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The consequences of daily cyclic hypoxia on a European grass shrimp: from short-term responses to long-term effects

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Running title: Physiological and molecular effects of cyclic hypoxia

ABSTRACT:

1. Salt marshes are a key coastal environment for their important role as nursery habitats for marine and estuarine fish and crustaceans. Salt marshes are variable environments where species can experience daily cyclic hypoxic stress, characterized by profound variations in oxygen partial pressure (pO_2) from supersaturated conditions (~42kPa) to extremely hypoxic conditions (~3kPa) in ~12-hours.
2. Here, under laboratory conditions, we assessed the physiological consequences of exposing the shrimp *Palaemon varians,* a species commonly found in the salt marshes of northern Europe, to the daily cyclic hypoxic regime currently experienced in its habitat in August (7.1±1.8 hours day^{-1} below 4.0kPa). In the laboratory adults were kept at water pO_2 <4.5kPa for 7-hours each night and in normoxic conditions for the rest of the time.
3. We recorded an acceleration of *P. varians*' moult cycle, which was 15% shorter in animals kept in cyclic hypoxia compared to animals in normoxia. Similarly, the pattern of expression of two cuticular proteins over an entire moult cycle indicated an effect of cyclic hypoxia on moult stage-related genes. After 16 days, morphological changes to the gills were detected, with shrimps in cyclic hypoxia having a 13.6% larger lamellar surface area (measured in μm^2/mg animal) than normoxic animals, which could improve gas exchange capacity. Overall, phenotypic and morphological data indicate that faster moulting is triggered in response to cyclic hypoxia, with the benefit that gill modifications can be prompted more

rapidly in order to meet oxygen requirements of the body.

4. On the first experimental day, in cyclic hypoxic exposed animals, we recorded a 50% decrease in feeding rates (during hypoxic conditions) in comparison to normoxic animals. Similarly, ammonium excretion was reduced by 66-75% during the 1st and 21st experimental day. Body size was reduced by ~4% after 28 days. Females that reproduced in cyclic hypoxic conditions reduced the amount of yolk in each egg by ~24%. Overall, results underline how, in a decapod shrimp living in a key coastal environment, many physiological parameters are impaired by a cyclic hypoxic regime that is currently found in its natural habitat.

INTRODUCTION:

Salt marshes are a key habitat of conservation importance along many temperate coastal fringes for their important role as nursery habitats for estuarine fish, crustacean species and other nekton (Cattrijsse & Hampel 2006). They can support a large number of breeding, feeding and roosting birds, as in the case of the Lymington salt marshes, UK (Oliphant 2013). Another important characteristic of salt marshes is that these habitats can be characterised by a substantial diel variability in the main physiochemical parameters, particularly temperature and oxygen partial pressure (pO_2). Smith and Able (2003) demonstrated that pO_2 could fluctuate every day from supersaturated conditions (>21 kPa) during the day to hypoxic conditions (~1 kPa) during the night, a phenomenon that is known as daily cyclic hypoxia.

The succession of hypoxic and normoxic periods characterises the most fundamental difference between cyclic and chronic hypoxia. Whilst, under chronic hypoxia no recovery time (i.e. normoxic periods) from stressful conditions are given to the exposed organism, under cyclic hypoxic conditions organisms have the possibility to restore normal homeostasis (Coiro, Poucher & Miller 2000; Sokolova *et al.* 2012). As a consequence of this, under cyclic hypoxia, different molecular and

physiological mechanisms can be activated in comparison to chronic hypoxia. In fact, as demonstrated by Li and Brouwer (2013b), exposing grass shrimp *Palaemon pugio* (a species only found in the American continent) to cyclic hypoxia for 10 days induced an up-regulation of *hemocyanin* mRNA (respiratory pigment) and a down-regulation of *vitellogenin* mRNA (the precursor protein of egg yolk), while 10-days exposure to chronic hypoxia induced a down-regulation of *hemocyanin* and no change in *vitellogenin*. Further, physiological processes (e.g. growth, reproduction) can be differently affected by cyclic and chronic hypoxia as demonstrated by Coiro *et al.* (2000) and Brown-Peterson *et al.* (2008). Overall, the different outcomes of chronic or cyclic hypoxia underline why it is more appropriate to assess the impact of cyclic (rather than chronic hypoxia) on those species which are subjected to cyclic hypoxia in their natural habitat.

