Diel oxygen fluctuation drives the thermal response and metabolic performance of coastal marine ectotherms

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Abstract

Coastal marine systems are characterised by high levels of primary production that result in diel oxygen fluctuations from undersaturation to supersaturation. Constant normoxia, or 100% oxygen saturation, is therefore rare. Since the thermal sensitivity of invertebrates is directly linked to oxygen availability, we hypothesised that (a) the metabolic response of coastal marine invertebrates would be more sensitive to thermal stress when exposed to oxygen supersaturation rather than 100% oxygen saturation and b) natural diel fluctuation in oxygen availability rather than constant 100% oxygen saturation is a main driver of the thermal response. We tested the effects of oxygen regime on the metabolic rate, and haemocyanin and lactate levels, of velvet crabs (*Necora puber*) and blue mussels (*Mytilus edulis*), under rising temperatures (up to 24°C) in the laboratory. Oxygen supersaturation and photosynthetically induced diel oxygen fluctuation amplified animal metabolic thermal response significantly, demonstrating that the natural variability of oxygen in coastal environments can provide considerable physiological benefits under ocean warming. Our study highlights the significance of integrating ecologically relevant oxygen variability into experimental assessments of animal physiology and thermal response, and predictions of metabolic performance under climate warming. Given the escalating intensity and frequency of climate anomalies, oxygen variation caused by coastal vegetation will likely become increasingly important in mitigating the effects of higher temperatures on coastal fauna.

Keywords: Oxygen supersaturation, Marine invertebrates, Macroalgae, Thermal response, Ocean warming refugia, Climate change

Introduction

Climate warming is a critical driver of worldwide ocean deoxygenation, having resulted in an overall loss of 1–2% of dissolved oxygen since the mid-20th century (1,2). Global temperature rise not only reduces oxygen solubility in water, but also increases the oxygen requirements of ectotherms (3). Although coastal waters conform to this trend, they often exhibit particularly high levels of primary production and can act as refugia from a combination of oxygen depletion and high temperatures and also from long-term oxygen depletion (4). This effect occurs over large spatial scales because, typically, productive coastal systems are interconnected, allowing them to form a mosaic of favourable habitats. The activity of primary producers can cause dynamic and dramatic shifts in the environmental conditions of coastal waters, namely pH (5), CO_2 (6) and, more recently, their role in generating significant diurnal and seasonal fluctuations in oxygen has been revealed (7). As a result, productive coastal waters exhibit oxygen supersaturation on a daily basis (8), while at night the cessation of photosynthesis and plant respiration cause concentrations to plummet as low as 10% (7).

A large number of ecologically and economically important marine species, from micro- to mega-fauna, have evolved in, or in close association with, coastal environments that are characterised by diel oxygen fluctuations, spending at least part of their life cycle in shallow waters (9–11). It is well known that productive marine coastal habitats can provide shelter from predation and are frequently used as nursery habitats as well as feeding grounds for adults (12,13). But in addition, the generation of hyperoxic conditions in such habitats has other advantages. Oxygen supersaturation can reduce exposure to pathogens (14) and enhance resistance to marine pollutants (15,16), and is also associated with increased clutch size in marine invertebrates (17). The mechanistic link between oxygen availability and thermal tolerance has been described by Pörtner (18). More recently, the association between oxygen supersaturation and thermal sensitivity has been explored, with evidence that exposure to oxygen supersaturation can improve the ability of marine animals to extract oxygen from water (7) and improve aerobic capacity during warming (19).

When driven by primary production, oxygen supersaturation is generally coupled on a diel basis with undersaturation, or hypoxia. This cyclical switch from undersaturation (during the night) to oxygen supersaturation (during the day) reflects the actual conditions experienced by the majority of aquatic animals living in productive marine systems (20). While effects of oxygen supersaturation and, to a greater extent, undersaturation (e.g. impacts on the early development, behaviour, growth and survival of marine animals (21–23)) are well known, to

our knowledge no studies have addressed the effects of the diel fluctuations of oxygen saturation. Rather, typically experimental designs consider oxygen levels to be stable at whichever of these extremes was studied.

To gain a more realistic understanding of the potential consequences of ocean warming and heatwaves for species' survival, it is necessary to address the question of how naturally occurring diel fluctuation of dissolved oxygen affects the responses of coastal animals to warming. Metabolic rates are widely used as a proxy for this, since animals tend to avoid reduced fitness or even mortality by maintaining aerobic performance and fulfilling the oxygen demand induced by increased metabolism (24,25). We addressed this question by testing two hypotheses: (a) that thermal response of metabolism is altered when animals are exposed to oxygen supersaturation rather than 100% oxygen saturation (experiment 1) and (b) that natural diel fluctuation in oxygen availability induced by primary producers rather than constant 100% oxygen saturation is a main driver of the thermal response of coastal invertebrates (experiment 2).

Methods

The study was carried out during November–December 2019 at the St Abbs Marine Station research facility on the east coast of Scotland (55° 53' 52''N, 2° 7'44"E). The oxygen and temperature conditions in the algae beds at the study site (from the period October–December 2019) were recorded with a PME miniDOT dissolved oxygen and temperature logger and light sensor (ONSET HOBO - UA-002-08) (Supp. Fig. 1).

Animal collection and experimental set-up

Two model coastal invertebrates were selected as study species: *Necora puber* (Linnaeus, 1767) (velvet crab) and *Mytilus edulis* (Linnaeus, 1758) (blue mussel) (Fig. 1 A,B). Similar-sized animals were collected: crabs at St Abbs (carapace width 80–120 mm) and mussels at Musselburgh, 40 miles farther north (shell length 50–60 mm). All specimens used in experiments appeared healthy, with no signs of shell or carapace damage. Mussels were hand-collected and crabs (only male specimens to avoid possible confounding effects of sex) were provided by local fishermen. Fouling was removed from mussel shells to avoid any interference with respiration measurements. Animals were acclimated in the laboratory under natural light/dark conditions (due to a Perspex roof) and ambient temperature seawater for 14 days prior to the experiments(approx. 10 h light: 14 h dark, 9–11°C, 100–104% oxygen saturation, salinity: 34.23–34.38 ppt). The two species were kept in separate flow-through

fiberglass tanks. Crabs were kept in a 973 L tank and provided with shelter to reduce stress. Mussels were held fully submerged in a 550 L tank. Crabs were provided a diet of either cooked mussels, or fish, or squid every 2–4 days. Mussels were fed algae (*Nannochloropsis* sp.) *ad libitum* at the start of the acclimation period and were subsequently allowed to filter natural sea water.

Two experiments (oxygen supersaturation and diel oxygen fluctuation - details below) were performed in a photoperiod- and temperature-controlled room, using separate treatment aquaria (110 L) for each experiment filled with ambient UV-treated seawater (salinity: 34.23–34.38 ppt). All aquaria were equipped with a PME miniDOT dissolved oxygen and temperature logger and light sensor (ONSET HOBO - UA-002-08). Water temperature in each aquarium was controlled with two thermometers (Aquastar, Germany) and two heating rods (Aquastar, Germany) connected to a control system (IKS Aquastar, Germany).

