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Impact of ocean acidification on thermal tolerance and acid–base regulation of *Mytilus edulis* (L.) from the North Sea

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ABSTRACT

Anthropogenic climate change confronts marine organisms with rapid trends of concomitant warming and CO_2 induced ocean acidification. The survival and distribution of species partly depend on their ability to exploit their physiological plasticity during acclimatization. Therefore, in laboratory studies the effects of simulated future ocean acidification on thermal tolerance, energy metabolism and acid–base regulation capacity of the North Sea population of the blue mussel *Mytilus edulis* were examined. Following one month of pre-acclimation to 10 °C and control CO_2 levels, mussels were exposed for two weeks to control and projected oceanic CO_2 levels (390, 750 and 1120 μatm) before being subjected to a stepwise warming protocol between 10 °C and 31 °C (+3 °C each night). Oxygen consumption and heart rates, anaerobic metabolite levels and haemolymph acid–base status were determined at each temperature. CO_2 exposure left oxygen consumption rate unchanged at acclimation temperature but caused a somewhat stronger increase during acute warming and thus mildly higher Q_{10}-values than seen in controls. Interestingly, the thermally induced limitation of oxygen consumption rate set in earlier in normocapnic than in hypercapnic (1120 μatm CO_2) mussels (25.2 °C vs. 28.8 °C), likely due to an onset of metabolic depression in the control group following warming. However, the temperature induced increase in heart rate became limited above 25 °C in both groups indicating an unchanged pejus temperature regardless of CO_2 treatment. An upper critical temperature was reached above 28 °C in both treatments indicated by the accumulation of anaerobic metabolites in the mantle tissue, paralleled by a strong increase in haemolymph PCO_2 at 31 °C. Ocean acidification caused a decrease in haemolymph pH. The extracellular acidosis remained largely uncompensated despite some bicarbonate accumulation. In all treatments animals developed a progressive warming-induced extracellular acidosis. A stronger pH drop at around 25 °C was followed by stagnating heart rates. However, normocapnic mussels enhanced bicarbonate accumulation at the critical limit, a strategy no longer available to hypercapnic mussels. In conclusion, CO_2 has small effects on the response patterns of mussels to warming, leaving thermal thresholds largely unaffected. High resilience of adult North Sea mussels to future ocean acidification indicates that sensitivity to thermal stress is more relevant in shaping the response to future climate change.

1. Introduction

Coastal regions host a variety of ecosystems that are increasingly threatened by anthropogenic climate change. Current climate change confronts marine organisms with multiple stressors such as increasing temperature, CO_2 and hypoxia (Pörtner et al., 2014). Temperature is the main factor shaping the performance of marine animals as it affects organisms in all life-stages and at all levels of biological organisation. There is evidence that temperature-induced impacts on marine ectothermic animals are based on unifying principles of physiological response, which led to the concept of oxygen- and capacity-limited thermal tolerance (OCLTT; for review, see Pörtner, 2010). This concept links physiological principles of thermal tolerance to climate-driven ecosystem changes. According to the OCLTT concept, the oxygen supply of an organism is maximal in the optimum temperature range (T_O) between upper and lower pejus thresholds, with performance being maximal close to upper pejus limits. This range characterizes the ecological thermal tolerance range where availability of aerobic energy is maximal for all physiological functions including growth, development and reproduction and therefore determines the geographical distribution of species and populations. In temperate zone species surpassing either an upper or lower pejus temperature (T_P) leads to a mismatch between oxygen uptake and demand as circulation and/or ventilation reach their capacity limits resulting in internal hypoxemia of the organism and thus

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progressively reduced aerobic performance and whole animal fitness, visible in a reduction of growth, reproduction etc. The next thresholds are the critical temperatures (T_C) when oxygen supply to tissues becomes insufficient to maintain energy expenditure resulting in a transition to anaerobic energy metabolism.