In arthropods, the respiratory system grows primarily at moults because it is covered by the rigid exoskeleton (Lundquist *et al.* 2017), whereas tissue mass increases between moults (and so does oxygen demand) (Kivelä, Lehmann & Gotthard 2016). In hypoxic conditions, a mismatch between oxygen supply and oxygen demand can develop (Massabuau & Abele 2012). Hence, as demonstrated by Greenberg and Ar (1996), insect larvae reared in hypoxic conditions moult more frequently and develop a larger respiratory system in comparison to larvae reared in normoxia (e.g. VandenBrooks *et al.* (2017)), in order to increase oxygen supply to the tissue and sustain oxygen demand of the body (Callier & Nijhout 2011, 2013). In insects, the increased moult frequency (due to hypoxia) is coupled with a reduction in body mass (Greenberg & Ar 1996; Callier & Nijhout 2011), probably due to reduced feeding or increased maintenance costs associated with the exposure to stressful conditions. Insects and crustaceans possess the same generalised structural body plan (Roer, Abehsera & Sagi 2015): body and gills are covered by a rigid exoskeleton that is periodically replaced at ecdysis. Therefore, it can be hypothesised that an acceleration of the moult cycle coupled with the development of larger gills will be observed in adult shrimp from salt marshes exposed to cyclic hypoxia.

At physiological level, the length of exposure to cyclic hypoxia can trigger different responses and effects on the species (Truebano *et al.* 2018). In the short-term, responses aim to maintain O_2 delivery to the tissue with molecular, metabolic and/or respiratory/circulatory adjustments (Brown-Peterson *et al.* 2008; Truebano *et al.* 2018) and aim to suppress aerobically expensive processes (e.g. excretion or feeding (Wei *et al.* 2009)) to reduce overall oxygen demand. After prolonged exposure, other mechanisms that imply functional modifications can be triggered (e.g. the enlargement of the respiratory system mentioned above). In addition to these morphological changes, exposure to cyclic hypoxia can have, in the long-term, negative consequences on physiological processes such as growth (Davidson, Targett & Grecay 2016) or reproduction (Cheek *et al.* 2009) with potential repercussions on the biology and ecology of the species (Fenberg & Roy 2008).

Cyclic hypoxia is currently experienced by a vast number of species from freshwater ponds, estuaries, lagoons and marshes worldwide; however, the incidence of studies reproducing cyclic hypoxic conditions is still rare and it is confined almost exclusively to species from the American continent. Within the Lymington marshes, UK, the ditch shrimp *Palaemon varians* Leach (1814), was chosen as a model due to its key ecological role in the ecosystem (Aguzzi *et al.* 2005). In fact, in European marshes, export of organic material is largely absent (Cattrijsse & Hampel 2006) and decaying marsh flora are processed by detritivorous species, such as *P. varians*, which plays a fundamental role in transfer of nutrients and energy in the ecosystem (Welsh 1975; Aguzzi *et al.* 2005). We demonstrated that the exposure to a daily cyclic hypoxic regime (of seven hours per day in hypoxia) induced changes in the transcriptome of the entire cephalothorax (not limited to cuticle and hypodermis but including hepatopancreas and all major organs) after 7 days. Further, it activated physiological adaptations resulting in an acceleration of the moult cycle and in morphological changes to the gills within 16 days of cyclic hypoxia. Finally, we demonstrated how the cyclic hypoxic regime reduced adult body size and ammonium excretion within 28 days of exposure, and affected reproduction in adult *P. varians* after 40 days exposure.

MATERIALS AND METHODS:

A detailed description of all materials and methods is available in Supporting Information. In all experiments, statistical significance was identified at $p \leq 0.05$.

Sampling site

The population of *Palaemon varians* originates from the salt marsh of Lymington (UK, location: 50° 44'19.8" N and 50° 44'22.2" W) and adult *P. varians* used in experimental work were all net-caught from this site. To assess daily cyclic oxic conditions and describe some of the variability that can occur in the ditch, pO_2 data was continuously measured in three discrete weeks (August 4th – 11th 2016; February 16th – 23rd 2017; May 14th – 22nd 2017). pO_2 was measured using a custom oxygen and temperature logger placed adjacent to the bottom of the channel, where animals are more frequently found.

General experimental protocol

Hypoxia was defined as pO_2 < critical oxygen pressure (p_{crit}). P_{crit} was determined according to Mueller and Seymour (2011) by using the "highest vertical distance method" (further details are provided in Supporting Information). P_{crit} was determined at 22 ˚C as this temperature is frequently measured in the marsh during summer (from June to September) at night (from 2000 to 0800 BST, Fig. S1A in Supporting Information). At 22 ˚C a mean p_{crit} of 4.55 ±0.63 kPa (n=10, Table S1) was obtained.

To perform all subsequent experiments, two flow-through experimental systems were built (one system for cyclic hypoxic treatment, one for normoxic treatment). All experimental work in these systems was conducted in filtered seawater at 33 PSU and 22 ˚C. Hypoxia was achieved by bubbling N_2 in the experimental tanks and water pO_2 was continuously measured and logged with Microx TX 3 (PreSens) sensors. Water and N_2 flow-rate were adjusted in order to obtain $pO_2<p_{crit}$. Animals were subjected daily to hypoxic conditions (pO_2 < 4.5 kPa) for 7 hours (from 0230 to 0930 hrs) and they were kept in normoxic conditions (by bubbling air into the system) for the rest of the day (Fig. S1B). In the normoxic flow-through system air was bubbled to prevent the development of hypoxic conditions. For each experiment, the duration (in terms of days) of the exposure, the mean±SD pO_2 level recorded during hypoxic periods in each tank and the sampling date in which animals were collected from the field is reported in Table S2. In all experiments max shrimp density was 18 animals per tank (volume = 12L).