Oxygen was supplied to tanks either in the form of an air bubbler (100% oxygen saturation) or via photosynthetic activity of macroalgae (oxygen supersaturation treatment and diel oxygen fluctuation treatment). Fresh, fertile macroalgae (Fig. 1C,D), *Fucus vesiculosis* (Linnaeus, 1753) (Bladderwrack), was collected on the first day of the experiment in Coldingham Bay (0.5 km south of St Abbs harbour), rinsed in UV-treated seawater to remove epibionts, and the same wet-weight of algae (2 kg, measured on a balance) was added to each aquarium allocated to the oxygen supersaturation treatment (Experiment 1) and the diel oxygen fluctuation treatment (Experiment 2). Two water pumps (Q116 aquarium pump, 320 Lh^{-1}) ensured circulation of oxygen within each aquarium for all treatments. LED growth lamps (KINGBO, 400-760 nm full spectrum) were set-up over each aquarium, including the 100% oxygen saturation treatments, to promote algal photosynthesis (light intensity was recorded as 5000 lux +/- 500 in both experiments). Lastly, mussels (in a protective cage, to avoid disturbance from crabs) and crabs (free-swimming) were added to each treatment aquarium. Algae and aquarium brushes provided shelter for individual crabs to prevent aggressive intraspecific behaviour.

Experiment 1 - Thermal response under constant oxygen supersaturation

This experiment was performed to test the effect of naturally occurring oxygen supersaturation on the respiration and thermal response of metabolism of the two coastal invertebrates, using primary producers as the source of oxygen supersaturation. Animals were acclimated in treatment aquaria (see above) under 100% oxygen saturation (air bubbler) or oxygen supersaturation (algae), and both treatments were under permanent light conditions at ambient

temperature (9°C) for 24 h, before animals were transferred to Perspex custom-made respirometry chambers equipped with an oxygen sensor spot glued to the inner wall. Prior to being transferred to the chambers, animals were cleaned with sterile seawater to help remove epibionts and microorganisms. Animals were acclimated for 12 h in the respirometry chambers, under light conditions, to recover from handling stress, at treatment oxygen levels (i.e. oxygen supersaturation (160% saturation) or 100% saturation), supplied by flow-through water from their respective treatment aquaria using pumps (Eheim, Germany), before starting a temperature ramp of 1ºC per hour (following 7,24) from 9–24ºC. Oxygen consumption (hereafter referred to as MO_2 in the Methods and Results) was recorded using intermittent respirometry at 9ºC, 14ºC, 19ºC and 24ºC for each animal in each treatment (n = 8, i.e. eight respiration chambers with one mussel each and eight chambers with one crab each for 100% oxygen saturation and oxygen supersaturation). MO_2 was measured by connecting each closed chamber, via an optical fibre, to a single channel oxygen transmitter Fibox (Microx) 4 (PreSens, Regensburg, Germany). To avoid disturbance to animals, chambers were covered in dark foil and positioned so that the optical fibre could be connected without subjecting animals to visual stimuli or movement. MO_2 was calculated as the linear decline in oxygen saturation in chambers. During measurements, while chambers were closed, oxygen saturation was not allowed to drop below 10% of the saturation level of the corresponding treatment the duration of measurement was approximately 30 s. All tubes and connectors were rinsed with bleach (10%) and UV-treated filtered seawater between each experimental run to remove any microorganisms that could affect oxygen consumption measurements. An empty chamber provided a control for microbial respiration at each temperature (following Fusi et al. (26)), which was found to account for less than 0.4% of animal oxygen consumption.

After measuring MO_2 at 24ºC, animals were carefully removed from their chambers. Haemolymph, approximately 200 µl, was extracted from crabs from the arthrodial membrane at the base of a fifth walking leg with a 25-gauge needle for measurement of haemocyanin concentration (27). Haemocyanin concentrations were calculated by diluting 10 µl haemolymph with 990 µl chilled oxygenated buffer (Tris-Hcl Buffer pH 7.4 50mM), before reading absorbance spectrophotometrically (Helios Epsilon, Thermo Fisher Scientific; 335 nm wavelength; (28)), three times for each sample. Haemocyanin concentration was calculated following Harris and Andrews (29), using the molar extinction coefficient $E1_{cm}$ mM = 17.26. Handling time from removing the animal from the chamber to completing haemolymph extraction was kept to 60 s. In order to obtain the internal volume of each respiration chamber, animals were weighed, and their volume calculated by means of water displacement in a

175 graduated cylinder before release. No deaths were recorded during acclimation, experiments
176 or handling.
177
178 *Experiment 2 - Thermal response under diel oxygen fluctuation*
179 This experiment was performed to test the effect of diel oxygen fluctuation on the respiration
180 and thermal response of metabolism of the two coastal marine invertebrates. For this second
181 experiment, we exposed one set of animals (n = 16 mussels and n = 16 crabs) to diel oxygen
182 fluctuation (achieved with the use of a growth lamp set to a 12 h:12 h light:dark cycle, and
183 setting the room lighting to the same cycle) in aquaria with macroalgae, and another set of
184 animals (n = 16 mussels and n = 16 crabs) exposed to constant 100% oxygen saturation
185 provided only with an air bubbler but no algae (Supp. Fig. 2). Animals were exposed to their
186 respective treatments for six days prior to measurements and the experiment was replicated at
187 9ºC, 16ºC and 21ºC using new animals for each temperature (i.e. a total of 48 of each crabs and
188 mussels). For the treatment tanks at 16ºC and 21ºC, animals were gradually acclimated to the
189 higher temperatures at a warming rate of 0.5ºC per hour. The room temperature was initially
190 maintained at 9ºC and subsequently increased to mirror that of the experimental temperature.
191 The oxygen levels in the diel oxygen fluctuation tank varied between 30% and 180% during
192 the experiment, and in the 100% saturation tanks, oxygen levels were 100% (+/- 4%; Supp.
193 Fig. 3). Every two days, half of the aquarium water was replaced with fresh UV-treated
194 seawater, first heated to the correct temperature, to ensure animals and oxygen levels were not
195 detrimentally affected by a build-up of urea (pH was monitored daily) or a loss of nutrients
196 hindering photosynthesis.
197 As for Experiment 1, animals were transferred to respirometry chambers and allowed
198 to recover from disturbance for 12 hours prior to measurements, and conditions in the chambers
199 were identical to those in the respective treatment tanks, with a water pump continuously
200 circulating water between the tanks and chambers. MO_2 was measured, as described above, at
201 the beginning of the light phase of the diel cycle after overnight (12 h) acclimation in individual
202 respiration chambers, at the point where oxygen saturation levels were identical between the
203 two treatments, i.e., 100% oxygen saturation. After measuring MO_2 for each individual animal,
204 chambers were kept closed and MO_2 was recorded every 2 min as oxygen decreased in each
205 chamber to 5-10% saturation in order to calculate the critical oxygen pressure, the partial
206 oxygen pressure below which oxygen consumption significantly declines, hereafter referred to
207 as PO_{2crit}.

Haemocyanin and lactate were measured from haemolymph of the eight crabs which were not placed in the respirometry chambers from each treatment and temperature after the six-day acclimation to diel oxygen fluctuation. Haemolymph could not be taken from the same animals used for MO_2 measurements because these individuals were used for measurement of $PO2_{crit}$. Haemocyanin was measured following the protocol described above. Lactate concentration was measured using a drop of venous haemolymph (extracted as described above), following Giomi et al. (7) with a Lactate Pro 2 Analyser (www.lactatepro.com.au).