While temperature is the main factor driving current ecosystem changes (Poloczanska et al., 2014), impacts of increasing CO_2 concentrations (causing ocean acidification, i.e. reductions in seawater pH, carbonate levels and carbonate saturation values) are expected to become increasingly involved, especially in upwelling areas (e.g. Barton et al., 2012). Future ocean acidification has the potential to disturb life-sustaining processes in marine organisms like growth, reproduction, energy metabolism and acid–base regulation. Especially calcifying organisms, like bivalves, are projected to be adversely affected due to the additional challenges involved in forming and protecting their calcareous shells and skeletons under the altered seawater carbonate chemistry (Fabry et al., 2008; Doney et al., 2009; Kroeker et al., 2010). Furthermore, the capacity for acid–base regulation is crucial for the scope of whole animal performance (Pörtner, 2008), but, in contrast to fish and crustaceans, bivalves and echinoderms are regarded to be weak acid–base regulators (for review, see Melzner et al., 2009; Parker et al., 2013). These circumstances gain increasing attention as several calcifiers are important species in costal ecosystem functioning and moreover for shellfish economies. In case of the North Sea, beds of *Mytilus edulis* provide substratum for various epibionts and offer shelter and food for a diverse community of organisms. A glimpse into the future is provided by massive die-offs of shellfish larvae due to shifts towards high-CO_2 upwelling conditions over the last years in coastal regions of the northeast Pacific Ocean (Barton et al., 2012).

One main strategy of marine invertebrates to survive short periods of elevated CO_2 exposure passively is the so-called metabolic depression with an associated reduction in ventilation (Guppy et al., 1994; Langenbuch and Pörtner, 2002; Michaelidis et al., 2005). However, this strategy may result harmful in the long term. Michaelidis et al. (2005) found permanently depressed aerobic metabolism in *Mytilus galloprovincialis* over 90 d of severe hypercapnia at ~5000 μatm leading to a 50% reduction in growth rate. Over a similar time period moderate hypercapnia of ~2000 μatm caused a significant reduction of oxygen consumption, clearance and ingestion rates in clams *Ruditapes decussatus* (Fernández-Reiriz et al., 2011). Further studies found no effect of CO_2 levels projected by 2100 (≤2000 μatm) on oxygen consumption rates of bivalves (Lannig et al., 2010; Fernández-Reiriz et al., 2012; Liu and He, 2012; Schalkhausser et al., 2012). In *M. edulis* from the Baltic (Kiel Fjord) no reduction in oxygen consumption was found even at 2500 μatm (Thomsen and Melzner, 2010). However, elevated background CO_2 levels in the fjord indicate that this population may be pre-adapted such that CO_2 levels applied during experimental exposures were not high enough to elicit the respective response. This pre-adaptation might not exist in populations from habitats with CO_2 levels similar to the atmosphere.

Recently, it could be shown that acute warming combined with hypercapnia exacerbates the warming induced constraints on aerobic energy metabolism and performance of decapod crustaceans (Metzger et al., 2007; Walther et al., 2009; Zittier et al., 2013). Hypercapnia caused a narrowing of the thermal window due to a downward shift of the upper critical temperature (Metzger et al., 2007; Walther et al., 2009).

The aim of the present study was to test a potential net effect of hypercapnia on thermal tolerance and acid–base regulation capacity of a commercially important marine bivalve, the blue mussel *M. edulis* from the North Sea. The impact of realistic CO_2 scenarios for two weeks was investigated before animals were exposed to acute warming. Oxygen consumption, heart rate, anaerobic metabolite accumulation in mantle tissue and acid–base status of haemolymph and extrapallial fluid were measured to elucidate the combined effects of the two drivers on performance.

2. Material & methods

2.1. Animal collection and maintenance

Wild type adult mussels, *M. edulis* (50 to 90 mm shell length) were collected from the subtidal zone around Helgoland, German Bight in June 2009 after the main larval peak. Seawater CO_2 levels of this area are relatively stable throughout the year and similar to atmospheric levels (average ≤ 400 μatm) (e.g. Thomas et al., 2007). Mussels were transported in tanks that were constantly flooded with North Sea water to the Alfred-Wegener-Institute for Polar and Marine Research (AWI, Bremerhaven, Germany) within 24 h after collection by the research vessel Uthörn. Mussels were separated, cleaned from epibionts and maintained in aerated and filtered natural seawater from the North Sea at 10 °C and a salinity of 32 in the aquarium facility of the AWI. Following pre-acclimation for at least one month, mussels were randomly placed in one of four tanks per group (15 L, max. 18 animals) and incubated for 2 weeks under different CO_2 concentrations (see below). Mussels were fed daily ad libitum with freshly hatched *Artemia* larvae (as suitable diet, e.g. Davenport et al., 2000) and a commercial living algal blend containing *Nannochloropsis*, *Phaeodactylum tricornutum* and *Chlorella* (DT's Live Marine Phytoplankton, Coralsands, Germany). To avoid interference with postprandial metabolism (e.g. Bayne and Scullard, 1977; Gaffney and Diehl, 1986) feeding was terminated three days before experimentation. All animal tanks (acclimation, incubation and experimental tanks) were cleaned daily from faeces and pseudofaeces.