In experiments investigating inter-moult duration, changes in the expression of cuticular genes throughout an entire moult cycle and gill histology, a synchronous population (composed of similar-size animals that had all moulted within 12 hours) was used. No animal selection was made according to the stage of the moult cycle during experiments involving RNA-seq, growth, feeding, excretion and reproduction.

Transcriptome response to hypoxia

A pilot RNA-seq experiment was performed on the cephalothorax of animals to identify changes in gene expression after seven days of cyclic hypoxic exposure. Animals (n=8 per treatment) were randomly sampled at 1030 hrs after seven days of exposure to experimental conditions (cyclic hypoxia or normoxia). RNA was extracted using a TRI-Reagent™ (Sigma Aldrich) protocol from whole cephalothoraxes. Library preparation followed Illumina TruSeq RNA-Library Preparation Kit.v2

(Illumina, California). Paired-end 115bp reads were sequenced on an Illumina HiSeq-2500. Raw sequencing data were imported into the CLC-Genomic Workbench v.8.5 (CLC Bio, Aarhus, Denmark) to generate an annotated reference transcriptome, to calculate gene expression levels and to perform hypergeometric tests on categories.

Statistically significant gene expression changes 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 expression of some up-regulated genes was further confirmed by means of quantitative-PCR analysis (following protocols described in "Gene expression of cuticular genes" section) and tested with t-test.

Changes in inter-moult duration

Shrimps from a synchronous population (where individuals had all moulted within 12 hours: "ecdysis1_day0") were individually tagged with coloured numbered tags (Queen-bees marking kit – Abelo, UK) and were randomly allocated to the cyclic hypoxic (n=27) or normoxic (n=27) treatment and maintained in experimental conditions until their next ecdysis (when the tag was lost on the shed exoskeleton: "ecdysis2_dayN"). Inter-moult duration (ecdysis2_dayN – ecdysis1_day0) was compared between treatments using Wilcoxon rank-sum test and Mann-Whitney test.

Gene expression of cuticular genes during moult cycle

Shrimps from a synchronous population were randomly allocated to the cyclic hypoxic (n=54) or normoxic (n=54) treatment and exposed to experimental conditions for up to 16 days. Every other day, from the day of ecdysis (day 0) up to 16 days after this event (day 16), cephalothoraxes from animals (n=6 per treatment) were collected and flash frozen (between 1000 and 1030 hrs). RNA was extracted from whole cephalothorax, DNase treated and reverse transcribed. qPCR reactions were performed using primer-sets reported in Table 1. To assess if cyclic hypoxia was able to alter the expression of the selected genes, the overall pattern of expression of the cyclic hypoxic group was compared with the pattern of the normoxic group using a general additive model (GAM). Differences between models were compared using "Akaike's Information Criterion" – AIC – values (Sakamoto, Ishiguro & Kitagawa 1986).

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

Shrimps from a synchronous population were randomly allocated to the cyclic hypoxic (n=5) or normoxic (n=5) treatment and exposed to experimental conditions for 18 days to make sure they completed one entire moult cycle in experimental conditions. After 18 days, the cephalothorax from each animal was fixed in Bouin's solution, dehydrated and embedded. Longitudinal sections of gill from each specimen were stained with haematoxylin and eosin. Micrographs were analysed using ImageJ software (Schneider, Rasband & Eliceiri 2012) to determine lamellar width, length, perimeter and density (Fig. S2). All parameters were corrected for differences in body mass using animal's wet weight and tested using t-test.

Changes in body size:

Shrimp were kept in cyclic hypoxia (6 experimental tanks, n=17 shrimps per tank) for 28 days and changes in wet weight over time were compared with a control population kept in normoxia (6 experimental tanks, n=17 shrimps per tank) for 28 days. On day 0, and then every seven days to 28 days, all animals were weighed using an analytical microbalance. Absence of systematic differences between experimental replicates were tested using a nested ANOVA. To test for changes in wet weight over time between treatments, linear regression models were compared using extra sum-of-squares F test.

Feeding and excretion:

Feeding and excretion of adult *P. varians* were quantified after 1 and 21 days of exposure to cyclic hypoxia or normoxia.