Statistical analysis

PO_{2crit} was calculated by plotting MO_2 against PO_2, using a piecewise linear regression function in SigmaPlot v.11, then calculating the breakpoint (following Giomi et al. (7)).

To test MO_2 under oxygen supersaturation, an analysis of covariance was performed using a linear mixed model (lme4) to test the effects of the factors 'Temperature' (as our continuous explanatory variable) and 'Treatment' (fixed, 2 levels: 100% oxygen saturation, oxygen supersaturation) with MO_2 as our response variable. Individual ID was included as a random factor in the mixed model to account for the non-independence of repeated measures across the temperature ramp.

A 1-way ANOVA was used to test the effect of 'Treatment' (fixed, 2 levels) on the dependent variable haemocyanin at 24ºC (crabs only). To explore thermal response under oxygen diel fluctuation, an analysis of covariance was used to test the effects of temperature as our continuous explanatory variable and the factor 'Treatment' (fixed, 2 levels: constant 100% oxygen saturation, diel oxygen fluctuation) on the response variables MO_2, PO_{2crit}, haemocyanin (crabs only) and lactate (crabs only). Prior to statistical testing, normality and homogeneity of variances of the data were confirmed using the Shapiro-Wilkes and Levene's tests, respectively. All statistical tests were performed in R (R Studio Version 1.1.463).

Results

In both experiments, a significant interaction between temperature and treatment was observed in the majority of cases. In general, oxygen treatment significantly affected the variables tested at intermediate to high temperatures, but not at low temperatures.

Experiment 1 - Thermal response of metabolism under constant oxygen supersaturation

Crab oxygen consumption (MO_2) was affected by a significant temperature × oxygen treatment interaction (second polynomial regression analysis, $F_{2,58} = 4.11$, $P < 0.05$; Supplementary Table 1). Overall crab MO_2 was highest at 14ºC and 19ºC, and at these two intermediate temperatures individual crabs exposed to oxygen supersaturation had a significantly higher MO_2 than those exposed to 100% oxygen saturation (Fig. 2A). Mussel MO_2 was found to be affected by a significant temperature × oxygen treatment interaction (second polynomial regression analysis, $F_{2,58} = 19.94$, $P < 0.0001$; Supplementary Table 1). The same pattern was observed as for crabs, with overall MO_2 highest at 14ºC and 19ºC and individuals exposed to oxygen supersaturation had a significantly higher MO_2 than those exposed to 100% oxygen saturation at these temperatures (Fig. 2B).

Crabs produced significantly higher levels of haemocyanin at 24ºC when exposed to oxygen supersaturation than 100% oxygen saturation (Fig. 3; 1-way ANOVA, $F_{1,14} = 31.53$, $P < 0.0001$; Supplementary Table 1).

Experiment 2: Thermal response of metabolism under diel oxygen fluctuation

Crab and mussel oxygen consumption (MO_2) were both significantly affected by a temperature × oxygen treatment interaction (second polynomial regression analysis, $F_{2,41} = 6.41$, $P < 0.01$ and $F_{2,42} = 6.82$, $P < 0.01$, respectively; Supplementary Table 2). When exposed to diel fluctuation in oxygen, both crabs and mussels had significantly higher MO_2 at 16ºC and 21ºC, compared to animals exposed to constant 100% oxygen saturation (Fig. 4A and B).

Due to the switch to anaerobic metabolism at 21ºC, PO_{2crit} was analysed for the temperatures below this, i.e., 9ºC and 16ºC. Crab PO_{2crit} was significantly affected by a temperature × oxygen treatment interaction (linear model, $F_{1,28} = 53.29$, $P < 0.0001$; Supplementary Table 2). At 16ºC crabs exposed to diel oxygen fluctuation had a significantly lower PO_{2crit} than those exposed to constant 100% oxygen saturation (Fig. 4C). Mussel PO_{2crit} was unaffected by either temperature or oxygen fluctuation or their interaction (Fig. 4D).

Crab lactate was significantly affected by a temperature × oxygen treatment interaction (linear model, $F_{1,44} = 127.49$, $P < 0.001$; Supplementary Table 2); those exposed to diel fluctuation in oxygen had significantly lower lactate concentrations at 16ºC and 21ºC than crabs exposed to constant 100% oxygen saturation (Fig. 5A). Lactate could not be detected in mussel haemolymph.

Crab haemocyanin was also significantly affected by a temperature × oxygen treatment interaction (linear model, $F_{1,44} = 6.33$, $P < 0.05$; Supplementary Table 2). Crabs exposed to diel

fluctuation in oxygen had significantly higher haemocyanin levels at 21ºC than crabs exposed to constant 100% oxygen saturation (Fig. 5B; Tukey p-pht, $P < 0.001$).

Discussion

Primary producer communities in shallow coastal waters offer several crucial ecosystem services to their associated animal communities. They provide a pH-controlled environment that can mitigate the effects of ocean acidification (30), and reduce pathogen activity through the production of biocides (14). Here we show that natural diel oxygen fluctuation and oxygen supersaturation due to photosynthesis drive changes in the sensitivity of ectotherm metabolic rate to temperature. Our findings highlight that the current experimental standards may be biased by the fact that animals are typically tested under ecologically unrealistic constant oxygen levels, while oxygen availability is actually highly variable, especially in productive coastal areas.

Ectotherms commonly sustain aerobic metabolism when faced with thermal stress, thus meeting the increased oxygen demand induced by heat (24,25,31). Natural levels of oxygen at the Scottish study location can reach around 160% saturation due to photosynthesis (Supp. Fig. 1). In our experiments, blue mussels and velvet crabs experiencing such levels of oxygen supersaturation showed increased metabolic rates under warming compared to animals in the absence of algae and experiencing constant oxygen levels at around 100% saturation (Experiment 1). Importantly, significant differences between oxygen treatments appeared as temperatures rose above 9°C, with increased metabolic rate in the study species recorded only under relatively high temperatures (beyond 14°C). Both mussels and crabs were able to maximise their metabolic activity under oxygen supersaturation at these temperatures, while no effect on animal metabolic rate was seen at low temperatures (9°C). Tropical animals kept under constant oxygen supersaturation showed a similar response when exposed to higher than ambient temperatures (7).

We found that exposure to diel fluctuation in the oxygen environment, including extremes of oxygen supersaturation and undersaturation, modified the metabolic performance of crabs at elevated temperatures by increasing rates of oxygen uptake (MO_2, Experiment 2). Crabs exposed to diel oxygen fluctuation had a lower PO_{2crit} than those exposed to 100% oxygen saturation at 16°C, indicating a more efficient ability to extract dissolved oxygen at a lower environmental oxygen tension. While at 9°C animals could largely sustain oxygen metabolic demands, 16°C represented a rarely encountered condition for these temperate crabs.

