' 15" N 1° 22' 22" W).

From personal observations on the field, it was very difficult to collect *Palaemon varians* with hand net around the sampling site during July and August, whereas it was easily collected from January to June and from September to January. A possible explanation could be that cyclic hypoxic conditions experienced during July and August might be different (and maybe more fatiguing) from the conditions experienced in the rest

of the year, probably due to the higher summer temperatures (frequently ≥22 ˚C, Fig. 2.4). This might result in a temporary relocation of the species away from the sampling site during July and August to a part of the marsh were the environmental conditions are more suitable for the species.

While on the one hand it could be hypothesised that species living in variable environments, such as marshes, should be able to tolerate a wide variety of environmental conditions (i.e. generalist species), on the other hand, the rapid climatic changes that we are currently experiencing raise questions as to the capability of organisms that live in such variable habitats to further tolerate an even greater variability due to global warming. As explained by Tomanek (2010), species living in highly variable environments, such as the Lymington salt marshes, are likely to be more affected by climate change (in comparison to species from moderately variable environments). In this context, a +1 or +2 ˚C increase in water temperature, according to climate change predictions, could cause summer temperatures ≥ 22 ˚C to be recorded more frequently (from a current frequency of 15%, to a possible frequency of 32% or 48%, Fig. 2.4). This would further exacerbate the hypoxic conditions currently experienced in the marsh by extending in time the duration and severity of the summer cyclic hypoxia, currently experienced only in July and August, and would force the species to relocate for longer periods during the year, potentially causing biological repercussions on the species and ecological repercussions on the ecosystem (as discussed in the following chapters).

2.5.2 Laboratory determination of *Palaemon varians*' p_{crit} and short-term (8-hour) hypoxic exposure:

In the literature many different strategies have been used to calculate p_{crit}. In fact, in spite of its definition (p_{crit} as the pO_2 value at which an oxy-regulator is no longer able to maintain oxygen consumption rate (MO_2) constant (Tang, 1933; Herreid, 1980; Mueller & Seymour, 2011)), the visual identification of p_{crit} by simply plotting pO_2 and MO_2 together is not possible. For over 30 years the principal way to identify p_{crit} has been via "broken stick regression" (BSR) (Marshall, Bode, & White, 2013). This method implies fitting two linear regressions because it assumes two different functional responses of an organism to decreasing pO_2: above p_{crit} MO_2 is assumed to be constant, hence it is described by a flat linear function; below p_{crit}, MO_2 is assumed to decrease in a linear manner with pO_2. As

recently advocated by Marshall et al. (2013), rates of respirations are not likely to have these two different functional responses characterized by an abrupt transition between them, rather they are likely a continuous transition between these phases, hence they are better described by nonlinear functions (e.g. logarithmic, power or one-phase association). In agreement with Marshall et al. (2013), I decided to use a nonlinear approach to estimate p_{crit} from pO_2-MO_2 data. Among the different nonlinear methods developed to calculate p_{crit}, the greatest difference method, proposed by Mueller and Seymour (2011), was chosen because of its more user-friendly approach that is prone to fewer errors (Mueller & Seymour, 2011). In accordance with Mueller and Seymour (2011), as a preliminary step before applying the greatest difference method, MO_2-pO_2 data were fitted with different mathematical models (i.e. linear and nonlinear models) and the (nonlinear) natural logarithm model was chosen because it gave the best fit (in terms of R^2 values).

As reported in the literature, p_{crit} is directly dependent on temperature (Herreid, 1980), with higher critical values at higher temperatures (Dupont-Prinet et al., 2013). For the studied population of *P. varians*, a p_{crit} value of 4.55 kPa at 22 ˚C was obtained. Previously Nielsen and Hagerman (1998) calculated a p_{crit} value of 6.56 ±0.6 kPa at 24 ˚C on a population of *P. varians* originating from the Roskilde Fjord area, Denmark, whereas Hagerman and Uglow (1984) had previously reported a critical value of 2.0 ±0.6 kPa at 20 ˚C for the same Danish population. Results from this work and the other studies were plotted in Figure 2.9. In agreement with Herreid (1980), an almost perfect linear trend could be fit between this work and the two published studies, and gave and additional proof of the accuracy of the critical oxygen value calculated at 22 ˚C in this work using the greatest difference method.

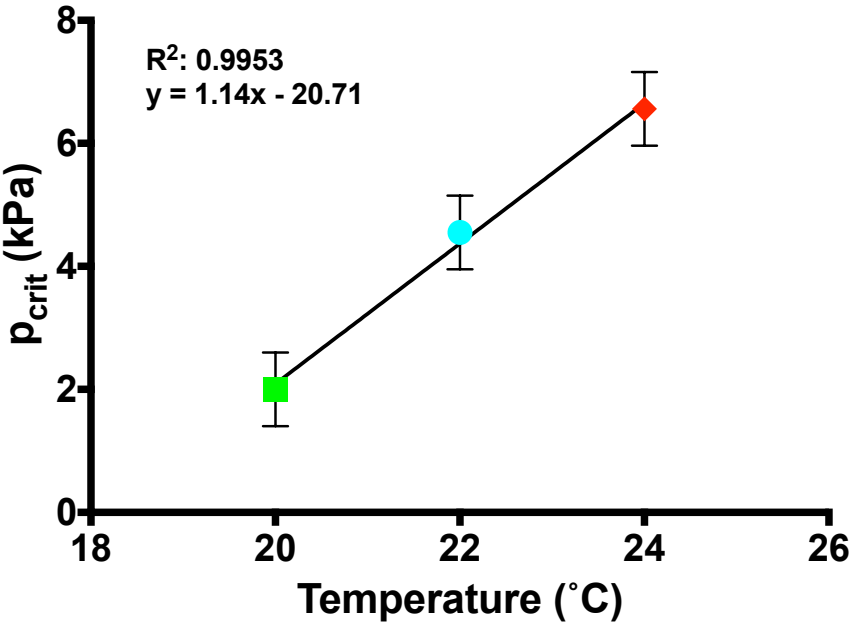

Figure 2.9: P_{crit}'s dependence on temperature. Graph shows results from three studies where *P. varians*' p_{crit} was calculated: 20 ˚C (green box, data from Hagerman and Uglow (1984)); 22 ˚C (blue circle, this work); 24 ˚C (red diamond, data from Nielsen and Hagerman (1998)). An almost perfect linear trend could be fit between the different data points, supporting the dependence of p_{crit} on temperature, as postulated by Herreid (1980).

By comparing the obtained p_{crit} value (and the other p_{crit} values from Figure 2.9) with pO_2 values recorded from the field in August (Fig. 2.5), it was estimated that hypoxic conditions were developing every night in the sampling site from a minimum duration of 2-hours to a maximum of 6 hours. This remark would support the previous observation (paragraph 2.5.1) in relation to the temporary relocation of the shrimps away from hypoxic zones; in fact, it could be argued that, whenever hypoxic conditions develop, it is more convenient (i.e. less energetically expensive) for the animals to "migrate" than to experience hypoxic stress, if relocating is possible, in agreement with Gamble (1971).

In the scientific community there is a general consensus that the p_{crit} is a proxy for hypoxia tolerance, with hypoxia tolerant species having lower p_{crit} (Herreid, 1980; Childress & Seibel, 1998; Chapman & McKenzie, 2009; Mueller & Seymour, 2011; Seibel, 2011) and living in environments that frequently incur in pO_2 fluctuations (Taylor & Spicer, 1987). In example, a p_{crit} of 0.5 kPa was obtained for the squat lobster *Pleuroncodes monodon* (Kiko et al., 2015), living in the Oxygen Minimum Zone off Peru (for comparison, this value is reported in Table 2.4 together with p_{crit} values for other decapod crustaceans taken from the literature). The low p_{crit} value of *P. varians* compared to the other members of the Palaemonid family supports the hypothesis of p_{crit} as a proxy

for hypoxia tolerance. In fact, within the Palaemonid family, *P. varians* possesses a lower p_{crit}, in comparison to the congeneric species *P. serratus*, *P. adspersus* and *P. longirostris* (which are all found in subtidal or estuary habitats characterized by a smaller degree of variability (Taylor & Spicer, 1989; Taylor, 1990; Nielsen & Hagerman, 1998)), while *P. varians*, *P. elegans* and *P. pugio* all have a similar p_{crit} value (being commonly found in rock pools (Morris & Taylor, 1985) or salt marshes (Welsh, 1975; Aguzzi et al., 2005).

Table 2.4: Comparison of p_{crit} values from the literature for some decapod crustaceans.

Species	**p_{crit} (kPa)**	**Temperature (˚C)**	**Salinity (PSU)**	**Reference**
Carcinus maenas	7.8-10.4	15	NA	(Taylor, 1976)
Cancer pagurus	7.9-10.4	10	32	(Bradford & Taylor, 1982)
Nephrops norvegicus	5.2	10	31	(Hagerman & Uglow, 1985)
Palaemon elegans	2	10	NA	(Morris & Taylor, 1985)
P. adspersus	7.7	24	NA	(Nielsen & Hagerman, 1998)
P. serratus	8	10	32	(Taylor & Spicer, 1989)
P. longirostris	6	10	32	(Taylor & Spicer, 1989)
P. varians	4.55	22	32	This work
Palaemonetes pugio	5.3	30	25	(Cochran & Burnett, 1996)
Munida rugosa	6.6	10	NA	(Zainal, Taylor, & Atkinson, 1992)
M. sarsi	7.4	10	NA	(Zainal et al., 1992)
Lithodes santolla	4.0-9.0	12	31	(Paschke et al., 2010)
P. monodon	0.5	13	NA	(Kiko et al., 2015)
Pandalus borealis	F: 4.8	8	28	(Dupont-Prinet et al., 2013)
P. borealis	M: 2.9	8	28	(Dupont-Prinet et al., 2013)

From a physiological point of view p_{crit} is important because significant physiological modifications, such as the onset of anaerobic metabolism, happen when this threshold is reached (e.g. in ovigerous *P. pugio*, cardiac output is no longer maintained when p_{crit} is reached and a redistribution of haemolymph across the body is observed

(Guadagnoli & Reiber, 2005)). Nielsen and Hagerman (1998) measured lactate content in haemolymph of *P. varians* and observed how lactate content increased up to 4-hour from the beginning of hypoxia and then sharply decreased. In contrast, I observed that lactate content in muscle tissue increased up to 6-hour before declining (Fig. 2.7). Even if it is plausible that the different tissues analysed were responsible for the different results, it is also possible that the change might be ascribed to the pO_2 levels used in the experiments, in accordance with Taylor and Spicer (1987). In the present work a pO_2 of ~3 kPa was used, whereas Nielsen and Hagerman (1998) tested water pO_2 of 0.66 kPa, well below the critical oxygen value of 6.55 kPa (at 24 ˚C). Hence, it could be argued that in this latter condition the severity of hypoxia negatively affected the capacity of the body to sustain anaerobic metabolism, since the availability of anaerobic substrates limits the duration of survival and the usage of anaerobic metabolism is proportional to the severity of hypoxia, as suggested by Richards (2011) and Taylor and Spicer (1987).

Another physiological consequence of exposure to hypoxic conditions below p_{crit} is the so-called "oxygen debt", when MO_2 is increased to restore normal homeostasis of the body after stressful conditions (Herreid, 1980). The higher MO_2 after 6-hours of hypoxic exposure, concurrent with the peak in lactate content, indicated that oxygen debt is only appreciable when lactate is approaching the highest concentrations within the tissues. Therefore, it could be hypothesised that *P. varians* is able to manage modest rises in lactate content without significantly altering its oxygen consumption rates. While Bridges and Brand (1980) reported how in some crustacean species oxygen debt is dependent on the length and severity of the hypoxic period, evidence seems to support a wide variety in this physiological response among species (Taylor, 1982; Zou, Du, & Lai, 1996). In fact, the lack of a statistical difference in MO_2 between hypoxic and normoxic animals after 8-hour (Fig. 2.7) is in agreement with Zou et al. (1996), which reported that the oxygen debt is not directly proportional to the duration of hypoxia.

As previously discussed, it is possible that within the Lymington salt marshes hypoxic conditions ($pO_2 < p_{crit}$) might currently develop for up to 6-hours during the nights in August. As shown in the laboratory, while such exposure would not lead to mortality in the animals, it would cause a significant increase in lactate content in the muscle and would trigger an oxygen debt in the animals. Given the periodicity in pO_2 fluctuations within the marsh (Fig. 2.5), it is possible that animals in the field are forced to similar hypoxic conditions for several consecutive nights. Therefore, important questions rise in relation to the physiological consequences of these exposures and will be addressed in the next chapters.

Overall, results presented in this section had a double relevance: i) they assessed how, exposing adults to water pO2 < p_{crit} (at 22 ˚C, 4.55 kPa) and its duration (up to 8-hours) were able to trigger a physiological response in the animals without causing mortality (no mortality was recorded in the short hypoxic exposure experiment). In fact, the assessment of a physiological response (i.e. anaerobic metabolism) was a condition in order to verify the accuracy of the p_{crit} calculated at 22 ˚C; ii) they assessed the efficacy of the flow-through experimental system in housing the animals and in realising the desired hypoxic conditions ($pO_2 < p_{crit}$), a necessary requirement for the experimental work presented in the next chapters where the effects of prolonged, daily cyclic hypoxia on the physiology of the animals were tested using this experimental system.

2.6 Conclusions:

Results presented here show how temperature and pO_2 are highly variable within the Lymington salt marsh ecosystem on a seasonal and diurnal scale, with amplitude of pO_2 oscillations being smaller during winter and greatly increased in late spring and late summer.

Data presented here underline how the critical oxygen value determined at 22˚C is able to trigger, in the short term, whole body physiological responses identified here as an increase in MO_2 and accumulation of lactate in the muscle. Even if the exposure of animals for 8-hours to $pO_2 \leq p_{crit}$ did not cause mortality, the observed physiological changes can be seen as stress responses typically associated with short term hypoxic exposures at a sub-lethal level associated with the involvement of anaerobic metabolism (i.e. lactate is the end product of lactic fermentation when oxygen is not available for cellular respiration).

Although our knowledge of the response of aquatic organisms to chronic hypoxia has steadily increased, surprisingly little is known about the mechanisms of adaptation of estuarine and coastal organisms to cyclic hypoxia (Brown-Peterson et al., 2008). In this context, the exposure of animals to short term cyclic hypoxia (7-hours in hypoxia followed by 17-hours in normoxia) for long periods of time, in a similar fashion to what was currently measured in Lymington, will allow the characterization of the mechanisms of adaptation (Chapter 3) to cyclic hypoxia in *P. varians* and of the degree of impairment on the major physiological processes (Chapter 4) of this decapod shrimp.

Chapter 3 Cyclic hypoxia accelerates the moult cycle in the Atlantic ditch shrimp *Palaemon varians* and induces changes in gill morphology

Summary:

While the acute effects of hypoxia are well characterized, the physiological adaptations initiated by long-term cyclic hypoxia are still not established. To gain mechanistic insight on how environmental hypoxia scales through physiology to produce ecosystem effects, adult *P. varians* were exposed to oxygen fluctuations down to their critical oxygen partial pressure (p_{crit}) for 7-hour intervals each night for up to 18-days, mimicking field conditions. Using Illumina RNA sequencing, changes in the expression of cuticular transcripts (i.e. cuticular proteins, Post-moult protein - *PMP*, Calcification associated peptide - *CaAP*, and peptide DD5 - *DD5*) were identified. These transcript data were supported by observations of an altered phenotype: hypoxic-exposed shrimp demonstrated a 15% reduction in the length of the inter-moult period and showed that the overall expression pattern (from ecdysis to 16 days after) of the cuticular markers *PMP* and *DD5* was accelerated in hypoxia-exposed shrimps. Further, morphological changes to the gills of hypoxic exposed animals were observed. These changes induced significant increases in gill lamellar length and surface area compared to normoxic shrimps (on average, 13.6% longer and larger lamellae in hypoxic shrimps). Collectively results clearly show how long-term cyclic hypoxia is able to initiate specific physiological responses in crustaceans, underlining the extent to which the respiratory limitation induced by hypoxia triggers a morphological change in a decapod crustacean.

3.1 Introduction:

As previously discussed (Chapter 2), salt marshes and other coastal environments can be subjected to diel fluctuations in water pO_2 and species living in these environments are likely to experience cyclic hypoxic conditions on a daily basis. An acute hypoxic exposure below p_{crit} was able to trigger metabolic responses (i.e. an increase in lactate content and oxygen consumption rates, Chapter 2) in the shrimp *Palaemon varians* but, on the long term, other physiological responses will likely initiate as a consequence of cyclic hypoxia.

As shown by Greenberg and Ar (1996) and Callier and Nijhout (2011), insect's larvae (e.g. the beetle *Tenebrio molitor*, the moth *Manduca sexta*) reared in hypoxic conditions exhibit an acceleration of the moult cycle and have smaller body weight in comparison to larvae reared in normoxia. Moult is an essential process for all arthropods: with this process animals shed the old exoskeleton and develop a new exoskeleton in which they can grow inside (Tom et al., 2014). Because it is covered by the rigid exoskeleton, the tracheal respiratory system of insects grows primarily at moults (Callier & Nijhout, 2013; Kivelä, Lehmann, & Gotthard, 2016), whereas tissue mass increases quite a lot between moults (and so does oxygen demand) (Kivelä et al., 2016). Hypoxic conditions can reduce oxygen supply to the detriment of oxygen demand (Massabuau & Abele, 2012; Ern et al., 2016). In this case insects' larvae can accelerate moult cycle to allow the development of a larger tracheal system able to deliver more oxygen to the tissue and sustain oxygen demand of the body (Callier & Nijhout, 2011, 2013). Insects and crustaceans possess the same generalised structural body plan (i.e. bauplan) (Roer, Abehsera, & Sagi, 2015): body and gills are covered by a rigid exoskeleton which is periodically replaced. Therefore it can be hypothesised that a similar acceleration of the moult cycle coupled with the development of larger gills will be observed in crustaceans exposed to hypoxia.

Partial evidence in support for this hypothesis was given by Brown-Peterson et al. (2008): while studying the effects of a 14 days exposure to cyclic hypoxia on gene expression of the grass shrimp *Palaemonetes pugio*, they reported the up-regulation of cuticular proteins (the major component of the exoskeleton, (Roer et al., 2015)), which might indicate an activation of the moult cycle (Brown-Peterson et al., 2008). Interestingly this up-regulation of cuticular proteins was specific to cyclic hypoxia: in fact, it was not observed as a consequence of chronic hypoxia in *P. pugio* exposed to moderate (7.0 kPa)

or severe (4.2 kPa) chronic hypoxia for 14 days (Brouwer et al., 2007; Brown-Peterson et al., 2008) or in other studies involving chronic hypoxia (Brouwer et al., 2004; Li & Brouwer, 2009b, 2009a; Lai et al., 2016).

While a vast literature has elucidated the molecular, physiological and behavioural mechanisms that fish and invertebrates use to cope with chronic and long-term hypoxia (Chapter 1, Sections 1.5 and 1.8.2) (Gamble, 1971; Herreid, 1980; Wood & Shelton, 1980; Childress & Seibel, 1998; Wannamaker & Rice, 2000; McMahon, 2001; Gorr et al., 2004; Seibel, 2011), the physiological adaptations initiated by long-term cyclic hypoxia are still not established especially when animals are cyclically exposed below their critical oxygen level (p_{crit}). In this Chapter, in order to understand how the impact of prolonged cyclic hypoxic exposure scales through physiology to produce whole-organism effects, adult *P. varians* were exposed to daily cycles of hypoxia (water pO_2 < species' p_{crit}) of seven hours per day for up to 18 days and physiological responses were investigated at different levels of biological organisation. Initially, I observed changes in the transcriptome of the cephalothorax. Then, at the phenotype level, I investigated changes in the length of the inter-moult period and, at molecular level, I examined the gene expression pattern of two cuticular markers throughout an entire moult cycle. Finally changes in gill morphology were studied. Results clearly showed hitherto unreported consequence of cyclic hypoxia in crustaceans, underlining the extent to which a physiological limitation triggers a morphological change in a decapod crustacean.

3.2 Specific chapter hypothesis:

1. The exposure of adult *P. varians* to cyclic hypoxia for 7 days will induce changes in the transcriptome of the animals.
2. An acceleration of the moult cycle will be observed in adults exposed to cyclic hypoxia from the day of moult (i.e. day of ecdysis).
3. Larger gills will develop in animals kept for the entire duration of one moult cycle in cyclic hypoxia.

3.3 Materials and Methods:

3.3.1 Sampling site, animal collection and maintenance

Adult *Palaemon varians* were net-caught in the Lymington salt marshes in June 2015 and 2016. Within one hour from collection, adult *P. varians* were recovered to the National Oceanography Centre Southampton inside 10L water buckets filled with water from the channel. Adults were kept in 150L aquaria with UV-sterilised and filtered seawater, filtration systems and air stones, and slowly acclimated (+1˚C/day) to the experimental temperature of 22˚C. Animals were fed three times/week with commercial shrimp granules (shrimp naturals – Sera, Germany) and water was changed once per week. One week before the experiment, animals were haphazardly distributed into the experimental tanks.

3.3.2 General experimental design

Experiments were performed using the custom flow-through experimental system described in Chapter 2, Section 2.3.3. Experimental temperature was set to 22 ˚C, based on the considerations drawn in Chapter 2, Section 2.4.1. Hypoxia was achieved by independently bubbling nitrogen into each 1L flask for 7 hours every night. Water pO_2 level was continuously measured with Microx TX 3 (PreSens) sensor calibrated following Oliphant et al. (2011). During hypoxia, water pO_2 level was set to be lower than the species' p_{crit} (at 22˚C: 4.55 ±0.63 kPa, as described in Chapter 2, Section 2.4.2, see Supplementary Table 1 – Appendix A for mean pO_2 values recorded during hypoxic periods in each tank). Between hypoxic periods, air was bubbled into the system to ensure 100% O_2 saturation (Fig. 3.1). An identical system was built for control animals and air was continuously bubbled into the water to ensure constant 100% O_2 saturation (normoxia).

No animal selection was made according to the stage of the moult cycle during the first experiment (when shrimp's cephalothorax was sampled for RNA-seq). In subsequent experiments investigating intermoult duration, changes in the expression of cuticular genes throughout an entire moult cycle and gill histology, a synchronous population (composed of animals that had all moulted within 12 hours) was used. In all experiments, initial

shrimp density was between 16 and 18 shrimps per tank and animals were sampled from different tanks in order to maintain a constant density in all the tanks.

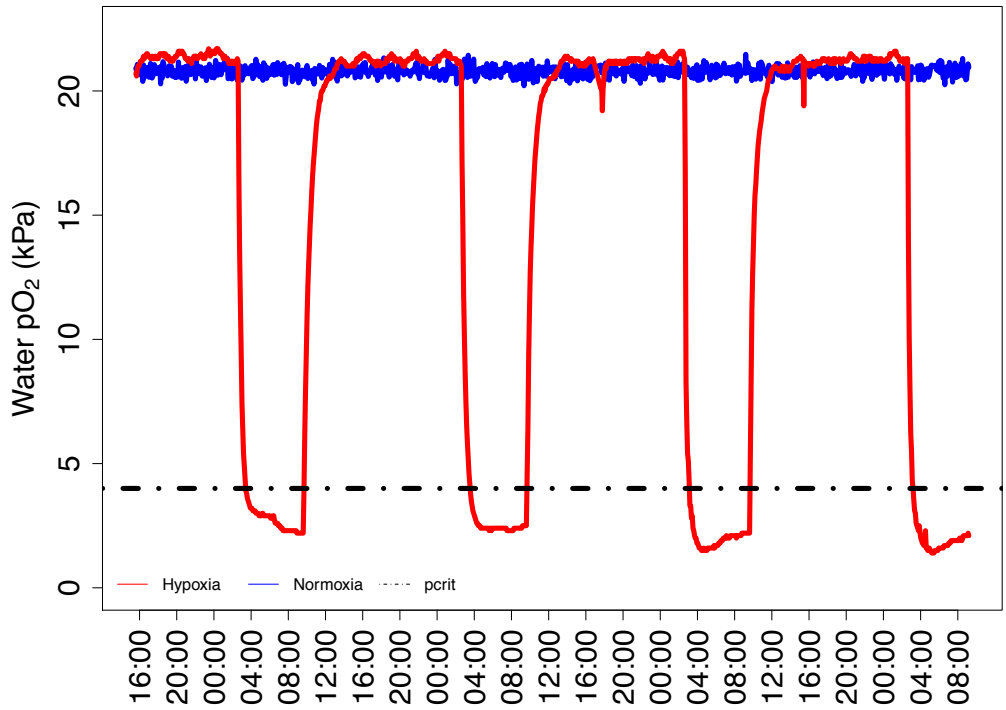

Figure 3.1: Schematic representation of normoxic (blue line) and daily cyclic hypoxic regime (red line) during all laboratory experiments. At 22˚C, 21 kPa ~ 100% air-saturation. Dash-dotted black line represents the critical pO_2 (4.55 kPa, ~21.6% air-saturation) for the studied population at 22˚C.

3.3.3 Transcriptome response to hypoxia

A pilot RNA-seq experiment was performed on cephalothorax of animals to identify genes putatively involved in tolerance of hypoxic conditions. Eight animals per treatment (normoxia and hypoxia) were randomly sampled after seven days of exposure to experimental conditions and cephalothorax snap frozen in liquid N_2. For this experiment no animal selection was made according to the stage of the moult cycle, therefore animals were not necessarily in the same moult stage.

Total RNA was extracted from whole cephalothorax using a TRI-Reagent™ (Sigma Aldrich) protocol according to the manufacturer's recommendations. RNA concentration was determined using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific). Experion™ (Bio-Rad, UK) electrophoresis was used to assess RNA integrity and only samples with RQI>9 were used. For both treatments (hypoxia and normoxia),

RNA from samples was pooled (i.e. two pools, hypoxia and normoxia, where created, each comprising RNA from eight samples). Library preparation followed Illumina TruSeq RNA Library Preparation Kit v2 (Illumina, California) according to the manufacturer's protocol. Paired-end 115bp reads were sequenced on a single lane of an Illumina HiSeq 2500 platform at the IBERS Translational Genomics facility, Aberystwyth University. Raw sequencing data was imported into the CLC Genomic Workbench v.8.5 (Qiagen) environment, where reads were trimmed to remove residual sequencing adapters, low quality bases and ambiguous nucleotides. The reference transcriptome for the subsequent gene expression study was obtained with the *de novo* assembly tool, using automatically set *bubble size* and *word size* parameters. The assembly was further refined by removing contigs displaying a very low sequencing coverage (<5 X), as these could be the results of excessive fragmentation of longer transcripts, mis-assembly or contamination from exogenous RNAs, altogether contributing to background noise, as suggested by Carniel et al. (2016). The quality of the assembled transcriptome was tested, in terms of completeness and integrity, with BUSCO v.3 (Simao et al., 2015), using the core set of metazoan single copy orthologous genes as a reference.

The RNA-seq analysis tool of the CLC Genomics Workbench was then used to calculate gene expression levels in the two samples. Trimmed reads were first mapped to the reference transcriptome with 0.75 and 0.98 as *length fraction* and *similarity fraction* parameters. To allow comparability of expression values between the two samples, read counts were normalized by totals, assuming a virtual number of 1 million reads per sample.

The reference transcriptome was annotated with the Trinotate pipeline (Grabherr et al., 2011). Briefly, the protein translation of each contig were predicted with TransDecoder, assuming a minimum ORF length of 100 codons. Contigs and virtually translated proteins were BLASTed against the UniprotKB database to detect significant homology with known sequences (based on an-e-value threshold of $1x10^{-5}$), and corresponding Gene Ontology annotations were subsequently extracted. At the same time, amino acidic sequences were screened with InteroProScan (Quevillon et al., 2005) to annotate conserved protein domains contained.

Statistically significant gene expression changes, both in terms of up-regulation and down-regulation between the hypoxia and control samples, were detected by the use of a Kal's Z-test (Kal et al., 1999). Contigs were considered as differentially regulated for proportion Fold Change values > 2 or < -2, supported by FDR-corrected p-values <= 0.05.

The subsets of up-regulated and down-regulated genes were separately subjected to hypergeometric tests (Falcon & Gentleman, 2007) on Gene Ontology and Pfam

annotations, to detect significantly over-represented terms which might be indicative of alterations of biological pathways underpinning the observed gene expression changes.

The expression of some up-regulated genes was further confirmed by means of quantitative-PCR analysis (following protocols described below in "Gene expression pattern" section). Statistical significance was identified at $P<0.05$ as determined by t-test after testing for normality (Shapiro test) and homogeneity of variances (Bartlett test) using R statistical software (R Core Team (2014)).

3.3.4 Changes in inter-moult duration

To quantify inter-moult duration in hypoxia and normoxia a synchronous population of animals (that had all moulted within 12 hours, in total n= 54 shrimps), that were previously acclimated at 22˚C in normoxic conditions, was used. On the first day of the new moult cycle, animals were individually tagged with coloured numbered tags (Queen bees marking kit – Abelo, UK), glued on the cephalothorax with cyanoacrylate glue (Fig. 3.3A). They were then randomly allocated to the hypoxic (n= 27 shrimps) or normoxic (n= 27 shrimps) treatment (hence they were exposed to either daily cyclic hypoxia or to normoxia) and maintained in experimental conditions until next ecdysis (when the tag was lost on exuviae). Thus, the number of days between two consecutive moults could be determined, as well as the cumulative frequency of moulted animals over time.

3.3.5 Gene expression of cuticular genes during moult cycle

To investigate changes in gene expression during the moult cycle, animals (n= 108 shrimps) were acclimated at 22 ˚C in normoxic conditions, similarly to animals from section 3.3.4. Each day, all moulted animals were isolated and maintained individually from the rest of the population and randomly allocated to the hypoxic (n= 54 shrimps, n= 18 shrimps/tank) or normoxic (n= 54 shrimps, n= 18 shrimps/tank) treatment (hence they were exposed to either daily cyclic hypoxia or to normoxia). Every other day, from the day of ecdysis (day 0) up to 16 days after this event (day 16), six animals were sampled per treatment and snap frozen in liquid N_2. Total RNA was extracted from whole cephalothorax and concentration and integrity assessed as above.

A volume containing 1.5 µg of total RNA was treated with Promega RQ1 RNase-free DNase (Promega Corporation, Hants, UK) according to the manufacturer's protocol. Total

RNA (0.68µg) was reverse transcribed in a 20µl reaction using Superscript III reverse transcriptase (Invitrogen, UK) and oligo(dT)$_{20}$ primers.

All qPCR reactions were performed on a LightCycler 96 (Roche, Switzerland). Each 25 µl reaction contained 12.5 µl of Precision Plus 2× qPCR Master mix (Primer-Design, UK) with SYBR green, and 1 µl of template cDNA. qPCR conditions were: 1 cycle of 95°C for 5 min, 40 cycles of [95°C 10 s, 60°C 1 min], followed by 72˚C for 45 sec. Each reaction was run in duplicate (technical replicate) with the addition of three inter-run calibrators in each 96-plate. After each run a melt curve analysis was performed in order to demonstrate the specificity of the qPCR products.

Primer-sets used are reported in Table 3.1. All primer-sets tested generated a single and discrete peak by melt curve analysis. As specified by the MIQE guidelines (Bustin, 2010), all primer-sets had an efficiency of between 90-105% and linearity greater than r^2=0.98 across four 10-fold serial dilutions.

Table 3.1: List of primes used for quantitative-PCR

ID primer	**Reference contigs**	**Final conc (nM)**	**5'-3' sequence**	**Amplicon size**
PMP FOR	contig_16319	300	AATTCAGCAGCCCAAAGTGG	113
PMP REV		300	CAGGCAGACATGAACTCAGC	
DD5 FOR	contig_23547	300	ACACTATGCATTCGTGGCTG	116
DD5 REV		300	CAGGAACTGGAGGTCCAACA	
CaAP FOR	contig_10413	300	ATCGTGGACTTCGAGTTGGA	106
CaAP REV		300	AATACTCGTTGCCGTCAGGT	
EF1-alfa FOR	contig_41	300	ACAGCACTGAGCCCAAGTAT	115
EF1-alfa REV		300	GAAATGGGAAGGATTGGCACA	
RPL8 FOR	contig_226	900	TCCCGGTCGTGGTGCACCTATT	179
RPL8 REV		900	GACGGCCTCGGTCACCAGTCTTT	

Elongation factor 1-alfa gene (*eef1A*) and ribosomal protein L8 gene (*rpl8*) were used as reference genes in all experiments after assessing their stability as housekeeping genes with qBase+ software (Biogazelle, UK). Indications from the transcriptome (on up-regulated contigs in relation to hypoxia) were used to select three cuticular markers: post-

moult protein - *PMP* (Roer et al., 2015), calcification-associated peptide - *CaAP* (Inoue et al., 2004), and peptide DD5 - *DD5* (Ikeya et al., 2001). All three marker genes were selected because they are highly expressed by the epithelium during the post-moult phase of the moult cycle and contribute to the deposition and calcification of the newly formed exoskeleton (Ikeya et al., 2001; Inoue et al., 2004; Roer et al., 2015)). Their expression throughout an entire moult cycle was then characterized.