Feed ingestion was quantified in animals (n=11 shrimps/treatment) placed in artificial sea-water at the same temperature and pO_2 as their respective experimental aquaria (water pO_2=3.0 kPa for the cyclic hypoxic group; water pO_2=21 kPa for the normoxic group). Each adult was fed (~4.5% its wet weight) with commercial pellet and, after 150 minutes, uneaten feed was dried, weighed and used to calculate feed ingestion rate.

Ammonium excretion was quantified in adults (n=14-18 adults/treatment) placed in artificial sea-water at the same temperature and pO_2 as their respective experimental aquaria (water pO_2=3.0 kPa for the cyclic hypoxic group; water pO_2=21 kPa for the normoxic group). After 210 minutes, water samples were collected and ammonium ions were measured with a Hach method 8155 “Ammonia Salicylate Method” (Hach, Colorado USA).

Two-way ANOVA with 'treatment' and 'time' as factors was used to assess differences in feed ingestion and ammonium excretion, followed by Tukey's multiple comparisons test.

Reproduction:

Reproductive pairs of a male and a female with ovaries in primary vitellogenesis (n= 12 pairs in hypoxia, 10 pairs in normoxia) were housed in retention chambers up to 40 days. During the experiment, reproductive success (ratio between gravid and non-gravid females), relative fecundity (egg number/body weight) and egg dry weight were compared with control pairs kept in normoxia and assessed using Chi-square test and t-test.

RESULTS:

Environmental variability

During 2016 and 2017, oxygen and temperature measurements were taken over one-week period in August (4^{th} – 11^{th} 2016), February (16^{th} – 23^{rd} 2017) and May (14^{th} – 22^{nd} 2017) (Fig. 1). Daily temperature variations of ~2 ˚C were recorded in February and increased to ~5.5 ˚C in August. Diel pO_2 oscillations were less pronounced in February, while they became very marked in May and August, where pO_2 could vary from ~40 kPa to ~3.5 kPa within 12-h. In May, the amplitude of the oscillations seemed "irregular" (with peaks in pO_2 that changed considerably every day), in contrast to August where a ~39 kPa difference between adjacent peaks and troughs was detected every 12-hours. In August, hypoxic conditions below 4.0 kPa lasted, on average, 7.1 ±1.8 hours day^{-1}.

Transcriptome response to hypoxia

A pilot RNA-seq experiment was performed to identify genes putatively involved in tolerance to cyclic hypoxic conditions in treatment groups of *P. varians*. Pooled cDNA libraries generated from cyclic 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, further reduced to 59,370 reference sequences upon the removal of poorly covered fragments (Table S3). This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFPG00000000. The version described in this paper is the first version, GFPG01000000.

The completeness and integrity of the reference transcriptome were evaluated with BUSCO, which revealed that only 5% of the assembled transcripts were absent and 4% were fragmented. 91% of the benchmarking single copy orthologous genes conserved across all metazoans were present as full-length transcripts, highlighting the high completeness and integrity of the assembled transcriptome.

Differential expression analysis identified a total of 399 differentially expressed contigs in the cyclic hypoxic treatment (214 up-regulated and 187 down-regulated) in the day 7 transcriptome. Among the annotated up-regulated contigs, overall the transcriptome was dominated by genes involved with cuticle synthesis (e.g. cuticular proteins, Table S4). Further, we identified proteins expressed in the post-moult phase of crustaceans and involved in deposition and calcification of the newly formed exoskeleton: post-moult protein (*PMP*), calcification-associated peptide (*CaAP*), peptides *DD5* and *M28* (Ikeya *et al.* 2001; Inoue *et al.* 2004; Roer *et al.* 2015). Among the down-regulated transcripts, the majority were identified as chitinase enzymes (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 dominant annotation among the down regulated contigs (Table S5).

MIQE-compliant quantitative PCR confirmed the up-regulation of those post-moult cuticular genes from the transcriptome data (Fig. 2). Contigs coding for *PMP, CaAP* and *DD5* in cyclic 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).

Changes in inter-moult duration

Moulting frequency distributions among treatments differed (Wilcoxon rank-sum test W=239, p=0.03), with the frequency distribution of cyclic hypoxic animals being shifted to the left (anticipated) in comparison to normoxia (Figure 3A and B, respectively). In addition, median inter-moult duration times (hypoxia 12 and normoxia 14 days) were different (Mann-Whitney, U=239, p=0.03, n=54).

Gene expression of cuticular genes during moult cycle

Following results from RNA-seq experiment, three cuticular markers (*PMP, CaAP* and *DD5*) were selected and their expression levels were quantified on alternate days (from ecdysis (day 0) to day 16) on synchronous populations exposed to cyclic hypoxia or normoxia (Fig. 4). All three markers were selected because their expression is cyclic with a peak in expression during post-moult phase of the moult cycle (just after ecdysis, day 0, Fig.4) (Ikeya *et al.* 2001; Inoue *et al.* 2004; Roer *et al.* 2015). To test whether the overall pattern of expression differed between treatments, a general

additive model (GAM) was run for each gene (Table 2). Comparison of AIC showed that for *PMP* and *DD5* the model fitting independently the treatments (cyclic hypoxia and normoxia) was preferred (i.e. better described the process that generated the data) over the simpler model fitting all data points together (without treatments) (ΔAIC_{PMP}: 8.6; ΔAIC_{DD5}: 3.2). Hence, due to the effect of the treatment, in the cyclic hypoxic group the overall pattern of expression for *PMP* and *DD5* was different in comparison to the normoxic group.