Water in this region only reaches this temperature on a few hot summer days (Marine Scotland, https://data.marine.gov.scot/dataset/scottish-coastal-observatory-st-abbs-site). Although our study was performed on animals acclimated to colder winter temperatures, and experimental timing may have underestimated the thermal sensitivity of these species, qualitative responses to temperature would likely be comparable in the warmer summer months as has been demonstrated for tropical species inhabiting warm environments (7).

Our measurements of haemocyanin and lactate levels likewise indicate an increased metabolic response to temperature under conditions of oxygen supersaturation (Experiment 1) and diel oxygen fluctuation (Experiment 2). Haemocyanin is the oxygen-carrying protein in a number of crustaceans and molluscs and critical for normal physiological function (32). Environmental factors affect haemocyanin concentrations in blue crabs (*Callinectus sapidus*), with low salinity and oxygen undersaturation causing a decrease in haemocyanin (32). In another crustacean, the Norway lobster (*Nephrops norvegicus*), the extent to which oxygen undersaturation resulted in an increase in haemocyanin was crucially dependent on both initial haemocyanin levels, so that individuals which had an initially high haemocyanin level showed no change, or even a decrease in haemocyanin (33). This decrease was linked to energy allocation, i.e., haemocyanin is regulated to the minimum level required for successful respiratory gas transport so that the energy required for protein synthesis may be allocated to more essential processes than the production of an excess of haemocyanin (34). A relationship between thermal tolerance and haemolymph oxygen transport was also found in the eurythermal crab *Carcinus maenas* (27), providing evidence that oxygen storage by haemocyanin has an increasingly important role in sustaining cardiac performance under warming by enhancing aerobic metabolism and increasing thermal tolerance. In our study, velvet crab haemocyanin was unaffected at temperatures that these animals naturally experience in the field. At average temperatures, and in fully oxygenated seawater, portunid crabs rely on the oxygen dissolved in haemolymph alone (27). In cases where we observed an increase in haemocyanin concentration during oxygen supersaturation, or diel oxygen fluctuation, we interpret this as a molecular mechanism to increase the oxygen scavenging efficiency of the whole system.

Lactate is a stress marker in many invertebrates and is commonly used as an indicator of anaerobic respiration resulting from hypoxic conditions (35), thermal stress (7,36) or the combination of the two (37). High levels of lactate indicate that an animal is relying increasingly on anaerobic metabolism, likely associated with more stressful conditions (38). We found that velvet crabs exposed to constant 100% oxygen saturation at temperatures

342 elevated above ambient, specifically 16ºC and 21ºC, displayed significantly higher lactate
343 levels than those exposed to diel oxygen fluctuation. As with the results for metabolic rate, this
344 clearly indicates that these crabs experience less physiological stress at higher temperatures
345 under conditions of cyclical diel oxygen fluctuation in water from under- to over-saturation.
346 Mussel metabolic rates were affected by temperature and/or oxygen level in a similar
347 manner to those of crabs. However, in contrast to the crabs, mussel PO_{2crit} was unaffected by
348 either temperature or oxygen treatment. The ability of these sedentary animals to close their
349 shells for extended periods, depressing their metabolism or relying on anaerobic pathways,
350 provides an alternative strategy for coping with low oxygen availability, for example during
351 low tide, from that of highly active species, such as crabs, which strive to maintain their
352 metabolic rates (41). Oxygen saturation levels of 20% have been previously found to have no
353 effect on resting metabolic rate of blue mussels from the southern Baltic Sea, but did affect
354 gaping activity (40). Mussels have evolved under highly variable oxygen conditions in which
355 regular submersion and emersion are superimposed on diel fluctuations in oxygen tension, and
356 a constant oxygen environment is not a natural setting for them. Although constant 100%
357 oxygen saturation is not detrimental to mussels, as demonstrated in the vast majority of studies
358 adopting steady rearing conditions, diel oxygen fluctuation led to a larger scope of metabolic
359 rate, which in turn determines a more efficient capacity for thermal response (18). To our
360 knowledge, this important aspect is not incorporated into published studies based on conditions
361 of 100% oxygen saturation.
362 Animals living in marine habitats exhibiting high primary productivity have evolved in
363 an inherently dynamic environment, particularly in terms of oxygen availability, and many
364 species, including those in our study, have evolved in close association with primary producers
365 under these fluctuating environmental conditions. These animals are physiologically adapted
366 to cope with the predictable variability in their environment (10,20,41); critically, their
367 metabolic performance is tailored to oxygen variation, resulting from changing temperature
368 $and levels of photosynthesis. We demonstrate that assessments of animal physiology and
369 thermal response under warming should account for ecologically relevant oxygen variability
370 rather than employing a stable experimental oxygen level, but studies so far have not done so
371 (20).
372 Coastal animals are adapted to persist in a naturally variable environment, having
373 evolved strategies to exploit predictable environmental change and mechanisms to anticipate
374 future expected changes (42). We suggest that the species investigated in our study, and likely
375 the majority of coastal ectotherms, normally exploit predictable daily oxygen supersaturation

to cope with nightly oxygen undersaturation. However, the environment is being altered at an unusual rate due to anthropogenic activity, compromising feedback mechanisms, and the ability of communities to adapt to different fluctuation regimes remains overlooked (42). Interestingly, the importance of behavioural and evolutionary mechanisms involved in the plasticity of species' thermal tolerance is unrelated to latitude or thermal seasonality, and it has been proposed that in cases where species are limited in their behavioural thermoregulation, greater plasticity in physiological traits will be favoured (43), it is possible that exposure to environmental fluctuations contributes to this.

The intensified response of coastal fauna to higher temperatures driven by oxygen supersaturation mediated by primary producers for parts of the day shown here is likely to become increasingly important under future climate change. While primary producers will have their own specific responses to ocean warming and acidification, it is likely that coastal habitats such as seagrasses, kelp forests, mangroves and coral reefs will be important as refugia in which oxygen variation can drive the metabolic performance of animals in a changing world. Upwelling areas have already been identified as climate refugia for marine macroalgae (44), as these areas show comparatively lower trends of warming. The thermal response of plants and animals results from the complex interaction of several factors, beyond temperature alone. Here we demonstrate that the provision, and resultant variability, of oxygen by primary production are important drivers of the thermal responses of coastal animals. By producing periodic oxygen supersaturation, coastal primary producers sustain the increasing metabolic demand of animals under warming, and are therefore likely to have an increasingly important effect on both permanent residents and transient animals that use these habitats as nursery sites under climate warming (e.g. 45). This makes the loss of macrophytes through ocean heatwaves and long-term warming (46), which will increase with further ocean warming, detrimental to the associated biota (47). The disappearance of diverse communities of macroalgae in coastal waters is a threat to biodiversity not only through habitat loss (48,49), but also through reduced oxygen variability and the effects of this on animal thermal responses.