After testing for stability using with geNorm analysis using qBase+ software, the geometric mean of the two reference genes was used to normalise gene of interest expression. Calibrated, normalised relative quantities (CNRQs) were calculated using qBase+ software.

To test whether the overall pattern of expression of target genes was different between treatments, a general additive model (GAM) in R (Team, 2014) was run for each gene, using package 'mgcv' (Wood, 2006). For each gene, one GAM model fitting separately GE response in hypoxia and normoxia was compared with a simpler GAM model fitting together all data points (independently from the treatment). For each gene, "Akaike's Information Criterion" – AIC – values (Sakamoto, Ishiguro, & Kitagawa, 1986) between models (the model with treatments and the model without) were compared. A difference in the 2-9 range between AIC values of models was considered significant and the model with lower AIC was chosen (Burnham, Anderson, & Huyvaert, 2011). For each gene, when the "complex" GAM model (with a distinct fit for GE response in hypoxia and normoxia) was statistically preferred over the simpler model, the rate of change in gene expression was additionally determined. Rate of change (Δ CNRQ over time) was determined by calculating the first derivative of the GAM model in hypoxia and and the GAM model in normoxia over time.

3.3.6 Changes to phenotype in *Palaemon varians*: gill modification in response to cyclic hypoxia

Animals (n= 10) were acclimated at 22 ˚C in normoxic conditions, similarly to animals from section 3.3.4. Daily, all moulted animals were isolated and maintained individually from the rest of the population, randomly allocated to the hypoxic (n= 5 shrimps) or normoxic (n= 5 shrimps) treatment (hence they were exposed to either daily cyclic hypoxia or to normoxia) and maintained for 18 days to make sure they completed one entire moult cycle in experimental conditions.

Prior to fixation the wet weight and total length of each individual was recorded. Animals were chilled on ice and the cephalothorax dissected with a single transverse cut between thorax and abdomen. The cephalothorax was fixed in Bouin's solution (BDH Gurr) for 24 hours at room temperature. Samples were dehydrated in a graded ethanol series (50%, 70%, 90%, 100% and 100% anhydrous ethanol) for 24 hours at each stage, cleared in xylene and xylene-paraffin for eight and twelve hours respectively, and then embedded in paraffin wax.

Longitudinal, 5-µm sections of gill were sectioned from each sample (n=5 animals per treatment) using a rotary microtome (Leitz Wetzler, model 1212), mounted on glass slides and stained with haematoxylin and eosin (Cellpath Ltd). All microscope analysis was carried out using an Olympus BH-2-RFCA research microscope fitted with with a Nikon Coolpix E4500 microscope camera. Images were analysed using ImageJ software (Schneider, Rasband, & Eliceiri, 2012). For each sample, longitudinal sections of the body produced transverse sections of gill-plates (or lamellae (Sun et al., 2015)). Lamellae from the sixth gill of each animal were analysed: the best-fitting ellipse was determined using the "fit ellipse" function in ImageJ, which returns major axis (lamellar length) and minor axis (lamellar thickness) of the ellipse. Lamellar perimeter (lamellar surface area) was calculated with the formula:

$$2*\pi* \sqrt{[(MA/2)^2 + (ma/2)^2 /2]}$$

where "MA" is the major axis and "ma" the minor axis. Lamellar density was calculated measuring the space between 15 consecutive lamellae, divided by the number of lamellae. For each parameter, mean values throughout the thickness of the gill were calculated and then normalized using the individual's wet weight. Statistical difference between controls and hypoxic gills was assessed using t-test, with a cut-off p-value <0.05.

3.4 Results:

3.4.1 Transcriptome response to hypoxia

A pilot RNA-seq experiment was performed to identify genes putatively involved in tolerance of hypoxic conditions. Pooled libraries generated from hypoxic and normoxic

treatments (n=8 animals for each), returned 31,461,523 and 25,253,636 raw reads, respectively. The raw Illumina reads were deposited at the NCBI Sequence Read Archive (accessions SRX2894799, SRX2894801), as a part of the BioProject PRJNA389547. The complete *de novo* assembled transcriptome generated from trimmed sequencing data contained 105,325 contigs, which were subsequently reduced to 59,370 reference sequences upon the removal of poorly covered fragments (Supplemental table 2). This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFPG00000000. The version described in this Chapter is the first version, GFPG01000000.

The completeness and integrity of the reference transcriptome were evaluated with BUSCO, which revealed that the large majority (91%) of the benchmarking single copy orthologous genes conserved across all metazoans were present as full-length transcripts. Only 5% of the transcripts were absent and 4% were fragmented. Overall, this highlights the high completeness and integrity of the assembled transcriptome compared to the expected complement of protein-coding transcripts from the *P. varians* genome. The relatively low fraction of missing transcripts is likely to correspond to developmentally regulated genes, unlikely to be expressed in adult individuals, and other tightly regulated sequences which are only expressed in particular situations or life stages.

Differential expression analysis identified a total of 399 differentially expressed contigs (214 up-regulated and 187 down-regulated) in the day 7 transcriptome. As evidenced by the annotation enrichment analyses, overall the transcriptome was dominated by genes involved with cuticle synthesis (Supplementary table 3): among the annotated up-regulated contigs, 45% were identified as cuticular proteins of crustaceans. A further 9% were identified as proteins highly expressed in the post-moult phase of crustaceans, involved in deposition and calcification of the newly formed exoskeleton (i.e. post-moult protein (*PMP*), calcification-associated peptide (*CaAP*), peptides *M28* and *DD5* (Ikeya et al., 2001; Inoue et al., 2004; Roer et al., 2015; Tynyakov et al., 2015)) and 5% were involved in calcium deposition/reabsorption (Pinoni & Mananes, 2004; Glazer et al., 2015). In addition, the up-regulation of three contigs homologous to important metabolic enzymes was identified: glucose-6-phosphate transporter (*G6PT*), representing the rate-limiting step for G6P hydrolysis into glucose (Lord-Dufour et al., 2009); gamma-butyrobetaine dioxygenase (*GBBH*), responsible for carnitine biosynthesis and therefore lipid metabolism (Rebouche, 1982); phosphoenolpyruvate carboxykinase (*PEPCK*), involved in gluconeogenesis (Schein et al., 2005).

Among the down-regulated transcripts, 12% of the contigs were chitinase enzymes, involved in chitin degradation (Abehsera et al., 2015) and a down-regulation of a vitellogenin transcript was observed. Similar results were obtained from hypergeometric tests on annotations, as extracellular processes involving cuticle and chitin-binding processes clearly emerged as over-represented among the up-regulated genes, in contrast with chitinase activity, which was the dominating annotation among the down regulated (Supplementary table 4).

Quantitative PCR confirmed the up-regulation of those post-moult cuticular genes from the transcriptome data (Fig. 3.2). Contigs coding for *PMP, CaAP* and *DD5* in hypoxia were all up-regulated according to qPCR data (~246, 31 and 15-fold, respectively in comparison to normoxia, whereas fold change calculated from RNA-seq was 189, 5.3 and 4.7, respectively).

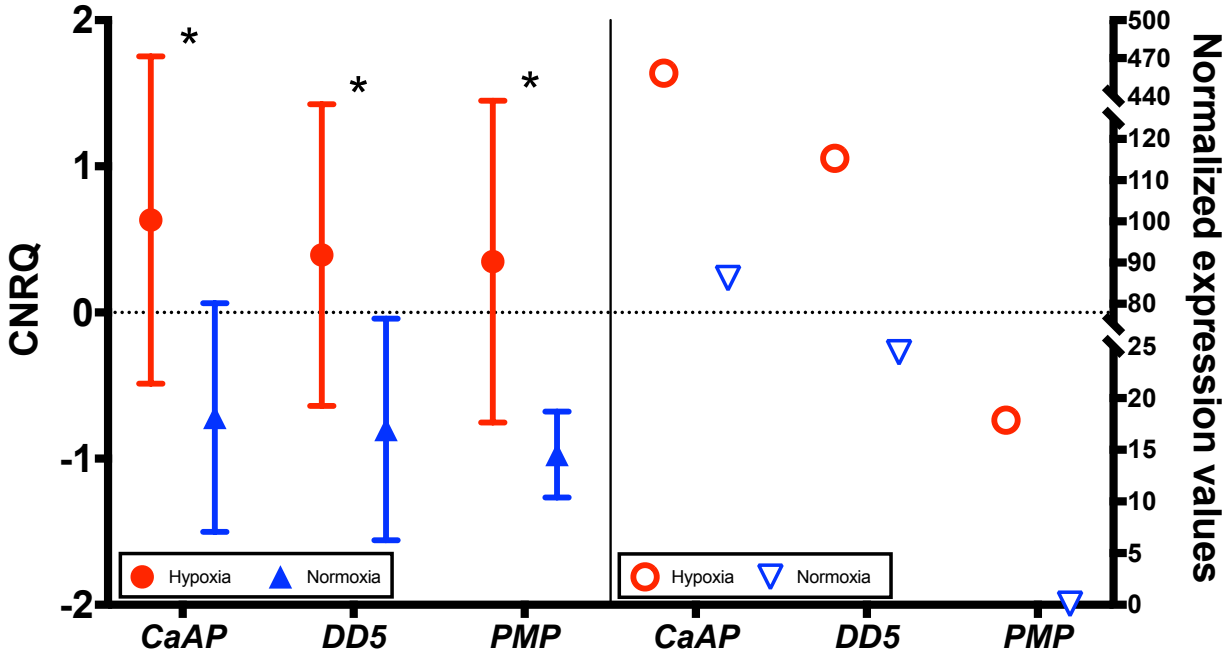

Figure 3.2: Normalized expression values of three cuticular contigs (right) differentially expressed from the RNA-seq experiment. Proportional fold change for *CaAP*, *DD5*, *PMP* was 5.3, 4.7 and 189, respectively. Real-time PCR (left) of the same cuticular genes differentially expressed from the RNA-seq experiment. Gene expression (on a $\log_{10}$ scale) is normalized to multiple reference genes (*EF1-alfa* and *RPL8*), giving calibrated, normalized, relative quantities (CNRQ) ± Standard deviation, SD. Significance values between treatments were determined by the parametric t-test (* $P < 0.05$, n=7 for each treatment). *CaAP*: calcification associated peptide; *DD5*: peptide DD5; *PMP*: post-moult protein.

3.4.2 Changes in inter-moult duration

In order to validate the causal relationship between hypoxia and moulting, the duration of *P. varians* intermoult period was quantified in cyclic hypoxia and control conditions by tagging each animal (see Fig. 3.3A). The frequency distribution of moulted animals in each treatment and the intermoult duration times are shown in Fig. 3.3B and C, respectively. Moulting frequency distributions among treatments statistically differed (Wilcoxon rank sum test W=239, p-value= 0.03), with the frequency distribution in hypoxia being shifted to the left (anticipated) in comparison to normoxia. In addition, median intermoult duration times (hypoxia 12 and normoxia 14 days) were statistically different (Mann-Whitney test, U=239, p-value= 0.03, n=54). Jefferies (1964) reported an intermoult duration of 14.6 days at 20 ˚C, and the results are concordant with our normoxic group. The hypoxic treatment, though, showed an accelerated moult cycle, with median intermoult time 15% shorter than normoxic animals.

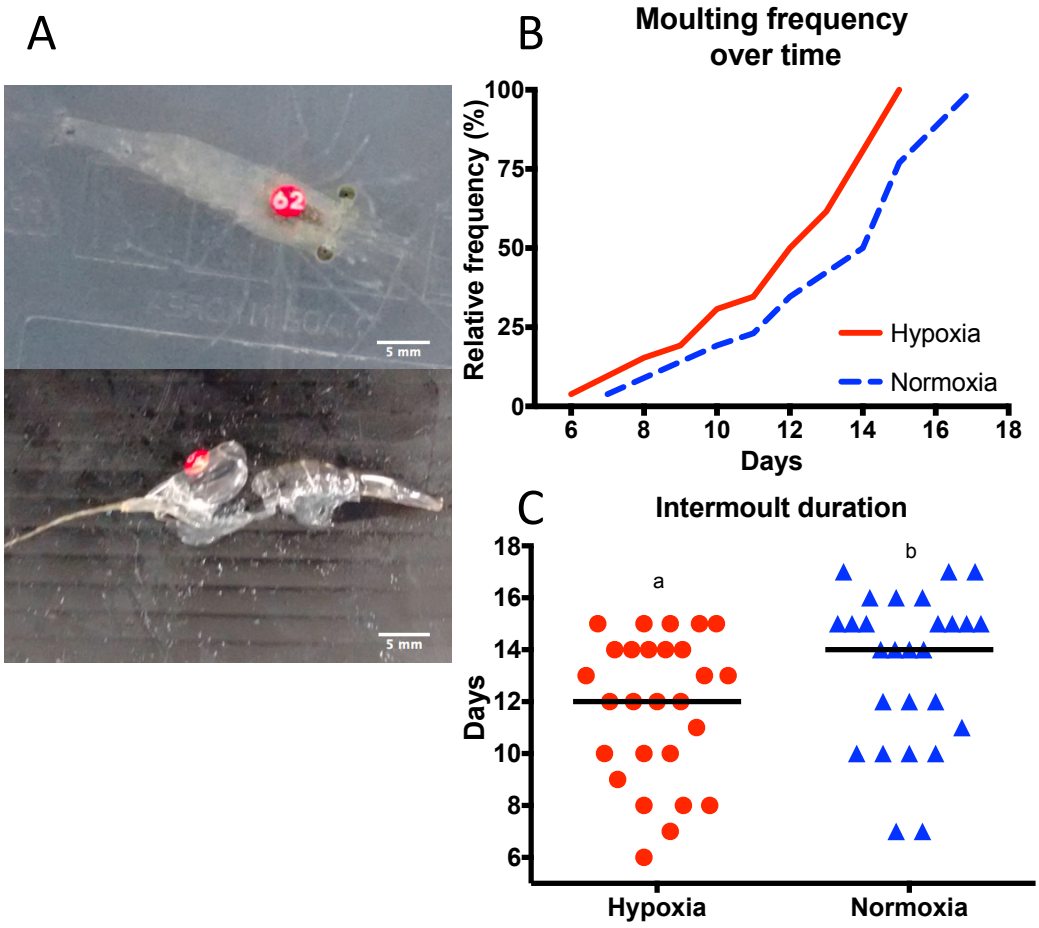

Figure 3.3: **A)** Tagged animal (up) and exuviae still carrying an old tag (bottom). **B)** Frequency of moulted animals over time within the two treatments (hypoxia n=26; controls n=28). **C)** Scatter plot showing intermoult duration (days) in the two treatments. Horizontal line represents the median for each treatment.

Different letters indicate significance values between treatments (non-parametric Mann-Whitney test, $P<0.05$, $n=54$).

3.4.3 Gene expression of cuticular genes during moult cycle

In the experiment of moulting synchrony, the expression levels of the post-moult cuticular markers *PMP, CaAP* and *DD5* were quantified on alternate days from ecdysis (day 0) to day 16 (Fig. 3.4) in normoxic and cyclic hypoxic conditions. All three markers were selected because they are highly expressed by the epithelium during the post-moult phase of the moult cycle and contribute to the deposition and calcification of the newly formed exoskeleton (Ikeya et al., 2001; Inoue et al., 2004; Roer et al., 2015)). Their expression in the epithelium is cyclic, with a peak in expression during post-moult phase (Luquet & Marin, 2004; Roer et al., 2015). As can be seen, expression levels of *PMP, CaAP* and *DD5* were highest in the post-moult phase (just after ecdysis on day 0) in both treatments (day 0, Fig. 3.4). They then gradually decreased during post-moult and intermoult up to day 6-8, whereafter expression increased again up to day 12-14. To test whether the overall pattern of expression was different between treatments, a general additive model (GAM) was run for each gene (Table 3.2). Comparison of AIC showed that for *PMP* and *DD5* but not *CaAP* the model fitting independently the treatments (hypoxia and normoxia) was statistically preferred over the simpler model fitting all data points together (without treatments) (ΔAIC_{PMP}: 8.6; ΔAIC_{DD5}: 3.2). Hence for *PMP* and *DD5*, overall pattern of expression differed between hypoxic and normoxic animals. To better appreciate those differences in pattern between treatments, from the GAM model we derived the rate of change in gene expression (Δ CNRQ, Fig. 3.5). For any time-point, a negative rate of change ($CNRQ_{day\ n} - CNRQ_{day\ n-1}$) indicated a decreased expression level, whilst a constant rate of change over two or more days indicated no variation in gene expression levels over the same interval time. Overall, the expression was more sensitive in hypoxia compared to normoxia. Rate of change in hypoxic animals increased more steeply (up to day 9) and decreased more sharply (from day 11) than in controls. In addition, in normoxic animals, rate of change of *PMP* was almost null from day 9, indicating almost no variation in gene expression levels (Fig. 3.4 and 3.5).

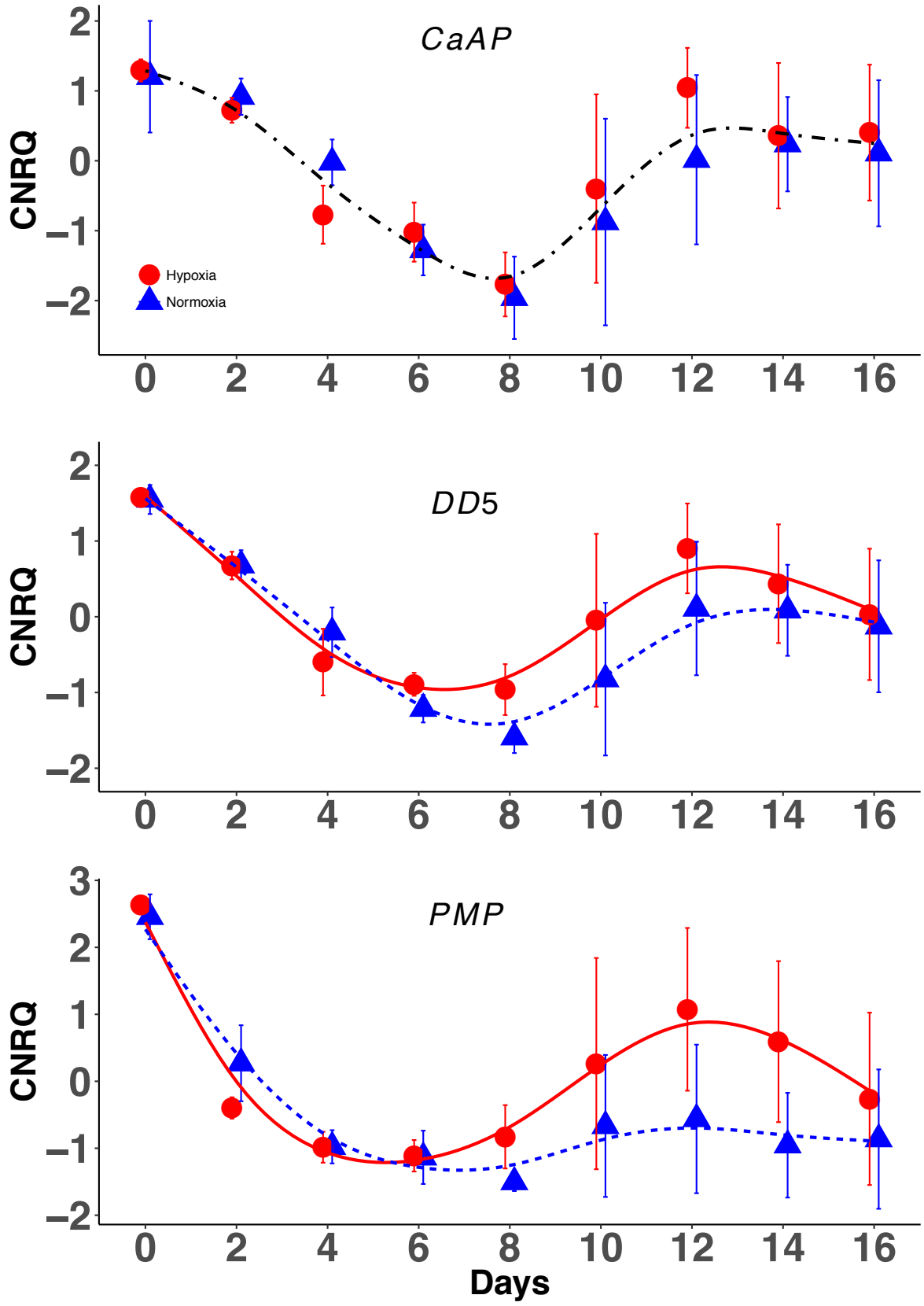

Figure 3.4: Expression of GOIs at different time points from ecdysis (day 0) to 16 days after, during the cyclic hypoxic experiment, in different treatments (n=6 for each treatment for each time point). Expression (on a $\log_{10}$ scale), normalized to two reference genes (*EF1-alfa* and *RPL8*), giving calibrated, normalized, relative quantities (CNRQ) ± Standard deviation, SD. For *DD5* and *PMP*, plotted lines represent GAM models fitted for each treatment (solid: hypoxia, dashed: normoxia) with the formula: Y~smooth(X,

by=treatment). For *CaAP*, dot-dashed line represents a GAM model fitted without treatments, with the formula: Y~smooth(X).

Table 3.2: Descriptive statistics of GAM models used. Model 1: Y~ smooth (X); Model 2: Y~smooth(X,by=treatment). To compare overall gene expression pattern, for each gene, a GAM model fitting independently gene expression data ("X") for each treatment – Model 2 – was compared with one simpler GAM model fitting all gene expression data, regardless of the treatments – Model 1. Akaike's "An Information Criterion" – AIC – values between models were compared and the model with lower AIC was chosen. "df" represent the number of parameters in the model.

	***CaAP*:**		***DD5***		***PMP***	
	df	**AIC**	**df**	**AIC**	**df**	**AIC**
Model 1:	7.64	267.18	7.10	209.14	7.06	293.97
Model 2:	12.26	269.22	11.31	205.85	10.85	285.30

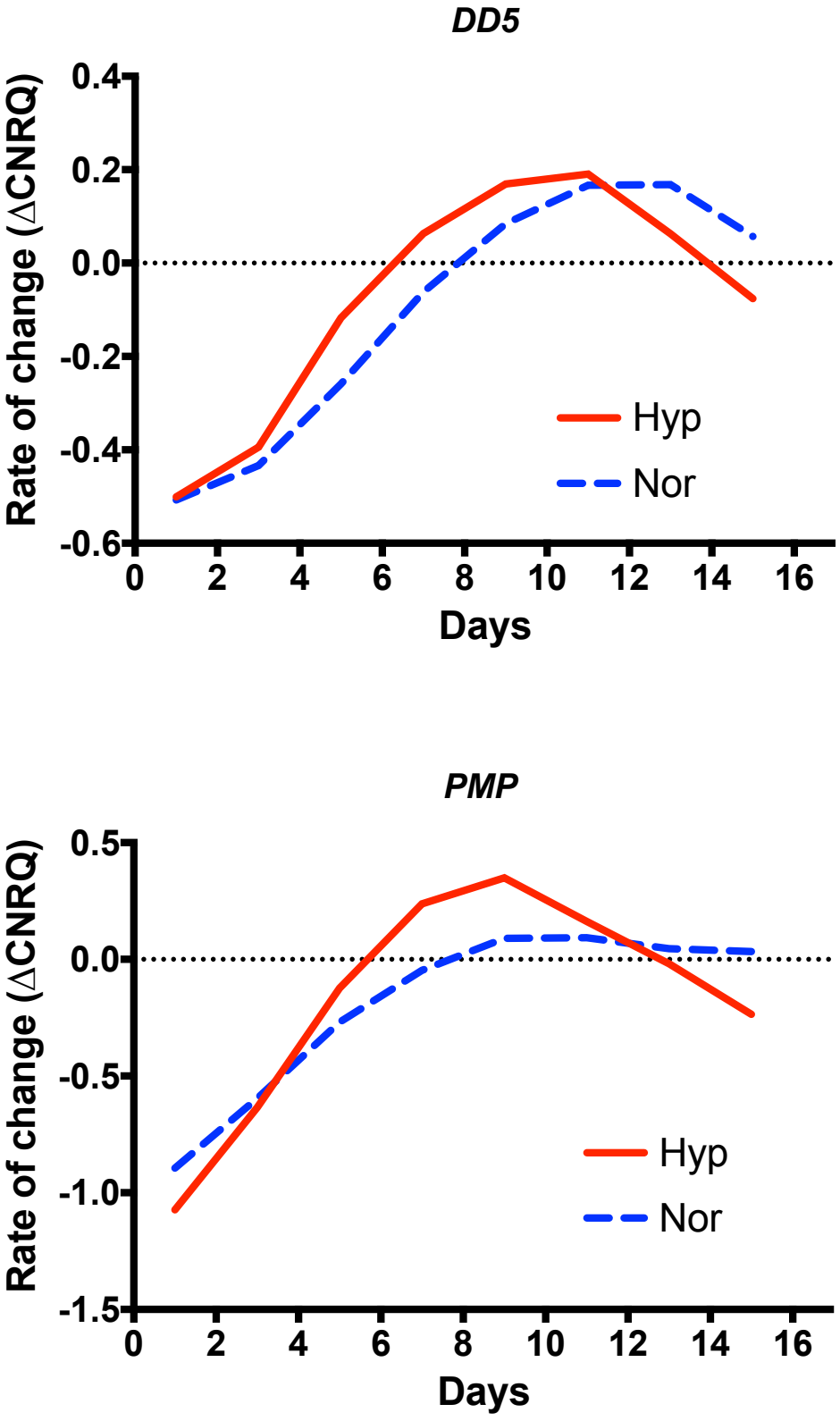

Figure 3.5: First derivative showing the rate of change in gene expression (Δ CNRQ) over time for the genes who had a statistically different pattern (resulted from GAM analysis): *DD5* and *PMP*. For any time-point, a negative rate of change ($CNRQ_{day\ n}$ - $CNRQ_{day\ n-1}$) indicates a decreased expression level; a constant rate of change over two or more days, indicates no variation in gene expression levels over the same interval time.

3.4.4 Changes to phenotype in *Palaemon varians*: gill modification in response to cyclic hypoxia

Results from histological analysis are shown in Fig. 3.6 and Fig. 3.7. Hypoxic shrimps had longer lamellae with a longer perimeter (t-test, p-values< 0.05, n=10 for each test, Fig. 3.7A, C), but no change in lamellar width and density was observed. (t-test, p-values> 0.05, n=10 for each test, Fig. 3.7B, D). The lamellae of hypoxic animals were, on average, 13.6% longer than control shrimp and, since perimeter of lamellae corresponds to their surface area, hypoxic shrimp had a larger (on average 13.6%) mean lamellar surface area for gas exchange (Hughes, 1983; Felgenhauer, 1992).

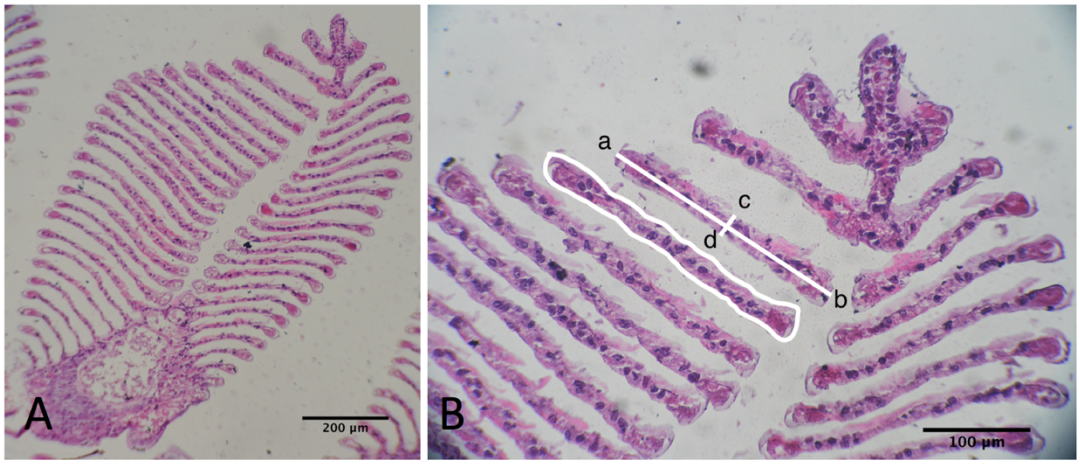

Figure 3.6: Histological images of gill from *P. varians*. **A)** Micrograph showing the entire gill (20x magnification). **B)** Detail of the gill (40x magnification) showing the lamellar perimeter (white) to highlight lamellar section. From the section of each lamellae, the best fitting ellipse was determined with ImageJ, together with its major axis (lamellar length – ab) and minor axis (lamellar width – cd).

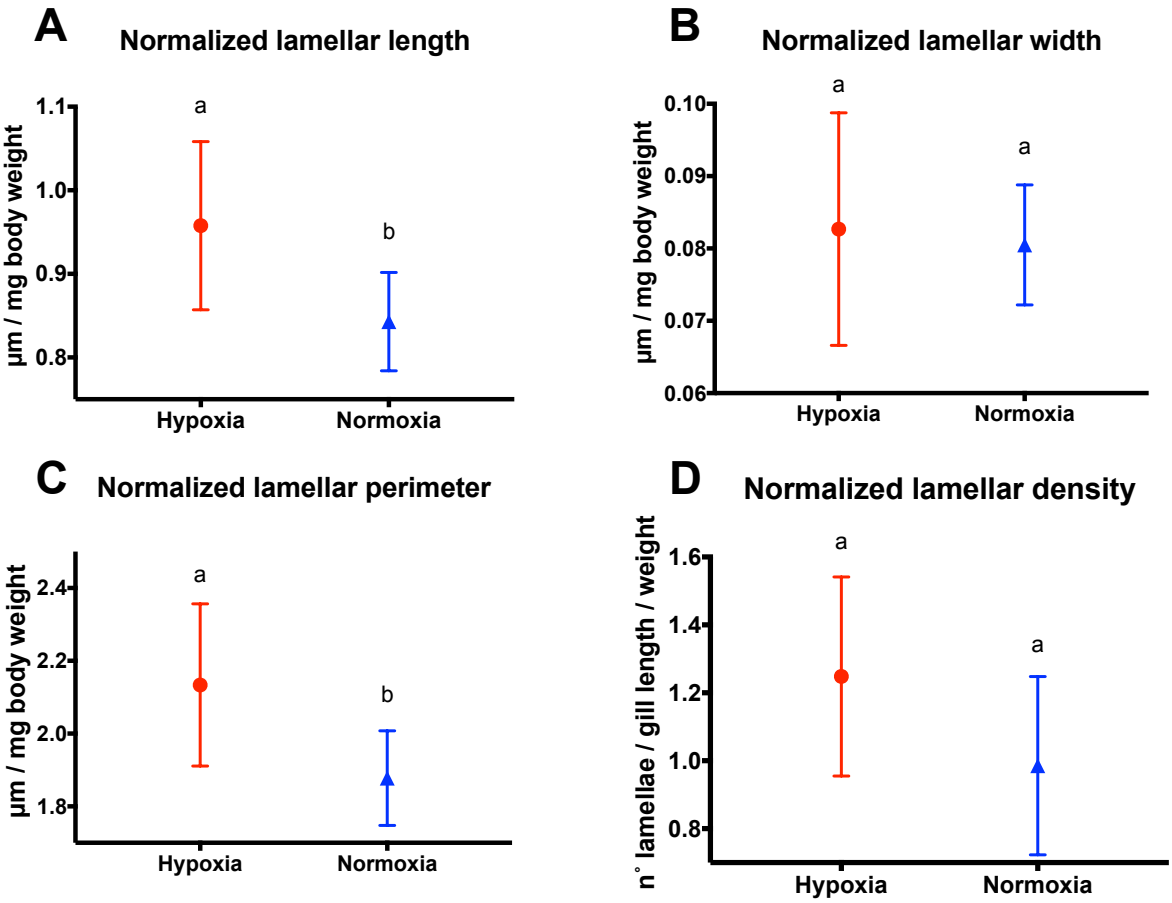

Figure 3.7: Morphological analysis on *P varians*' gills exposed to hypoxia or normoxia for 16 days from ecdysis. For all graphs, mean ±SD are plotted. All data were normalized using wet body weight from each animal. Different letters indicate significance values between treatments (unpaired t-test, $P<0.05$, n=5 for each treatment).