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

Cyclic hypoxic shrimps had longer lamellae (t-test=2.20, p=0.053, n=10, Fig. 5) with a longer perimeter (t=2.21, p=0.055, n=10), but no change in lamellar width (t=1.82, p=0.12, n=10) and density (t=1.49, p=0.17, n=10) were observed.

Changes in body size:

The absence of systematic differences between experimental replicates was confirmed by nested ANOVA ("tank nested within treatment", $F_{10,144}$=0.71, p-val=0.71). The linear regression of the weight-time relationship in cyclic hypoxia was statistically different from that in normoxia (extra sum-of-squares F test, $F_{1, 971}$ =7.182, p=0.01, Fig. 6). The rate of decrease in weight over time was -0.6 in the cyclic hypoxic exposed animals and -0.26 in the normoxic animals (a negative growth as a consequence of captivity was similarly reported by Brown-Peterson *et al.* (2008) when keeping *P. pugio* for over 30-days in experimental tanks). At day 28, mean weight of hypoxic animals was on average ~4% smaller than mean weight of normoxic animals.

Feeding and excretion:

There was a significant effect of treatment and time on feed ingestion ($F_{1,40}$= 12.35, p=0.001 and $F_{1,40}$=6.22, p=0.02, respectively), with no interaction effect ($F_{1,40}$= 0.16, p=0.69). Feed ingestion was reduced in cyclic hypoxic compared to normoxic animals at day 1, but, at day 21 no difference could be detected (Fig. 7A).

Two-way ANOVA revealed an effect of treatment on ammonium excretion ($F_{1,51}$= 77.57, p<0.0001). Cyclic hypoxic animals had a lower excretion rate in comparison to normoxic animals at day 1 and day 21 (Fig. 7B).

Reproduction:

The reproductive success (ratio of gravid to non-gravid females), was not different between cyclic hypoxia and normoxia (Chi-square: 1.564, df=1, p=0.21, Fig. 8A). Similar results were obtained for the relative fecundity of females, which did not show statistical difference (unpaired t-test= 0.35, df=10, p=0.72, Fig. 8B). Egg dry weight normalised to female body weight was lower (~24%) in cyclic hypoxic females compared to controls (unpaired t-test= 2.344, df= 119, p=0.03, Fig. 8C).

DISCUSSION:

Cyclic hypoxia is currently experienced by a vast number of species in different ecosystems worldwide. This study assessed the consequences of exposing the shrimp *Palaemon varians* to the daily cyclic hypoxic regime currently experienced in its habitat in August. In the short-term a reduction in feeding and ammonium excretion were detected in adults exposed to cyclic hypoxia while, in the long-term, an acceleration of the moult cycle and an increase in gill surface area were

found. Further, a long-term exposure to cyclic hypoxia resulted in a decrease in adult body size and a decrease in the dry weight of eggs produced by females. These results emphasize how a cyclic hypoxic regime mimicking field conditions is able to impact the physiology of a key coastal invertebrate at many levels of biological organization.

Environmental variability:

In this study, rapid changes in pO_2 (within 12 hours) were found in all sampling weeks, with maximum fluctuations of ~42 kPa in August. A similar magnitude of diel variability in pO_2 has been reported for other salt marshes in USA (e.g. (Smith & Able 2003; Cheek *et al.* 2009)). Diel variability found in Lymington is probably due to a combination of abiotic and biotic factors: water channels are characterized by stagnant waters (that prevent mixing and limit gas exchange with the atmosphere) and by a thick algal coverage (that increases pO_2 during the day via photosynthesis and, together with animals, decreases pO_2 during the night via respiration). Indeed, it proved difficult to collect *P. varians* with hand net at the sampling site during July and August, whereas the species was easily collected during the rest of the year (unpubl. obs.). It is possible that cyclic hypoxic conditions of July and August were more stressful in comparison with conditions experienced in the rest of the year, probably due to the higher summer temperatures (frequently ≥20 ˚C), forcing *P. varians* to temporarily relocate to more suitable environmental conditions.

Transcriptome response to hypoxia:

Differential expression analysis from whole cephalothorax identified 399 differentially expressed contigs in asynchronously-moulted shrimp subjected to cyclic hypoxia. The majority of the differentially expressed genes were involved with cuticle deposition (i.e. cuticular proteins, *PMP*, *DD5*), cuticle re-arrangement (i.e. chitinases) and cuticle calcification (i.e. *CaAP,* gastrolith proteins).