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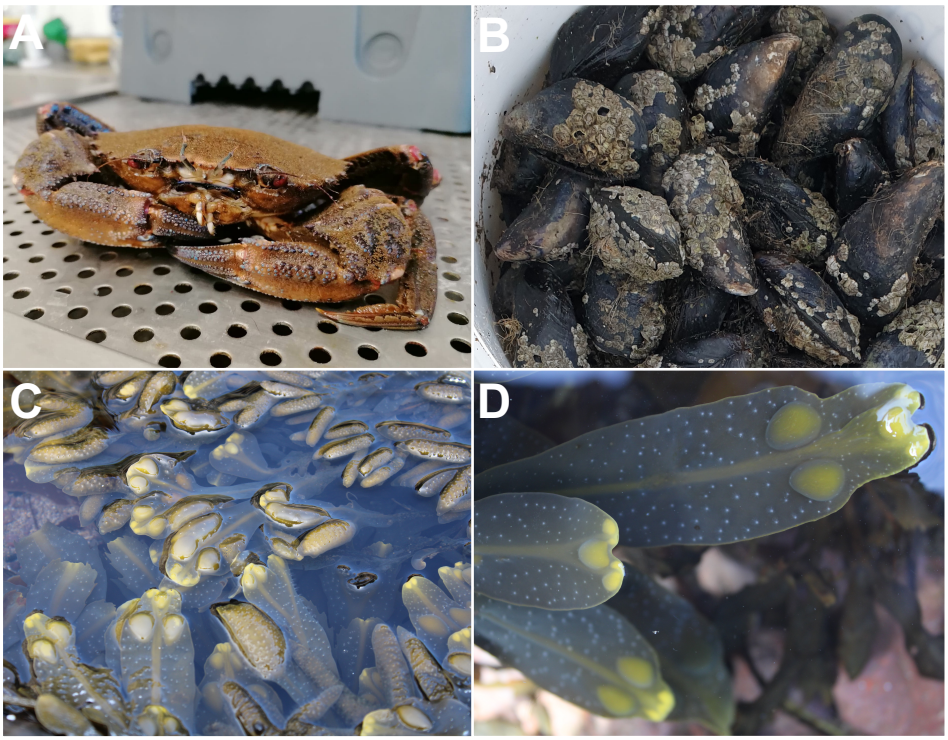

552

553 **Figure 1 –** (A) velvet crab (*Necora puber*), (B) blue mussels (*Mytilus edulis*) and (C,D)
554 bladderwrack (*Fucus vesiculosis*). Photos: (A) J.M. Booth, (B) E. Chapman, (C,D) C. Rochas.

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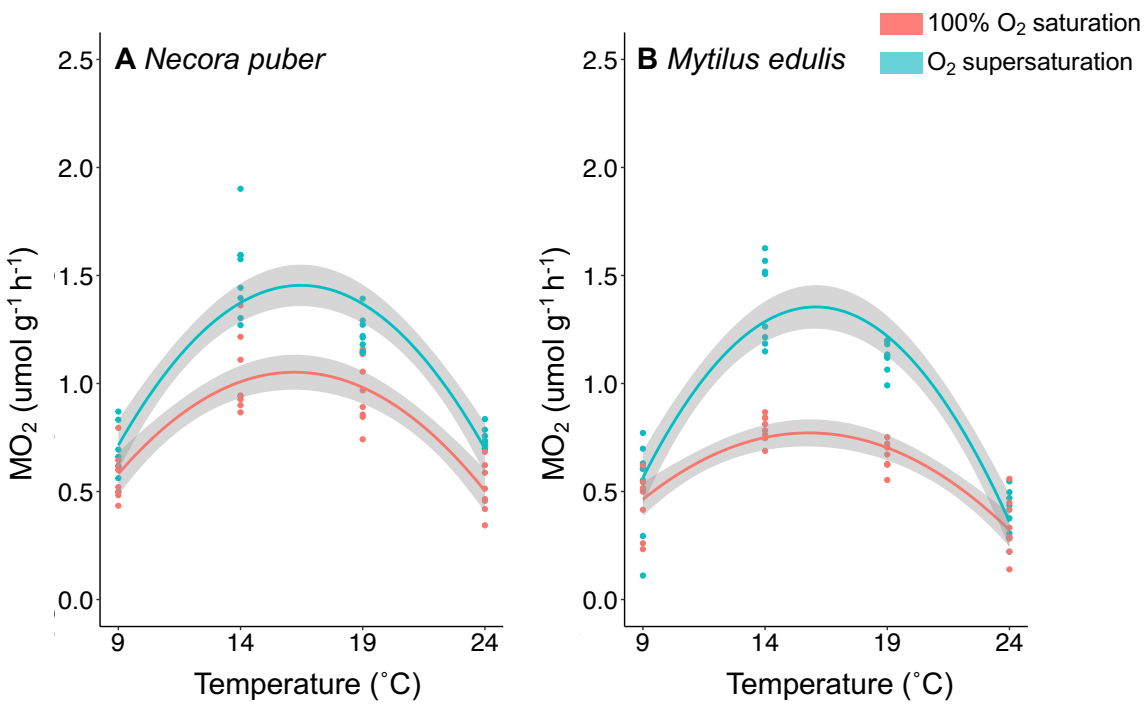
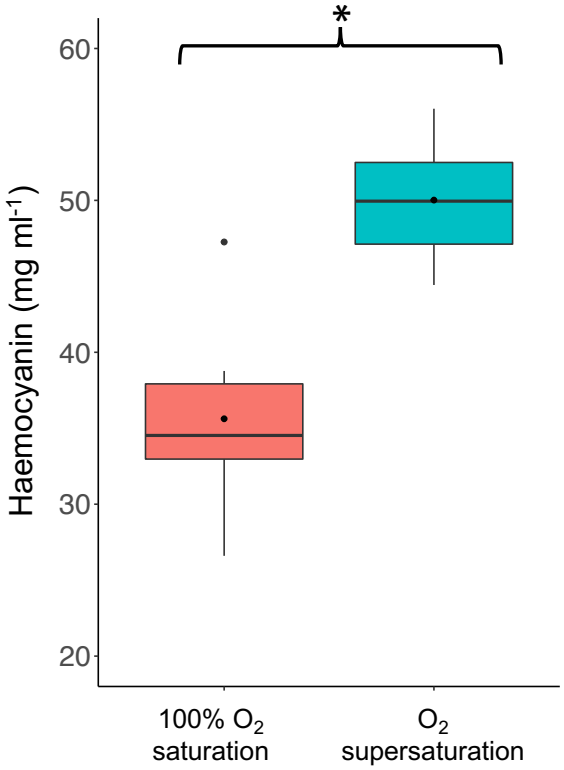


Figure 2 – Oxygen consumption (MO_2) by (A) *Necora puber* and (B) *Mytilus edulis* over a temperature ramp from 9ºC to 24ºC under 100% oxygen saturation (red) and oxygen supersaturation (blue). Data represent MO2 in umol per gram wet weight per hour.



563

564 **Figure 3 –** Haemocyanin concentrations in *Necora puber* haemolymph after a temperature
565 ramp from 9ºC to 24ºC under 100% oxygen saturation (red) and oxygen supersaturation (blue).
566 Asterisks represent significant differences between the treatments ($P < 0.05$).