3.5 Discussion:

3.5.1 Transcriptome response to hypoxia

Differential expression analysis identified 399 differentially expressed contigs, in shrimp subjected to hypoxia. Many of the differentially expressed genes are known to be involved with cuticle deposition (i.e. cuticular proteins), cuticle re-arrangement (i.e. chitinases) and cuticle calcification (i.e. *CaAP,* gastrolith proteins) and included three important metabolic enzymes (*PEPCK*, *G6PT*, *GBBH*). Up-regulation of *PEPCK* has been reported in *Palaemonetes pugio* (Li & Brouwer, 2009a; Brown-Peterson et al., 2011; Li &

Brouwer, 2013b) under chronic and cyclic hypoxia, whereas up-regulation of *G6PT* and *GBBH* has never been reported in crustaceans under these experimental conditions. The up-regulation of *G6PT, GBBH* and *PEPCK* indicates metabolic pathways to support glucose homeostasis (*G6PT, PEPCK*) and lipid metabolism (*GBBH*). As reported by Lord-Dufour et al. (2009), *G6PT* regulates functions such as glycemia and lactic acidemia by promoting glycogenolysis and making glucose available (Chou et al., 2002), whereas *GBBH* produces carnitine, essential for the transport of activated fatty acids across the mitochondrial membrane during mitochondrial beta-oxidation (Paul, Sekas, & Adibi, 1992). However, the up-regulation of these metabolic enzymes does not represent a substantial shift in metabolic pathways (Pillet et al., 2016). Apart from the up-regulation of *PEPCK*, we did not find up-regulation of other key enzymes involved in anaerobic pathways, such as pyruvate kinase or lactate dehydrogenase, after seven days of cyclic hypoxia (in agreement with Li and Brouwer (2013b)). This conclusion is in line with that of Brown-Peterson et al. (2011) and agrees with observations made on *Palaemon varians* in the Lymington marshes. It has been argued that the large daily fluctuation in pO_2 during the summer might have driven adaptations (Demers et al., 2006) in *P. varians* such that, after an initial shock response to hypoxia, the metabolism reaches a new steady state after several days (Brouwer et al., 2005). Therefore, in populations inhabiting sites experiencing large pO_2 fluctuations, the expression of conventional hypoxia sensitive genes (i.e. *LDH* and *PK*) is not altered (Brown-Peterson et al., 2011). Finally the down-regulation of a vitellogenin transcript has been reported in many studies in chronic and cyclic hypoxic conditions, including: *Daphnia magna* (Lai et al., 2016), *Palaemonetes pugio* (Li & Brouwer, 2009a, 2013a) and also fish (e.g. *Fundulus grandis* (Cheek et al., 2009)).

The moulting cycle of crustaceans can be broadly divided into four stages: inter-moult, early pre-moult, late pre-moult and post-moult (Drach, 1939). Pre-moult and post-moult are separated by ecdysis (when animals shed the old cuticle), which marks the beginning of a new cycle. The rigid exoskeleton, which is composed of organic components (i.e. chitin and proteins (Nousiainen et al., 1998; Roer et al., 2015)) impregnated with calcium carbonate (Roer et al., 2015), is replaced during moulting to permit the individual's growth. Therefore, the expression of moult-related genes is inevitably related to the phases of the moult cycle (Tom et al., 2014; Abehsera et al., 2015; Roer et al., 2015): i.e. the expression of cuticular proteins peaks between post-moult and intermoult whereas chitinases peak at ecdysis (Tom et al., 2014; Roer et al., 2015).

In our data, the increased expression of cuticular contigs (in comparison with controls) and the low expression of chitinases suggest that hypoxia might have an effect on

the moult cycle of *P. varians*. This assumption is supported by the up-regulation of *PMP, CaAP* and *DD5* genes, which are expressed during post-moult phase by the epithelium and play an important role in deposition and calcification of the newly formed exoskeleton (Ikeya et al., 2001; Inoue et al., 2004; Roer et al., 2015). In spite of the numerous reports of differential responses of crustaceans to hypoxia in relation to moult stage (Charmantier, Soyez, & Aquacop, 1994; Mugnier & Soyez, 2005; Mugnier et al., 2008), only a limited few have reported the involvement of moult as a consequence of chronic hypoxia (Clark, 1986; Wei et al., 2008) and none as a consequence of daily cyclic hypoxia.

3.5.2 Changes in inter-moult duration

In order to validate the mechanistic relationship between hypoxia and moulting, the duration of *P. varians* intermoult period was quantified. Results showed a median intermoult time 15% shorter (Fig. 3.3) in hypoxic animals compared to normoxic animals. These phenotypic data not only confirm an effect of hypoxia on the moult cycle of *P. varians*, but also reveal that exposure to cyclic hypoxia accelerates the moult cycle.

It is interesting to note that these conclusions differ from those reported by Clark (1986) where inhibition of moulting was documented in *Penaeus semisulcatus* exposed to chronic hypoxia (~4 kPa for 17 days). However, this difference might be explained by the different experimental conditions tested: chronic hypoxia (constant hypoxia) and cyclic hypoxia (succession of hypoxia and normoxia), or by the absolute minimum pO_2. Since moulting is an energetically expensive process, it is possible that under continuous chronic hypoxia the shrimp, as a stress response, undergo prolonged metabolic depression (Wei et al., 2008) that completely inhibits moulting. In contrast under conditions of cyclic hypoxia, the hypoxic/normoxic turnover allows, in normoxia, “recovery time” to restore homeostasis and results in an accelerated moult cycle.

Hypoxia has been correlated with smaller body sizes and slower larval development times for many insect larvae (Frazier, Woods, & Harrison, 2001; Harrison, Kaiser, & VandenBrooks, 2010; Callier & Nijhout, 2013). In addition, a similar acceleration of moult cycle as a consequence of hypoxia has been demonstrated in different insect larvae (i.e. *Manduca sexta* (Callier & Nijhout, 2011) and *Tenebrio molitor* (Greenberg & Ar, 1996)): larvae in hypoxia moult sooner and at a smaller body size in comparison to normoxic larvae. The correlation between hypoxia and moult in insects has been explained by the anatomical structure of insect’s tracheal system, which is covered

with cuticle and therefore does not grow during inter-moult (Callier & Nijhout, 2013; Kivelä et al., 2016). Under 'normal' conditions moult represents a physiological mechanism to support growth and increase O_2 supply by expanding the tracheal system (i.e. a larger body mass requires a larger tracheal system). Under hypoxic conditions, however, the resulting oxygen limitation is a functional constraint for the body (i.e. not enough oxygen can be taken up from the environment) to which moulting provides a solution (Callier & Nijhout, 2013). Therefore insects in hypoxia moult when they reach a smaller body size (in terms of weight) in comparison to insects reared in normoxia (Callier & Nijhout, 2011).

3.5.3 Gene expression of cuticular genes during moult cycle

In the experiment of moulting synchrony, the expression levels of *PMP, CaAP* and *DD5* were quantified on alternate days (Fig. 3.4) and differences in the overall pattern of expression were tested using a GAM model (Table 3.2). Analysis showed a different pattern among treatments for *PMP* and *DD5* (Fig. 3.4) with a more responsive expression in hypoxia (e.g. rate of change in gene expression in hypoxic animals increased sooner and more steeply and then decreased more sharply than in controls, Fig. 3.5). A possible explanation for the observed difference is that, in hypoxic conditions, hypoxia triggered a physiological stimulus to moult in a narrower time frame (compared to controls) and the "normal" inter-individual variability in intermoult duration was diminished. On the other hand, in normoxic conditions the physiological stimulus to moult was relaxed, resulting in greater inter-individual variability, with the overall result that rates of change in gene expression were less marked. Collectively these results support the previous conclusion regarding an alteration of moult cycle as a consequence of hypoxia.

As mentioned above, insects grown in hypoxia show an accelerated moult cycle (Callier & Nijhout, 2013) and develop a larger tracheal system (Callier & Nijhout, 2011). The reason is that tracheal system of insects is covered with cuticle, and its size can be changed only with moulting. Therefore if insects experience an oxygen limitation due to hypoxia, the result is an enlargement of the tracheal system to counteract this limitation (Callier & Nijhout, 2011). Crustacean gills, like insect tracheae, are covered with cuticle (Felgenhauer, 1992), therefore a similar physiological response to insects is supported.

3.5.4 Changes to phenotype in *Palaemon varians*: gill modification in response to cyclic hypoxia

In order to test the hypothesis that hypoxia would alter gill structure, morphological gill modifications in *P. varians* were quantified. Results showed average lamellar surface area for gas exchange was 13.6% larger (Fig. 3.7) in hypoxic animals compared to normoxic animals.

As described by Hughes (1983) the gill surface is a crucial site for exchanges of respiratory gases. Gas transfer is described by Fick's law (Massabuau & Abele, 2012) and it is directly proportional to the respiratory surface area. Hence, the observed increase is functional for animals to cope with the lowered O_2 tension during hypoxic periods. Hughes (1983) observed that changes to the gills (in decapod crustaceans such as *Nephrops norvegicus*, *Homarus gammarus* and *Cancer pagurus*) scaled to individual body sizes were mainly a function of increasing the area of gill lamellae, rather than increasing the number of lamellae. Hence, the observed lack of change in lamellar density is not unexpected and in terms of energy expenditure, one might argue that it is less expensive to elongate existing lamellae, rather than creating new lamellae to increase lamellar density and enlarge gas exchange surfaces area.

Data presented here support the hypothesis that in response to cyclic hypoxia moulting represents a 'solution' to meet oxygen requirements of the body (Callier & Nijhout, 2013), by providing the animals with larger gills at an earlier timepoint. It has been argued that the morphological alteration of the gills represents an appreciable energetic investment (Callier & Nijhout, 2011) and the results herein agree with this statement: in fact, within a single, shorter moult cycle (~ 12 days), gill size was increased by 13.6%.

3.6 Conclusions:

In this Chapter it was shown how 16 days exposure to cyclic hypoxia was able to trigger physiological adaptations in *Palaemon varians* that accelerated its moult cycle, which was ~15% shorter, and induced morphological changes in lamellar surface area, which was ~13.6% larger. It can be hypothesised that this adaptation in *P. varians*, never reported for crustaceans, allows the species to live in such a variable environment as Lymington salt marsh, with considerable fluctuations in temperature and, most importantly, oxygen (from 200% air saturation to nearly 0% every 12 hours). Further, the acceleration of the moult cycle constitutes a physiological mechanism specifically initiated by long-term cyclic hypoxia, as it was not described as a consequence of chronic hypoxia. In the next Chapter the consequences of longer exposures to cyclic hypoxia will be investigated on other physiological processes, such as maintenance, growth, feeding and reproduction.

Chapter 4 Effects of daily cyclic hypoxia on physiological processes in *Palaemon varians*: maintenance and growth, feeding and excretion, reproduction and larval development

Summary:

Although our knowledge of the response of aquatic organisms to chronic hypoxia has steadily increased, surprisingly little is known about the impairment of the main physiological processes (e.g. growth, reproduction, feeding, larval development) in estuarine and coastal species following long-term exposure to cyclic hypoxia. In order to quantify this, six physiological processes, namely maintenance, growth, feeding, excretion, reproduction and larval development, were assessed in adults and larvae of the ditch shrimp *Palaemon varians* exposed to daily cyclic hypoxia for a period up to 40 days. Adults exposed to normoxia had ~4% heavier body weight and were ~1.4% longer (in terms of total length) compared to cyclic hypoxic animals after 28 days of exposure. Significant reductions in feed ingestion and ammonium excretion rates were found in hypoxic adults after 1 and 21 days. In the reproductive experiment, which lasted 40 days, ovigerous females produced eggs with a median volume 26% smaller and a dry weight 24% lighter than females in which the ovarian development was conducted in normoxic conditions. Finally, while larval development duration was not delayed by cyclic hypoxia, larval dry weight was smaller in cyclic hypoxic larvae. Collectively, results suggest that the long-term cyclic hypoxic regime tested here (which mimicked a natural cycle measured in the marsh were by *P. varians* is commonly found) was able to induce an effect on the different physiological processes tested, even if the magnitude of impairment depended on the physiological process.

4.1 Introduction:

Globally there is an increasing concern in relation to hypoxia in aquatic environments as this phenomenon has been associated with declines in habitat quality (Buzzelli et al., 2002; Wu, 2002) and alterations in the biology of the species (Brouwer et al., 2007; Landry et al., 2007; Cheek, 2011; Li & Brouwer, 2013a; Davidson et al., 2016). The physiological mechanisms shown in Chapters 2 (i.e. anaerobic metabolism) and 3 (i.e. accelerated moult cycle and morphological modifications to the gills) were triggered as a consequence of the stressful condition following hypoxic exposure. According the model proposed by Sokolova et al. (2012), stressful conditions alter the "normal" energy allocation by increasing the costs of maintenance (i.e. all physiological mechanisms that maintain homeostasis of the body, e.g. disposal of anaerobic end products) to the detriment of other energetically expensive processes such as growth, reproduction, excretion or feeding (e.g. (Bell, Eggleston, & Wolcott, 2003; Wei et al., 2008; Wei et al., 2009; Kiko et al., 2016)). The result is that long-term stressful conditions (e.g. long-term chronic or cyclic hypoxia) can, in example, reduce growth or affect reproduction (e.g. (Pichavant et al., 2001; Stierhoff et al., 2006; Cheung et al., 2008)) in a species-specific way (as shown in Sections 1.6.1 and 1.7) and in a "stressor-specific" way. In fact, as reported by Coiro et al. (2000), growth in *Palaemonetes vulgaris*' juveniles was less affected by cyclic hypoxia than by chronic hypoxia; in other words juveniles in cyclic hypoxia grew from ~10 to ~ 50% more than juveniles in chronic hypoxia. Similar results were obtained by Stierhoff et al. (2006) on the flounders *Pseudopleuronectes americanus* and *Paralichthys dentatus*.

The different effects that are elicited by the two types of hypoxia (i.e. chronic and cyclic) can also lead to opposite results; in fact, while Brouwer et al. (2007) reported an increased fecundity in *Palaemonetes pugio* exposed to chronic hypoxia (compared to females kept in normoxia), Brown-Peterson et al. (2011) reported a decreased fecundity in a population of *P. pugio* exposed to cyclic hypoxia in the field (in comparison to a population from a normoxic site, see Chapter 1, Tab. 1.1 for details). While the consequences of chronic hypoxia on the physiology of ecthoterms are better characterized (Coiro et al., 2000; Pichavant et al., 2001; Brouwer et al., 2007; Landry et al., 2007; Cheung et al., 2008; Wei et al., 2008; Li & Brouwer, 2009b; Motyka et al., 2017), the effects of long-term cyclic hypoxia are still poorly understood and the studies addressing this question have shown some degree of variability between species, see Chapter 1 Tab. 1.1.

In the light of the concepts expressed before, the fluctuations in pO_2 that *P. varians* is experiencing on a daily base in Lymington suggested that cyclic hypoxia might be influencing the biology of this species. For all these reasons the effects of long-term cyclic hypoxia on the main physiological processes (maintenance and growth, reproduction, larval development, feeding and excretion) have been investigated in the coastal organism *P. varians* by using a cyclic hypoxic scheme that mimics the "natural" cyclic hypoxia experienced by the species in its habitat, the Lymington salt marsh.

4.2 Specific chapter hypothesis:

1. Prolonged cyclic hypoxia will increase maintenance costs in hypoxic-exposed animals. These increased costs will manifest in lower body weight and smaller body length over time in hypoxic animals compared to normoxic animals.
2. Cyclic hypoxia will reduce feed ingestion and ammonium excretion rates.
3. Prolonged cyclic hypoxia will affect the reproductive parameters of *P varians*; in particular, cyclic hypoxia will alter the number of ovigerous females and their fecundity, and will alter the volume and dry weight of eggs carried by ovigerous females.
4. Daily cyclic hypoxia will increase maintenance costs in hypoxic-exposed larvae. These increased costs will delay larval development by increasing development duration and affecting juvenile dry weight.

4.3 Material and methods:

4.3.1 Animal collection and general experimental protocol:

Animals were hand-net collected from a channel in the Lymington salt marsh, UK. In the laboratory, animals were held in 150L aquaria and water temperature was gradually increased (+1˚C/day) starting from the field temperature at the time of collection, up to the experimental temperature of 22 ˚C and they were kept at 22 ˚C for two weeks prior to the beginning of the experiment. This temperature was chosen to represent summer temperature because it is currently experienced in the water of the channel during summer nights (see Chapter 2). During acclimation and throughout the experiments, animals were fed three-times per week using commercial shrimp granules (shrimp naturals – Sera, Germany) at a ration of one granule per shrimp (~ 4.8% of animal's mean wet weight) and water was changed one time per week (~20% of the volume). One week before the beginning of the experiment, animals were moved into the experimental flow-through system previously described (Chapter 2).

During all experiments with adults, hypoxic animals were kept in daily cyclic hypoxia (water pO_2 < 4.55 kPa) for 7 hours, from 0230 to 0930 hrs, and kept in normoxia (water pO_2 ~21 kPa) for the rest of the day (see Supplementary Table 1 – Appendix A for mean pO_2 values recorded during hypoxic periods in each tank). This daily regime was retained for several weeks (exact duration was experiment-specific). Normoxic animals (controls) were always kept in normoxia for the entire duration of the experiments.

To assess the impact of cyclic hypoxia on larval development, a population of larvae were subjected to daily hypoxia and compared to a control population kept in normoxia. Larvae were kept individually in 200mL glass bottles filled with filtered sea-water at 22˚C inside an incubator with 12:12 light:dark cycle. In order to obtain cyclic hypoxia, treatment water was changed two times per day in both treatments: in the cyclic hypoxic treatment, water with pO_2= 5.5kPa (to simulate mild hypoxic conditions, mean hypoxic level 4.9 ±0.6 kPa) was added in all bottles in the morning (around 0930 hrs) and replaced with normoxic water (pO_2= 21kPa) seven hours later (around 1730 hrs). In the normoxic treatment (control), normoxic water was used for morning and afternoon water changes. Larvae were starved during the first larval instar. On moulting to the second larval instar, *Artemia* sp. nauplii were introduced in excess into the bottles every other day, in late afternoon (around 1800 hrs).

4.3.2 Maintenance and growth:

To investigate variations in wet weight, animals were kept in cyclic hypoxia for 28 days and changes in wet weight and total length were compared with a control population kept in normoxia for 28 days. Prior to the experiment, in order to reduce variability in size and weight, a population of experimental animals with a wet weight range between 0.190g and 0.300g (±0.0005g) was selected (as they represent the most frequent size in Lymington, personal observation). No selection criteria for moult stage was applied; hence animals were not necessarily in the same moult stage (i.e. all in intermoult or pre-moult). The random animal allocation to the treatment (hypoxia, n= 102 animals in total, or normoxia, n=102 animals in total) and to the experimental tanks (12 tanks in total, n= 17 animals per tank) was achieved by using a custom Python (Python Software Foundation, https://www.python.org) script to generate random numbers. Initial density was 17 animals per tank, final density was between 15 and 18 animals per tank.

On the day preceding the start of the experiment (day 0) and every seven days up to 28 days, animals were weighed using an analytical balance (Denver Instrument si-234 Colorado - USA, weight ± 0.0001g) and their total length was measured with a using digital vernier calliper (accuracy 0.01 mm). Animals were starved for two days before their wet weight was determined in order to avoid bias due to ingested food.

For each time point (days 0, 7, 14, 21 and 28) differences in the frequency distribution of weight between treatments (hypoxia and normoxia) were tested using Wilcoxon Rank sum test, using R statistical software (Team, 2014). For all time points, mean weight and total length of each tank (n=15-17 animals) were calculated.

The absence of systematic differences between the twelve experimental replicates (i.e. tanks) was tested on weight data collected at day 28 by using a mixed model nested Anova with the factor “tank” nested within the factor “treatment” (i.e. hypoxia and normoxia). This assessment was necessary to validate the accuracy of the experimental replicates. Initially weight data were tested for normal distribution with Shapiro-test. Because normal distribution criteria were not met, weight data were transformed, dividing the final weight (i.e. weight at day 28) of each shrimp ($fw_{\text{tank"a"}}$) by the mean initial weight of the tank in which the shrimp was kept (i.e. tank “a” mean weight at day 0: $MW_{\text{tank"a"}}$), using this formula:

Transformed data = $(fw_{\text{tank"a"}} / MW_{\text{tank"a"}})$

Nested Anova was run using a random sub-set of 13 shrimp per tank (in order to have a balanced number of biological replicates (i.e. shrimp) for each experimental replicate), and the whole process of random selection and Anova test was repeated 1000 times, to avoid bias due to random selection. Statistical difference was identified at p-value <0.05.

To test whether the slope of the weight-time (or length-time) relationship changed between treatments, a linear regression model fitting separately data from hypoxia and from normoxia (Model 2) was compared with a simpler model fitting all data points, regardless of the treatment (Model 1). The same intercept was used for all models, since a population with a narrow weight range was used and random animal allocation was performed prior to the experiment. An extra sum-of-squares F test from the software GraphPad Prism v7.0 was used to compare the models, and Model 1 was preferred over Model 2 unless the outcome of the F test was not significant ($p < 0.05$).

4.3.3 Feeding and ammonium excretion:

Adult *P. varians* were maintained as described in the previous sections and exposed to the same experimental conditions as above, with a stocking density of 17 animals per tank.

In order to quantify feed ingestion, adults (n=11 animals/treatment/time point) were individually placed in retention chambers (one adult per chamber) made with 10cm Petri dish bottoms and 15cm high mesh (0.5 mm radius) collars (Brouwer et al., 2007). They were then left to acclimate for 24-h before the test was performed. During the test each adult was fed in the morning (09:00 AM) with one commercial granule (shrimp naturals – Sera, Germany) per shrimp and, after 150 minutes, uneaten feed was carefully collected and individually stored at -80 ˚C. All samples were dried in an oven at 70 ˚C. Dry weight was measured with an analytical balance (Denver Instrument si-234 Colorado - USA, weight ± 0.0001g). Feed ingestion was calculated as follows:

$$I = [(DW_{granule} - DW_{uneaten\_feed\_a}) / DW_{granule}] / BM_a$$

$DW_{granule}$ is the mean dry weight (mg) of commercial granules kept in water for 150 minutes and dried in oven. $DW_{uneaten\_feed\_a}$ is the uneaten feed from animal “a”, BM_a is the wet weight (g) of animal “a”.

In order to quantify ammonium excretion, adults (n= 14-18 animals/treatment/time point) were individually placed in glass bottles with 200 mL of artificial sea-water at the

same temperature and salinity of the experimental aquaria. After 210 minutes, water samples were collected and ammonium ions were measured with a Hach method (Hach, Colorado USA) (ammonium salycilate and ammonium cyanurate) following the manufacturer's protocol (Fass et al., 1994; Qing et al., 2016).

For both treatments (hypoxia and normoxia), feed ingestion and ammonium excretion tests were performed in two conditions: with hypoxic-exposed animals kept in hypoxia and normoxic-exposed animals kept in normoxia (Experimental conditions) and with both treatments kept in normoxia (Normoxic conditions). All tests were always performed at 0900 AM.

Two-way ANOVA with Tukey's multiple comparisons were used to assess differences in feed ingestion at different sampling dates (Experimental conditions and Normoxic conditions) and ammonium excretion at different sampling dates. Data collected in Experimental conditions was analysed separately from data collected in Normoxic conditions. For all analysis statistical significance was identified at $P<0.05$.

4.3.4 Reproduction:

Adult *P. varians* were collected in March 2016. For breeding, *P. varians* were sexed using a stereomicroscope and males were identified by the presence of the appendix masculine on the second pleopod pair, which is absent in females (Oliphant, 2013). Females (n= 22 in total) containing white ovaries in primary vitellogenesis (Bouchon, 1991a) were used during the experiment. Acclimation and feeding followed the protocol described in Section 4.3.1.

Breeding and spawning in *P. varians* are induced by long day length (Bouchon, 1991b). Hence day length was increased by two hours per day until a 18:6 light:dark cycle was achieved. Reproductive pairs of ditch shrimps (in total 10 pairs in hypoxia and 12 pairs in normoxia) were housed in retention chambers made with 10cm Petri dish bottoms and 15cm high collars of mesh (Brouwer et al., 2007). In each tank (n= 2 tanks per treatment) used during the experiment, up to six retention chambers were accommodated in vertical position (with the Petri dish at the bottom of the tank), in order to maximise water flow throughout the chamber.

In order to quantify the impact of cyclic hypoxia on reproduction, reproductive pairs were kept in cyclic hypoxia for 40 days and changes to reproductive success, relative fecundity, egg size and egg dry weight were compared with control pairs kept in normoxia.

Pairs were checked daily for egg production, and females were determined gravid when the presence of pleopodal eggs for two consecutive days was confirmed (Brouwer et al., 2007). All eggs from gravid females were gently removed using a pair of tweezers, counted and the total length and wet weight of each female were recorded. Relative fecundity was calculated as the number of pleopodal eggs divided by the wet weight of the female. A Chi-square test was used to test alteration in the reproductive success (number of reproductive couples versus non-reproductive couples). To calculate egg volume, a picture of the eggs was taken using a Leica S8AP0 dissection stereomicroscope (with lighting provided by a Schott KL 1500 LCD halogen cold light with swan-neck attachment), and the volume was determined with ImageJ (Schneider et al., 2012) by using the "fit ellipse" function. The "fit function" would determine the best fitting ellipse and would return the Major (MA) and minor (ma) axes of the fitted ellipse. Major and minor axes were used to calculate egg volume with the formula (Oliphant, 2013):

Volume= 4/3 * π * ½ * MA * ¼ * (ma^2)

Egg dry weight was measured (n =6 eggs per female were collected) after samples had been freeze-dried for 24 hours using a Thermo Scientific Heto PowerDry LL33000 freeze dryer. Samples were weighed for dry weight using a Sartorius microbalance ME5.

Previously, Oliphant (2013) showed some variability between eggs (in terms of egg volume and dry weight) carried by the same female. To test the presence of differences in egg volume between females, a mixed model nested Anova was set up using the fixed factor treatment (with two levels, hypoxia and normoxia) and a random factor female (originally with 4 hypoxic and 7 normoxic females) nested in treatment. The mixed model was balanced by randomly selecting 4 hypoxic and 4 normoxic females; for each female 15 eggs were randomly selected and, with this balanced subset, the nested Anova was run. To avoid bias due to random selection, the process was repeated 1000 times. Statistical difference was identified at p-value <0.05.

A similar model was used to test differences in egg dry weight between females, with the only difference that a sub-sample of 5 eggs per female was used.

Results from both nested Anova models showed a great amount of variability within females, suggesting that further analysis (i.e. comparing the effect of the hypoxic treatment on egg volume and dry weight) should consider each egg as a replicate, rather than using a mean value for each female. To assess statistical differences in relative fecundity, egg volume and dry weight among treatments, student t-tests or Mann-Whitney tests were used (after testing for normality - Shapiro test - and Homogeneity of Variances -

Bartlett test) using statistical software R (Team, 2014). For all analysis statistical significance was identified at $P<0.05$.

4.3.5 Larval development:

Ovigerous *P. varians*, identified by the presence of eggs or embryos attached to the pleopods under the abdomen, were collected in Lymington (June 2016) and transported to NOCS. Ovigerous *P. varians* were isolated in 1 L plastic buckets in LMS Model 230 Series 2 Cooled Incubators (accuracy = ± 0.5 °C) set to the field temperature at the time of collection (~20˚C) and 12:12 light:dark cycle. Temperature was increased 1˚C/day up to 22˚C.

Embryonic development of broods was assessed and staged according to Müller (2004). Of the ovigerous *P. varians* collected, only those with embryos close to hatching (stages VII and VIII, see below) were used (n= 7 in total), in order to minimize any possible effect of captivity on embryonic development. On hatching, n= 20 actively swimming larvae were separated from each mother using a 3 ml plastic pipette and randomly allocated to the hypoxic or normoxic treatment (n=10 eggs for each treatment from each female). Larvae were kept individually in 200mL glass bottles filled with filtered sea-water at 22˚C inside an incubator with 12:12 light:dark cycle.

Larvae were checked daily (morning and late afternoon), their development was classified according to Fincham (1983). The time required to reach juvenile stage was recorded. Additionally, it was annotated whether the larvae received food or not the evening before metamorphosis. Upon reaching the juvenile stage, individuals were blotted dry on tissue paper, transferred to pre-weighed tine capsules and frozen at -80 °C. Dry weight measurements followed the protocol described for egg dry weight (Section 4.3.4).

Differences in survival rate were assessed with Chi-square test. A Mann-Whitney test was used to test differences in duration of development. As a consequence of the fact that animals received food every other day and because being fed, or not, on the day of metamorphosis could have potentially altered juvenile dry weight, two-way ANOVA with treatment and food as factors was used to assess differences in juvenile dry weight followed by Tukey's multiple comparisons. For all analysis statistical significance was identified at $P<0.05$.

4.4 Results:

4.4.1 Maintenance and growth:

For each sampling day, frequency distributions of wet weight and body size (by means of total length – TL) among different treatments were plotted (Fig. 4.1 and Fig. 4.2, respectively) with the respective p-value from the Wilcoxon Rank sum test (Tab. 4.1). Overall, for wet weight and TL, there was a gradual separation of the cumulative frequency curves of the two treatments that was proportional to the length of the experiment (Tab. 4.1). The gradual separation of the two curves was coupled with a decrease in p-value that, for wet weight, almost reached the significance threshold of 0.05 by day 28 (W= 3938, p-val=0.06, Tab. 4.1). A similar separation of the cumulative frequencies was detected also for TL, even if the separation became evident by day 28 (W= 3800, p-val=0.03).

Table 4.1: Summary of the Wilcoxon rank sum test (W) with continuity correction used to compare frequency distributions of wet weight and total length for each time-point.

	Wet weight:		**Total length:**	
Day 0	W = 4871	p = 0.37	W = 7042.5	p = 1
Day 7	W = 4656.5	p = 0.35	W = 4844.5	p = 0.54
Day 14	W = 4384.5	p = 0.17	W = 4571	p = 0.32
Day 21	W = 4167	p = 0.10	W = 4721.5	p = 0.56
Day 28	W = 3938	p = 0.06	W = 3800	p = 0.03

The absence of systematic differences between the experimental replicates was confirmed by nested Anova: during the simulations with a sub-set of samples (1000 simulations with 13 samples per treatment) no significant effect could be detected in the factor “tank” nested within the factor treatment (see Supplementary Table 5 – Appendix A for a sub-set of the 1000 simulations): in fact, out of 1000 simulations, lowest p-value for the factor tank nested within treatment was 0.16.