The expression of these genes is inevitably related to the phases of the moult cycle (e.g. see Tom *et al.* (2014) and Abehsera *et al.* (2015)). The increased expression of cuticular contigs after 7-days exposure suggested that cyclic hypoxia might exert an effect on the moult cycle of *P. varians*, similarly to that reported for insect larvae reared in hypoxia (Kivelä *et al.* 2016). This conclusion was supported by the up-regulation of *PMP, CaAP* and *DD5* genes, markers highly expressed during post-moult phase in crustaceans (Ikeya *et al.* 2001; Inoue *et al.* 2004; Roer *et al.* 2015).

Changes in moulting cycle and gill morphology of synchronously moulted shrimp:

Exposure to cyclic hypoxia accelerated the moult cycle of adult *P. varians* (inter-moult time was 15% shorter compared to normoxic animals), in accordance with Greenberg and Ar (1996) who reported an accelerated moult cycle in larvae of the beetle *Tenebrio molitor* reared in hypoxia.

Gene expression (GE) analysis of synchronously-moulted shrimp showed how the differences in the overall pattern due to the treatment (modelled with model 2) were greater than the natural variability of the population (modelled with model 1). The overall expression pattern (over 16 days) of the post-moult markers *PMP* and *DD5* was accelerated in cyclic hypoxia compared to normoxia. In agreement with our results, Brown-Peterson *et al.* (2008) showed that exposing *Palaemonetes pugio* to 14 days of cyclic hypoxia induced an up-regulation of cuticular proteins (similar to this study), whereas chronic hypoxia for 14 days exclusively induced a down-regulation of electron-transport chain proteins and no up-regulation of cuticular proteins. These different GE patterns found by Brown-Peterson *et al.* (2008) might be explained by the different experimental conditions tested: chronic hypoxia and cyclic hypoxia. Since moulting is an energetically expensive process, it could be hypothesised that shrimps, when subjected to continuous stress from chronic hypoxia, undergo prolonged metabolic depression that completely inhibits moulting (as reported by

Wei *et al.* (2008)). In contrast, in cyclic hypoxia, the turnover hypoxia-normoxia allows "recovery time" to restore homeostasis and stimulates an acceleration of the moult cycle.

The lamellar length of *P. varians* gills (expressed as µm/mg_animal) of cyclic hypoxic animals was, on average, 13.6% longer than control shrimp. Animals after one moult cycle in cyclic hypoxia had a 13.6% larger lamellar surface area. In agreement with our findings, gill remodelling as a consequence of chronic hypoxia has been demonstrated in several fish species where an increase in lamellar surface area has been reported during exposure to hypoxia (Nilsson 2007). The observed increase in surface area represents a functional solution for animals to cope with the lowered pO_2 during hypoxic periods. In fact, oxygen movements across the gills are directly proportional to the respiratory surface area (Massabuau & Abele 2012). Hence, a 13.6% larger surface area would improve the capacity for gas exchange. Overall, data presented here indicate that faster moulting is triggered in response to cyclic hypoxia, with the benefit that gill modifications can be produced more rapidly in order to meet oxygen requirements of the body, in accordance with Callier and Nijhout (2013).

Effects of cyclic hypoxia on body size, feeding, reproduction and ammonium excretion:

Body size is generally considered a fundamental biological trait affecting reproduction, predator-prey relationships and competition with other species (Fenberg & Roy 2008). In *P. varians*, the rate of decrease in wet weight over time was faster in cyclic hypoxic animals in comparison to normoxic animals. After 28-days exposure to cyclic hypoxia the average body weight was ~4% smaller than the average weight of normoxic animals. Li and Brouwer (2013a) demonstrated that a field population of *P. pugio* from a cyclic hypoxic salt marsh was ~10% shorter and ~22% lighter in comparison to a population from another site not experiencing cyclic hypoxia. In the laboratory

growth depression has been demonstrated in some fish species (Stierhoff, Targett & Miller 2006; Davidson *et al.* 2016) and in *Palaemon vulgaris* larvae (a species only found in the American continent) (Coiro *et al.* 2000). Coiro *et al.* (2000) identified between 15% and 70% reduction in growth in *P. vulgaris* larvae under a 12-h hypoxia and 12-h normoxia cyclic hypoxic regime (in contrast to 7-h hypoxia and 17-h normoxia of our work). Hence, it could be argued that longer times in normoxia (as the ones in this study) facilitate the recovery from hypoxia by reducing the impact of hypoxic stress on physiological processes, while shorter normoxic periods result in greater physiological impairment.