2.2. Incubation and experimental set up

For CO_2 incubations, systems were set up in a temperature control room (10 °C) using several reservoirs (450 L) and header tanks (210 L) to provide different treatment conditions according to projected scenarios of oceanic CO_2 levels (today: 390 and towards the year 2100: 750 and 1120 μatm). Water was circulated between the reservoir and the header tank; both were continuously bubbled with the respective air–CO_2 mixture via a HTK gas system (Hamburg, Germany). From the header tank, water was supplied to the animal tanks (15 L) at a flow rate of ~120 mL min^{-1}, thereby providing stable CO_2 conditions. It was then collected in a 210 L basin re-equilibrated by continuous bubbling and re-circulated to the reservoir. Water was exchanged two times a week after disconnecting the reservoir from the system. Subsequently, the system was refilled and equilibrated for 24 h while the water from the basin was re-circulated into the header tank.

The experimental setup comprised two animal tanks (80 L, starting with 36 animals) and a reservoir (for water exchange), all temperatures were feedback-controlled by a thermostat (LAUDA RP 845, Lauda-Königshofen) and continuously bubbled with the respective air–CO_2 mixture produced by a MKS mass flow controller (MKS Instruments Deutschland GmbH, München). Each animal tank contained four respiration chambers. Water was exchanged before each temperature rise.

Temperature (T), salinity (S), pH, and total alkalinity (TA) were measured daily in all animal tanks (incubation, experimental and reservoirs tanks for water changes) for determining the water chemistry. Measurements were carried out using a salinometer (WTW LF197 combination temperature and salinity probe) and a pH meter (NBS scale, Mettler-Toledo pH meter). TA was analysed by potentiometric titration (METROHM Prozessanalytik GmbH&Co, Germany). The partial pressure of CO_2 in seawater (PCO_2) was calculated based on the measured parameters using the CO2SYS program (Pierrot et al., 2006) after equilibrium constants of Mehrbach et al. (1973) for the CO_2/bicarbonate/carbonate system, as refitted by Dickson and Millero (1987) and used for KSO_4 as provided by Dickson (1990) (incubation: Table 1, experimentation: Table 2).

Table 1
Carbonate chemistry of seawater during the incubation of blue mussels, *M. edulis* at different CO_2 concentrations.

PCO_2 µatm (set)	T °C (set)	pH_{NBS}	PCO_2 µatm	T °C
390	10	8.14 ± 0.03	443 ± 30	10.1 ± 0.5
750	10	7.91 ± 0.02	758 ± 74	10.1 ± 0.5
1120	10	7.81 ± 0.02	1037 ± 93	9.7 ± 0.1

2.3. Preparation of animals and experimental protocol

After CO_2-exposure mussels were transferred into the experimental setup. Eight mussels per treatment (N = 72) were used for parallel measurements of oxygen consumption and heart rate. Shell thickness was reduced by grinding and a plethysmograph infrared sensor (Vishay Semiconductors, CNY70) was placed above the pericardial cavity for non-invasive heart rate measurements. The sensors were integrated through the lid of the respiration chamber, superglued to the shell and covered with dental wax. Mussels were then placed into respiration chambers within the experimental tanks. In order to monitor recovery from handling stress recordings of oxygen consumption and heart rate were started immediately. After stable reading was obtained (after 3 to 7 h depending on individual; see Fig. 1) data were collected for analyses. Other mussels of the respective treatment (for the sampling of tissues and body fluids) were left undisturbed at least overnight after being transferred to the experimental tanks.