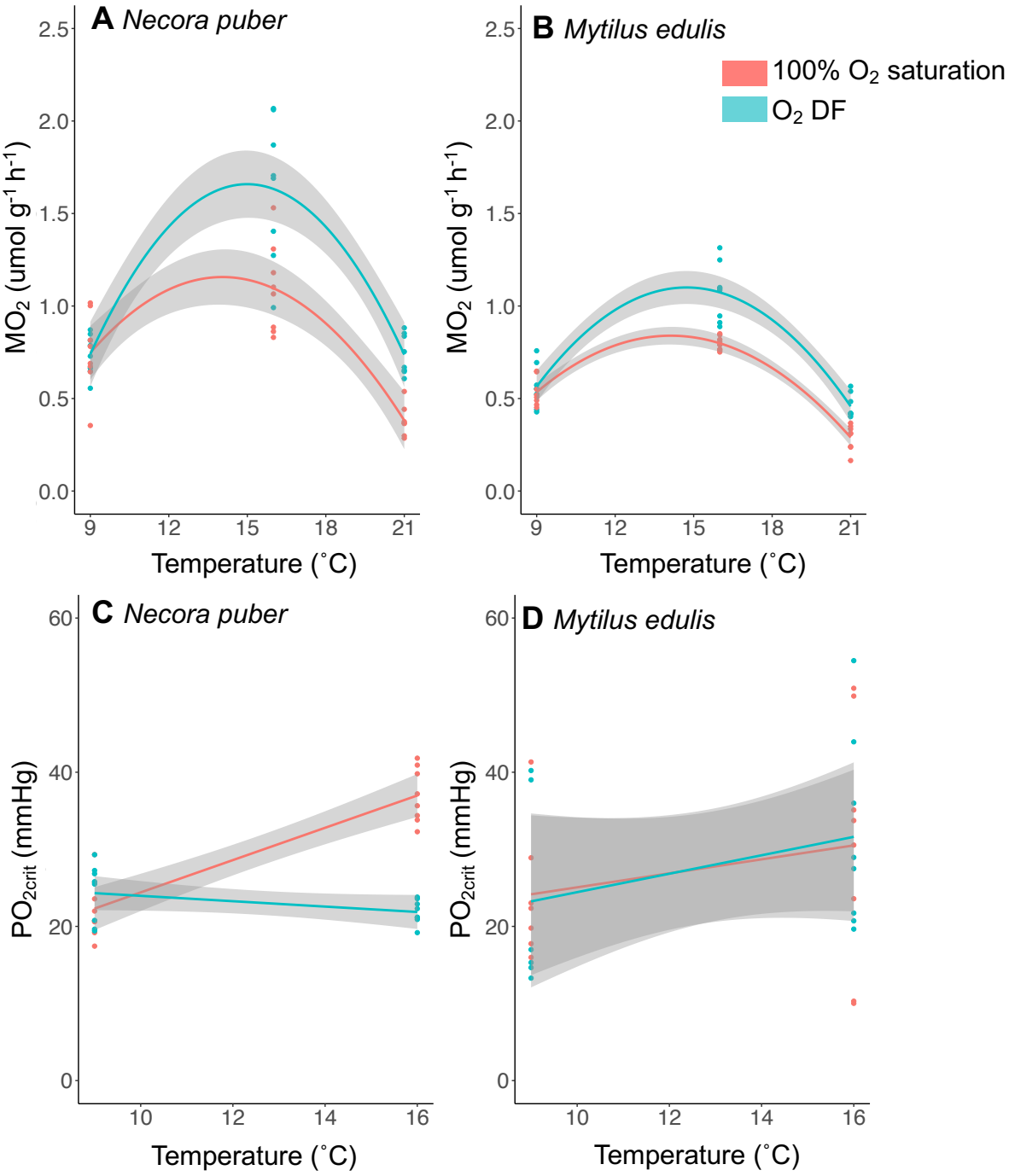
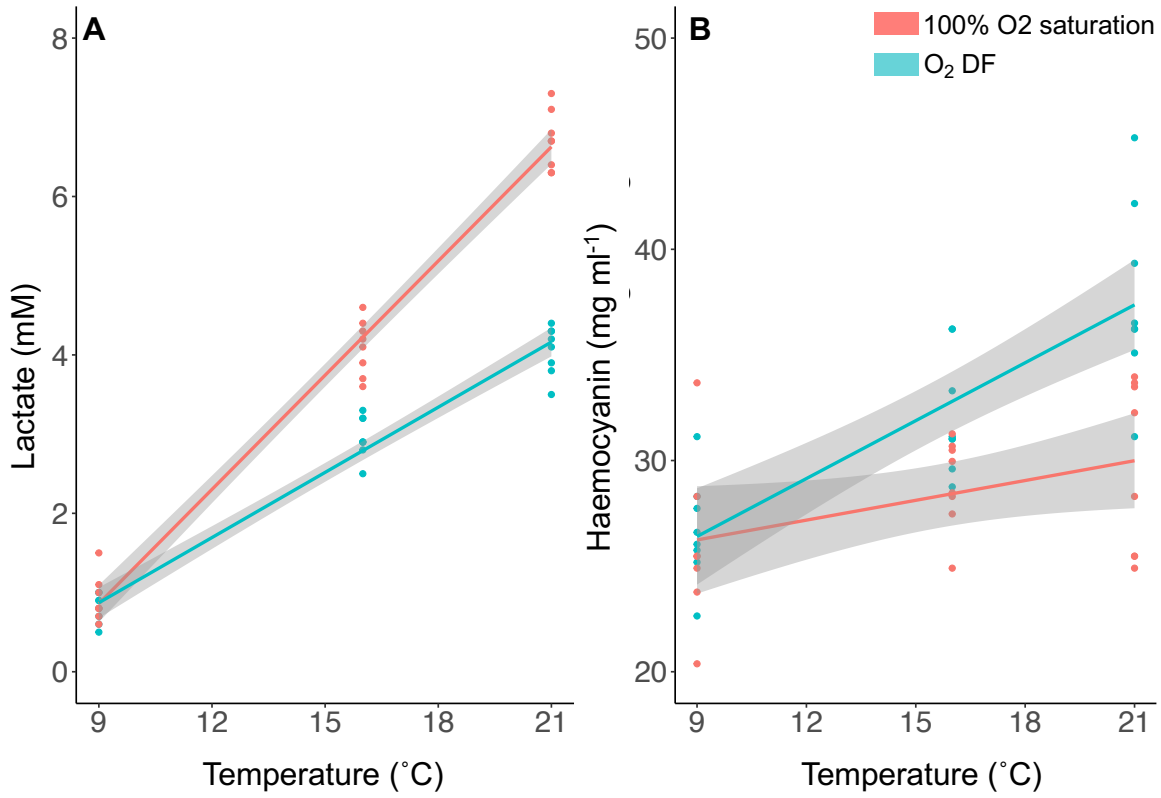


Figure 4 – Oxygen consumption (MO_2) by (A) *Necora puber* and (B) *Mytilus edulis* and PO_{2crit} in (C) *Necora puber* and (D) *Mytilus edulis* at 9ºC, 16ºC and 24ºC under 100% oxygen saturation (red) diel oxygen fluctuation (O_2 DF, blue) after 6 days exposure to treatment. Data represent MO_2 in umol per gram wet weight per hour.



573

574 **Figure 5 –** (A) Lactate and (B) haemocyanin concentration in *Necora puber* haemolymph at
575 9ºC, 16ºC and 24ºC under 100% oxygen saturation (red) and diel oxygen fluctuation (O_2 DF,
576 blue) after 6 days exposure to treatment.