Feeding is a fundamental biological process that requires aerobic conditions (Wei *et al.* 2009) and therefore it can be negatively affected by hypoxia, as documented in relation to chronic hypoxia (Brandt *et al.* 2009). Indeed, on the first experimental day of cyclic hypoxic exposure, food ingestion was significantly reduced in cyclic hypoxic-exposed animals when food was provided during hypoxia. However, this difference in food ingestion during hypoxia was not detectable at day 21, arguably as a result of long-term effects such as the increased gill surface area or an increased concentration of O_2-binding proteins (e.g. Truebano *et al.* (2018)) that could mitigate the hypoxic stress.

In *P. varians* a significant reduction in ammonium excretion was found in cyclic hypoxic-exposed animals (~66-75% in comparison to normoxic animals) during the 1st and 21st day of cyclic exposure. The reduced excretion rates in cyclic hypoxic-exposed shrimps after 21 days indicated that excretion was still suppressed (in contrast to food ingestion). From an ecological perspective, the substantial reduction in ammonium excretion could have important consequences on ecosystems populated by *P. varians* (i.e. salt marshes), in particular on nutrient cycling and energy fluxes. *P. varians* has been reported to play a key ecological role in macerating detritus and dead marsh plants

and in excreting large quantities of ammonia (Welsh 1975; Escaravage & Castel 1990; Aguzzi *et al.* 2005), thus making energy available at a variety of trophic levels and supporting significant growth of microflora (Welsh 1975). Therefore, a reduced excretion of ammonia due to cyclic hypoxia in the environment may alter nutrient turnover and, in turn, affect the microbial community of the salt marsh.

Reproduction is another energetically expensive process that is frequently affected when animals experience stressful conditions (Petes, Menge & Harris 2008; Sokolova *et al.* 2012). The down-regulation of a vitellogenin transcript was found in the transcriptome of cyclic hypoxic animals after 7 days of cyclic hypoxia (Table S3), and was reported for *P. pugio* following 5 and 10 days of cyclic hypoxia (Li & Brouwer 2009, 2013b). A significant reduction (~24%) in egg dry weight was found in females that reproduced in cyclic hypoxic conditions, suggesting a trade-off between fecundity and egg size, in accordance with Berrigan (1991). Egg dry weight is considered a proxy for the level of maternal resources invested into eggs, also known as per offspring investment (POI) (Oliphant & Thatje 2014). This trait is of fundamental importance within life history biology (J Marshall & Uller 2007), as higher POIs are generally associated with greater offspring fitness (Giménez & Torres 2004). A reduced POI would translate into a smaller energy reserve, potentially delaying larval development. In accordance with Truebano *et al.* (2018), our results suggest that shrimp sacrifice offspring quality in order to maintain offspring number. In contrast to our results, Brown-Peterson *et al.* (2008) and (2011) demonstrated in the lab and in the field that *P. pugio* exposed to cyclic hypoxia reduced its fecundity (from 30% to 60%, according to the cyclic hypoxic regime) without changing POI. It could be hypothesised that the observed differences between *P. varians* and *P. pugio* are the result of constraints dictated by the different larval development processes of the species. While the development of *P. varians* comprises only 5 larval stages (Oliphant 2013) the development of *P. pugio* encompasses 11 different larval stages (Broad 1957) hence, for *P. pugio,* lowering fecundity and retaining POI could be functional in cyclic hypoxia as it would not affect the long larval development process. Overall these different results underline a

species-specific variability in response to cyclic hypoxia and emphasize the importance of this study, given the paucity of studies that have hitherto assessed the impact of cyclic hypoxia on European crustacean species.

CONCLUSIONS:

Many important coastal ecosystems can be affected by cyclic hypoxia on a daily basis but rather little is known of the consequences on the physiology and/or ecology of species living in these habitats (especially in Europe). This study assessed changes to the physiology of the decapod shrimp *Palaemon varians* induced by a long-term daily cyclic hypoxic regime (water pO_2< 4.5 kPa for 7 hours day^{-1}), mimicking a cyclic hypoxic regime measured in *P. varians*' habitat (7.1 ±1.8 hours day^{-1} below 4.0 kPa).

In the short-term adult *P. varians* responded by reducing their feeding rate by ~50% and ammonium excretion by ~66% and, after 1 week, 399 differentially expressed genes were identified in its transcriptome. Long-term effects involved an acceleration of the moult cycle (~15% shorter in cyclic hypoxic animals) and a 13.6% increase in gill surface area after one moult cycle. Finally, a longer exposure to cyclic hypoxia reduced adult body size by ~4% and decreased the amount of yolk that female allocated in each egg by ~24%.

Overall, data emphasise how different physiological processes were affected by this cyclic hypoxic regime that mimicked environmental conditions within the Lymington salt marshes and influenced the physiology of a decapod crustacean species.

Conflict of interest:

Authors declare no conflict of interest.