Measurements were started at control temperature (10 °C) and temperature was increased by 3 °C every night. Temperature was increased up to 22 °C (=1st run) under all CO_2 conditions (390, 750, 1120 µatm; all data shown). As data analyses revealed that individuals did not reach their critical temperatures, a 2nd run up to 31 °C was carried out two months later under control (390 µatm) and high CO_2 conditions (1120 µatm). These data are displayed for the temperature interval from 25 °C to 31 °C. Mussels used in the 2nd run were treated in the same way (incubation time, feeding procedure, handling, etc.) and displayed similar size and weight ranges when compared to the 1st run. Occasional measurements of all parameters were performed below 25 °C during the 2nd run and confirmed the comparability of both data sets.

Mussels were kept unfed during the whole temperature trial to avoid postprandial rise in metabolism. Reduced food amount can modulate the stress sensitivity of animals — especially during long-term experiments (Melzner et al., 2011) but pre-experiments performed over one week at constant temperature (10 °C) under the respective CO_2 levels did not reveal temporal variation indicating that animals of the present study were post-absorptive but not yet depressing metabolism due to starvation. That the lack of food might induce an adverse effect when high temperatures are reached is questionable as food induced metabolism will also cause a lowering of thermal limits. However, all animals were treated in the same way making the data of normocapnic and hypercapnic animals comparable and differences in the response can, thus, be attributed to the CO_2 levels applied. Moreover, all mussels were in good shape also at the end of the 2nd run, as confirmed by condition indices (CI, calculated as: dry meat weight [g] × 1000 / (shell length [cm])^3) of 4.96 ± 1.03 at 390 µatm and 4.67 ± 0.91 at 1120 µatm that lay well in the range of previously findings for *M. edulis* (cf. Lundebye et al., 1997).

Table 2
Carbonate chemistry of seawater during the experimentation with blue mussels, *M. edulis* (acute warming protocol, 3 °C/night) at different CO_2 concentrations (390, 750, 1120 µatm). Total alkalinity was very stable throughout the experimental period with 2411 ± 17, 2414 ± 10 and 2412 ± 27 µatm/kg SW, respectively.

T °C (set)	390 µatm			750 µatm			1120 µatm		
	T °C	pH_{NBS}	PCO_2 µatm	T °C	pH_{NBS}	PCO_2 µatm	T °C	pH_{NBS}	PCO_2 µatm
10	10.1	8.35	344.8	10.1	7.94	752.2	10.3	7.76	1202.9
13	12.8	8.20	410.4	13.1	7.94	850.0	13.2	7.79	1124.9
16	16.3	8.19	406.8	16.0	7.95	770.0	16.4	7.84	1020.4
19	18.9	8.20	412.6	18.7	7.99	723.1	19.2	7.83	1082.3
22	22.0	8.15	487.5	22.3	7.97	868.6	21.9	7.85	1063.0
25	24.9	8.05	600.6				24.9	7.76	1195.9
28	27.8	8.11	496.1				27.8	7.75	1410.4
31	30.9	8.09	527.8				31.1	7.80	1170.8

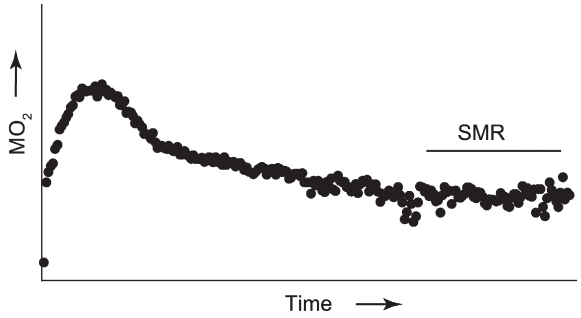

Fig. 1. Example of oxygen consumption (MO_2) pattern of individual *Mytilus edulis* over time after preparation for experimental analyses. The initial MO_2 increase reflects the time span the system needs to equilibrate. The adjacent stabilized phase indicates metabolic rate elevation after handling stress. The subsequent decrease in MO_2 reflects the recovery phase and return to SMR (standard metabolic rate). SMR was reached after 3 to 7 h depending on the individual and the time span until first valve opening occurred after handling.

Oxygen consumption and heart rates were recorded online throughout the experimental period. Data from the first 5 h after temperature rise were discarded to disregard short-term acclimation. In addition, samples of body fluids and tissue were collected at each temperature (N = 6–8) from parallel incubations of non-monitored mussels. Samples of body fluids (haemolymph and extrapallial fluid) were removed with gas-tight sterile syringes. Haemolymph was withdrawn from the posterior adductor muscle and extrapallial fluid was sampled with a long (8 cm) needle gently inserted between the shell and the pallial attachment of the mantle. To avoid contact with the atmosphere, both fluids were immediately analysed for acid–base parameters and gas concentrations (see below). Afterwards, the mantle tissue was excised, freeze-clamped and stored in liquid nitrogen until analysis of anaerobic metabolites.