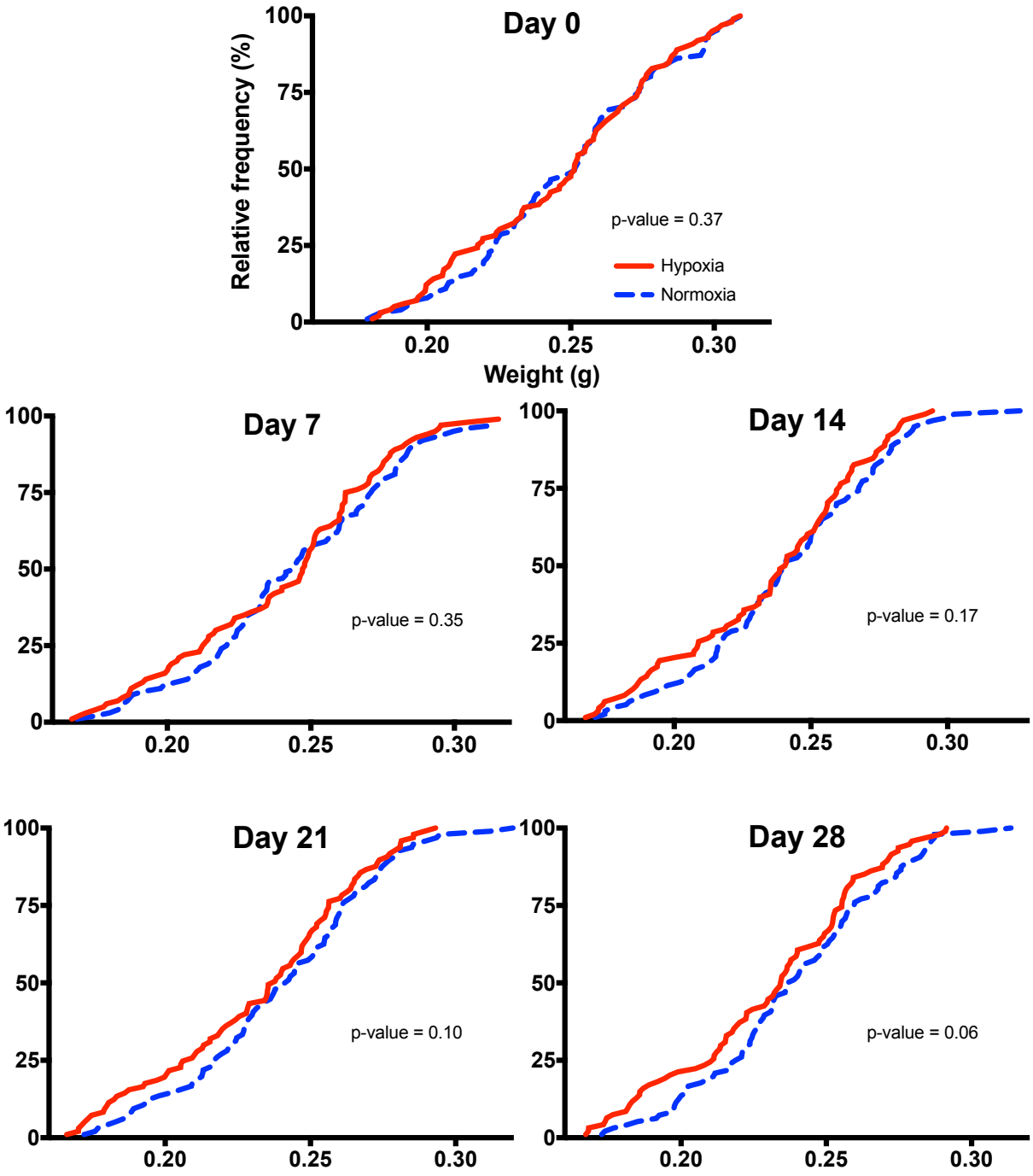

Figure 4.1: Weight frequency distributions at different time-points in hypoxic-exposed animals (red line) and normoxic animals (blue dotted line). N= 95 – 100 animals per treatment per time point. Wilcoxon Rank sum test p-value for each comparison is reported in the respective plot.

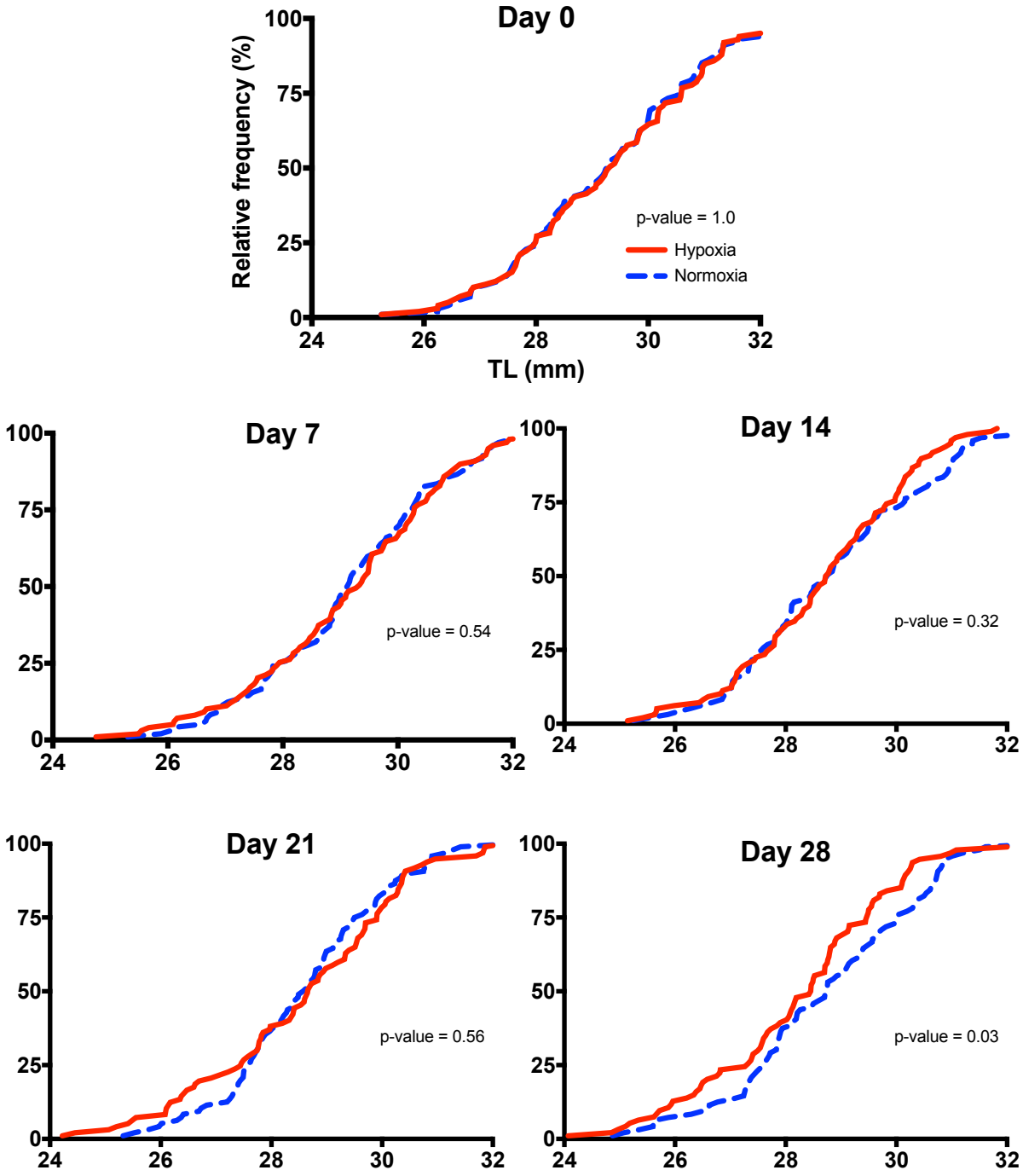

Figure 4.2: Total length (TL) frequency distributions at different time-points in hypoxic-exposed animals (red line) and normoxic animals (blue dotted line). N= 95 – 100 animals per treatment per time point. Wilcoxon Rank sum test p-value for each comparison is reported in the respective plot.

Results from extra sum-of-squares F test to compare slopes between treatments clearly evidenced how the model with two different slopes (one for each treatment – Model 2) was preferred over the model with one shared slope (Fig. 4.3A; p =0.01, Tab. 4.2A). Hence in the experimental conditions the rate of decrease in wet weight over time was different in the hypoxic exposed animals (i.e. they were losing weight more quickly) in

comparison to normoxic animals. In particular, at day 28, mean weight value of hypoxic animals was on average ~4% smaller than mean weight of normoxic animals (at day 28).

Similarly, body size (Fig. 4.3B, Tab. 4.2B) data supported the hypothesis that two different slopes (one for each treatment) produced a better fit than a single curve (p-value=0.03). Also for body size the slope was different between treatments, and was more negative in hypoxic animals compared to normoxic animals.

Finally, with data from day 28, the weight-length relationship among different treatments was calculated (Fig. 4.3C), but no different slope between treatments could be identified (Tab. 4.2C).

Table 4.2: Extra sum-of-squares F test to compare fit models for **A.** Weight, **B.** Total length, **C.** Weight-length relation. In **A**, **B**, **C** the null hypothesis was: one shared slope for the entire dataset (without considering the treatments); in contraposition to the alternative hypothesis that two different slopes could be fit, one for each treatment (hypoxia and normoxia). DFn: Degrees of freedom numerator; DFd: Degrees of freedom denominator.

Comparison of Fits **H0: Same slope for all data set** **H1: Slope different for each data set**					
A. Weight		**B. Total length**		**C. Weight-length relation**	
Extra sum-of-squares F test	7.182	Extra sum-of-squares F test	4.368	Extra sum-of-squares F test	0.6315
DFn, DFd	1, 971	DFn, DFd	1, 969	DFn, DFd	2, 96
P value	0.01	P value	0.03	P value	0.53

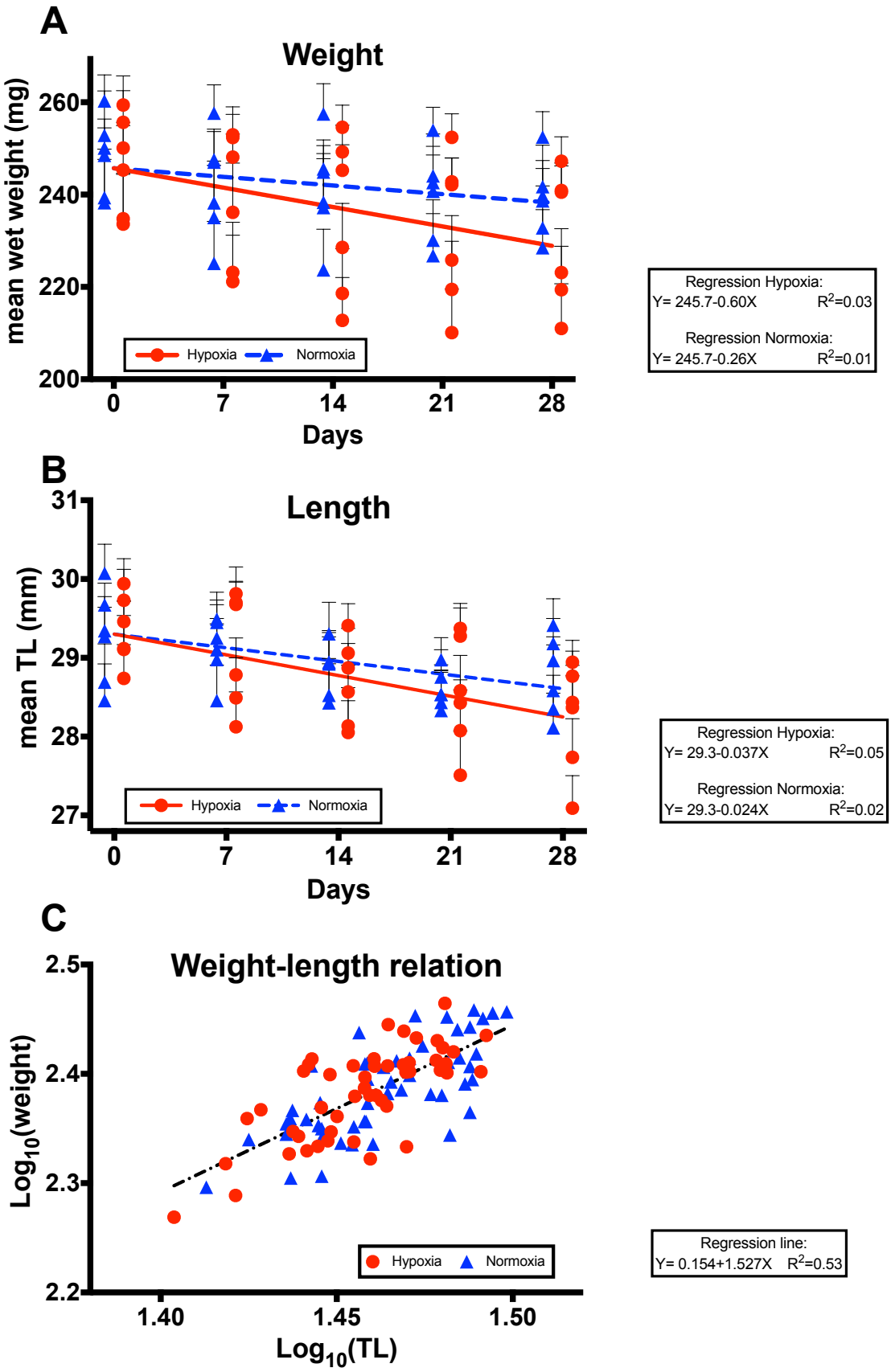

Figure 4.3: A. Mean wet weight +SEM (n=15-18 for each point) from each experimental tank, over time. Lines indicate the best fitted weight-time relationship for each treatment (hypoxia: red continuous line; normoxia: blue, dashed line) calculated considering all data points (and not the mean values only). B. Mean total length +SEM (n=15-18 for each point) from each experimental tank, over time. Lines indicate the best

fitted length-time relationship for each treatment calculated considering all data points (and not the mean values only). C. Weight-length relationship calculated after 28-days of experiment in each treatment. Dotted-dashed line indicates the best fitting line with no distinction for the treatments.

4.4.2 Feeding and excretion:

The impact of cyclic hypoxia on feed ingestion is shown in Fig. 4.4. There was no significant difference in feed ingestion in normoxic conditions between any day of the experiment (Tab. 4.3B, Fig. 4.4B). On the other hand (Tab. 4.3A, Fig. 4.4A), feed ingestion was statistically lower between hypoxic and normoxic animals in experimental conditions during the first cyclic hypoxic exposure (day 1), but, by day 21, no difference could be detected.

In experimental conditions hypoxic animals had a constantly lower excretion rate over time, in comparison to normoxic animals (Tab. 4.3C, Fig. 4.4C). Vice versa, no difference in ammonium excretion was found in normoxic conditions between any day of the experiment (Tab. 4.3D, Fig. 4.4D).

Table 4.3: Two-way ANOVA table used to test an effect of cyclic hypoxia on feed ingestion (**A** and **B**) and ammonium excretion (**C** and **D**). In **A** and **C** tests were done in normoxic-conditions (for the normoxic treatment) and in hypoxic-conditions (for the hypoxic treatment) towards the end of the daily hypoxic period. In **B** and **D**, both treatments were tested in normoxic conditions. SS: Sum of squares. MM: Mean sum of squares. DFn: Degrees of freedom numerator; DFd: Degrees of freedom denominator

A. Feed ingestion, Experimental cond - ANOVA Table				
	SS	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.1558	0.1558	F (1, 40) = 0.1575	0.6936
Days	6.224	6.224	F (1, 40) = 6.293	0.0163
Treatment	12.35	12.35	F (1, 40) = 12.49	0.0010
Residual	39.56	0.989		

B. Feed ingestion, Normoxic cond - ANOVA Table				
	SS	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.03229	0.03229	F (1, 40) = 0.04428	0.8344
Days	0.157	0.157	F (1, 40) = 0.2152	0.6452
Treatment	0.02346	0.02346	F (1, 40) = 0.03217	0.8586
Residual	29.17	0.7292		

C. Ammonia excretion, Experimental cond - ANOVA Table				
	SS	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.0001565	0.0001565	F (1, 51) = 0.241	0.6256
Days	0.002162	0.002162	F (1, 51) = 3.33	0.0739
Treatment	0.05036	0.05036	F (1, 51) = 77.57	<0.0001
Residual	0.03311	0.0006492		

D. Ammonia excretion, Normoxic cond - ANOVA Table				
	SS	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.00009087	0.00009087	F (1, 43) = 0.0583	0.8103
Days	0.001138	0.001138	F (1, 43) = 0.7302	0.3975
Treatment	0.0002013	0.0002013	F (1, 43) = 0.1291	0.7211
Residual	0.06702	0.001559		

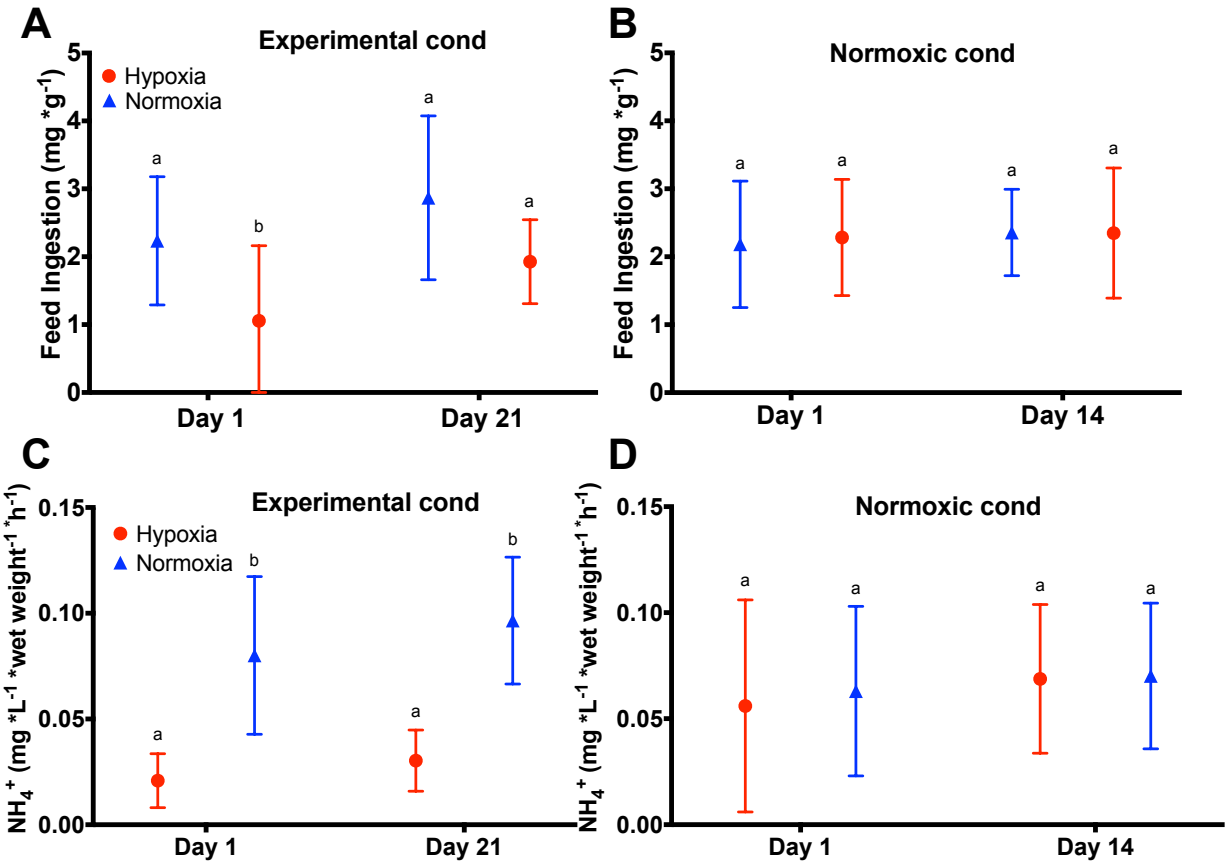

Figure 4.4: **A,B.** Feed ingestion rates after different days of exposure to cyclic hypoxia or normoxia. **C,D.** Ammonium excretion rates after different days of exposure to cyclic hypoxia or normoxia. "Experimental cond" indicates that tests were done in hypoxia for the hypoxic-exposed animals and in normoxia for the normoxic animals. "Normoxic cond" indicates that measurements were recorded under normoxic conditions for all animals for all treatments. For all graphs mean ± SD are plotted. Different letters indicate significance values between treatments.

4.4.3 Reproduction:

There was no effect of the experimental replicates (i.e. tanks) on the number of reproductive females: in the cyclic hypoxic treatment, 4 females managed to reproduce (2 females per experimental replicate, Chi-square= 0, p-value = 1); in the normoxic treatment, 8 females managed to reproduce (4 females per experimental replicate, Chi-square= 0, p-value = 1). The reproductive success, in terms of ratio between gravid and non-gravid females, was not statistically different between cyclic hypoxia and normoxia (Chi-square: 1.564, df=1, p-value=0.21, Fig. 4.5A). Similar results were obtained from the relative fecundity of the females, which also did not show statistical difference (unpaired t-test= 0.359, df=10, p-value=0.72, Fig. 4.5B).

The mixed model nested Anova revealed a statistical difference (p-val<0.05) in egg volume between females in 100% of the models (1000 models out of 1000, see Supplementary Table 6 – Appendix A for the tabular results from 20 models). Similar results were obtained for egg dry weight, with more than 95% of the models showing statistical significance of the factor female nested in the factor treatment. To picture the variability shown with the mixed model nested Anova, egg volume and egg dry weight from each female were plot in Fig. 4.5C, 4.5D.

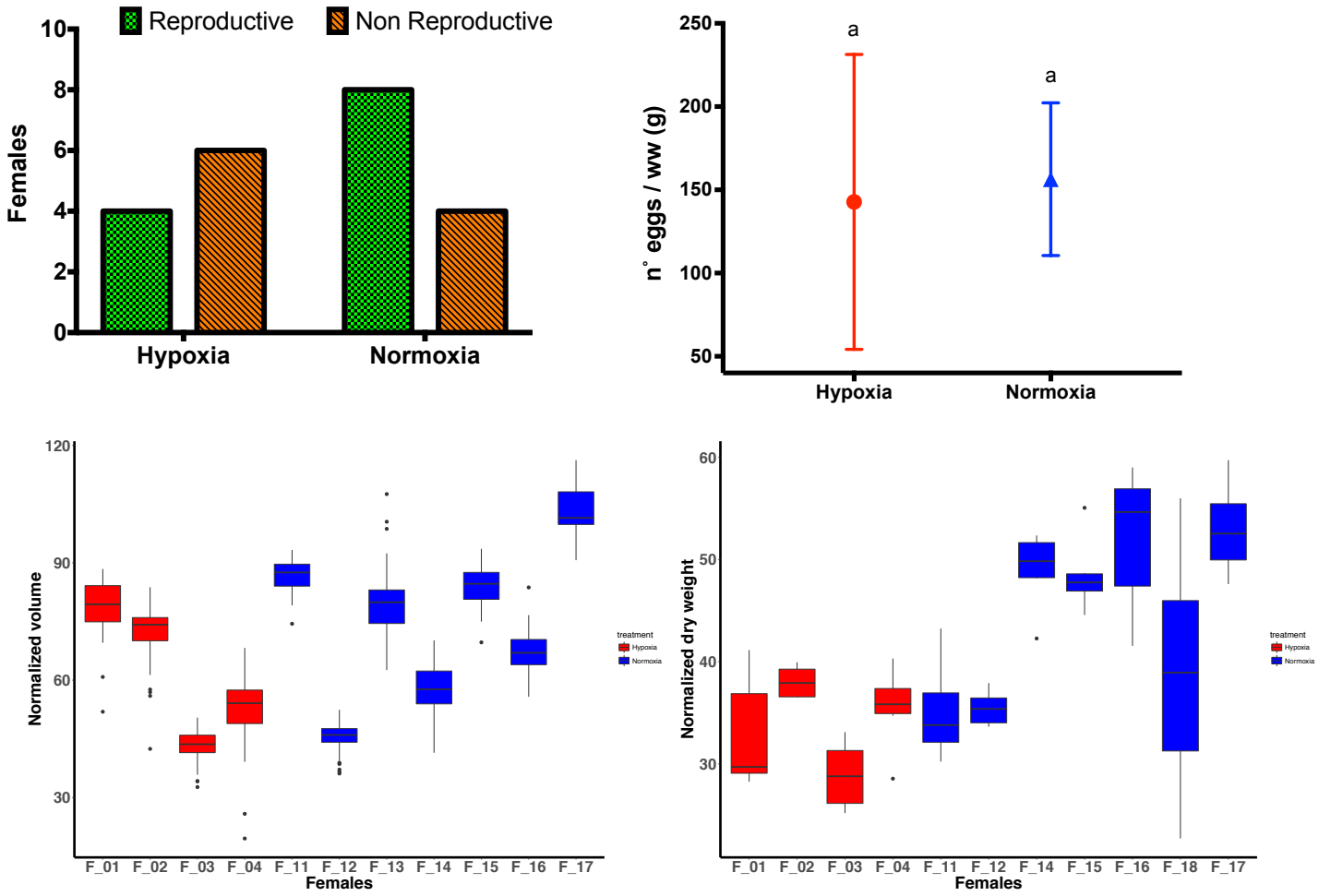

Figure 4.5: Impact of cyclic hypoxia on: **A.** the number of ovigerous females; **B.** the relative fecundity of females; **C.** the volume of bred eggs; **D.** the dry weight of eggs from all the females in the experiment. In **C,D** each female is identified with a different number. Same identifiers were used for the same female in the two graphs. In B. Mean ±SD are plotted and different letters indicate significance values between treatments.

When testing for differences in egg volume and dry weight between treatments, egg volume and egg dry weight were both found statistically smaller in cyclic hypoxic females compared to controls (Mann-Whitney U= 59612, n_{Hyp} = 264; n_{Nor} = 525, p-value<0.001

and unpaired t-test= 2.344, df= 119, p-value=0.03 respectively, Fig. 4.5C, 4.5D). Females kept in cyclic hypoxia produced eggs with a median volume 26% smaller and a dry weight 24% lighter than females in which the ovarian development took place in normoxic conditions.

4.4.4 Larval survival and development:

Throughout the experiment, no difference in larval survival was detected (Chi-square= 0, df= 1, p-value=1, Fig. 4.6A). Similarly, the length of the development process was not impacted by cyclic hypoxia (Mann-Whitney U= 1664, n_{Hyp} = 65, n_{Nor}= 63, p-value=0.055, Fig. 4.6B).

Every other day during development all larvae were given food, which is essential in order to successfully complete larval development (Oliphant & Thatje, 2014). Because animals were fed every other day and because being fed on the day of the final moult could have potentially altered juvenile dry weight, a two-way ANOVA with treatment and food as factors was performed (Tab. 4.4) and revealed a statistically significant interaction between factors. Tukey's multiple comparisons revealed that normoxic larvae that received food on the day before metamorphosis had a heavier dry body mass in comparison to all other groups (Fig. 4.6C).

Table 4.4: Two-way ANOVA table to test the effects of cyclic hypoxia and the presence/absence of food on wet weight of P. varians juveniles. SS: Sum of squares. MM: Mean sum of squares. DFn: Degrees of freedom numerator; DFd: Degrees of freedom denominator

Juveniles' dry weight - ANOVA Table				
	SS	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.010	0.010	F (1, 120) = 7.909	0.006
Food	0.007	0.007	F (1, 120) = 5.677	0.019
Treatment	0.004	0.004	F (1, 120) = 3.1	0.081
Residual	0.157	0.001		

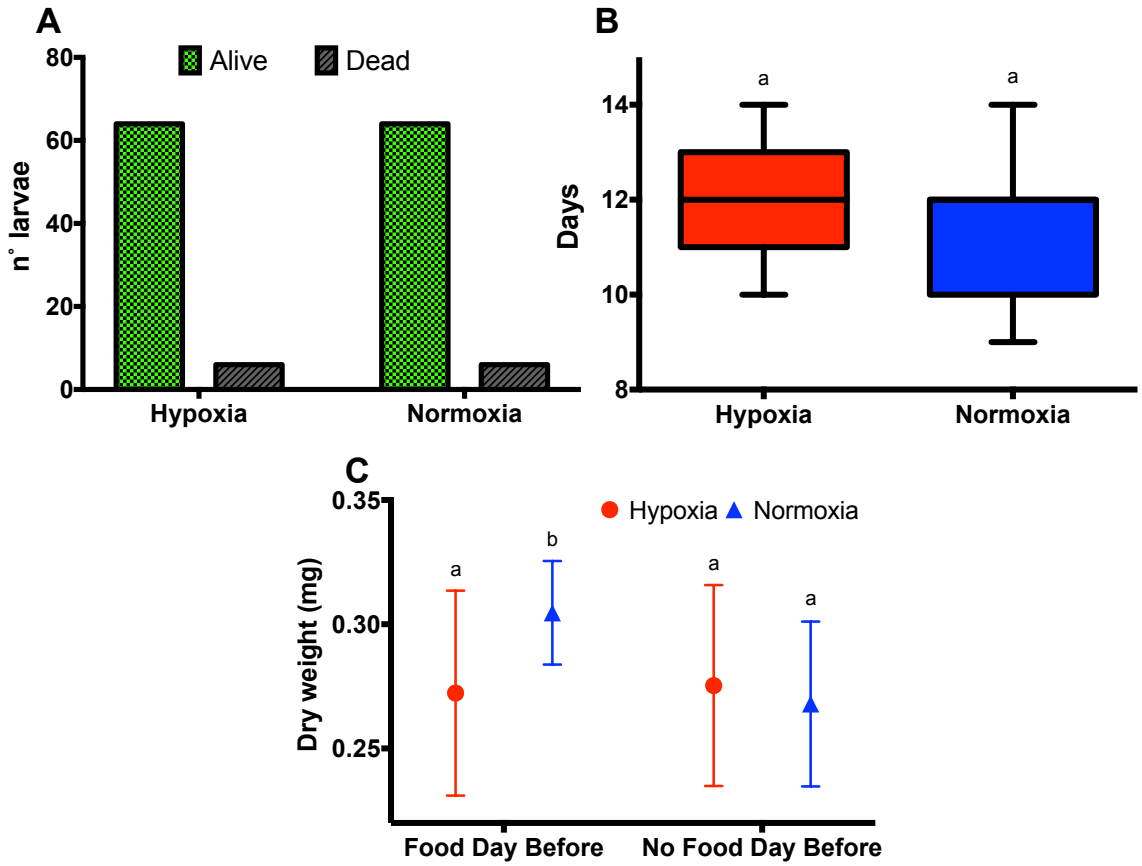

Figure 4.6: Impact of cyclic hypoxia on: **A.** the proportion of larvae that successfully completed development; **B.** the duration of development (n_{Hyp} = 65, n_{Nor}= 63); **C.** the dry weight of juveniles (n = 20 – 40 per treatment per food level) when larval development was completed. In **C.** mean ±SD are plotted. Different letters indicate significance values between treatments.

4.5 Discussion:

4.5.1 Maintenance and growth:

Body size (body length and body mass) is thought to be one of the most important traits for an organism, because it correlates with fundamental aspects of organism's biology and ecology (Fenberg & Roy, 2008). In order to understand the extent to which daily cyclic hypoxic conditions, currently found in Lymington, impact on animal's wet weight and total length over time, adult *P. varians* were kept either in cyclic hypoxia or normoxia for 28 days.

The rate of decrease in wet weight over time was different in the hypoxic exposed animals (i.e. they lost weight more quickly) in comparison to normoxic animals; as a consequence of that, at the end of the experiment, the average body weight of hypoxic animals was ~4% smaller than the average weight of normoxic animals. Similar results were obtained in relation to body length, with hypoxic animals being ~1.4 % smaller than normoxic animals, whereas no overall change in Weight-Length Relationship (WLR) was observed. While a small decrease in body weight over time was observed also in normoxic animals (also reported by Brown-Peterson et al. (2008) in normoxic exposed *P. pugio* kept in the laboratory), it could be argued that this decrease was due to captivity (Pecl & Moltschaniwskyj, 1999; Carmichael & Brush, 2012) or the food regime adopted (McLeese, 1972) or food quality (Linan-Cabello, Paniagua-Michel, & Zenteno-Savin, 2003; Carmichael & Brush, 2012). Captivity and feeding regime were fixed between the treatments group and, therefore, the difference in weight between the two treatments at the end of the experiment was only attributable to the hypoxic stress.

It could be argued that the observed difference in weight (~ 4%) was mainly the result of two different factors (possibly acting in a synergistic fashion): an increased maintenance cost (as proposed by Sokolova et al. (2012)) and a reduced feeding behaviour (in agreement with (Wei et al., 2008; Remen et al., 2012; Davidson et al., 2016)). In this section I will focus on maintenance, as the effects of hypoxia on feeding will be discussed in the next section. An altered physiological status due to stressful conditions would increase the cost of maintenance processes, which would use energy to restore normal conditions (Sokolova et al., 2012). For example, exposing *P. varians* to hypoxic conditions for 6-hours caused a significant increase in lactate content (Chapter 2) and the additional energetic costs associated with restoring homeostasis would be classified as maintenance

costs. Similarly, it could be argued that the acceleration of the moult cycle and the morphological changes to the gills (reported in Chapter 3) are all energetically expensive adaptations that ultimately affected maintenance costs. In fact, if, on the one hand, these mechanisms would improve the efficacy of gas transfer (therefore compensating the hypoxic stress), on the other hand the costs associated with these mechanisms could have arguably affected the wet weight and body length of the animals. Data presented by Harrison et al. (2010) and by Frazier et al. (2001) showed how insects reared in hypoxic conditions invested more energy in expanding their tracheal system (the equivalent of the crustacean gills) and moulted at a smaller body size in comparison to insects reared in normoxia, in accordance with the lower body weight and the morphological changes to the gills observed in *P. varians*. The ability to actively regulate growth and body mass in order to deal with unfavourable environmental conditions (i.e. cyclic hypoxia), as initially noted by Cui (1989), is known as compensatory growth (Wei et al., 2008) and has been well documented in aquatic metazoans (see Wei et al. (2008) for references).