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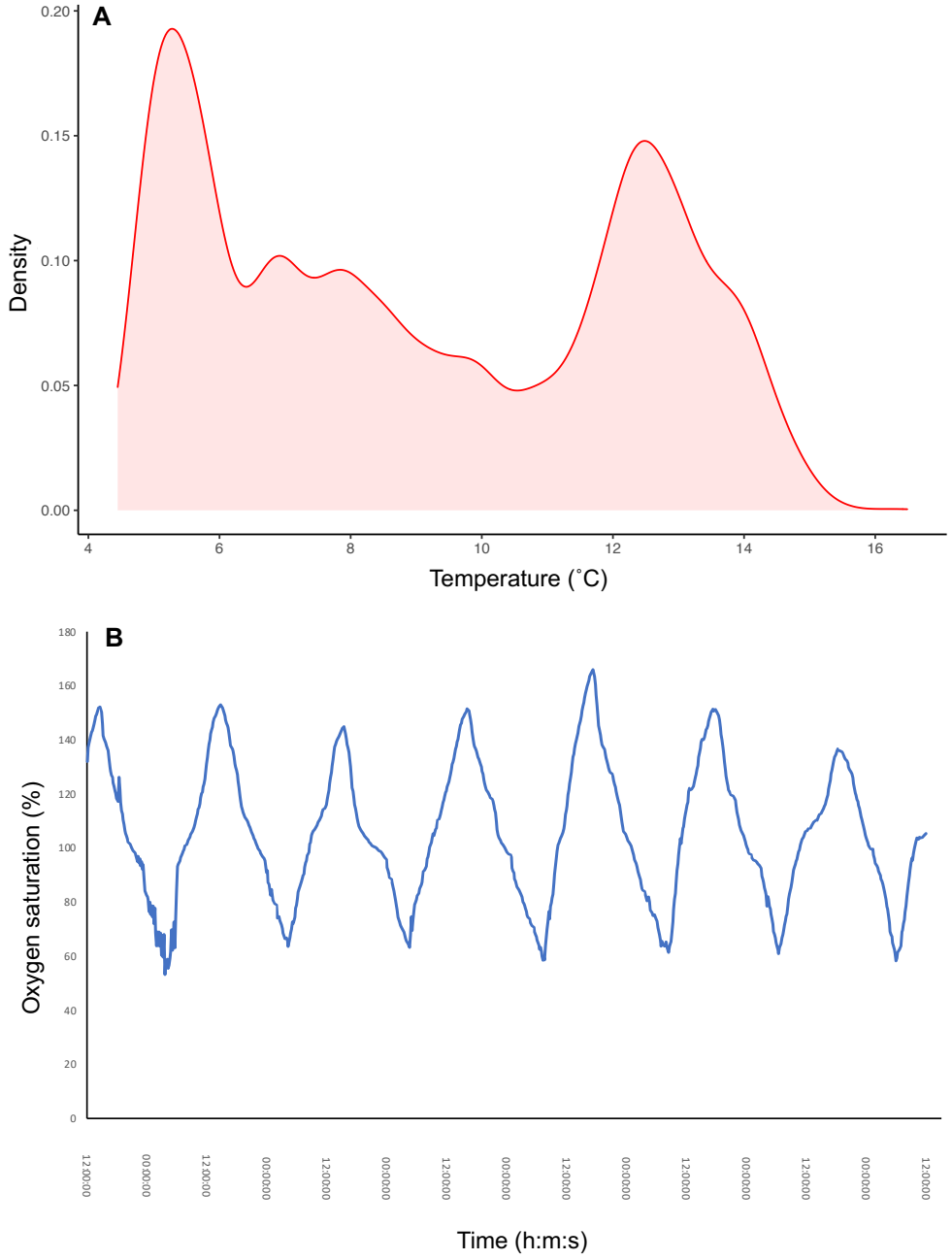
578

Supplementary Material

Diel oxygen fluctuation drives the thermal response and metabolic performance of coastal marine ectotherms

JM Booth[1†*], M Fusi[2†*], F Giomi[3], ECN Chapman[4], K Diele[2,4] & CD McQuaid[1]



584

585 **Supplementary Figure 1**. (A) Frequencies of the seawater temperature and (B) diel fluctuation
586 of oxygen saturation measured with a miniDOT oxygen and temperature logger near the
587 boundary layer of the algal habitats where the animals used in the experiment live. Data in the
588 density plot were collected over the course of the 12 months prior to the experiments, while
589 oxygen data are a subsample (two weeks from October 2019 are shown).

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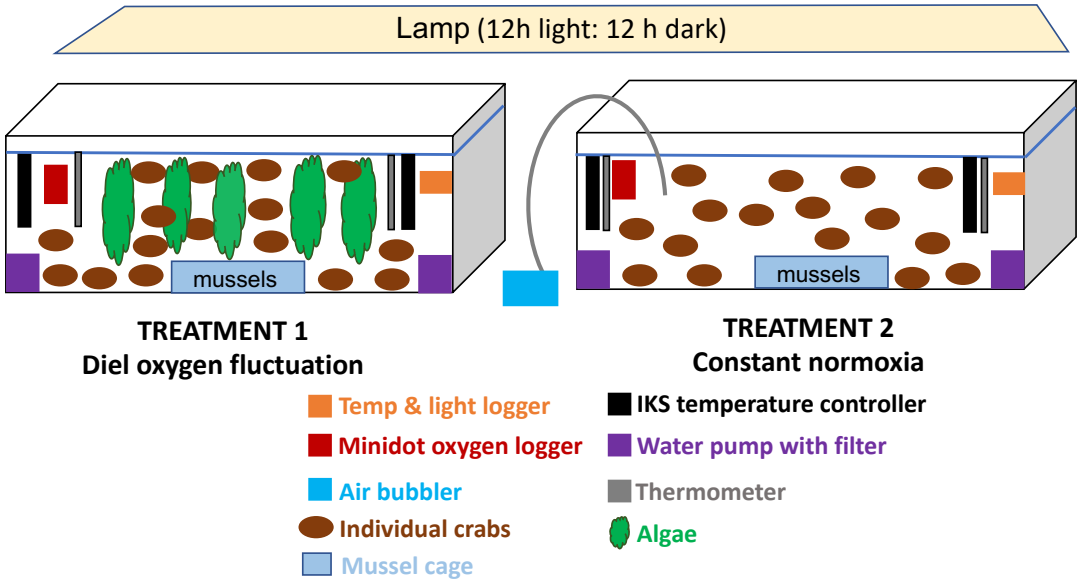

593

594 **Supplementary Figure 2 –** Thermal response under diel oxygen fluctuation: experimental set-
595 up. The experiment was repeated at 9ºC, 16ºC and 21ºC. Oxygen was provided by algae
596 (treatment 1) or an air bubbler (treatment 2).