2.4. Determination of oxygen consumption and heart rate

Oxygen consumption (MO_2) measurements were conducted following Van Dijk et al. (1999) using flow-through respirometry. Briefly, the flow rate (3–46 mL min^{-1}) was set in a way that mussels consumed less than 20% of the O_2 from the water. Throughout the experiment only readings of lowest metabolic rates, stable for at least 40 min (standard metabolic rate, SMR, see Fig. 1) were used for analyses. After the experiment, mussels were dissected to determine shell-free dry weight (DW). MO_2 was measured using oxygen optodes with integrated temperature compensation (TX-3, PreSens GmbH, Regensburg). Optodes were calibrated in oxygen-free (0%, N_2 bubbled) and air-saturated (100%) seawater. The 100%-oxygen values were checked daily to compensate for temperature and hardware drifts. Once air saturation values deviated from calibrations by more than 2%, recorded data were corrected and a new two-point calibration was performed. Values given as % air saturation were converted to

$$PO_2(\text{kPa}) = (P\text{atm} - PH_2O) \times 0.2095 \times (\%\,\text{air saturation})/100,$$

where *P*atm is the atmospheric pressure (kPa), PH_2O is the temperature-specific water vapour pressure (kPa), calculated after

Dejours (1975), and 0.2095 is the fraction of oxygen in air. MO_2 was calculated as follows:

$$MO_2\left(\mu mol\ O_2 h^{-1} g\ DW^{-1}\right) = (\Delta PO_2 \times \beta O_2 \times V_{fl})/DW,$$

where ΔPO_2 is the difference in oxygen partial pressure between inflowing and outflowing water (kPa), βO_2 is the temperature-specific oxygen capacity of water (μmol O_2 L^{-1} kPa^{-1}), V_{fl} is the flow rate (L h^{-1}) and DW is the shell-free dry weight (g) of the mussel. Heart rate was recorded using a PowerLab system with Chart v4.1.1 Software (AD Instruments, Spechbach, Germany). Averaged heart rate was determined at each temperature by counting the peaks expressed as beats per minute (bpm) over 30–90 s intervals within a 3 h period. Unfortunately, some heart recordings became unreliable at high temperatures possibly due to grounding problems, reducing the available sample size above 22 °C.

2.5. Determination of gas and acid–base status in haemolymph and extrapallial fluid

Haemolymph and extrapallial fluid were analysed immediately after sampling. PO_2, PCO_2 and pH were measured using a blood gas analyser (MT 33, Eschweiler, Germany) calibrated at the specific experimental temperature. Total CO_2 concentration (CCO_2, Table 3) of body fluids was determined by gas chromatography (Agilent 6890N GC System, Agilent Technologies, USA). Bicarbonate concentrations ($[HCO_3^-]$) were calculated from measured CCO_2 (mM) and PCO_2 (Torr) as follows:

$$[HCO_3^-](mM) = CCO_2 - (\alpha CO_2 \times PCO_2),$$

where αCO_2 is the solubility of CO_2 in the body fluid (mM $Torr^{-1}$) calculated after Heisler (1986).