In the field, Li and Brouwer (2013a) showed how a population of *P. pugio* from a cyclic hypoxic marsh was ~10% shorter (in body length) and ~22% lighter (in body weight) in comparison to a population not experiencing cyclic hypoxia. Similar results were obtained on *Palaemonetes vulgaris* larvae (Coiro et al., 2000) and in some fish species, mainly the summer flounder *Paralichthys dentatus* (Stierhoff et al., 2006; Stierhoff et al., 2009; Davidson et al., 2016). Coiro et al. (2000) quantified an impairment of growth between 15% and 70% in *P. vulgaris* larvae according to the cyclic hypoxic regime tested, in contrast to a ~4% reduction in *P. varians* adults from this study. Unfortunately Coiro et al. (2000) only tested hypoxic cycles that had equal time in hypoxia and normoxia (i.e. 6-h hypoxia and 6-h normoxia or 12-h hypoxia and 12-h normoxia, in contrast to 7-h hypoxia and 17-h normoxia of this work). Hence, their experimental conditions were very different from this study. Indeed, it could be argued that longer times in normoxia (as the ones in this study) can facilitate the recovery from hypoxia, therefore smoothing the impact of this stress on physiological processes. In fact, as demonstrated by Coiro et al. (2000) and Stierhoff et al. (2006), growth is generally less impaired in animals exposed to cyclic conditions in comparison to chronic conditions, in which there are no normoxic periods (i.e. no recovery periods). Overall, in accordance to the results presented here, all studies showed a significant reduction in growth as a consequence of cyclic hypoxia, even if the magnitude of reduction differed between species and between cyclic hypoxic regimes (see Chapter 1, Tab. 1.1).

Hitherto a plethora of studies have reported an impairment in growth as a consequence of chronic hypoxia in several fish species (Pichavant et al., 2001; Landry et al., 2007; Stierhoff et al., 2009) and crustaceans (Seidl, Paul, & Pirow, 2005; Wei et al., 2008). Interestingly, Coiro et al. (2000) reported how predicting the effects of cyclic hypoxia on a physiological process by knowing the effects of chronic hypoxia on the same process leads to an underestimation of the effects. In this context, the paucity of studies directly assessing the impacts of cyclic hypoxia on growth of decapod crustaceans is a limiting factor that prevents understanding how the species responds to this stressor.

A reduction in body size has been argued to have an important influence on reproductive output (mainly impacting relative fecundity), on predator-prey relationships and on competition with other species (Fenberg & Roy, 2008; Caruso et al., 2014), with potential consequences at all levels of the ecosystem. In their study, Coiro et al. (2000) tested different cyclic hypoxic regimes (i.e. different pO_2 levels and different duration of the cycles) and found that all conditions caused a significant reduction in growth in comparison to controls, with the magnitude of decrease being proportional to both the duration of the hypoxic period and the level of pO_2 tested (i.e. lower pO_2 and/or longer hypoxic periods had a bigger impact on growth). Davidson et al. (2016) and Stierhoff et al. (2006), while studying the flounder *P. dentatus,* found the same correlation between the level of hypoxia in a cyclic regime and the impact on growth. In addition, Stierhoff et al. (2006), demonstrated that the impact of a cyclic hypoxic regime on organism growth is proportional to the water temperature. Within the context of climate change, the relevance of these conclusions is extremely important. While the cyclic hypoxic regime tested in this study mimicked "natural conditions" currently experienced in Lymington by the species, it could be argued that the body weight will further decrease in the future as hypoxic conditions are predicted to increase in severity (i.e. lower pO_2 levels) and duration thus having a greater impact on maintenance.

4.5.2 Feed ingestion and ammonium excretion:

Feeding and digestion are two fundamental biological processes that require aerobic conditions (Wei et al., 2009) for their execution (in particular digestion) and therefore they can be negatively affected by hypoxia, as widely documented in relation to chronic hypoxia (Llanso & Diaz, 1994; Bell et al., 2003; Siikavuopio et al., 2007; Brandt et al.,

2009). On the first experimental day of cyclic hypoxic exposure, feed ingestion was significantly reduced in hypoxic-exposed animals when food was provided during hypoxia, in accordance with the hypothesis that the hypoxic stress would reduce oxygen-demanding activities (Wei et al., 2009). However, this difference in feed ingestion during hypoxia was no longer detectable at day 21. Arguably, this different behaviour could be the result of physiological changes (e.g. the morphological changes to the gills, discussed in Chapter 3) that were triggered during the experiment. In this context, it is interesting to note that several authors also have described a reduction in feed ingestion as a consequence of chronic or cyclic hypoxia, but none of them reported that feed ingestion resorted back to "normal" levels (i.e. no difference between hypoxic and normoxic animals) after some weeks of exposure (Pichavant et al., 2001; Siikavuopio et al., 2007; Brandt et al., 2009; Remen et al., 2012; Davidson et al., 2016).

If food was provided when the hypoxic period was ceased (i.e. in normoxic conditions), feed ingestion between treatments did not differ (neither at day 1 nor at day 14). This is an indication that adults were quickly able to recover a normal feeding behaviour, once hypoxia terminated. As reported by several authors (Wei et al., 2008; Remen et al., 2012), often hyperphagia (i.e. a higher consumption of food) can be observed when animals are put back in normoxic conditions after a period of hypoxia, as a mechanism to "compensate for the hypoxia-induced growth depression" (Wei et al., 2008). In contrast to Wei et al. (2008) and Remen et al. (2012), no hyperphagia was observed in *P. varians*. This could be due to the different type of hypoxic stressor studied (i.e. Wei et al. (2008) reported hyperphagia in the Chinese shrimp *Fenneropenaeus chinensis* after 10 days of chronic hypoxia), or it could be a species-specific mechanism that is more evident in less hypoxic tolerant species (i.e. Remen et al. (2012) described it in the post-smolt Atlantic salmon, *Salmo salar*, exposed to cyclic hypoxic conditions of ~8.4 kPa (~40% air saturation) while concluding that ~15 kPa (70% air saturation) already represented a threshold for reduced growth).

As mentioned in the previous paragraph, it could be hypothesised that a reduction in growth observed as a consequence of hypoxia is caused not only by an increase in maintenance costs but also by a reduction in feeding (Wei et al., 2008). However, there is still no agreement on how the two factors interact to produce an effect on growth when animals are exposed to hypoxia, as reported by Pichavant et al. (2001). In fact, while Stierhoff et al. (2006) and Davidson et al. (2016) hypothesised that a reduced feed intake was directly responsible for the reduced growth of flounders *P. dentatus* and *Pseudopleuronectes americanus*, data from *P. varians* seem to disagree. In fact, while after

21 days in cyclic hypoxia no difference could be detected in feed intake between hypoxic and normoxic animals, a significant reduction in growth was however observed after 28 days, suggesting that the increased maintenance costs rather than the altered feeding would play a more important role in growth reduction.

Similarly to feeding, another energetically demanding process is ammoniacal excretion and a number of studies have demonstrated its suppression as a consequence of different stressors, such as salinity (Rosas et al., 1999; Diaz et al., 2001), temperature (Leung, Chu, & Wu, 1999; Kiko et al., 2016) or hypoxia (Rosas et al., 1999; Kiko et al., 2015; Kiko et al., 2016). While Rosas et al. (1999) showed a reduction in ammonium excretion in juveniles of white shrimp, *Penaeus setiferus*, only when the hypoxic stressor was concomitant with changes in salinity, in *P. varians* a significant reduction in ammoniacal excretion was found in hypoxic-exposed animals (~ 66 – 75% in comparison to normoxic animals) during the 1st and 21st hypoxic period (i.e. day 1 and day 21, Fig. 4.4). Results from *P. varians* are in agreement with Kiko et al. (2015) and (2016) who demonstrated a reduction in ammonium excretion rates due to hypoxia in calanoid copepods and euphausiids (key components of marine zooplankton who perform diel vertical migrations inside OMZs) and in the squat lobster *Pleuroncodes monodon* (that is able to tolerate anoxic conditions off Peru). It is interesting to note that, while all the above mentioned species from Kiko et al. perform diel migrations in OMZs and hence they must possess physiological adaptations to survive in such conditions, yet their ammonium excretion is lowered in hypoxic conditions in a similar way to what observed in *P. varians*. The reduced excretion rates of ammonium in hypoxic-exposed animals during hypoxia after 21 days indicated that excretion was still suppressed (in contrast to feed ingestion). Further, when the hypoxic period was ceased (i.e. in normoxic conditions), ammoniacal excretion did not differ between hypoxic and normoxic-exposed animals (neither at day 1 nor at day 14), indicating that the impaired excretion was a temporary condition persisting only during hypoxia.

With the exception of the aforementioned studies (Rosas et al., 1999; Kiko et al., 2015; Kiko et al., 2016), in which the effects of acute hypoxia on ammonium excretion were investigated, so far, no study has evaluated the effects of daily cyclic hypoxia on ammonium excretion on marine invertebrates. This is in contrast to the vast literature that showed how feeding can be impaired due to hypoxia. From an ecological point of view, the fact that ammonium excretion, contrary to feeding, was still lower after 21 days of exposure, could arguably have important consequences on the ecosystems populated by *P. varians* (i.e. UK salt marshes), in particular on nutrient cycling and energy fluxes. In fact,

as reported by Escaravage and Castel (1990a) and Aguzzi et al. (2005), *P. varians* plays a key ecological role in macerating detritus and dead marsh plants and excreting large quantities of ammonia (Welsh, 1975; Aguzzi et al., 2005), thus making energy available at a variety of trophic levels and supporting heavy growth of microflora (Welsh, 1975). Therefore, it could be hypothesized that a decrease in nutrient turnover caused by a reduced excretion of ammonia could affect the microbial community with possible implications also at higher levels of the trophic chain.

4.5.3 Reproduction:

Reproduction is an essential process because it permits the propagation of the species, but it is also energetically expensive (Sokolova et al., 2012) and hence it is frequently affected when animals experience stressful conditions (Wu, 2002; Petes, Menge, & Harris, 2008; Sokolova et al., 2012). While the proportion of females that successfully hatched eggs and their fecundity were not altered during the experiment, a significant reduction in egg volume and egg dry weight (both scaled to female body weight) were found. In other studies on the closely related species *Palaemonetes pugio*, different results were obtained when animals were exposed to chronic (Brouwer et al., 2007) or cyclic hypoxia (Brown-Peterson et al., 2008; Brown-Peterson et al., 2011). As reported by Brouwer et al. (2007), exposure to chronic hypoxia (4 kPa at 27 ˚C) for 4 weeks did not alter the proportion of females that successfully hatched eggs but increased their fecundity. It could be argued that the different results obtained by Brouwer et al. (2007) in comparison to this work might depend on the type of stressor (i.e. chronic versus cyclic hypoxia) (Landry et al., 2007). In fact, while in cyclic hypoxia the presence of normoxic periods (where animals could recover, (Coiro et al., 2000)), might have reduced the overall level of stress experienced by the organisms, the constant hypoxic stress in chronic hypoxia might have driven a change in physiology to maximise reproductive output in such conditions, as hypothesised by Brouwer et al. (2007). This is in agreement with Brown-Peterson et al. (2008), who reported a decrease in fecundity (an opposite result in respect to Brouwer et al. (2007)) in *P. pugio* exposed to diel cyclic hypoxia for 77-days. Interestingly, Brown-Peterson et al. (2011), while comparing the cyclic hypoxic conditions from two different bay systems in Florida, identified that relative fecundity and percentage of gravid females decreased (~ 34% and ~60% respectively) in one site experiencing more

severe diel hypoxia (from 2.3 to 21 kPa, with 2.9 h per day below 5 kPa), whereas only the percentage of gravid females was decreased (~40%) in the site experiencing moderate cyclic hypoxia (from 4.1 to 21 kP, with 0.7 h per day below 5 kPa). Taken together, results suggest a variability in response to hypoxia which depends on the type of stressor (i.e. chronic or cyclic) and, in the case of cyclic hypoxia, on its severity (i.e. hours per day spent in hypoxic conditions and minimum pO_2 experienced during hypoxia).

The level of maternal resources invested into eggs, known as per offspring investment (POI) (Oliphant & Thatje, 2014), is of fundamental importance within life history biology (J Marshall & Uller, 2007), as higher POIs are generally associated with shorter development times (Giménez & Torres, 2004). In addition to a lowered fecundity, Brown-Peterson et al. (2008) reported no change in larval starvation resistance (a proxy for egg's yolk content and hence POI (Oliphant, 2013)) as a consequence of cyclic hypoxia. In contrast to Brown-Peterson et al. (2008) and Brown-Peterson et al. (2011), an opposite pattern was observed in this study, where no change in fecundity but a decrease in egg volume and egg dry weight were found. It could be argued that the observed differences between the species are the result of constraints dictated by the different larval development processes of *P. pugio* and *P. varians*. While the development of *P. pugio* encompasses 11 different larval stages (Broad, 1957), the development of *P. varians* is shorter and comprises only 5 larval stages (Oliphant, 2013). In the light of these differences it could be hypothesised that supporting POI by lowering fecundity is functional for *P. pugio* in hypoxic conditions (as demonstrated also with an *in situ* experiment from Brown-Peterson et al. (2011)) as it would not delay the long larval development process, whereas, in *P. varians*, a decrease in POI would have less impact on the shorter larval development process. In this way, by reducing egg volume (~26% in comparison to eggs from normoxic females) and dry weight (~24%) *P. varians*' fecundity would not change and females would be able produce the same number of offspring. Similar examples of opposite reproductive strategies have been already reported in relation to hypoxia. In example, while the Atlantic croaker *Micropogonias undulatus* reduced yolk content in relation to hypoxia (Tuckey & Fabrizio, 2016), the bivalve *Macoma balthica* increased yolk content in each egg (i.e. increased POI) (Long et al., 2014).

In spite of the great attention to the impacts of hypoxia, hitherto only a limited number of studies have addressed the question "how does cyclic hypoxia affect reproduction in marine organisms" (Brown-Peterson et al., 2008; Cheek et al., 2009; Brown-Peterson et al., 2011; Cheek, 2011; Bera et al., 2017). To the best of my knowledge this is the first study assessing the impact on reproduction from long-term daily cyclic

hypoxia on a decapod species from Europe. The opposite reproductive strategy between *P. varians* (this study) and the closely related American species, *P. pugio* (Brown-Peterson et al., 2008), emphasizes the relevance of this work in outlining how the reproductive strategies can differ even between closely related species and outlines the need to test the effects of cyclic hypoxia on other coastal decapod species, in order to gain a better understanding of how coastal species respond to this phenomenon.

4.5.4 Development:

Larval development is a complex process that starts after hatching and involves a series of anatomical changes terminating with the juvenile phase (Oliphant, 2013). In this study, larvae from *P. varians* were raised either in cyclic hypoxia or in normoxia from the stage of zoea 1 (the first larval stage after hatching) up to the juvenile stage (at the end of the larval development) (Fincham, 1983; Oliphant, 2013). At the end of the experiment no difference in larval survival was observed between the normoxic and hypoxic treatment. In a similar way, developmental duration was not affected by cyclic hypoxia. It could be argued that the lack of difference in survival and development duration depended on the experimental conditions tested (i.e. the severity of the diel hypoxic regime used). Although Coiro et al. (2000) demonstrated an impairment of growth in *P. vulgaris* larvae exposed to cyclic hypoxia but the experimental conditions were very different. Whilst the *P. vulgaris* larvae were kept at ~ 4 kPa (below the species p_{crit}) and were only allowed 12 hours per day to restore normal homeostasis in normoxia (i.e. recovery time, (Coiro et al., 2000)), in this work *P. varians* larvae were kept slightly above the p_{crit} (~ 5.5 kPa) and had longer recovery time (18 hours). The lack of a difference in development duration is also an indication of the tolerance to hypoxia of *P. varians* larvae, which, as postulated by Miller, Poucher, and Coiro (2002), Yannicelli and Castro (2013) and Alter et al. (2015), is directly comparable with the environmental variability experienced by the species (for reference see Chapter 2).

Every other day during development all larvae were given food, which is essential in order to successfully complete *P varians*' larval development (Oliphant & Thatje, 2014). Research on larvae from the squat lobster *Pleuroncodes monodon* led Yannicelli and Castro (2013) to conclude that the effect of hypoxia on larval development "would rather result from an impairment of acquiring/metabolizing external sources of energy rather than

from direct cellular malfunction". This conclusion seems to agree with the higher dry weight of normoxic animals fed the day before metamorphosis, compared to hypoxic animals. In cyclic hypoxic conditions larvae might build, during hypoxia, some oxygen debt (i.e. consequence of lactate accumulation (Alter et al., 2015)) that might delay oxygen-expensive processes like feeding and digestion, and might in turn be the reason for the observed reduction in juvenile's dry weight.

4.6 Conclusions:

Many important coastal ecosystems can be affected by cyclic hypoxia on a daily basis and little is known in relation to the consequences of such phenomenon (in contrast to a vast literature on chronic hypoxia) on the physiology and/or ecology of species living in these habitats. As pointed out during the discussion, it is very difficult to estimate the effects of cyclic hypoxia from chronic hypoxia because these two stressors either lead to dissimilar effects on the physiology of the species or they exert similar effects but with a different magnitude. It is therefore very important to study cyclic hypoxia directly, and not by estimating its effects from chronic hypoxia exposure.

In an attempt to fill some of the current gaps, this study assessed changes on some physiological processes of the decapod shrimp *Palaemon varians* induced by a long-term daily cyclic hypoxic regime, currently detected in *P. varians*' habitat. Results outlined a significant impairment of all the tested physiological processes, even if the magnitude of the effects differed between processes. After 28 days of daily cyclic hypoxia, an increased cost of maintenance (identified as a ~4 % reduction in body weight) was observed in hypoxic animals, coupled with a ~1.4 % decrease in body length. While feed ingestion, initially suppressed under cyclic hypoxic conditions, was not different from normoxic animals after 21 days, ammoniacal excretion was still reduced by ~ 66 – 75 % in hypoxic animals after 21 days. Ovigerous females in cyclic hypoxia were able to maintain a similar fecundity in comparison to normoxic females, but they reduced the amount of yolk in each egg (also known as POI) by ~ 24%. The implications linked with these results vary at different levels. In fact, while it could be argued that the reduction in growth could likely affect *P. varians*' predator-prey interactions, the reduced ammoniacal excretion could arguably alter nutrient cycling and energy transfer within the ecosystem. Overall the results

emphasise how all the studied physiological processes are affected by this cyclic hypoxic regime, which mimicked current environmental conditions within the Lymington salt marshes.

Chapter 5 The effects of long-term acclimation to cyclic hypoxia on thermal and copper (Cu^{2+}) tolerance

Summary:

In their environment, organisms are constantly facing variations of the main physiochemical parameters, therefore it is important to understand the effects deriving from the occurrence of two or more stressors on physiological performances of marine and freshwater organisms. In its environment *Palaemon varians* is experiencing daily cyclic hypoxia but other sources of acute stress (i.e. thermal stress and copper, Cu^{2+}, pollution) can affect its physiology. In order to understand how acute thermal tolerance and Cu^{2+} tolerance are impaired after a long-term exposure to daily cyclic hypoxia in the shrimp *P. varians*, animals were exposed for 28 days to cyclic hypoxia and then critical thermal maximum CT_{max} and copper Cu^{2+} toxicity (30 mg L^{-1}) were assessed (in normoxic conditions). Cyclic hypoxic animals showed an increased CT_{max} (ranging from ~0.9 to ~1.7˚C) in comparison to normoxic animals. Mortality due to Cu^{2+} was higher in the normoxic acclimated group exposed to Cu^{2+} (~55%) compared to the cyclic hypoxic acclimated group exposed to Cu^{2+} (~26%). In addition, a higher gene expression of metallothioneins *Mt* (genes involved in detoxification) was found in the hypoxic group exposed to Cu^{2+} in comparison to the normoxic group exposed to Cu^{2+}, suggesting a higher detoxification ability in the hypoxic acclimated group. Results suggest that long-term daily cyclic hypoxia is able to improve thermal tolerance and reduce Cu^{2+} mortality in adult *P. varians*. A possible explanation might be found in the morphological changes to the gills (i.e. greater lamellar surface area) induced by cyclic hypoxia. These changes would provide a greater efficiency in gas exchange and in turn sustain respiratory functions for a longer time when animals are facing heat shock; or could alleviate the magnitude of damage to the gills as a consequence of Cu^{2+} and therefore prevent (or at least delay) the onset of internal anoxia and the consequent death due to Cu^{2+}.

5.1 Introduction:

The environmental conditions that depict natural habitats are not shaped by a single factor only (e.g. oxygen, or temperature, or pH) but are characterized by a continuous co-variation of multiple parameters (Sperling, Frieder, & Levin, 2016). Environmental data presented in Chapter 2 are a typical example with daily variations in temperature and pO_2. In the past, research was primarily focussed on the manipulation of a single environmental variable in order to assess organismal performance under changing conditions, yet "in nature, organisms are simultaneously exposed to multiple environmental variables, including stressors that may affect different physiological systems" (Todgham & Stillman, 2013).

Global warming is currently altering the ecosystems worldwide (Portner & Farrell, 2008) and temperature and O_2 are considered two of the major environmental variables that affect the overall biology of marine animals (Burleson & Silva, 2011). The range of temperatures tolerated by an organism is ecologically important because it sets the fundamental thermal niche (Ern et al., 2016) and therefore limits the habitats that can be colonized by the species. The upper limit of the thermal niche is traditionally referred to as the critical thermal maximum (CT_{max}) and is the temperature where animal function ceases due to the collapse of one or more vital physiological functions (Portner, 2010; Burleson & Silva, 2011). It has been hypothesised that thermal tolerance (i.e. CT_{max}) is limited by the capability of the cardiorespiratory system to supply oxygen to the tissues and hence oxygen availability (e.g. hypoxia) could change CT_{max} (Verberk et al., 2016). In this context, a plethora of studies assessed how acute hypoxia affects CT_{max} with contrasting outcomes (see Verberk et al. (2016) for an exhaustive review). In example acute hypoxia did not affect CT_{max} in the prawn *Penaeus monodon* (Ern et al., 2015), while it reduced CT_{max} in the crayfish *Astacus astacus* (Ern et al., 2015) and in the isopod *Porcellio scaber* (Stevens et al., 2010). In such a variable environment as the Lymington marshes, cyclic hypoxic conditions develop regularly during the night when temperatures are decreasing (Chapter 2, Figure 2.5) whereas thermal stress can be experienced occasionally during the day (when temperatures increase). Hence, in its environment, *P. varians* is likely to experience the two stressors in succession, rather than simultaneously. In the context of a warming world where thermal stress is expected to exacerbate, it is therefore important to elucidate how acclimation to cyclic hypoxia can have an effect on thermal tolerance by altering CT_{max}.

In addition to climate change, marine habitats are under increasing threat from human activities (Doney et al., 2012) that cause environmental contamination due to heavy metals (Lorenzon, Francese, & Ferrero, 2000). Copper is among the most toxic of the heavy metals in freshwater and marine biota (Eisler, 1998), and it has been identified as the metal which poses the greatest threat to organisms in UK waters (Donnachie et al., 2014; Fitzgerald et al., 2016). Copper releases to the biosphere come mostly from activities such as mining, smelting, industrial emissions, municipal wastes and sewage sludge (Eisler, 1998). Copper contamination in coastal waters also derives from its usage in antifouling paint (Claisse & Alzieu, 1993; Schiff et al., 2007; Dafforn, Lewis, & Johnston, 2011) or in compounds used as biocides (Eisler, 1998). Copper concentration in rivers and coastal habitats in not always constant in time (Şeker & Kutlu, 2014): a survey conducted on the estuary harbour of Southampton and on the Ocean Village marina (Southampton, UK) showed how the concentration of labile Copper (i.e. free copper ions and inorganically bound copper) doubled during Spring and Summer 2001/2002, in comparison to the concentrations measured in Autumn and Winter 2001/2002 (e.g. from 0.2 $\mu g\ L^{-1}$ in Winter and Autumn to 0.45 $\mu g\ L^{-1}$ in Spring and Summer) (Jones & Bolam, 2007).

Copper is particularly important for its elevated toxicity to molluscs crustaceans (Hebel, Jones, & Depledge, 1997; Eisler, 1998). The primary lethal effect of copper in gastropod mollusks is caused by disruption of the transporting surface epithelium (Eisler, 1998). In crustaceans, copper decreases hemocyanin-oxygen affinity (Truchot & Boitel, 1992), reduces the activity of glycolytic enzymes, alters sodium-potassium ATPase activity (Eisler, 1998) and, among all, induces anatomical and cytological damage to the gills (Spicer & Weber, 1992; Hebel et al., 1997; Eisler, 1998; Soegianto et al., 1999): these changes include thickening of the epithelium (Spicer & Weber, 1991, 1992; van Heerden, Vosloo, & Nikinmaa, 2004) with edema (Alkobaby & Abd El Wahed, 2017), formation of necrotic areas and gaps between cuticle and epithelium (Soegianto et al., 1999) that affect the ability to uptake oxygen from the water, eventually resulting in internal hypoxia (Spicer & Weber, 1992).

Animals have evolved three main cellular mechanisms in order to defend themselves from the toxic effects of heavy metals: *i*) mechanisms balancing metal excretion rates with uptake; *ii*) intracellular sequestration mechanisms (involving proteins known as metallothioneins (Coyle et al., 2002)) followed by elimination through the lysosomal endomembrane system; *iii*) intracellular sequestration processes in specific vacuoles, producing solid metallic phosphorous granules (Brown, 1982) which subsequently undergo exocytosis (Depledge & Rainbow, 1990; Ahearn, Mandal, &

Mandal, 2004). Metallothioneins are cysteine rich, low molecular weight proteins that, in uncontaminated conditions, act as reservoir of cations such as copper (Ahearn et al., 2004), but, when heavy metal contamination is present, are able to sequestrate the excess ions, preventing damage to the cellular machinery (Coyle et al., 2002; Ahearn et al., 2004). In fact, free copper ions in the cytoplasm are able to trigger the production of Reactive Oxygen Species (ROS), such as the superoxide (O_2^-) radical, which would in turn cause lipid peroxidation (Barata et al., 2005; Rhee et al., 2011; Atli & Grosell, 2016). One of the most extensively studied enzymes involved in protection against ROS is Superoxide Dismutase (SOD), which catalyzes the dismutation (or partitioning) of O_2^- into either ordinary molecular oxygen (O_2) or hydrogen peroxide (H_2O_2) (Rhee et al., 2011).

Hitherto a plethora of studies that evaluated the co-occurrence of Cu^{2+} and acute hypoxia (Spicer & Weber, 1992; Fitzgerald et al., 2016; Sappal et al., 2016; Fitzgerald, Katsiadaki, & Santos, 2017) or chronic hypoxia (Eriksson & Weeks, 1994; Mustafa, Davies, & Jha, 2012) and results generally report an increased Cu^{2+} toxicity in concomitance with acute or chronic hypoxia. Given the temporal variability in Cu^{2+} concentration during the year (Jones & Bolam, 2007), *P. varians* could be subjected to environmental Cu^{2+} pollution after being exposed to cyclic hypoxia. In this context, no study has evaluated how a cyclic hypoxic acclimation could alter Cu^{2+} tolerance in decapod crustaceans.

For all the above considerations, this Chapter will focus on how thermal and Cu^{2+} tolerance are affected in *P. varians* following a 28 days period of acclimation to cyclic hypoxia.

5.2 Specific chapter hypothesis:

1. Prolonged exposure to daily cyclic hypoxia (28 days) will alter the critical thermal maximum of animals in comparison to control animals
2. Prolonged exposure to daily cyclic hypoxia (28 days) will alter Cu^{2+} tolerance of animals.

5.3 Material and Methods:

5.3.1 The effect of prolonged daily cyclic hypoxia on critical thermal maximum CT_{max}:

Adult *Palaemon varians* collected from Lymington salt marsh were exposed to either the normoxic- or daily cyclic hypoxic-conditions described in Chapter 3 for 28 days (see Supplementary Table 1 – Appendix A for mean pO_2 values recorded during hypoxic periods in each tank). Stocking density was 18 animals per tank. At the end of this period, animals from the two treatments were tested for CT_{max} in two times of the day: 2 and 6.5 hours after the end of the diel hypoxic period (n= 7 animals per treatment per time of day). For both treatments, the normoxic and hypoxic-treatment, assessment of CT_{max} was carried out in normoxic conditions in order to evaluate the effects of cyclic hypoxic acclimation on thermal tolerance. Briefly, animals were individually placed in a 500 mL beaker with 250 mL of oxygenated and filtered sea-water at 22 ˚C inside a water bath (Thermo Electron Corporation Haake W46 water bath). The water temperature was monitored to the nearest 0.1 °C using an electronic thermometer. Initial temperature was 22 ˚C, and it was increased at a constant rate of 0.33 ˚C min^{-1} according to (Ravaux et al., 2012; New et al., 2014). Behaviour in response to temperature was recorded with a GoPro Hero 3+ black camera in order to determine the onset of Loss of Equilibrium (LoE). LoE was defined as the water temperature at which the shrimp rested on the bottom in either an "upside-down" or a "side-ways" position for more than 2 s, (Ravaux et al., 2012; New et al., 2014). CT_{max} was defined as the first temperature at which LoE was observed (Ravaux et al., 2012). After the test animals were immediately snap frozen in liquid N_2 in order to extract RNA from their cephalothorax for gene expression analysis.

5.3.2 The effect of prolonged daily cyclic hypoxia on survival of acute Copper (Cu^{2+}) exposure:

Adult *Palaemon varians* were "acclimated" to normoxia or daily cyclic hypoxia for 28 days as described in section 5.3.1. After 28 days, survival of acute Cu^{2+} exposure was tested in normoxic conditions to assess the effects of cyclic hypoxic acclimation on acute Cu^{2+} survival. Individuals acclimated to hypoxia were placed in 10 L plastic aquaria (n= 7

per aquarium) and were exposed to artificial (32 PSU) seawater (n= 20 in total) or artificial seawater spiked with metal (n= 21 in total) by the addition of a stock solution of $CuSO_4 \cdot 5H_2O$ and incubated at 22 ˚C. An identical method was used for individuals acclimated to normoxic conditions. Stock solutions were prepared using deionised water and analytical reagent grade compounds. Exposure concentrations (Cu: 0 and 30 mg L^{-1}) were selected based on available lethal copper toxicity data in palaemonids (96 h lethal concentration to 50% of individuals: 37.0 mg L^{-1} in *Palaemonetes pugio* at 22 ˚C, (Curtis & Ward, 1981)). Mortality was assessed every 24 hours. Seawater oxygen saturation was determined at the end of each 24-hour period using an oxygen micro-optode connected to a PreSens Microx TX3 array, calibrated according to manufacturer's instructions. Oxygen saturation did not decrease below 70% in any treatment. After 6 days, surviving animals were immediately snap frozen in liquid N_2. Cephalothorax was used for gene expression analysis, whereas the abdomen was used to assess activity of the superoxide dismutase (SOD) enzyme.

5.3.3 Gene expression:

RNA was extracted from cephalothorax of animals (n= 8-9 animals per treatment per Cu^{2+} dose) previously snap frozen in liquid N_2. Total RNA was extracted using a TRI-Reagent™ (Sigma Aldrich) protocol according to the manufacturer's recommendations. RNA concentration and integrity were assessed as previously described in Chapter 3, section 3.3.3. Subsequently, RNA was DNase treated and reverse transcribed and cDNA was used to perform qPCR reactions as previously described in Chapter 3, section 3.3.5.