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Data accessibility:

Raw RNA-sequencing reads have been deposited on the NCBI Sequence Read Archive (SRX2894799–SRX2894801). This Transcriptome Shotgun Assembly project has been deposited at DDBJ/EMBL/GenBank under the accession GFPG00000000. The version described in this study is the first version, GFPG01000000. This project is associated with the BioProject ID PRJNA389547. Remaining raw data was deposited in the Dryad Digital Repository: https://doi.org/10.5061/dryad.d12635v (Peruzza *et al.* 2018)

Author contributions:

LP, ST and CH conceived the work. LP performed the research. AO and DW performed the sequencing. LP, MG, AO and AP performed the bioinformatics. LP wrote the manuscript. All authors edited the manuscript.

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Supporting information

Additional supporting information may be found in the online version of this article.

Figures and tables:

Table 1: Primers used for quantitative PCR in *Palaemon varians*. Linear range is expressed as the number of cDNA dilutions (produced by 10-fold serial dilution and started from the undiluted concentration 10^0 of cDNA) for which each primer pair showed the reported Efficiency and R^2.

Figure 1: Oxygen and temperature measured within one channel from the Lymington salt marsh in three discrete weeks: 4th – 11th August 2016; 16th – 23rd February; 14th – 22nd May.

Figure 2: Normalized expression values of three cuticular contigs (left) 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 (right) of the same cuticular genes: gene expression (on a $\log_{10}$ scale) is normalized to multiple reference genes (*EF1-alpha* and *RPL8*), giving calibrated, normalized, relative quantities (CNRQ) ±Standard deviation. Significance values between treatments are indicated by "*".

CaAP: calcification associated peptide; *DD5*: peptide DD5; *PMP*: post-moult protein.

Figure 3: **A)** Frequency of moulted animals over time within the two treatments (cyclic hypoxia n=26; normoxia n=28). **B)** Scatter plot showing inter-moult duration (days) in the two treatments. Horizontal line represents the median for each treatment. Different letters indicate significance values between treatments.

Figure 4: Expression of markers at different time points from ecdysis (day 0) to 16 days after in different treatments (n=6 for each treatment for each time point). Expression (on a $\log_{10}$ scale), normalized to two reference genes (*EF1-alpha* and *RPL8*), giving calibrated, normalized, relative quantities (CNRQ) ±Standard deviation. For *DD5* and *PMP*, plotted lines represent GAM models fitted for each treatment (solid: cyclic 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 2: Descriptive statistics of GAM models used. Model 1: GAM model fitting all gene expression data, regardless of the treatments. Formula: Y~smooth(X); Model 2: GAM model fitting independently gene expression data ("X") for each treatment. Formula: Y~smooth(X,by=treatment). Akaike's Information Criterion – AIC – values between models were compared and the model with lower AIC was chosen. df: number of parameters in the model.

Figure 5: Morphological analysis on *P. varians* gills. For all graphs, mean±SD are plotted. Different letters indicate significance values between treatments.

Figure 6: Mean body weight ±SEM (n=90-95 animals/treatment/day). Lines indicate linear regressions fitted for each treatment (cyclic hypoxia: red line. Formula: Y=245.7-0.6X; normoxia: blue dotted line. Formula: Y=245.7-0.26X).

Figure 7: **A)** Feed ingestion rates and **B)** ammonium excretion after different days of exposure to cyclic hypoxia or normoxia in *P. varians* (mean±SD, n=10-13 animals/treatment/day). Different letters indicate significance values between treatments.

Figure 8: Impact of cyclic hypoxia on **A)** proportion of reproductive females; **B)** relative fecundity (mean±SD, n=4 hypoxia, n=8 normoxia); **C)** egg dry weight (means±SEM, n=36 hypoxia, n=88 normoxia). Different letters indicate significance values between treatments.

Target:	**Reference contigs:**	**F / R primer conc. (µM):**	**5'-3' FOR sequence:**	**5'-3' REV sequence:**	**Amplicon size (bases):**	**Efficiency:**	**R^2:**	**Linear range:**
PMP	contig_16319	0.3/0.3	AATTCAGCAGCCCAAAGTGG	CAGGCAGACATGAACTCAGC	113	1.86	0.99	4
DD5	contig_23547	0.3/0.3	ACACTATGCATTCGTGGCTG	CAGGAACTGGAGGTCCAACA	116	1.91	0.98	4
CaAP	contig_10413	0.3/0.3	ATCGTGGACTTCGAGTTGGA	AATACTCGTTGCCGTCAGGT	106	1.85	1	4
EF1-alpha	contig_41	0.3/0.3	ACAGCACTGAGCCCAAGTAT	GAAATGGGAAGGATTGGCACA	115	1.88	0.99	5
RPL8	contig_226	0.9/0.9	TCCCGGTCGTGGTGCACCTATT	GACGGCCTCGGTCACCAGTCTTT	179	1.84	0.95	5

	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