2.6. Tissue extraction and determination of metabolites

Tissue succinate concentrations were determined as follows. Mantle tissue was powdered under liquid nitrogen using a mortar and pestle. Tissue powder (~300 mg) was homogenized (0 °C, 360 W) with ice-cold 0.6 M perchloric acid (PCA) added to a vol/wt ratio of 1 to 5. Precipitated protein was removed by centrifugation (0 °C, 2 min at 16,000 *g*). The extract was neutralized to a pH of ~7.5 with 5 M potassium hydroxide (KOH) and centrifuged again to remove precipitated potassium perchlorate. The supernatant was stored at −80 °C until further analysis. Samples were dried in a SpeedVac for ^1H NMR spectroscopy. Prior to measurements dried extracts were resolved in 500 μL D_2O containing 1% trimethylsilyl propionate (TSP) as internal reference and transferred to 5 mm NMR tubes, resulting in a final concentration of 0.3 g initial tissue powder per mL. Fully relaxed high-resolution ^1H NMR spectra were recorded using an inverse ^1H-broad band probe (^1H/BBI) on a 400 MHz 9.4 T WB NMR spectrometer with Avance electronics (Bruker Biospin GmbH, Germany) similar to Lannig et al. (2010). Acquisition parameters were as follows: TD = 16 k, NS = 128, DS = 2, SW = 4.8 k, AQ = 1.7 s, D1 = 12 s with a constant receiver gain of RG 203 to ensure comparability of samples. Spectra were post-processed automatically using TopSpin 2.5 (Bruker Biospin GmbH, Germany). All data were zero filled to 64 k and processed with an exponential multiplication of 0.5 Hz prior to Fourier transformation. After phase and baseline correction, spectra were calibrated to TSP at 0.0 ppm. Succinate concentrations were determined by analysing the area under a singlet peak at 2.4 ppm, corresponding to the chemical shift of succinate at pH 7.5, using the integration routine in TopSpin.

Table 3
Total carbon dioxide of haemolymph (CCO_2) in control (390 μatm) and CO_2 exposed (750 and 1120 μatm) blue mussels, *M. edulis* during acute warming (3 °C/night).

T °C	CCO_2 [mM] at given CO_2 level		
	390 μatm	750 μatm	1120 μatm
10	1.53 ± 0.19	1.83 ± 0.23	2.36 ± 0.06
13	1.47 ± 0.15	1.58 ± 0.36	1.82 ± 0.21
16	1.49 ± 0.12	1.57 ± 0.14	1.76 ± 0.11
19	1.55 ± 0.24	1.50 ± 0.22	1.62 ± 0.06
22	1.81 ± 0.21	1.77 ± 0.26	1.73 ± 0.28
25	1.69 ± 0.16		1.78 ± 0.15
28	1.79 ± 0.16		2.03 ± 0.17
31	4.05 ± 1.20		2.51 ± 0.47

2.7. Statistical analysis

Before data were processed with R outliers were removed by using Nalimov's test (Noack, 1980). Data were analysed with a Shapiro–Wilk-Test for normality. Two-way analysis of variance (ANOVA) in combination with Tukey's post hoc test was performed to analyse effects of temperature and CO_2 level and possible interactions thereof. Normality was not fulfilled for haemolymph PO_2 data, therefore, data were log-transformed prior to analysis. Arrhenius break temperature indicates the discontinuity in the temperature dependence of MO_2, at which a significant change in the slope of the plot occurs (Sokal and Rohlf, 1995). However, breakpoint analysis was impossible because a limited number of temperatures resulted above potential breakpoints in the warmth to be able to calculate linear regressions by the least-square method (Sokal and Rohlf, 1995). Hence, the phase change was determined using a sigmoidal function that describes the exponential increase of MO_2 with rising temperature, and the subsequent limitation in the warmth, when maximum curvature at a value of zero for the second derivate of the model indicates the breakpoint temperature. Differences were considered significant if $P < 0.05$. Values are presented as means ± SD, N = 5–8 unless stated otherwise.