Primer-sets used are reported in Table 5.1. All primers were tested according to the MIQE guidelines (Bustin, 2010). Candidate reference genes were tested by geNorm analysis using qBase+ software (Biogazelle, Belgium). The combination of the elongation factor 1-alfa (*eef1A*) and ribosomal protein L8 (*rpl8*) genes provided the best normalisation strategy for this study. After assessing housekeeping's stability, the geometric mean of the two reference genes was used to normalise gene of interest expression. Calibrated, normalised relative quantities (CNRQs) were calculated using qBase+ software. CNRQs were then plotted and subjected to statistical analysis.

Table 5.1: List of qPCR primers used. *Rpl8* and *EF1-alfa* were used as reference genes, after assessment of their stability, according to Bustin (2010). Linear range refers to the linear range of serial dilutions (1:10) of cDNA over which the standard curve is calculated. R^2 represents the degree of linearity of the standard curve.

ID primer	**Final conc (nM)**	**5'-3' sequence**	**Efficiency**	**R^2**	**Linear range:**
Rpl8 FOR	900	TCCCGGTCGTGGTGCACCTATT	1.84	0.99	6
Rpl8 REV	900	GACGGCCTCGGTCACCAGTCTTT			
EF1-alfa FOR	300	ACAGCACTGAGCCCAAGTAT	1.88	0.99	5
EF1-alfa REV	300	GAAATGGGAAGGATTGGCACA			
cHH FOR	50	CGGCCTGGCTAGGATAGAAA	1.95	0.99	4
cHH REV	300	GCCCGCTTCTTCAGATTCAG			
HIF FOR	300	GAGAGCGAGATCTTCACGGA	2.05	0.98	4
HIF REV	300	TGAGGAAAGCGATGGTGAGT			
Hsp70 FOR	50	CCAGCCGTCACCATCCAGGTGT	2.05	0.99	3
Hsp70 REV	50	GCGGTCGATGTCCTCCTTGCTG			
Ldh FOR	300	TGGGAATGATGCCCTTGAA	1.88	1	4
Ldh REV	900	GAATCTCGCCTTTCCCTTGTC			
SOD FOR	300	ATTTGCTTCGCAGCCATAGG	1.8	1	4
SOD REV	300	CTTTCGTATCGCCCACTTCG			
Mt FOR	300	TAAATGCGACTGCGCTTCTG	1.94	0.99	5
Mt REV	300	TGGCGCAGTCTTCTTTTGAG			

5.3.4 Superoxide dismutase (SOD) enzyme activity:

Frozen tissue from 8 animals per treatment per Cu^{2+} dose was initially weighed with an analytical balance (Denver Instrument si-234 Colorado - USA, weight ± 0.0001g) and then immediately homogenised at 4°C in Tris-HCL pH 7.6 buffer (volume used was four times the weight of the sample). Following 10 mins incubation at 4°C, the homogenates were centrifuged at 1000g for 10 min at 4°C and supernatant was retained for analysis. Total protein and enzyme activities were determined fluorometrically using a microplate reader (FLUOstar OPTIMA, BMG Labtech). Total protein was assessed at 584 nm absorbance using an assay kit employing the Lowry method with Peterson's modification

(Sigma-Aldrich TP0300). Superoxide dismutase (SOD) activity was assessed at 450 nm absorbance using an assay kit employing inhibition of xanthine oxidase activity (Sigma-Aldrich 19160). Total protein content was normalised on the wet weight of the corresponding sample. The normalized total protein content was used to normalize SOD activity.

5.3.5 Statistical analysis:

The effects of cyclic hypoxic acclimation on: CT_{max}, gene expression, and SOD activity were assessed using a Two-way ANOVA with "acclimation" and "time of day" as factors (in the case of CT_{max}) or with "acclimation" and "Cu^{2+} dose" in the case of Cu^{2+}; for all analysis post-hoc Tukey's multiple comparisons test were further used. Statistical significance was identified at p-value < 0.05.

Survival of animals exposed to Cu^{2+} was tested using Log-rank (Mantel-Cox) test and statistical significance was identified at p-value < 0.05.

5.4 Results:

5.4.1 The effect of prolonged daily cyclic hypoxia on critical thermal maximum CT_{max}:

The onset of CT_{max} in animals was behaviourally determined by measuring the temperature at which Loss of Equilibrium (LoE) was observed. CT_{max} was significantly affected by the interaction of the two factors (Acclimation and Time of day), Table 5.2.

Table 5.2: Two-way ANOVA table to test the effects of hypoxic acclimation on thermal tolerance by means of CT_{max}.

ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	3.21	1	3.21	F (1, 21) = 32.96	P<0.0001
Time of day	0.00	1	0.00	F (1, 21) = 0.006	0.94
Acclimation	1.76	1	1.76	F (1, 21) = 18.11	P<0.001
Residual	2.04	21	0.10		

When CT_{max} was assessed at 1200 hrs, no difference was detected between hypoxic-exposed and normoxic-exposed animals (mean CT_{max}: 36.9 ±0.4 ˚C and 37.1 ±0.3 ˚C, respectively, Fig. 5.1); on the other hand, when animals were tested in the afternoon (1630 hrs), CT_{max} was statistically higher in the hypoxic group, compared to the normoxic group (mean CT_{max}: 37.65 ± 0.2 ˚C and 36.4 ±0.3 ˚C, respectively). CT_{max} calculated at 1200 hrs in both groups was statistically lower when compared to CT_{max} of the "afternoon-hypoxic group", whereas it was statistically higher than CT_{max} of the "afternoon-normoxic group" (Fig. 5.1).

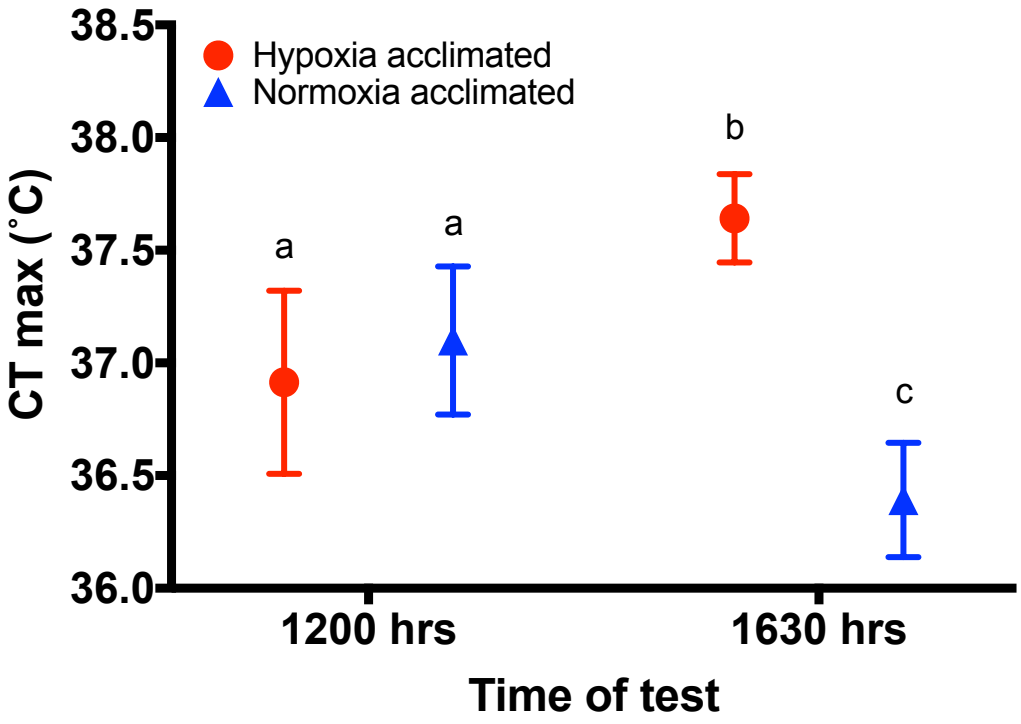

Figure 5.1: CT_{max} (means ±SD, n=7 per each treatment in each time point) calculated in the two groups (hypoxic- and normoxic-acclimated animals) at different times of the day. Different letters indicate statistical difference.

Gene expression analysis revealed different patterns across the analysed genes (Tab. 5.3 and Fig. 5.2): the relative expression of crustacean hyperglycaemic hormone (*cHH*) and heat shock protein 70 (*HSP70*) (two markers of generic stress in decapod crustaceans (Webster, Keller, & Dircksen, 2012; Morris, Thatje, & Hauton, 2013)) was ~50% higher in the hypoxic group at 1200 hrs, in comparison to every other group (Fig. 5.2), whereas no change in the relative expression of lactate dehydrogenase (*Ldh*) and hypoxia inducible factor (*HIF*) was found between any of the groups.

Table 5.3: Two-way ANOVA table on gene expression levels of **A)** *cHH*, **B)** *HSP70*, **C)** *Ldh* and **D)** *HIF*.

A. *cHH* Expression - ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.52	1	0.52	F (1, 22) = 5.776	0.03
Time of day	0.22	1	0.22	F (1, 22) = 2.415	0.13
Acclimation	0.45	1	0.45	F (1, 22) = 5.004	0.04
Residual	1.99	22	0.09		

B. *HSP70* Expression - ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.12	1	0.12	F (1, 21) = 1.258	0.27
Time of day	0.56	1	0.56	F (1, 21) = 5.876	0.02
Acclimation	0.43	1	0.43	F (1, 21) = 4.520	0.04
Residual	1.98	21	0.09		

C. *Ldh* Expression - ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.02	1	0.02	F (1, 23) = 0.212	0.65
Time of day	0.06	1	0.06	F (1, 23) = 0.522	0.48
Acclimation	0.01	1	0.01	F (1, 23) = 0.047	0.83
Residual	2.56	23	0.11		

D. *HIF* Expression - ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.18	1	0.18	F (1, 22) = 2.465	0.13
Time of day	0.14	1	0.14	F (1, 22) = 1.931	0.18
Acclimation	0.08	1	0.08	F (1, 22) = 1.11	0.30
Residual	1.58	22	0.07		

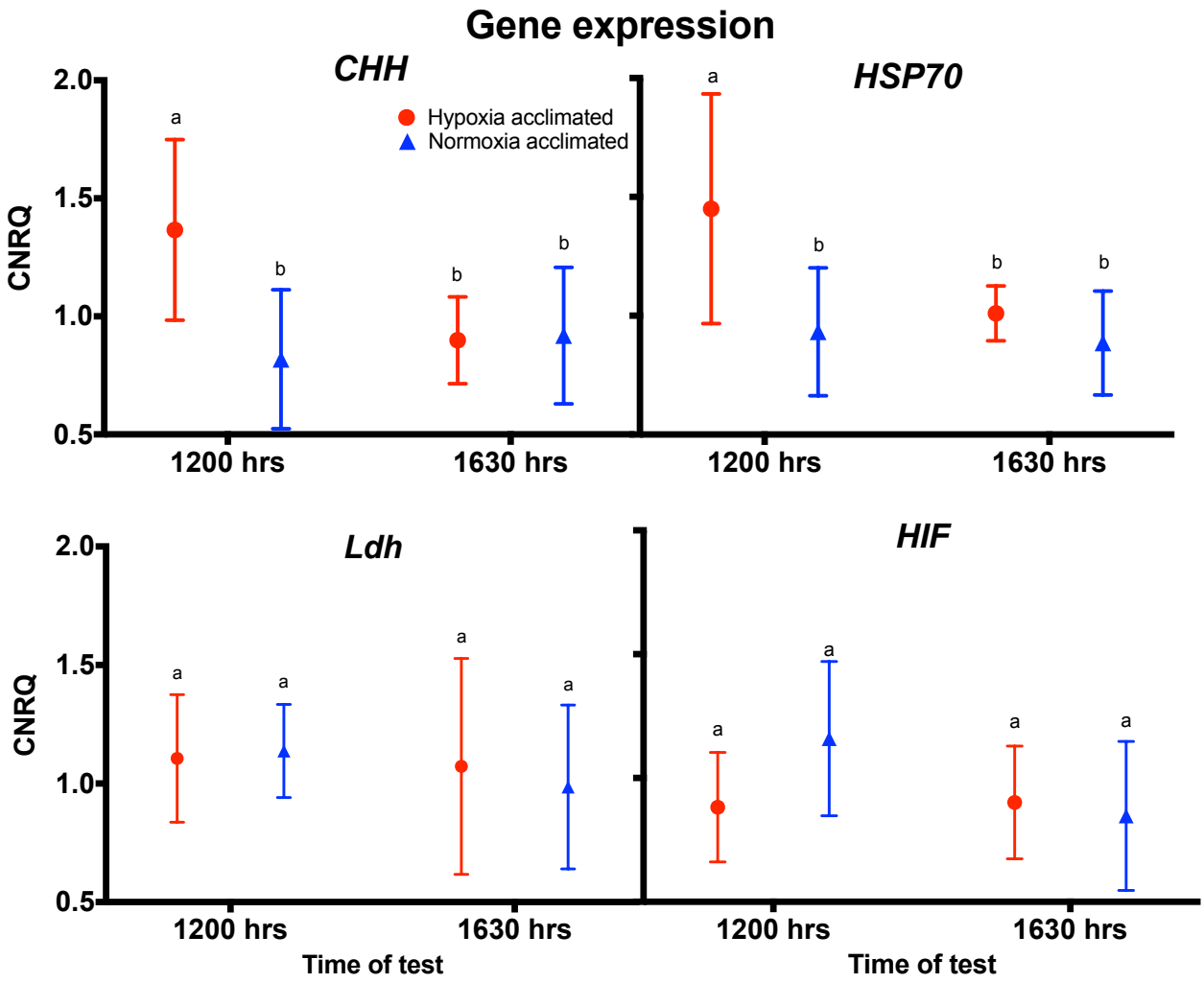

Figure 5.2: Gene expression of *cHH*, *HSP70*, *Ldh* and *HIF* (means ±SD, n=5-6 animals per each treatment per each time point) in the two groups (hypoxic- and normoxic-acclimated animals) at different times of the day. Different letters indicate statistical difference.

5.4.2 The effect of prolonged daily cyclic hypoxia on survival of acute Copper (Cu^{2+}) exposure:

Survival was not statistically different between the hypoxic- and normoxic-acclimated groups at 0 mg L^{-1} Cu^{2+} (Mantel-Cox test, χ^2: 1.11, df=1, p=0.29; Figure 5.3). At 30 mg L^{-1} Cu^{2+} survival was statistically different in the hypoxic-acclimated animals in comparison to normoxic-acclimated animals (Mantel-Cox test, χ^2: 4.2, df=1, p=0.04): in these conditions, 71% of the hypoxic-acclimated animals survived, in contrast only 43% of the normoxic-acclimated animals survived normoxic copper exposure at 30 mg L^{-1}.

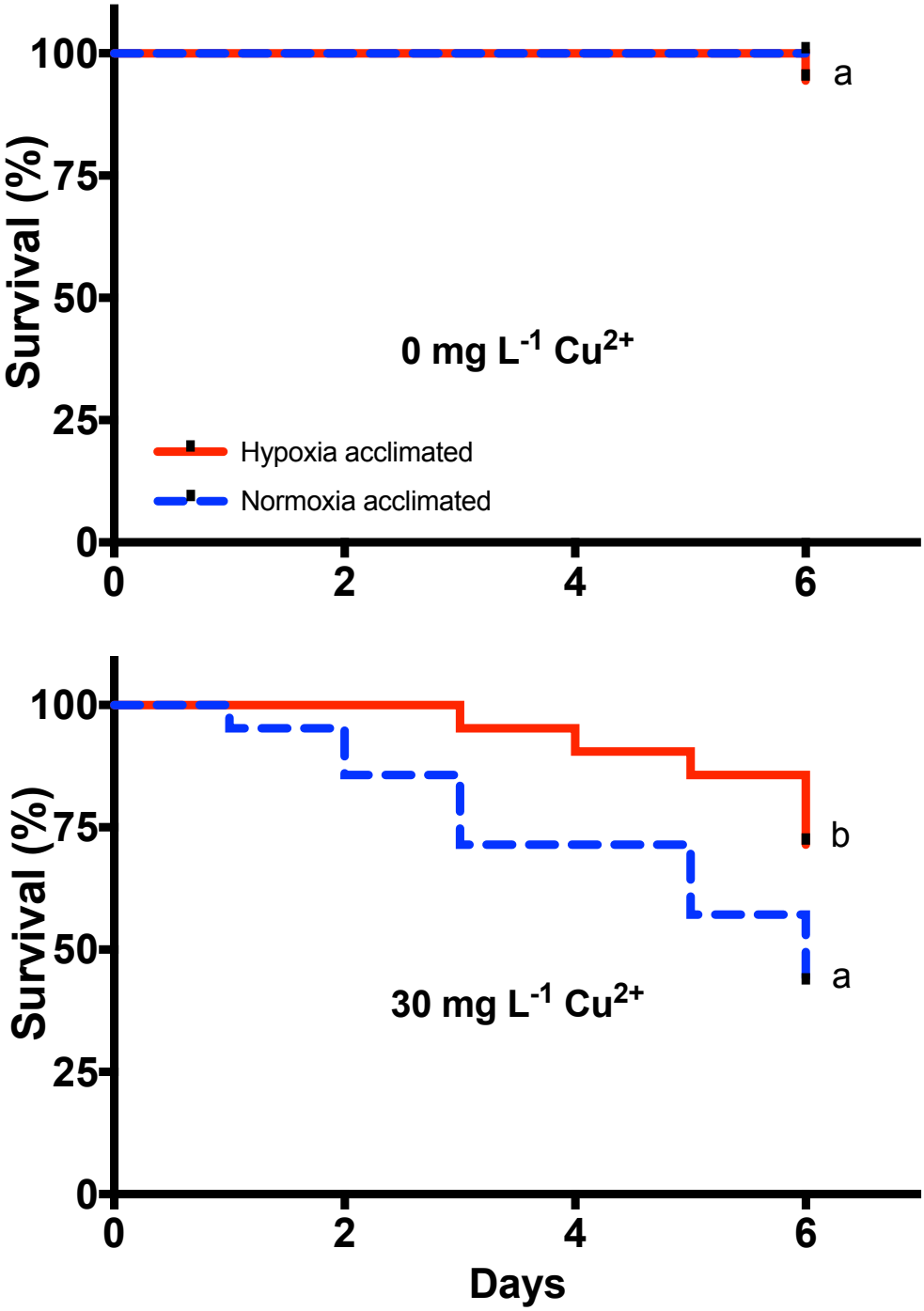

Figure 5.3: Survival of animals exposed to acute Cu^{2+} toxicity. n=21 per each combination of treatment (i.e. hypoxia and normoxia acclimated) and Cu^{2+} dose (i.e. 0 and 30 mg L^{-1}). Different letters indicate statistical difference.

The activity of the superoxide dismutase (SOD) enzyme, measured in the abdomen of animals exposed to 0 and 30 mg L^{-1} Cu^{2+}, was significantly higher in the hypoxic and normoxic groups exposed to 30 mg L^{-1} Cu^{2+} (Tab. 5.4, Fig. 5.4).

Table 5.4: Two-way ANOVA to test the effects of cyclic hypoxic acclimation and Cu^{2+} exposure on the activity of the enzyme SOD.

ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	55.45	1	55.45	F (1, 27) = 0.378	0.54
Cu^{2+} dose	630.30	1	630.30	F (1, 27) = 4.302	0.04
Acclimation	10.63	1	10.63	F (1, 27) = 0.072	0.79
Residual	3956.00	27	146.50		

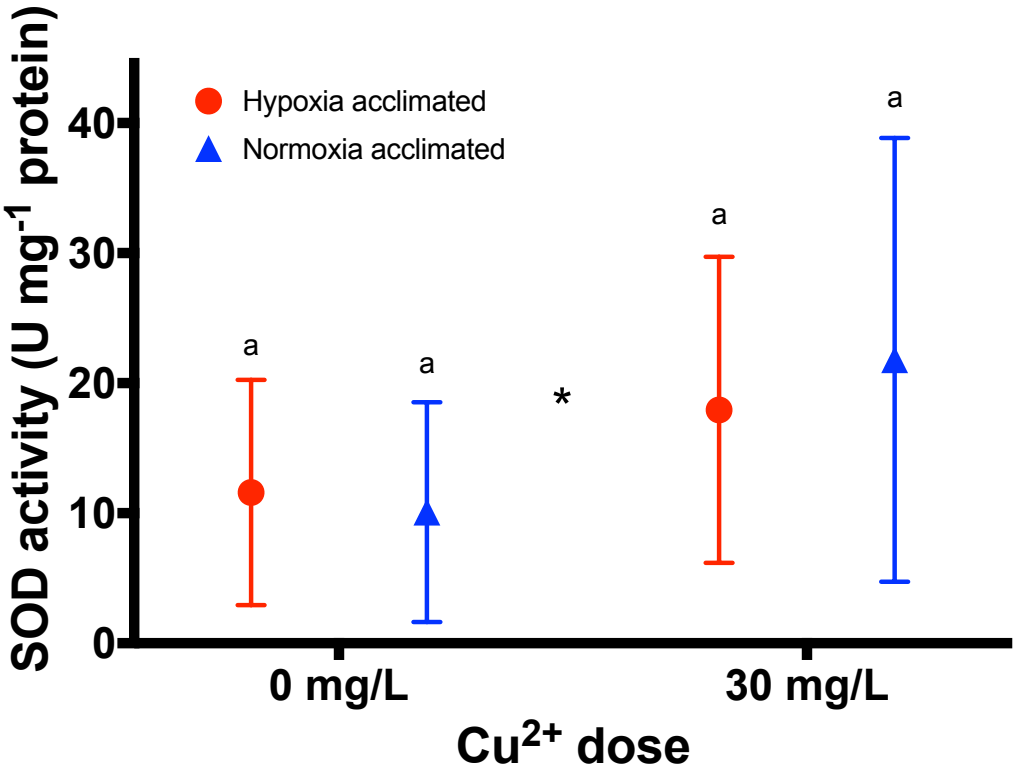

Figure 5.4: SOD activity (mean ±SD, n=9 per treatment in each dose). Different letters indicate statistical difference. Star (*) indicates a statistical difference between levels of the factor "Cu^{2+} dose", as shown in Table 5.4.

Gene expression analysis, carried on the cephalothorax of the animals, revealed a significant effect of Cu^{2+} dose on the expression of *Mt* and *SOD* and a significant effect of the treatment on the expression of *Mt* (Tab. 5.5).

Table 5.5: Two-way ANOVA table to test the effects of cyclic hypoxic acclimation and Cu^{2+} exposure on gene expression of: **A)** Metallothionein *Mt* and **B)** Superoxide dismutase *SOD*.

A. *Mt* Expression - ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.07	1	0.07	F (1, 30) = 0.902	0.35
Cu^{2+} dose	5.93	1	5.93	F (1, 30) = 76.53	P<0.0001
Acclimation	0.91	1	0.91	F (1, 30) = 11.74	0.002
Residual	2.33	30	0.08		

B. *SOD* Expression - ANOVA Table					
	SS	**DF**	**MS**	**F (DFn, DFd)**	**P value**
Interaction	0.00	1	0.00	F (1, 30) = 0.001	0.97
Cu^{2+} dose	8.82	1	8.82	F (1, 30) = 54.72	P<0.0001
Acclimation	0.08	1	0.08	F (1, 30) = 0.489	0.49
Residual	4.84	30	0.16		

The relative expression of both *Mt* and *SOD* was equal in hypoxic- and normoxic-acclimated animals treated with 0 mg L^{-1} Cu^{2+} (Fig. 5.5). Animals treated with 30 mg L^{-1} Cu^{2+} showed a significantly reduced expression of *Mt* and *SOD*, compared to animals treated with 0 mg L^{-1} Cu^{2+}, but, for *Mt*, the magnitude in reduction was statistically different in hypoxic-acclimated animals compared to normoxic-acclimated animals; in fact, the relative expression of hypoxic-acclimated animals was ~2.6 times higher than the expression of normoxic-acclimated animals (Fig. 5.5).

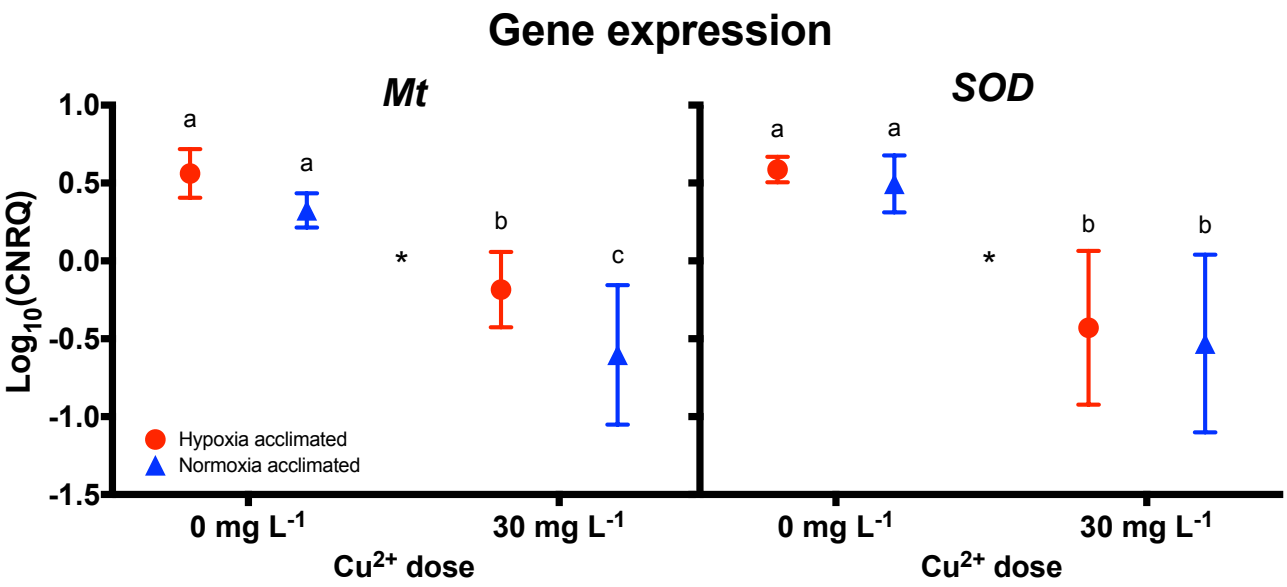

Figure 5.5: Gene expression of *Mt* and *SOD* (means ±SD, n=9 animals per each treatment per each time point) in the two groups (hypoxic- and normoxic-acclimated animals) at different times of the day. Different letters indicate statistical difference. Star (*) indicates a statistical difference between levels of the factor "Cu^{2+} dose", as shown in Table 5.5.

5.5 Discussion:

5.5.1 The effect of prolonged daily cyclic hypoxia on critical thermal maximum CT_{max}:

As has been demonstrated in the context of a single-stressor, cyclic hypoxia is able to induce changes to different physiological processes in the ditch shrimp *Palaemon varians* (Chapters 3 and 4). In its habitat, *P. varians*, continuously faces a co-variation of the main environmental parameters (i.e. temperature and oxygen, see Chapter 2 Fig. 2.5), underlining the ecological importance of research with multiple environmental parameters. Nonetheless, the effects of prolonged cyclic hypoxic acclimation on CT_{max} have never been studied on *P. varians* and so were evaluated by applying a cyclic hypoxic regime currently observed in the natural habitat of *P. varians*. CT_{max} was assessed in normoxic conditions and not during hypoxia because in Lymington the two stressors (i.e. hypoxia and thermal stress) do not tend to occur simultaneously; in fact, temperature decreases during the night while hypoxic conditions develop, whereas temperature increases during the day due to the

heating from the Sun while normoxic (or hyperoxic) conditions are present (see Chapter 2, Fig. 2.5).

In this work, acclimation to cyclic hypoxia had a positive effect on thermal tolerance of *P. varians*. In fact, hypoxic-acclimated animals (tested in the afternoon) showed an increase in CT_{max} in comparison to normoxic-acclimated animals (independently of the tested time Fig. 5.1). It could be argued that the increased thermal tolerance might be a consequence of the morphological modifications to the gills as a result of cyclic hypoxia (presented in Chapter 3). In fact, the observed increase in gill surface area would provide a greater efficiency in gas exchange (in accordance with Fick's law) and could in turn sustain respiratory functions and oxygen requirements of the body for a longer time when animals are facing heat shock, consequently delaying the onset of LoE.

To the best of my knowledge, this is the first study assessing the effects of cyclic hypoxic acclimation on CT_{max} in a marine ectotherm, while, hitherto, only three studies (Burleson, Carlton, & Silva, 2002; Burleson & Silva, 2011; Motyka et al., 2017) evaluated the effects of chronic hypoxic acclimation on CT_{max}. All previous studies have been conducted on fish, which possess a rather different physiology in comparison to crustaceans. It is therefore perhaps unsurprising that these fish studies produced contrasting results: in fact, while Burleson et al. (2002); (2011) reported how a seven day exposure to constant hypoxia (~ 10 kPa, at 22 ˚C) in channel catfish *Ictalurus punctatus* increased CT_{max} by providing the animals a greater heart rate, systolic pressure, and ventilation, Motyka et al. (2017) reported no change in CT_{max} of the steelhead trout *Oncorhynchus mykiss* after chronic hypoxic acclimation (~8.5 kPa for > 3 months).

On the other hand, a large number of studies have focussed on the interaction between acute hypoxia and $CT_{max,}$ in an attempt to prove the Oxygen and Capacity Limited Thermal Tolerance, OCLTT (Verberk et al., 2016; Pörtner, Bock, & Mark, 2017). In this case, published studies on fish and decapod crustaceans showed dissimilar results: in killifish, *Fundulus heteroclitus*, acute hypoxia (~ 1.6 kPa at 15 ˚C) decreased CT_{max}, while in red drum, *Sciaenops ocellatus*, in marine lumpfish, *Cyclopterus lumpus*, or in common perch, *Perca fluviatilis*, no change in CT_{max} was observed (Brijs et al., 2015; Ern et al., 2016). A similar variability as been observed in two decapod crustaceans: the giant tiger shrimp, *Penaeus monodon*, and the European crayfish, *Astacus astacus* (Ern et al., 2015; Verberk et al., 2016). In fact, while hypoxia (12 kPa) did not affect CT_{max} in *P. monodon*, it caused a significant reduction (~0.8 ˚C) in *A. astacus,* as reported by Verberk et al. (2016). Overall, the different outcomes between this work and the aforementioned studies on acute hypoxia and CT_{max} in decapod crustaceans could be ascribed to the type of

stressors applied (i.e. acute hypoxia instead of cyclic hypoxia), which have been shown to elicit mechanisms that are specific to the type of hypoxic stressor, as shown by Brown-Peterson et al. (2008) when comparing molecular responses to acute, chronic and cyclic hypoxia in the grass shrimp *Palaemonetes pugio*.

Here, CT_{max} for the hypoxic-acclimated group differed during the day with a lower value observed at 1200 hrs. This lower CT_{max} value might be explained by a higher level of stress (quantified by means of gene expression) in the animals at 1200 hrs, just after the suspension of the daily exposure to hypoxia. In fact, gene expression results have shown that expression of stress markers *cHH* and *HSP70* were significantly up-regulated in hypoxic-acclimated animals at 1200 hrs compared to the expression at 1630 hrs. As reported by Ravaux et al. (2012), only longer exposure times at temperatures near CT_{max} (~ 30 minutes at 34 ˚C, followed by 2-h recovery) would have triggered an up-regulation of *HSP70*. Hence, the observed up-regulation of *cHH* and *HSP70* identifies an altered physiological condition (of hypoxic-acclimated animals at 1200 hrs) that could be attributed to the temporal proximity between the end of the hypoxic exposure and the thermal challenge.

As reported by several authors, CT_{max} is dependent on the acclimation temperature and higher acclimation temperatures allow higher CT_{max} values (Ravaux et al., 2012; New et al., 2014). A work conducted on *P. varians* acclimated to different temperatures allowed Ravaux et al. (2012) to calculate an Acclimation Response Ratio ARR (defined as the change in the CT_{max} per degree change in acclimation temperature (Claussen, 1977)) of 0.50 (Ravaux et al., 2012). In this study, the calculated CT_{max} for normoxic-acclimated animals at 22 ˚C (36.8 ˚C) is ~1 ˚C higher than the CT_{max} calculated on *P. varians* acclimated at 20 ˚C (36 ˚C, (Ravaux et al., 2012)) and is therefore consistent with the predicted ARR determined by Ravaux et al. (2012).