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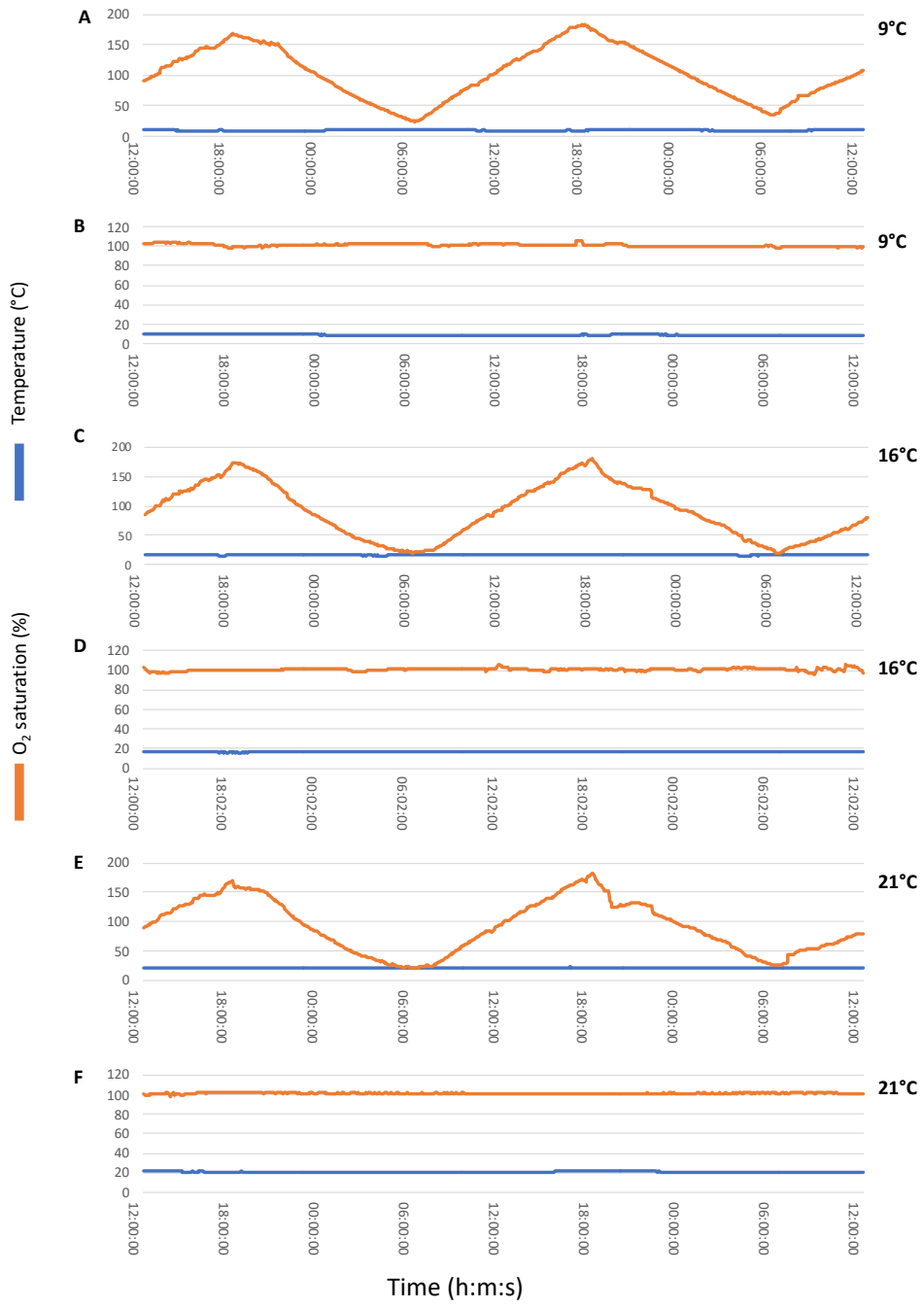

598

599 **Supplementary Figure 3**. Dissolved oxygen saturation (%) and temperature of treatment tanks
600 during the lab-based experiment. A sample of 48 hours is shown as a representation of the
601 conditions experienced by the animals under diel oxygen fluctuation (A, C, E) and 100%
602 oxygen saturation (B, D, F) at the three experimental temperatures (9, 16 and 21°C). Data were
603 collected with a miniDOT oxygen and temperature logger.

604

Supplementary Table 1 – ANOVA (analysis of variance) table for Experiment A, testing the effect of oxygen treatment (oxygen supersaturation, constant 100% oxygen saturation) and temperature on (A) crab MO2, (B) mussel MO2 and (C) crab haemocyanin at 24ºC. Statistically significant results are shown in bold.

(A) Crab MO2	DF	Mean Sq.	F	Pr(>F)
Temperature	2	2.51	107.44	**< 0.00001**
O2 Treatment	1	1.15	49.13	**< 0.00001**
Temperature:O2 Treatment	2	0.11	4.11	**0.02258**
Residuals	58			

(B) Mussel MO2	DF	Mean Sq.	F	Pr(>F)
Temperature	2	2.66	125.87	**< 0.00001**
O2 Treatment	1	1.42	67.44	**< 0.00001**
Temperature:O2 Treatment	2	0.42	19.94	**< 0.00001**
Residuals	58			

(C) Crab haemocyanin	DF	Mean Sq.	F	Pr(>F)
O2 Treatment	1	829.17	31.53	**< 0.00001**
Residuals	14	26.29		

Supplementary Table 2 – ANOVA (analysis of variance) table for Experiment B, testing the effect of oxygen treatment (oxygen diel variation, constant 100% oxygen saturation) and temperature on (A) crab MO2, (B) mussel MO2, (C) crab PO2crit, (D) mussel PO2crit, (E) crab lactate and (F) crab haemocyanin. Statistically significant results are shown in bold.

(A) Crab MO2	DF	Mean Sq.	F	Pr(>F)
Temperature	2	2.73	56.84	**< 0.00001**
O2 Treatment	1	1.01	21.09	**< 0.00001**
Temperature:O2 Treatment	2	0.31	6.41	**0.003825**
Residuals	41	0.05		

(B) Mussel MO2	DF	Mean Sq.	F	Pr(>F)
Temperature	2	1.32	150.44	**< 0.00001**
O2 Treatment	1	0.30	34.04	**< 0.00001**
Temperature:O2 Treatment	2	0.06	6.82	**0.002715**
Residuals	42	0.01		

(C) Crab PO2crit	DF	Mean Sq.	F	Pr(>F)
Temperature	1	298.90	27.21	**< 0.00001**
O2 Treatment	1	343.74	31.30	**< 0.00001**
Temperature:O2 Treatment	1	585.33	53.29	**< 0.00001**
Residuals	28	10.98		

(D) Mussel PO2crit	DF	Mean Sq.	F	Pr(>F)
Temperature	1	0.04	0.00	0.9906
O2 Treatment	1	48.93	0.18	0.6751
Temperature:O2 Treatment	1	14.89	0.05	0.8169
Residuals	28	272.71		

(E) Crab lactate	DF	Mean Sq.	F	Pr(>F)
Temperature	1	165.61	1706.64	**< 0.00001**
O2 Treatment	1	20.15	207.66	**< 0.00001**
Temperature:O2 Treatment	1	12.37	127.49	**< 0.00001**
Residuals	44	0.10		

(F) Crab haemocyanin	DF	Mean Sq.	F	Pr(>F)
Temperature	1	497.84	40.30	**< 0.00001**
O2 Treatment	1	220.60	17.86	**0.0001181**
Temperature:O2 Treatment	1	78.24	6.33	**0.0155714**
Residuals	44	12.35		