3. Results

Oxygen consumption rates (MO_2) of *M. edulis* (Fig. 2) were similar under normocapnia (390 μatm: 7.1 ± 2.0 μmol O_2 h^{-1} g DW^{-1}) and hypercapnia (750 μatm: 6.6 ± 1.4; 1120 μatm: 7.1 ± 1.1 μmol O_2 h^{-1} g DW^{-1}) at acclimation temperature (10 °C). Warming from 10 to 28 °C led to a progressive and significant increase in MO_2 under both normocapnia and hypercapnia, which resulted in a somewhat higher oxygen consumption rate under elevated CO_2 (390 μatm: 29.2 ± 11.0, $P < 0.001$, Q10 = 2.19; 1120 μatm: 36.8 ± 5.5 μmol O_2 h^{-1} g DW^{-1}, $P < 0.001$; Q10 = 2.49). After the last temperature rise from 28 °C to 31 °C only 43% (3 out of 7) of the animals under normocapnia and 63% (5 out of 8) under hypercapnia showed a further increase in oxygen consumption rate, while MO_2 values in the other animals decreased (390 μatm: 22.4 ± 2.3, 1120 μatm: 19.2 ± 11.4 μmol O_2 h^{-1} g DW^{-1}; Fig. 2, grey symbols). As calculating a mean from two different responses makes no sense it was focused on animals that can cope with the high temperature and data of decreasing MO_2 at 31 °C were excluded for statistical analyses. Mean values of increased oxygen consumption rates at 31 °C (390 μatm: 32.4 ± 12.7, $P < 0.001$, N = 3, Q10 = 2.06; 1120 μatm: 38.4 ± 8.2 μmol O_2 h^{-1} g DW^{-1}, $P < 0.001$, N = 5, Q10 = 2.23) were only slightly higher than those at 28 °C or even than those at 25 °C among normocapnic animals. Two-way ANOVA suggested a main effect of temperature ($P < 0.001$) and CO_2 ($P = 0.047$). The MO_2 data were best fitted by a five parameter sigmoidal function revealing that the upper breakpoint temperature (= maximum curvature) was lower under normocapnia (25.19 °C) than under 1120 μatm (28.84 °C) (Fig. 2, vertical lines).

Samples of tissue and body fluids were collected from non-monitored mussels (see Section 2.3) with shells open, when selected for sampling. No differences in the succinate levels of mantle tissue were found between the three CO_2 conditions throughout the entire experimental period. During warming from 10 °C to 28 °C no significant

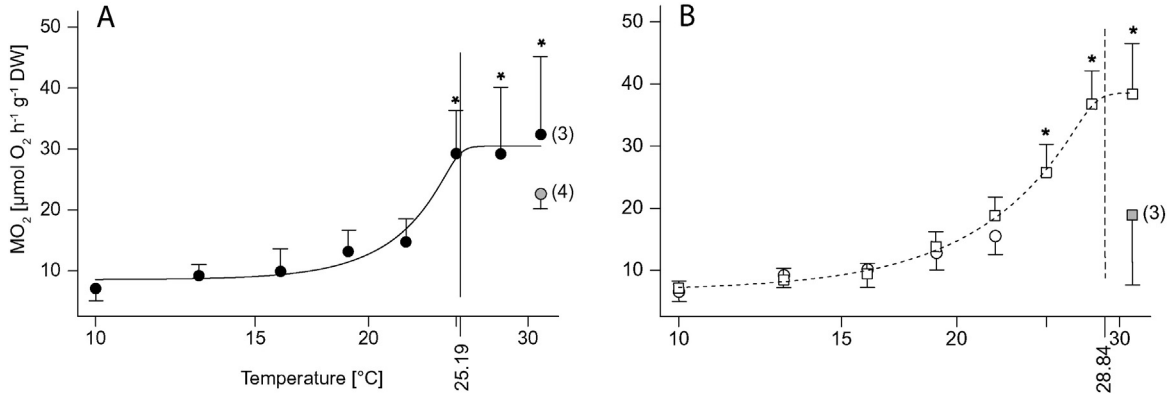

Fig. 2. Oxygen consumption rate (MO_2) in (A) control (390 µatm) and (B) CO_2 exposed (750 and 1120 µatm) blue mussels, *M. edulis* during acute warming (3 °C/night). Vertical lines indicate the breakpoint temperature, when a limitation in MO_2 rise occurs. Grey symbols indicate the mean value in animals that showed a decrease in MO_2 at the respective CO_2 level (for details see text). The number of animals is given in parentheses if below 5. * = significantly different from the respective data at 10 °C.

changes occurred (Fig. 3), however, the additional increase to 31 °C resulted in a significant accumulation of succinate under both normocapnia and hypercapnia (390 µatm: 0.12 ± 0.03 a.u. at 10° vs. 0.61 ± 0.33 at 31 °C, $P < 0.001$; 1120 µatm: 0.08 ± 0.03 a.u. at 10 °C vs. 0.61 ± 0.16 at 31 °C, $P < 0.001$). Accordingly, two-way ANOVA identified a main effect only for temperature ($P < 0.001$).