Overall, it could be argued that the different outcomes reported in the literature in comparison to this study might depend on several factors (i.e. the species studied and the type of stressor). In this thesis it was shown for the first time that cyclic hypoxic acclimation increased *P. varians* $CT_{max.}$ From the literature, despite the majority of studies being carried out on fish, a substantial variability is manifested between species in relation to hypoxia and CT_{max}, as reported by Verberk et al. (2016). Further, as has been shown, the effects of acute or chronic hypoxia on CT_{max} can be very different (suggesting how understanding of the interaction between stressor can be complex and challenging (Gunderson et al., 2015)), and the absence of studies evaluating the effects of cyclic hypoxic acclimation on CT_{max} remains a gap in our knowledge.

5.5.2 The effect of prolonged daily cyclic hypoxia on survival of acute Copper (Cu^{2+}) exposure:

In aquatic environments hypoxia is currently considered a major threat to biota (Kemp et al., 2005) and often co-occurs with pollutants (Fitzgerald et al., 2017) derived from human activities (in particular copper). As mentioned in the introduction, Cu^{2+} content can sharply vary during the year (Jones & Bolam, 2007; Şeker & Kutlu, 2014) as a result of human activities. These sharp variations could impose acute stress on aquatic organisms. Cyclic hypoxic stress could therefore be coincident with metal stress or the two stressors might be experienced in succession, one after the other. This Chapter will present preliminary results focussing on the exposure to cyclic hypoxia and Cu^{2+} in succession, not considering the simultaneous exposure.

Given the environmental variability of salt marshes (as shown in Chapter 2) and the temporal variability of Cu^{2+} concentration during the year (Jones & Bolam, 2007), species living in these habitats could possibly experience environmental Cu^{2+} pollution after being subjected to cyclic hypoxia; therefore it is important to understand how daily cyclic hypoxic acclimation can alter the tolerance of aquatic organisms to heavy metals such as Cu^{2+}. In this work, long-term cyclic hypoxia significantly increased the acute survival of animals exposed to normoxic 30 mg L^{-1} Cu^{2+} in comparison to controls, which were not conditioned to hypoxia. In spite of the fact that, after 6 days of Cu^{2+} exposure, the gills of both groups were blackened (a characteristic sign of histological damage associated with Cu^{2+} exposure, image not shown (Soegianto et al., 1999)), survival of the hypoxic acclimated animals was ~30% higher than controls.

It has been hypothesized that internal anoxia might develop after Cu^{2+} exposure due to histological alterations at the gills (Spicer & Weber, 1992; Malekpouri et al., 2016). In fact, Spicer and Weber (1992) demonstrated how in *Cancer pagurus* exposed to sub-lethal concentrations of Cu^{2+} (0.4 mg L^{-1}) for 7 days, an increase in the diffusion barrier thickness at the gills developed, causing a respiratory impairment. Hypoxic-acclimation induces the development of a greater lamellar surface area (as demonstrated in Chapter 3). It could be argued that this change, in hypoxic-animals, could alleviate the magnitude of damage to the gills as a consequence of Cu^{2+}, and therefore prevent (or at least delay) the onset of internal anoxia, therefore explaining the higher survival of hypoxic-acclimated animals. Future work is needed in order to quantify the magnitude of damage induced by Cu^{2+} in the

gills of exposed animals and elucidate how hypoxic-acclimation is able to increase the survival of animals.

To the best of my knowledge, results presented here constitute the first study that assessed how acclimation to a daily cyclic hypoxic regime (currently experienced in Lymington salt marshes) is able to alter the impact of Cu^{2+} on survival of crustaceans. A number of studies evaluated the co-occurrence of Cu^{2+} and acute hypoxia (Spicer & Weber, 1992; Fitzgerald et al., 2016; Sappal et al., 2016; Fitzgerald et al., 2017) or chronic hypoxia (Eriksson & Weeks, 1994; Mustafa et al., 2012). Chronic hypoxia (~7.6 kPa), without previous hypoxic acclimation, in combination with Cu^{2+} (500 mg kg dry wt. $^{-1}$) resulted in enhanced toxicological responses in the carp, *Cyprinius carpio* (Mustafa et al., 2012). A greater survival rate (~ 50%) was reported by Fitzgerald et al. (2016; 2017) in embryos of three-spined stickleback, *Gasterosteus aculeatus*, and in embryos of zebra fish, *Danio rerio*, constantly exposed to acute hypoxia (~10 kPa) and up to 0.1 mg L^{-1} Cu^{2+} (in comparison to embryos exposed to normoxia and Cu^{2+}) with no hypoxic acclimation; interestingly, this greater survival rate of hypoxic embryos was retained up to 48h after hatching but then, after this time, an increased mortality due to hypoxia and Cu^{2+} was reported (Fitzgerald et al., 2017). The short or absent acclimation time to hypoxia might have been one of the main reasons for the different outcome between my results and studies from the literature (e.g. (Mustafa et al., 2012)). In fact, since hypoxia and Cu^{2+} both impair respiratory physiology (the former by impairing gas exchanges, the latter by damaging the gills), it could be hypothesised that the improved tolerance to Cu^{2+} (observed in this study in hypoxic acclimated animals as a result of the morphological modifications to the gills) would be neutralized by the simultaneous exposure to Cu^{2+} and cyclic hypoxia, which would both affect respiration by impairing gas exchange. This would cause higher mortality in comparison to the hypoxic acclimated group exposed to Cu^{2+} alone.

In response to situations of stress (e.g. exposure to heavy metals), organisms have evolved a series of mechanisms able to sequester metallic ions into different forms (i.e. soluble or insoluble metal complexes or metallic granules) in order to prevent cellular damage (Pourahmad & O'Brien, 2000; Coyle et al., 2002; Ahearn et al., 2004), and prevent damage from reactive oxygen species ROS (Pourahmad & O'Brien, 2000). Among all the proteins involved in metal detoxification and protection from ROS, Metallothioneins (Mt) and superoxide dismutase (SOD) are probably the most studied. In this work an increased activity of the enzyme SOD was detected in hypoxic- and normoxic-acclimated treatments exposed to Cu^{2+} after 6 days, in comparison to treatments not exposed to Cu^{2+}. The data from this thesis contrast with those from a study by Barata et al. (2005) on the cladoceran

Daphnia magna exposed to Cu^{2+} for 2 days that showed no increased activity of the enzyme SOD in comparison to controls, and similarly Atli and Grosell (2016) who reported no change in SOD activity in the pond snail *Lymnacea stagnalis* exposed to Cu^{2+} after 48 hours. It could be argued that the different results between this and the previous studies depended on the tested Cu^{2+} concentration and on the duration of the Cu^{2+} exposure. In accordance with Geracitano, Monserrat, and Bianchini (2002), while a 2-day exposure was not able to trigger an increase in SOD activity on the polychaete *Laeonereis acuta* exposed to an acute dose of Cu^{2+}, a 14-day exposure resulted in an increased activity of SOD, therefore suggesting how the tested concentration and the duration of exposure might elicit different results. In this context, it could be hypothesised that the high Cu^{2+} concentration coupled with the exposure (6 days) of this study likely caused an increase in ROS within the cells, which in turn triggered the increased activity of SOD in the Cu^{2+} treatments.

Overall, gene expression levels of *Mt* and *SOD* decreased in treatments exposed to Cu^{2+} in *P. varians*. This decrease is commonly reported following acute metal exposures (Ren et al., 2011; Sappal et al., 2016). In fact, according to the model proposed by Sokolova et al. (2012), acute stress conditions are usually accompanied by a decrease in aerobic scope and a consequent reduction of energy expensive processes (i.e. transcription of genes and ion gradients, (Richards, 2011)) to sustain the increased homeostatic costs. The magnitude of reduction is species-specific and is proportional to the level and type of stress experienced (Sokolova et al., 2012). Therefore, in agreement with Sokolova et al. (2012), it could be argued that the reduced gene expression of *Mt* and *SOD* in hypoxic- and normoxic-acclimated animals exposed to Cu^{2+} (in comparison to the non-Cu^{2+} exposed groups) was a direct consequence of the stress conditions following Cu^{2+} exposure. Interestingly, within the groups exposed to Cu^{2+}, the decrease of *Mt* was less marked in hypoxic-acclimated animals in comparison to normoxic-acclimated animals. This difference in *Mt*'s expression levels could be the result of the morphological changes to the gills (discussed in Chapter 3) as a consequence of cyclic hypoxia; in fact, it could be hypothesised that, during Cu^{2+} exposure, the relatively larger gills of hypoxic acclimated animals provided a better capacity of extracting O_2 from the water, hence conferring a greater aerobic scope and allowing animals to trigger a greater transcriptional activity of *Mt* in comparison to normoxic acclimated animals exposed to Cu^{2+}. On the other hand, the lack of difference in the expression of *SOD* within Cu^{2+} exposed treatments could be explained by the observed increased enzymatic activity of SOD as a consequence of Cu^{2+}, which could have conferred protection from ROS without the need of additional

transcription of *SOD*. In conclusion, the morphological changes to the gills coupled with the higher expression of *Mt* in the hypoxic-acclimated group were probably responsible for the greater ability to counteract Cu^{2+} toxicity and ultimately contributed to the increased survival of the hypoxic-acclimated group.

5.6 Conclusions:

In order to understand the effects deriving from the occurrence of two or more stressors on physiological parameters of adult *Palaemon varians*, animals were exposed to long-term daily cyclic hypoxia, followed by either a normoxic acute thermal challenge or a normoxic acute Cu^{2+} toxicity test. Results presented here underline how, in response to long-term daily cyclic hypoxia, animals showed an increased CT_{max} (ranging from ~0.9 to ~1.7˚C). Interestingly, animals tested for CT_{max} within 2-hours from the end of the hypoxic period showed a higher level of stress (underlined by a differential expression of *HSP70* and *cHH*) than animals tested later during the day. These data indicate that whilst cyclic hypoxia can improve CT_{max}, this improvement appears a few hours after the end of the hypoxic period, suggesting that this positive effect of hypoxia on animal's CT_{max} might depend on when the they experience the heat shock.

Results from Cu^{2+} toxicity tests showed an increased survival to 30 mg L^{-1} Cu^{2+} in animals that had previously experienced cyclic hypoxia (survival ~ 75%), in comparison to controls (survival ~ 45%) and a higher gene expression of *Mt* (detoxifying enzymes) in the hypoxic-acclimated group, compared to the normoxic-acclimated. Since Cu^{2+} toxicity is thought to derive primarily from the development of internal anoxia due to the extensive damage to the gills, the observed increase in survival following acute Cu^{2+} exposure might be explained by morphological changes to the gills following cyclic hypoxia.

As it was shown here, complex interactions/responses can be revealed when considering the effects of multiple stressors on the physiology of organisms, and not all the effects can be considered negative. As such this work reinforces the need to understand complexity in the physiological responses to multi-factorial climate change experiments, bearing in mind that acclimatization to one stressor (i.e. cyclic hypoxia) can play a fundamental role in the physiological response to another stressor.

Chapter 6 Synthesis and Conclusions:

6.1 The consequences of daily cyclic hypoxia: from short-term responses to long-term effects

Since the 1980s it has been documented how vast areas of the sea could temporarily become hypoxic, resulting in mass mortality of organisms (Rosenberg, 1980; Stachowitsch, 1984; Boesch & Rabalais, 1991; Diaz & Rosenberg, 2008). Due to the vast impact of these events on the ecosystems and on fisheries, extensive research has been conducted on the effects of acute and chronic hypoxia on marine animals, allowing us to gain a detailed understanding of the mechanisms involved in the physiological response to this stressor (e.g. see (Herreid, 1980; Wu, 2002; Gorr et al., 2010)). Unfortunately, not all aquatic ecosystems experience chronic hypoxia; in fact, mainly in coastal habitats (i.e. estuaries, lagoons, marshes) pO_2 levels can fluctuate on a daily basis, resulting in what is termed has daily cyclic hypoxia (Guasch et al., 1998; Cheek et al., 2009; Li & Brouwer, 2013a).

Scientific interest around daily cyclic hypoxia has grown only in the last decade, when researchers realised how even a short exposure (in duration) to hypoxia, repeated every day, could produce physiological effects on organisms (Coiro et al., 2000; Brown-Peterson et al., 2011). This thesis has characterized the effects of daily cyclic hypoxia on some aspects of the physiology of the Atlantic ditch shrimp, *Palaemon varians*, exposed to this stressor for increasing duration, from hours to weeks. The results provided valuable new understanding of the adaptive mechanisms used by the species to cope with cyclic hypoxia (Chapter 2 and 3), together with insights in the long-term consequences on major physiological processes, such as growth and reproduction (Chapter 4 and 5).

A schematic of the main biological processes altered as a consequence of the length of exposure to cyclic hypoxia is shown in Figure 6.1. As expressed by Morris (2015), stressful conditions (i.e. cyclic hypoxia) are able to elicit a series of responses, depending on the duration and magnitude of the exposure to stress, which can be grouped into four categories: behavioural responses, metabolic responses, cellular stress responses (CSR) and homeostatic responses (Kültz, 2003; Kultz, 2005; Morris et al., 2013; Sokolova, 2013). Behavioural responses (e.g. escape, aerial surface respiration (Taylor & Spicer, 1987; Richards, 2011; Spicer, 2014)) are likely to be the first responses to hypoxia, as soon as a

pO_2 threshold is detected (Taylor & Spicer, 1989; Bumett & Stickle, 2001). In fact, as mentioned in Chapter 2, when water pO_2 approached p_{crit}, after initially adopting a brief exploratory behaviour, *P. varians* became fully quiescent (as shown by Taylor and Spicer (1987) on the congeneric *Palaemon elegans* and *P. serratus*). This behavioural response can be explained by the fact that, in concurrence with p_{crit}, basal metabolism cannot be sustained (Herreid, 1980; Richards, 2011). Hence energetically expensive processes (e.g. active movement, Fig. 6.1) are suppressed in order to preserve energy (Richards, 2011; Seibel, 2011), thus explaining the quiescent behaviour of the shrimps when exposed to $pO_2 < p_{crit}$.

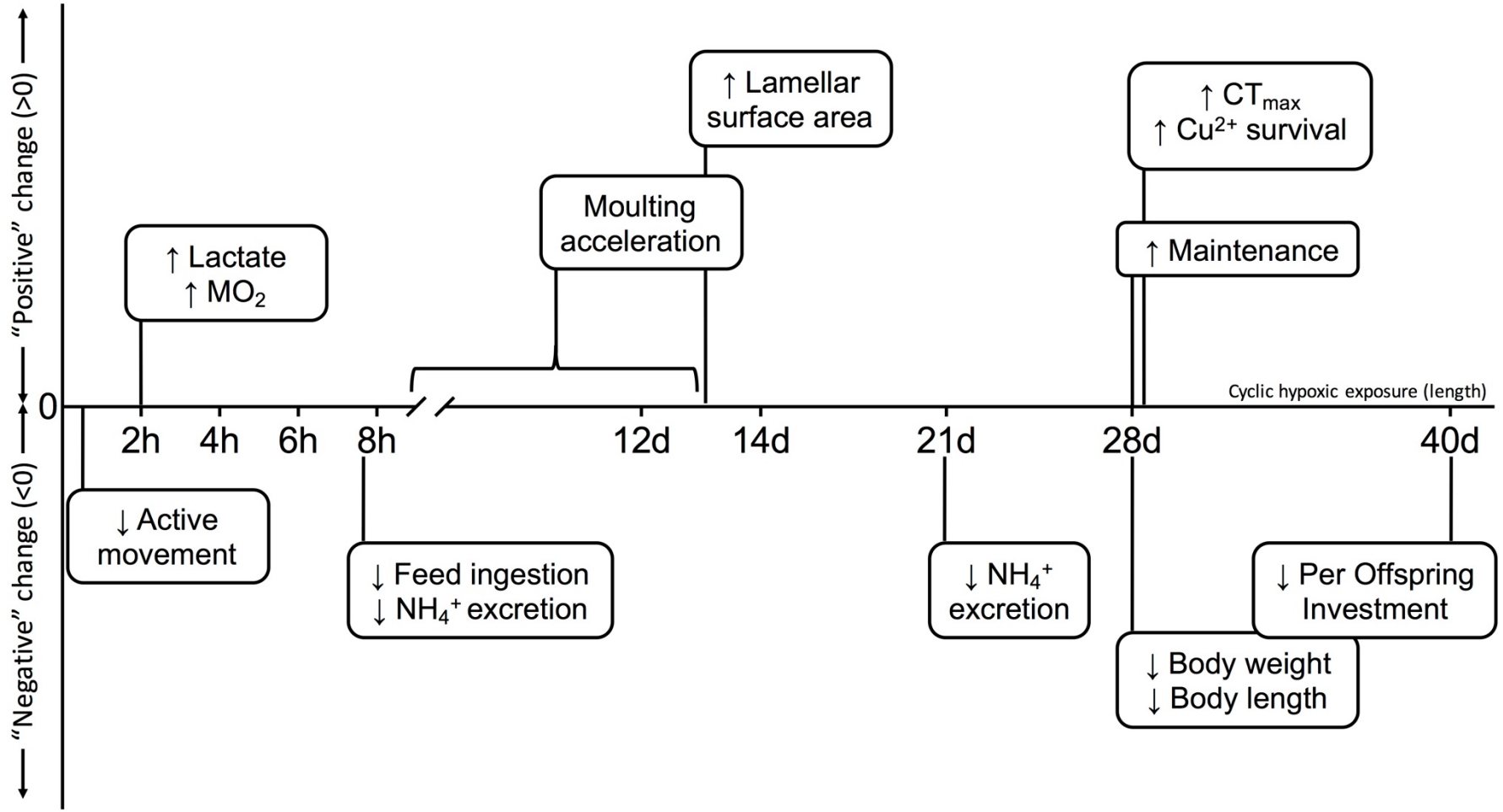
"Positive" change (>0)
"Negative" change (<0)
0
Cyclic hypoxic exposure (length)
2h
4h
6h
8h
12d
14d
21d
28d
40d
↑ Lactate
↑ MO2
↓ Active movement
↑ Lamellar surface area
Moulting acceleration
↓ Feed ingestion
↓ NH4+ excretion
↑ CTmax
↑ Cu2+ survival
↑ Maintenance
↓ NH4+ excretion
↓ Body weight
↓ Body length
↓ Per Offspring Investment

Figure 6.1: Schematic diagram illustrating the main biological processes (inside the boxes) of P. varians that resulted affected as a consequence of cyclic hypoxia. X-axis represents the duration of exposure to cyclic hypoxia, from 2 hours (2h) to 40 days (40d). Note that the x-axis is not continuous but there is a break denoting a change in the temporal scale of the axis from hours (h) to days (d). Y-axis represent the directionality of the observed change in comparison to normoxic animals (i.e. controls): “positive” change was defined as an increase in the parameter of hypoxic animals in comparison to controls; “negative” change was defined as a decrease in the parameter.

During the first short-term hypoxic exposure (presented in Chapter 2), metabolic responses and CSR could be described in *P. varians* as a consequence of the metabolic suppression following exposure to $pO_2 < p_{crit}$ (Sokolova, 2013). In fact, in this condition basal metabolism could not be sustained solely via aerobic metabolism (Herreid, 1980; Richards, 2011) and anaerobic metabolism was triggered (Fig. 6.1), as suggested by the increase in lactate content in the muscle and by the oxygen debt (i.e. increased oxygen consumption, MO_2) of hypoxic exposed animals demonstrated in Chapter 2. In this stressed status of metabolic suppression food ingestion and ammoniacal excretion were both substantially reduced (as demonstrated in Chapter 4) since they are both energetically expensive processes (Bell et al., 2003; Wei et al., 2008; Kiko et al., 2015; Kiko et al., 2016). Interestingly, when food ingestion and ammoniacal excretion were measured (in hypoxic conditions) after 21 days of cyclic hypoxic exposure, only ammoniacal excretion was altered. The presence of an impaired excretion after 21 days is an indication that *P. varians* was still experiencing stressful conditions (i.e. metabolic suppression) after 21 days. An additional proof of that was found in Chapter 5 when a higher expression of *HSP70* and *cHH* was discovered in hypoxic acclimated animals after the end of the 28th daily hypoxic exposure. The up-regulation of these two genes indicated the activation of the CSR (Kultz, 2005; Sokolova, 2013); in fact HSPs are amongst the most intensively researched general responses to stress in biology (Morris et al., 2013) and cHH is probably the most studied hormone involved with stress resistance in decapod crustaceans (Webster et al., 2012). All the aforementioned mechanisms were temporary (i.e. not permanent) modifications to the normal biological and physiological process, which were restored when the stressful conditions ceased, in agreement with Sokolova et al. (2012): in fact, during the daily normoxic period, lactate was reduced, feeding and excretion returned to normal levels and the expression levels of *HSP70* and *cHH* were not different from normoxic animals, as demonstrated in Chapters 2, 4 and 5, respectively.

The diel succession of hypoxic and normoxic periods characterizes the most fundamental difference between cyclic and chronic hypoxia. In fact, while in chronic hypoxia no recovery times (i.e. normoxic periods) from stressful conditions are given to the animals, in cyclic hypoxia animals have the possibility to restore normal homeostasis (Coiro et al., 2000; Sokolova et al., 2012). As a consequence of this, when exposing animals to long-term chronic or cyclic hypoxia, different physiological mechanisms can be activated and different results can be obtained (in comparison to

chronic hypoxia), as demonstrated by Coiro et al. (2000), Brouwer et al. (2005), Stierhoff et al. (2006) and Brown-Peterson et al. (2008). In particular, as demonstrated by Brown-Peterson, exposing *Palaemonetes pugio* to chronic hypoxia for 14 days exclusively induced a down-regulation of electron-transport chain proteins, whereas 14 days of cyclic hypoxia induced an up-regulation of cuticular proteins in *P. pugio* (Brown-Peterson et al., 2008). As demonstrated in Chapter 3, when *P. varians* were exposed to cyclic hypoxia for up to 16 days, this exposure induced an acceleration of the moult cycle at molecular and phenotypic level (i.e. animals moulted more quickly) that ultimately resulted in the development of morphological changes to the gills of hypoxic animals (Fig. 6.1), identifed as an increase in lamellar length and surface area. This set of phenotypic changes, which are not immediately triggered as behavioural responses or CSR, can be grouped as homeostatic responses (Kültz, 2003; Kultz, 2005). In fact, homeostatic responses are not transient in nature like CSR (and thus they are switched on until either acclimatisation has occurred or environmental conditions have reverted back (Morris, 2015)), they imply a functional change (Morris, 2015) and are stressor-specific (e.g.: in Brown-Peterson et al. (2008) only cyclic hypoxia induced gene expression changes in cuticular proteins, the sign of an induction of the moult cycle). As explained in Chapter 3, by producing larger gills, those morphological changes were able to improve gas exchange through the gills (which depend on Fick's law, as explained in Chapter 1) allowing animals to acquire more oxygen (Callier & Nijhout, 2013) in spite of the extremely low ΔpO_2 ($pO_{2\ water}$ - $pO_{2\ haemolymph}$) during hypoxic conditions. However, as previously pointed out hypoxic-acclimated animals showed evidence of CSR (i.e. up-regulation of *HSP70* and *cHH*) and metabolic response (i.e. lowered ammoniacal excretion) even after 28 days of cyclic hypoxia, underlining how the magnitude of the morphological changes to the gills was probably still insufficient to meet the requirement of the body, therefore triggering stressful responses.

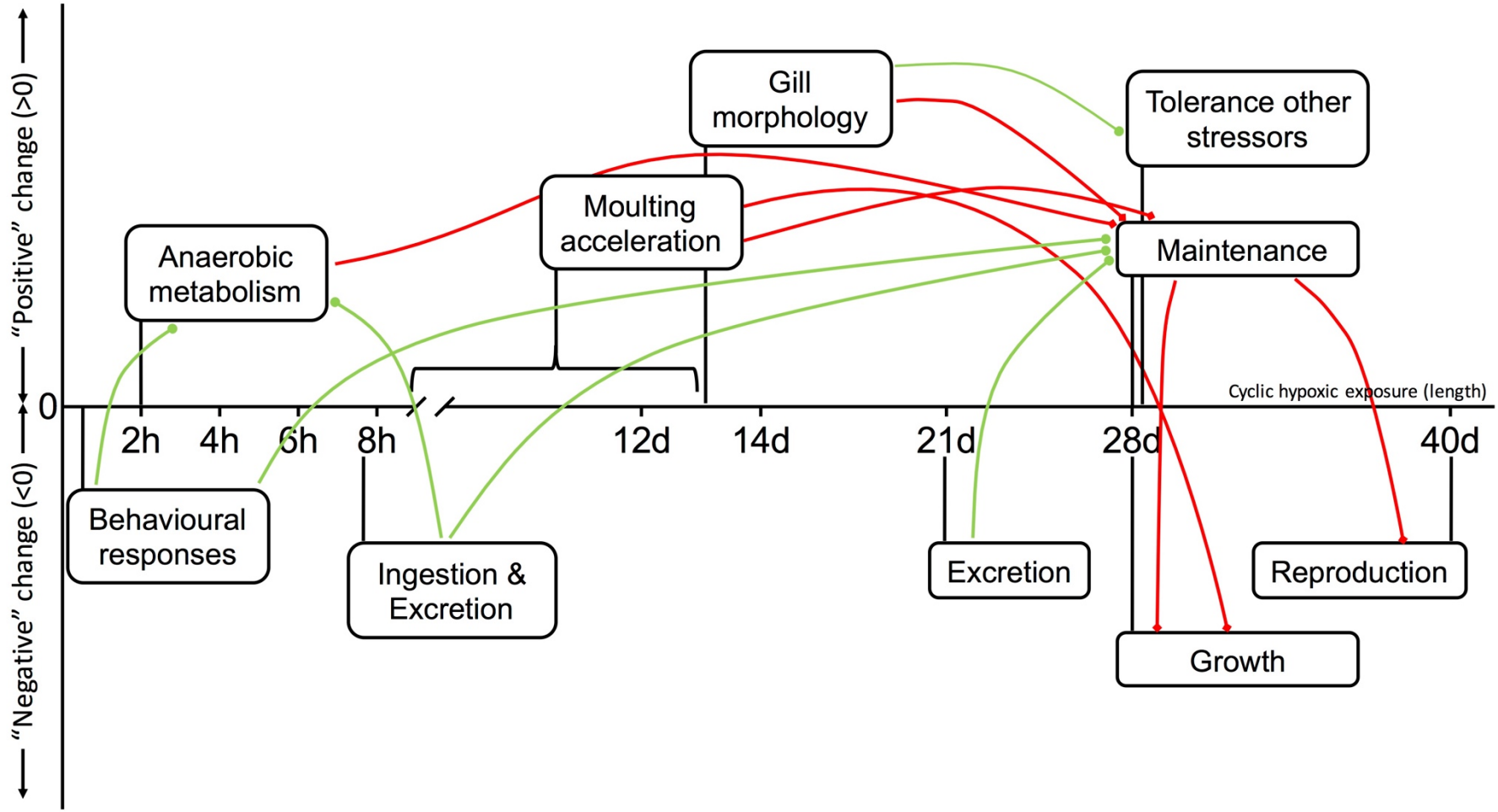
"Positive" change (>0)
"Negative" change (<0)
0
Cyclic hypoxic exposure (length)
2h
4h
6h
8h
12d
14d
21d
28d
40d
Gill morphology
Tolerance other stressors
Moulting acceleration
Anaerobic metabolism
Maintenance
Behavioural responses
Ingestion & Excretion
Excretion
Reproduction
Growth

Figure 6.2: Schematic illustrating how the different responses to cyclic hypoxia could exert an effect on other responses. Arrows point the direction of the effect (e.g. anaerobic metabolism triggers an increase in maintenance costs). Green arrows depict an effect that allows to save energy (by reducing energetically expensive processes) or to enhance a physiological process. Red arrows depict an effect that elicit an increase in energy expenditure or causes an impairment in a physiological process.

Figure 6.2 shows how the different physiological responses to cyclic hypoxia (discussed above) exerted an effect among each other and presents their possible contribution towards the observed changes in growth, reproduction and tolerance to other stressors. When *P. varians* was exposed to hypoxic conditions below its p_{crit}, a conservational strategy (as termed by Sokolova et al. (2012)) was adopted and the observed reduction in active movements, feeding and excretion allowed to preserve ATP reserves, therefore mitigating the magnitude of anaerobic metabolism (green lines, Fig. 6.2) and the overall costs of maintenance. The homeostatic responses identified in *P. varians* were energetically expensive mechanisms, as functional change was implied (as demonstrated by Callier and Nijhout (2011) in the tobacco hornworm, *Manduca sexta*); thus it could be argued that their application had a metabolic impact on the energetic reserve pool of the body (Sokolova et al., 2012). Further, an energetic cost had to be paid daily in order to restore normal homeostasis of the body following hypoxic exposure, as previously explained (e.g. to dispose of anaerobic end products) (Richards, 2011; Seibel et al., 2014). All these factors, in turn, translated into an overall increased maintenance cost (red lines, Fig. 6.2), which was quantified in Chapter 4 as a reduction in wet weight of *P. varians* exposed to cyclic hypoxia for 28 days. Finally, it could be argued that the increased maintenance costs negatively affected reproduction (red line, Fig. 6.2) by impairing female's Per Offspring Investment (as demonstrated in Chapter 4) and, in conjunction with the accelerated moulting cycle, had an effect on growth, quantified in Chapter 4 as a reduction in body length and wet weight, in accordance with Callier and Nijhout (2013).

It is interestingly to note how the morphological changes to the gills improved *P. varians*' thermal and Cu^{2+} tolerance, as discovered after 28 days of cyclic hypoxic exposure (green line, Fig. 6.2): cyclic hypoxic animals showed an increased thermal tolerance (ranging from ~0.9 to ~1.7˚C) in comparison to normoxic animals; mortality due to Cu^{2+} was higher in the normoxic acclimated group exposed to Cu^{2+} (~55%) compared to the cyclic hypoxic acclimated group exposed to Cu^{2+} (~26%). As explained in Chapter 5, evidence seems to suggest that both thermal and Cu^{2+} stress cause the onset of internal hypoxia. In this context, morphological changes to the gills would probably delay the onset of internal hypoxia by improving gas exchanges through the gills (according to Fick's law, Chapter 1) and were therefore identified as possible explanations for the observed results.

6.2 Ecological implications:

In Chapter 2 it was identified how, in its habitat, *P. varians* might be subjected to a relocation during summer months (i.e. July and August) probably in relation to the environmental conditions (i.e. high temperatures coupled with extreme daily hypoxic events) developing in the monitored part of the marsh. In the near future, it is likely that climate change may exacerbate the impact of cyclic hypoxia on the physiology of crustaceans from salt marsh environments (such as *P. varians*). In fact, under the widely accepted predictions of global warming (Christensen et al., 2007; Pachauri et al., 2014), an increase in water temperature will increase metabolic rates (and hence oxygen demand) of ectotherms (Clarke & Portner, 2010; Ern et al., 2016). This will likely translate into more severe hypoxic periods that will last longer and will likely expand to areas of the marsh that might currently experience less severe cyclic hypoxic oscillations: in fact, it could be hypothesised that while new areas of the marsh will likely develop the environmental conditions that currently cause the relocation of *P. varians*, the parts of the marsh which *P. varians* is currently avoiding only during summer months will likely become inhospitable for longer periods of the year.

As demonstrated by Welsh (1975), Escaravage and Castel (1990a) and Aguzzi et al. (2005), grass shrimps inhabiting salt marshes play a fundamental role "in the transfer of nutrients and energy among various tropic levels of coastal ecosystem" (Aguzzi et al., 2005). Their detritivorous feeding habit largely contributes to the maceration of detritus and dead marsh plants into a heterogeneous assortment of uneaten particles, which are subsequently invaded and decomposed by diatoms and bacteria (Welsh, 1975). Further, they excrete large quantities of ammonia and phosphate, which, together with the release of uneaten particles, is probably responsible for the heavy growth of microflora, as hypothesised by Welsh (1975). As shown in Chapter 4, ammoniacal excretion was reduced during hypoxia after 21 days in cyclic hypoxic conditions, contrary to feeding, which was not reduced. In contrast to these results, Aguzzi et al. (2005) showed how in a pond system next to the Guadalquivir estuary (Spain), where marked variations in pO_2 were observed, a feeding activity rhythm was discovered in *P. varians* in June and July, with a peak in the morning (likely in occurrence with normoxic periods) and minimal activity during the night (when hypoxic conditions could develop). Given the fact that digestion is an energetically expensive process, the rhythmicity reported by Aguzzi et al. (2005) seems to suggest how, in the field, *P. varians* would avoid feeding during hypoxic periods.

Therefore, in response to predicted climate change feeding and excretion could be further reduced, thus negatively affecting microbial and algal community and potentially creating marked changes in salt marsh ecosystems.

The observed consequences of cyclic hypoxia on maintenance, growth and reproduction were not immediately appreciable on the short-term (i.e. days) but became evident on the long-term (i.e. with exposure duration >28 days). While it could be argued that one reason for this derives from the extensive hypoxic tolerance of *P. varians* (greater than the congeneric *P. elegans*, *P. longirostris* and *P. serratus*, as shown in Chapter 1), another rationale can be found in the differences between chronic and cyclic hypoxia. In general, the effects of cyclic hypoxia on one physiological parameter are less evident than the effects from chronic hypoxia (as demonstrated by Coiro et al. (2000), Stierhoff et al. (2006) and Landry et al. (2007)). This difference in outcome is mainly due to the absence of recovery periods (i.e. normoxic periods (Coiro et al., 2000)) in chronic conditions. In fact, in cyclic conditions, normoxic periods can be used by the animals to restore normal homeostasis, in accordance to Sokolova et al. (2012). In all the experimental work carried out in this thesis a cyclic hypoxic regime that mimicked natural conditions was used. This regime provided a long recovery period (17 hours every day), which, in the light of the above considerations, is likely to be one of the factors responsible for the small (but significant) differences observed in growth and reproduction, in agreement with Coiro et al. (2000) and Landry et al. (2007). As a consequence of climate change, changes in the current cyclic hypoxic regime (experienced in the field) will likely occur, causing a shrinkage in the duration of normoxic periods and an expansion of hypoxic ones. This would translate into higher costs of maintenance (compared to the one observed in Chapter 4), in accordance with Sokolova et al. (2012), and greater growth and reproductive depression (in comparison to Chapter 4). As previously discussed in Chapter 4, body size is generally considered one of the most important traits of an organism (Fenberg & Roy, 2008) and reduction in body size might have important influences on reproductive output (mainly impacting relative fecundity), on predator-prey relationships and on competition with other species (Fenberg & Roy, 2008; Caruso et al., 2014). Given the ubiquitous distribution of salt marshes over the coasts of the UK and the important ecological role played by detritivorous species such as *P. varians*, cyclic hypoxia in conjunction with climate warming is therefore likely to cause significant consequences at all levels of these widespread ecosystems.

6.3 Current limitations in experimental set-up:

In the first part of Chapter 2, within the coastal habitat of the Lymington salt marsh (UK) the environmental variability in temperature and pO_2 was monitored in different seasons and was compared with nearby coastal habitats. Results showed a substantial variability in pO_2 and temperature on a daily scale (i.e. variations up to ~40 kPa within 12 hours were recorded in August) and on a seasonal scale (i.e. smaller variations in February, greater in May and August). Further, it has been demonstrated how variation in pO_2 and temperature was far greater in Lymington marshes than in nearby coastal habitats, underlining the difference between these habitats and the higher variability of salt marsh environments, in agreement with Jefferies (1964) and Marsden (1976).

Whilst the environmental variability was investigated only in relation to temperature and pO_2, it could be hypothesised that these are not the only parameters changing throughout the day. As demonstrated by Cochran and Burnett (1996) in a tidal saltmarsh in South Carolina, USA, and by Yates et al. (2007) in two distinct bays in Florida, USA, pCO_2 and pH can vary considerably during the day. In fact, during the night CO_2 is released through respiration in the water while photosynthetic fixation of CO_2 is stopped. This causes a raise in pCO_2 and a drop is pH, as shown by Cochran and Burnett (1996) who reported a difference of one pH unit within 12 hours in one marsh ecosystem. The increase in pCO_2, known as hypercapnia, can therefore co-occur with hypoxia (Cochran & Burnett, 1996; Melzner et al., 2013) and it could be argued that the two stressors together can elicit different effects in comparison to hypoxia alone (Cochran & Burnett, 1996; Hardy et al., 2012). Given the vast diel variation in pO_2 recorded in Lymington, it could be hypothesised that also in this habitat a diel variation in pCO_2 (and hence pH) is observed. Unfortunately, it was not possible to quantify the magnitude of these diel fluctuations in the field to gain a more comprehensive understanding of how the different environmental parameters vary during the day and throughout the seasons.

In its habitat, *P. varians* does not only experience daily hypoxia but, together with diel changes in water temperature, hyperoxic conditions ($pO_2 > 21$ kPa) develop during the day, as shown in Chapter 2. While temperature fluctuations can alleviate or exacerbate the effects of other stressors by acting on the metabolism of ectotherms (Gillooly et al., 2001; Clarke & Fraser, 2004), hyperoxia as suggested by Morris and Taylor (1985), does not seem to trigger the same responses in all organisms (e.g. in response to hyperoxia the heart rate of *Astacus leptodactylus* is decreased, whereas heart rate of *P. elegans* is independent

of pO_2 (Morris & Taylor, 1985)). Hence the daily succession of hypoxia and hyperoxia could arguably trigger different mechanisms and bring different outcomes than cyclic hypoxia alone. Given the paucity of studies in the literature focussing on the effects of long-term daily cyclic hypoxia, all the experimental work in this thesis has been conducted with a fixed temperature and without considering hyperoxic conditions. By doing so this work set the basis for future investigations that should take into account all (or some) the aforementioned variables (i.e. temperature, pH, pCO_2 and hyperoxia) in order to uncover the effects of multiple stressors on the physiology of decapod shrimps like *P. varians*.

In Chapter 3, *P. varians*' mechanisms of adaptation to long-term cyclic hypoxia were presented. The involvement of the moult cycle in relation to cyclic hypoxia was suggested by analysing the transcriptome from the cephalothorax of animals exposed for 7 days to experimental conditions. While on the one hand transcriptomics data permitted the identification of change in the moult cycle, on the other hand it is worth mentioning that ~ 53% of the differentially expressed (and non-redundant) contigs (214 out of 399) were categorized as "unknowns" (i.e. non-annotated) due to the absence of a positive identity from the major sequence databases (e.g. Pfam, UniProtKB, nr). It could be hypothesised that some other mechanisms in response to cyclic hypoxia still sit within the "unknowns". In fact, the excessive number of non-annotated contigs is currently the major bottleneck, especially when the tested species is a non-model organism (Clark & Greenwood, 2016), a limitation that currently prevents full characterisation of how organisms respond to stressors at a genetic level (Landry & Aubin-Horth, 2007). To overcome this limitation some solutions have been proposed, including the creation of Ecological Association Ontology (EAO) databases (Pavey et al., 2012; Primmer et al., 2013). The main idea of the EAO database is that " 'unknown genes' might share ecologically important functions in different species facing a similar environmental challenge. In such a case, they could be annotated according to their link with a particular ecologically relevant trait" (Pavey et al., 2012). In this way, the identification of systematic associations between unknown genes could help narrow identification of the the particular mechanisms involved (or at least helping selecting candidate genes) for traits of interest. Unfortunately, while some of these databases currently exist (see Pavey et al. (2012) for reference) their application is still limited.

In Chapter 5 the effects of a cyclic hypoxic acclimation on other physiological parameters (namely thermal tolerance and survival after acute Cu^{2+} exposure) were tested. The effects of hypoxic acclimation on both parameters was assessed in normoxic conditions. While it could be argued that a more realistic scenario would have required

testing these parameters in normoxic and hypoxic conditions, some considerations led the decision to apply such experimental design. As shown in Chapter 2, in the salt marshes of Lymington hypoxia develops during the night and it is generally coupled by a decrease in water temperatures rather than an increase (as in the case of determining the critical thermal maximum); therefore, in its environment *P. varians* is not likely to experience thermal stress coupled with hypoxic conditions as they tend to happen in separate moments of the day. As mentioned in Chapter 2, diel cyclic hypoxic conditions were detected during the sampling week in May and in August and it was noticed how, during July and August, *P. varians* seemed to relocate avoiding the extreme diel fluctuations in pO_2 of these months. In this context it could be hypothesised that as a consequence of its relocation (in order to avoid hypoxic conditions) *P. varians* would be forced to move to a different part of the marsh (i.e. a deeper part of the marsh or where water currents would alleviate hypoxia) where it could be subjected to other stressors, such as Cu^{2+}. The result of this hypothesis would be an initial exposure to cyclic hypoxia followed by the exposure to another stressor, in accordance to the experimental design used in Chapter 5.

6.4 Future directions:

This thesis has shown some of the physiological consequences deriving from exposure to cyclic hypoxia in the shallow water shrimp *Palaemon varians* by addressing some key aspects of its physiology. Very frequently, when we answer one question, we also realise that new questions arise. In fact, in addition to the questions that emerge from the limitations pointed out in the previous paragraph, there are additional uncertainties that can be addressed in future research, which are listed below:

***In situ* estimates of population size:** In Chapter 2 it was mentioned that *P. varians* might be subjected to a relocation during summer months in relation to the environmental conditions (i.e. high temperatures coupled with extreme daily hypoxic events) developing in the monitored part of the marsh. In order to test this hypothesis regular estimates of population size should be carried along different parts the marsh, coupled with monitoring of the environmental parameters (e.g. temperature and pO_2). By doing this it would be possible to understand which environmental conditions are sought by the animals as they

relocate and which environmental conditions trigger this behavioural response of migration in wild populations. Moreover, in the context of climate change, where an increase in temperature is predicted and hypoxic events are thought to extend in duration and severity, this information would be useful in predicting the magnitude of future relocations in the light of an expansion of unfavourable environmental conditions into new areas of the marsh, as the world warms.

Moult acceleration and gill morphology: In Chapter 3 it was proven how, in response to 14 days of cyclic hypoxia, shrimp would accelerate their moult cycle and would develop larger gills, as part of an homeostatic response to cyclic hypoxia (Kültz, 2003; Kultz, 2005). It was additionally shown how this homeostatic response was not able to fully balance the stress derived from cyclic hypoxia on longer exposures (e.g. excretion was still impaired after 21 days during hypoxia). In this context it would be interesting to see whether the exposure to cyclic hypoxia induces an acceleration of the moult cycle only once, or if the cycle is constantly accelerated until cyclic hypoxic conditions persist. Moreover, it would be interesting to quantify if animals continuously increase their gill surface area with every moult cycle while being exposed to cyclic hypoxia. Finally, in order to gain a better understanding of the extent of the morphological changes to the gills, a different approach (rather than histology) could be implied by using Computerized Tomography. By performing CT scan, a 3D reconstruction of the entire branchial system could be obtained. From this reconstruction it would be possible to gain a better estimate of the entire branchial surface area and reveal other potential anatomical adjustments to the gills.

Effects of cyclic hypoxia on reproduction and development (more): In Chapter 4 the effects of cyclic hypoxia on some aspects of reproduction were quantified, and it has been found that the maternal investment (POI) was impaired by cyclic hypoxia. A lower POI would translate as reduced energy reserves with which to complete embryogenesis and development, potentially causing a delay in development. In order to test this, the development of embryos from females that reproduced in cyclic hypoxia (with a lower POI) should be monitored and compared with embryos from normoxic females (with a higher POI). Embryonic development could be additionally carried out in normoxic or cyclic hypoxic conditions in order to determine whether cyclic hypoxia is able to affect the development of embryos.

6.5 Final conclusion:

As climate change is currently altering our planet, an increasing number of ecosystems and species is currently faced by stressful conditions like hypoxia or ocean acidification. This is particularly evident in coastal habitats where physiochemical conditions coupled with human input make these habitats more vulnerable to changes. This work has proven how, in spite of the great hypoxic tolerance of the ditch shrimp *Palaemon varians*, a cyclic hypoxic regime currently experienced in its environment was able to induce changes on *P. varians* at different levels of biological organisation, showing that even a brief exposure to hypoxia repeated daily can elicit physiological effects. Continued effort in elucidating the consequences of cyclic hypoxia on coastal species is important for our understanding of the responses of marine ectotherms to contemporary climate change, especially in those highly variable environments (e.g. lagoons, marshes) where species might be more affected by climate change than species from moderately variable environments.

Appendix A

Supplementary Table 1: pO_2 levels during hypoxic experiments. Sections refer to the specific Chapter's section of the thesis.

Mean pO_2 levels (kPa ±SD) during hypoxic trials for each replicate tank for each experiment:				
Experiment:	Section:	Tank 1	Tank 2	Tank 3
Short hypoxic exp	2.3.4	2.7 ±0.5	2.7 ±0.9	2.9 ±0.3
RNA-seq:	3.3.3	2.9 ±0.7	3.0 ±0.6	2.1 ±0.6
Intermoult duration	3.3.4	2.1 ±0.5	2.1 ±0.6	2.8 ±0.7
Gene expression during moult cycle	3.3.5	3.5 ±0.4	2.7 ±0.5	2.5 ±1.0
Gill modifications	3.3.6	3.5 ±0.4	2.7 ±0.5	2.5 ±1.0
Growth	4.3.2	2.9 ±0.9	3.1 ±0.6	3.0 ±0.9
Feeding/Excretion	4.3.3	2.7 ±0.4	2.8 ±0.3	2.9 ±0.2
Reproduction	4.3.4	2.8 ±0.8	2.8 ±0.9	2.9 ±1.1
Hypoxic acclimation	5.3.1	2.7 ±0.4	2.8 ±0.3	2.9 ±0.2
Hypoxic acclimation	5.3.2	2.7 ±0.4	2.8 ±0.3	2.9 ±0.2

Supplementary Table 2: Trascriptome statistics

Assembly Report with Scaffolded regions	
Total reads	95,513,752
Generated contigs	105,325
Minimum length	250 bp
Average length	907 bp
Median length	1491 bp
Maximum length	26718 bp

Mapping Report

Mapped reads	82.29%
Not mapped reads	17.71%
Read in pairs	75.02%

RNA-seq Experiment report		
Reference Transcriptome (n° contigs)		59,370
	Normoxia	Hypoxia
Mapping reads	68.64%	75.05%
Uniquely mapping reads	68.43%	74.82%
Not-uniquely mapping reads	0.21%	0.23%

Supplementary Table 3: List of putative responsive genes statistically up/down regulated in comparison to controls. Differential expression between treatment and control was calculated using a Kal's Z-test on proportions with FDR <0.05 and proportional fold change > |2|.

Upregulated

Description:	Contig ID	Proportional fold change:	UniprotKB accession number	GenBank
Chitinase 2	contig_13969	235.39	Q9W5U2	BAA14014.1
Post-moult protein 1	contig_16319	189.1	A0A0K2C0S2	AKZ75936.1
Cuticle protein	contig_54328	138.18	P81388	
Cuticle protein	contig_66705	58.45	P81388	
Chemosensory protein 3	contig_38778	36.64		ABH88168.1
Elongation of very long chain fatty acids protein	contig_47915	26.8	Q1HRV8	
Cuticle protein	contig_25634	20.73	P81576	
Cuticle protein	contig_60605	20.54	P81576	
Cuticle protein	contig_34320	16.45	P81577	
Cuticle protein	contig_58327	15.86	P81388	
Chemosensory protein 3	contig_62563	15.78		ABH88168.1
Cuticle protein	contig_35509	13	P81589	
RNA-binding protein like	contig_16807	12.71	Q5RBM8	
Chitinase	contig_25080	11.97	Q9W5U2	
Peptide M28	contig_20072	11.45	A0A0R6QQU2	
Cuticle protein	contig_5295	8.64	P81580	
Cuticle protein	contig_8285	8.46	P82119	
Cuticle protein	contig_719	8.42	P81589	
Cuticle protein	contig_14361	8.18	P81582	
Cuticle protein	contig_3086	8.1	P82119	
Cuticle protein	contig_54327	7.71	P81579	
Retinoid-inducible serine carboxypeptidase	contig_16170	7.49	Q9HB40	
Cuticle protein	contig_35105	7.32	P81579	
Gastrolith protein 18	contig_7251	7.28		ALC79580.1
Cuticle protein	contig_8790	7.15	P81585	

Cuticle protein	contig_7102	6.98	P81580	
Cuticle protein	contig_14360	6.94	P81582	
Cuticle protein	contig_8789	6.86	P81580	
Post-moult protein 1	contig_16101	6.84	/	AKZ75936.1
Cuticle protein	contig_747	6.56	P81580	
Cuticle protein	contig_7970	6.52	P81388	
Cuticle protein	contig_60747	6.48	P81584	
Cuticle protein	contig_8284	6.34		
Post-moult protein 1	contig_16100	6.22		AKZ75936.1
Cuticle protein	contig_795	6.2	P82119	
Post-moult protein 1	contig_16102	6.16		AKZ75936.1
Gastrolith protein 18	contig_11672	6.15		ALC79580.1
Cuticle protein	contig_718	5.95	P81589	
Glucose-6-phosphate transporter	contig_12820	5.9	O43826	
Cuticle protein	contig_6370	5.49	P81589	
Calcification associated peptide	contig_10413	5.31		BAD16776.1
Cuticle protein	contig_4477	4.96	P81583	
Larval cuticle protein	contig_5276	4.93		JAG02058.1
Cuticle protein	contig_107	4.92	P82119	
Gastrolith protein 30	contig_33893	4.9	O76217	
DD5 peptide	contig_23547	4.72	Q7M4F4	
Alkaline phosphatase	contig_791	4.51	Q92058	

Down-regulated

Description:	**Contig ID**	**Proportional fold change:**	**UniprotKB accession number**	**GenBank**
Vitellogenin	contig_15601	-31.37	Q6RG02	
Alpha-2 macroglobulin	contig_1092	-7.09		AEC50080.1
Peritrophin C	contig_2074	-6.12		ADE06398.1
Hemocyanin B chain	contig_1471	-5.69	P10787	
Serpin serine protease inhibitor	contig_7339	-5.26	Q8VHP7	

Insulin-like androgenic gland factor	contig_7365	-4.48		BAJ84108.1
Serine–threonine kinase	contig_5351	-4.3	Q9QYZ3	
Endochitinase	contig_3019	-3.92	P36362	
Cuticular protein analogous to peritrophins	contig_752	-3.87		JAN84453.1
Chitinase	contig_25081	-3.47	Q9W5U2	
Vigilin	contig_4806	-3.35	Q8VDJ3	
Serine–threonine kinase	contig_5352	-3.17	Q9QYZ3	
Chitinase	contig_8095	-2.96	H2A0L4	
Serine–threonine kinase	contig_1214	-2.92	Q9QYZ3	
Serine–threonine kinase	contig_7112	-2.89	P38692	
Serine–threonine kinase	contig_3557	-2.85	Q8VCT9	
Vigilin	contig_13004	-2.84	Q00341	
Fibrillin 2	contig_7351	-2.83	Q14246	
Serine–threonine kinase	contig_598	-2.81	Q54XJ4	
TATA box-binding protein-like 1	contig_271	-2.78	Q9YGV8	
Antimicrobial peptide (Crustin 7)	contig_466	-2.67		AOF80302.1
Serine–threonine kinase	contig_9498	-2.57	Q54PX0	
EGF-like module-containing mucin-like hormone receptor-like 1	contig_13978	-2.53	Q14246	
Hemocytin	contig_1311	-2.46		XP_020279864.1
EGF-like module-containing mucin-like hormone receptor-like 1	contig_7350	-2.45	Q14246	
Protein-tyrosine kinase	contig_597	-2.42	Q9YHZ5	
Pim 1	contig_5174	-2.42	Q924U5	
Innexin	contig_17765	-2.39	O61787	
Acyl-CoA : lysophosphatidylglycerol acyltransferase 1	contig_2915	-2.34	Q91YX5	
Chitinase	contig_3729	-2.29	Q9W5U3	

TPA zinc finger protein	contig_2262	-2.28	P10394
Proto-oncogene tyrosine-protein kinase ROS	contig_3558	-2.26	P11799
Protein-tyrosine kinase	contig_2031	-2.22	Q9RRH3
Chitinase	contig_1734	-2.2	Q9W5U4

Supplementary Table 4: Hypergeometric tests on categories, performed on each of the annotation categories of the sub-set of differentially expressed genes with the following criteria: p- value <0.01 and "Observed – Expected Difference" ≥3.

UP-REGULATED

eggNOG

Category:	Description:	p-value:
COG1819	Glycosyltransferase	4.03E-006

GO molecular function

Category:	Description:	p-value:
GO:0042302	Structural constituent of cuticle	3.33E-016
GO:0008061	Chitin binding	3.35E-003

InterPro

Category:	Description:	p-value:
IPR031311	Chitin-binding type R&R consensus	3.77E-015
IPR029277	Single domain Von Willebrand factor type C domain	3.98E-010
IPR012539	Crustacean cuticle	2.11E-015
IPR002557	Chitin binding domain	5.19E-010
IPR002213	UDP-glucuronosyl/UDP-glucosyltransferase	1.03E-004
IPR000618	Insect cuticle protein	4.56E-014

PFAM

Category:	Description:	p-value:
PF00379.20	Insect cuticle protein	5.95E-012
PF15430.3	Single domain von Willebrand factor type C	1.79E-009
PF00201.15	UDP-glucuronosyltransferase	1.29E-003
PF01607.21	carbohydrate-binding module (CBM)	2.79E-010
PF08140.8	Crustacean cuticle protein repeat	2.30E-013

DOWN-REGULATED

eggNOG

Category:	Description:	p-value:

COG3325	Chitinase	9.75E-005
COG0515	Serine Threonine protein kinase	2.82E-006

GO cellular components

Category:	Description:	p-value:
GO:0009897	External side of plasma membrane	1.52E-004

GO biological processes

Category:	Description:	p-value:
GO:0000272	Polysaccharide catabolic process	2.43E-007
GO:0007218	Neuropeptide signaling pathway	1.25E-003
GO:0007186	G-protein coupled receptor signaling pathway	2.30E-003
GO:0008203	Cholesterol metabolic process	5.02E-004
GO:0006032	Chitin catabolic process	2.86E-007

GO molecular function

Category:	Description:	p-value:
GO:0004867	Serine-type endopeptidase inhibitor activity	2.98E-003
GO:0004568	Chitinase activity	5.97E-007
GO:0008061	Chitin binding	2.53E-006
GO:0004672	Protein kinase activity	4.11E-003
GO:0004674	Protein serine/threonine kinase activity	4.10E-007
GO:0005524	ATP binding	1.48E-003

InterPro

Category:	Description:	p-value:
IPR011009	Protein kinase-like domain	2.01E-012
IPR004087	K Homology domain	1.24E-004
IPR004088	K Homology domain, type 1	7.31E-006
IPR001881	EGF-like calcium-binding domain	3.18E-003
IPR017441	Protein kinase, ATP binding site	4.55E-007
IPR013781	Glycoside hydrolase, catalytic domain	5.76E-003
IPR000719	Protein kinase domain	2.93E-011
IPR008271	Serine/threonine-protein kinase, active site	1.56E-008

IPR021109	Aspartic peptidase domain	1.51E-005
IPR013026	Tetratricopeptide repeat-containing domain	5.76E-003
IPR011990	Tetratricopeptide-like helical domain	5.95E-005
IPR002290	Serine/threonine/dual specificity protein kinase	7.31E-004
IPR018097	EGF-like calcium-binding, conserved site	1.89E-003
IPR001223	Glycoside hydrolase family 18, catalytic domain	7.15E-004
IPR002557	Chitin binding domain	1.09E-003
IPR014756	Immunoglobulin E-set	5.23E-003
IPR000152	EGF-type aspartate/asparagine hydroxylation site	1.89E-003

PFAM

Category:	Description:	p-value:
PF07645.12	Calcium-binding epidermial growth factor domain	1.34E-003
PF00013.26	K Homology (KH) domain	1.60E-006
PF07719.14	Tetratricopeptide repeat	6.06E-005
PF00704.25	Glycoside hydrolase family 18 (probably Chitinase II)	5.41E-003
PF13975.3	gag-polyprotein putative aspartyl protease	7.10E-007
PF00069.22	Protein kinase domain	1.97E-007

Supplementary Table 5: Nested ANOVA table assessing differences on growth between the experimental replicates (Tanks) after 28 days of cyclic hypoxia or normoxia. “Treatment:Tank” indicates that the experimental replicates were nested in the factor Treatment. Here 20 of 1000 Anova tests are reported. Each Anova was done with a random sub-set of 13 shrimps per tank (necessary to obtain an equal number of replicates in each level). In all 1000 tests, p-value for Treatment:Tank was never significant.

[[1]]	**Df**	**F value**	**p-value**	[[11]]	**Df**	**F value**	**p-value**
Treatment	1	3.15	0.08	Treatment	1	1.69	0.20
Treatment:Tank	10	0.71	0.71	Treatment:Tank	10	0.57	0.84
Residuals	144			Residuals	144		
[[2]]				[[12]]			
Treatment	1	1.89	0.17	Treatment	1	2.41	0.12
Treatment:Tank	10	0.69	0.73	Treatment:Tank	10	0.57	0.83
Residuals	144			Residuals	144		
[[3]]				[[13]]			
Treatment	1	1.63	0.20	Treatment	1	1.98	0.16
Treatment:Tank	10	0.69	0.73	Treatment:Tank	10	0.67	0.75
Residuals	144			Residuals	144		
[[4]]				[[14]]			
Treatment	1	5.03	0.03	Treatment	1	2.01	0.16
Treatment:Tank	10	0.34	0.97	Treatment:Tank	10	0.37	0.96
Residuals	144			Residuals	144		
[[5]]				[[15]]			
Treatment	1	2.20	0.14	Treatment	1	2.34	0.13
Treatment:Tank	10	0.85	0.58	Treatment:Tank	10	0.76	0.66
Residuals	144			Residuals	144		
[[6]]				[[16]]			
Treatment	1	2.07	0.15	Treatment	1	1.32	0.25
Treatment:Tank	10	0.36	0.96	Treatment:Tank	10	0.52	0.87
Residuals	144			Residuals	144		
[[7]]				[[17]]			
Treatment	1	2.75	0.10	Treatment	1	2.41	0.12
Treatment:Tank	10	0.79	0.64	Treatment:Tank	10	0.72	0.71
Residuals	144			Residuals	144		
[[8]]				[[18]]			
Treatment	1	1.49	0.22	Treatment	1	1.29	0.26
Treatment:Tank	10	0.36	0.96	Treatment:Tank	10	0.44	0.93
Residuals	144			Residuals	144		
[[9]]				[[19]]			
Treatment	1	0.81	0.37	Treatment	1	1.79	0.18
Treatment:Tank	10	0.80	0.63	Treatment:Tank	10	0.81	0.62
Residuals	144			Residuals	144		
[[10]]				[[20]]			
Treatment	1	1.46	0.23	Treatment	1	2.40	0.12
Treatment:Tank	10	0.47	0.91	Treatment:Tank	10	0.65	0.77
Residuals	144			Residuals	144		

Supplementary Table 6: Nested ANOVA table testing differences in egg volume between females. "Treatment:Female" indicates that the females were nested in the factor Treatment. Here 20 of 1000 Anova tests are reported. Each Anova was done with a random sub-set of 4 hypoxic and 4 normoxic females, with 15 eggs per female. In all 1000 tests, p-value for Treatment:Female was always significant (i.e. < 0.05).

[[1]]	**Df**	**F value**	**p-value**	[[11]]	**Df**	**F value**	**p-value**
Treatment	1	84.73	2.26E-15	Treatment	1	4.44	0.0373
Treatment:Female	6	56.45	<2e-16	Treatment:Female	6	85.5	<2e-16
Residuals	112			Residuals	112		
[[2]]				[[12]]			
Treatment	1	104.5	<2e-16	Treatment	1	377.73	<2e-16
Treatment:Female	6	206.5	<2e-16	Treatment:Female	6	72.99	<2e-16
Residuals	112			Residuals	112		
[[3]]				[[13]]			
Treatment	1	86.6	1.32E-15	Treatment	1	45.19	7.84E-10
Treatment:Female	6	141.8	<2e-16	Treatment:Female	6	150.94	<2e-16
Residuals	112			Residuals	112		
[[4]]				[[14]]			
Treatment	1	281.6	<2e-16	Treatment	1	55.88	1.84E-11
Treatment:Female	6	104.8	<2e-16	Treatment:Female	6	93.09	<2e-16
Residuals	112			Residuals	112		
[[5]]				[[15]]			
Treatment	1	192.1	<2e-16	Treatment	1	82.47	4.35E-15
Treatment:Female	6	135.6	<2e-16	Treatment:Female	6	59.87	<2e-16
Residuals	112			Residuals	112		
[[6]]				[[16]]	1	57.35	1.12E-11
Treatment	1	179.1	<2e-16	Treatment	6	114.85	<2e-16
Treatment:Female	6	141.2	<2e-16	Treatment:Female	112		
Residuals	112			Residuals			
[[7]]				[[17]]	1	92.52	2.51E-16
Treatment	1	68.09	3.39E-13	Treatment	6	144.41	<2e-16
Treatment:Female	6	60.08	<2e-16	Treatment:Female	112		
Residuals	112			Residuals			
[[8]]				[[18]]	1	58.27	8.21E-12
Treatment	1	64.2	1.17E-12	Treatment	6	114.33	<2e-16
Treatment:Female	6	151.4	<2e-16	Treatment:Female	112		
Residuals	112			Residuals			
[[9]]				[[19]]	1	7.687	0.00652
Treatment	1	234.84	<2e-16	Treatment	6	85.939	<2e-16
Treatment:Female	6	95.69	<2e-16	Treatment:Female	112		
Residuals	112			Residuals			
[[10]]				[[20]]	1	195.1	<2e-16
Treatment	1	72.63	8.26E-14	Treatment	6	115.1	<2e-16
Treatment:Female	6	142.6	<2e-16	Treatment:Female	112		
Residuals	112			Residuals			

Supplementary Table 7: Nested ANOVA table testing differences in egg dry weight between females. "Treatment:Female" indicates that the females were nested in the factor Treatment. Here 20 of 1000 Anova tests are reported. Each Anova was done with a random sub-set of 4 hypoxic and 4 normoxic females, with 5 eggs per female. p-value for Treatment:Female was significant (i.e. < 0.05) in 95% of the tests.

[[1]]	Df	F value	p-value	[[11]]	Df	F value	p-value
Treatment	1	44.366	6.87E-07	Treatment	1	29.553	1.38E-05
Treatment:Female	6	4.016	0.00634	Treatment:Female	6	8.628	4.65E-05
Residuals	24			Residuals	24		
[[2]]				[[12]]			
Treatment	1	19.09	0.000207	Treatment	1	21.021	0.000119
Treatment:Female	6	4.725	0.00262	Treatment:Female	6	7.966	8.48E-05
Residuals	24			Residuals	24		
[[3]]				[[13]]			
Treatment	1	22.874	7.22E-05	Treatment	1	46.95	4.37E-07
Treatment:Female	6	7.685	0.00011	Treatment:Female	6	13.39	1.24E-06
Residuals	24			Residuals	24		
[[4]]				[[14]]			
Treatment	1	73.89	8.64E-09	Treatment	1	22.601	7.76E-05
Treatment:Female	6	7.635	0.000116	Treatment:Female	6	5.491	0.00107
Residuals	24			Residuals	24		
[[5]]				[[15]]			
Treatment	1	77.516	5.56E-09	Treatment	1	5.63	0.026013
Treatment:Female	6	9.625	1.98E-05	Treatment:Female	6	6.47	0.000369
Residuals	24			Residuals	24		
[[6]]				[[16]]			
Treatment	1	58.488	6.94E-08	Treatment	1	106.6	2.62E-10
Treatment:Female	6	6.464	0.000372	Treatment:Female	6	12.3	2.60E-06
Residuals	24			Residuals	24		
[[7]]				[[17]]			
Treatment	1	87.44	1.79E-09	Treatment	1	62.002	4.17E-08
Treatment:Female	6	15.26	3.83E-07	Treatment:Female	6	6.688	0.000295
Residuals	24			Residuals	24		
[[8]]				[[18]]			
Treatment	1	9.251	0.00562	Treatment	1	49.814	2.69E-07
Treatment:Female	6	3.409	0.01413	Treatment:Female	6	8.124	7.33E-05
Residuals	24			Residuals	24		
[[9]]				[[19]]			
Treatment	1	62.816	3.72E-08	Treatment	1	32.57	7.03E-06
Treatment:Female	6	6.193	0.000495	Treatment:Female	6	12.23	2.72E-06
Residuals	24			Residuals	24		
[[10]]				[[20]]			
Treatment	1	35.387	3.87E-06	Treatment	1	44.8	6.36E-07
Treatment:Female	6	9.049	3.22E-05	Treatment:Female	6	15.07	4.28E-07
Residuals	24			Residuals	24		

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