Oxygenation and acid–base status determined in extrapallial fluids were similar to the ones in haemolymph (Table 4), therefore only haemolymph data are described here. Haemolymph PO_2 showed high inter-individual variability. Two-way ANOVA suggested a significant effect of temperature and of the interaction between temperature and CO_2 on haemolymph PO_2 ($P < 0.001$ and $P = 0.044$, respectively). However, post hoc analyses did not reveal significant differences during warming when compared to acclimation temperature (10 °C) or between the CO_2 treatments at a specific temperature likely due to high inter-individual variability (Fig. 4A). In normocapnic mussels haemolymph PO_2 shows a trend to decrease during warming from 116.4 ± 5.2 Torr at 10 °C to 85.0 ± 29.2 Torr at 31 °C, which resembles the decline under hypercapnia when initial PO_2 values at 10 °C were somewhat lower than in control PCO_2 incubations (750 µatm: from 102.6 ± 12.2 Torr at 10 °C to 80.2 ± 10.5 at 22 °C; 1120 µatm: from 100.1 ± 13.1 Torr at 10 °C to 87.2 ± 22.8 at 31 °C). Post hoc tests confirmed a significant decrease only between 13° and 31 °C under normocapnia, again due to the high variability of data.

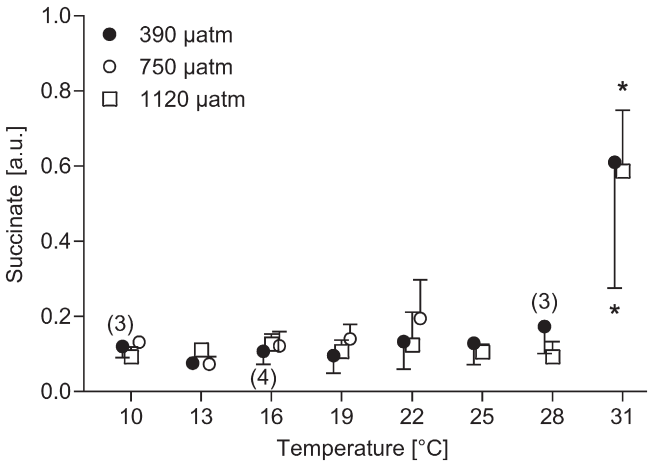

Fig. 3. Succinate content in mantle tissue of control (390 µatm) and CO_2 exposed (750 and 1120 µatm) blue mussels, *M. edulis* during acute warming (3 °C/night). The number of animals is given in parentheses if below 5. * = significantly different from the respective data at 10 °C.

Haemolymph PCO_2 did not show significant differences between CO_2 treatments throughout the experimental period (Fig. 4B). While no changes occurred during warming from 10 to 28 °C, further warming to 31 °C resulted in a significant increase of haemolymph PCO_2 in both normocapnic and hypercapnic animals, with a somewhat stronger increase in the former ones (390 µatm: 0.93 ± 0.23 at 10 °C vs. 10.83 ± 4.57 Torr at 31 °C, $P < 0.001$; 1120 µatm: 1.77 ± 0.52 at 10 °C vs. 7.79 ± 3.01 Torr at 31 °C, $P < 0.001$).

Haemolymph pH of *M. edulis* displayed different patterns depending on CO_2 treatment during acute warming between 10 °C and 31 °C (Fig. 4C). pH was significantly affected by temperature, CO_2 treatment and their interaction ($P < 0.001$, $P = 0.003$, $P = 0.016$, respectively). pH values remained highest under normocapnia. They fell significantly during the first temperature rise from 7.65 ± 0.06 at 10 °C to 7.39 ± 0.05 at 13 °C ($P = 0.005$), and remained relatively stable thereafter until they dropped significantly from 7.38 ± 0.08 at 22 °C to 7.01 ± 0.25 at 25 °C ($P < 0.001$), with no further change until 31 °C (7.02 ± 0.05). In contrast, haemolymph pH under 750 µatm of hypercapnia started with a value of 7.41 ± 0.12 at 10 °C ($P = 0.028$), significantly lower than under normocapnia, but reached 7.34 ± 0.11 at 22 °C, similar to the value seen under normocapnia. Mussels under hypercapnic conditions of 1120 µatm displayed a slight decrease from 7.48 ± 0.15 at 10 °C to 7.29 ± 0.06 at 22 °C followed by a drop to 7.10 ± 0.11 at 25 °C, significantly lower than at acclimation temperature ($P < 0.001$), with no further changes thereafter. A sudden drop in haemolymph pH between 22 °C and 25 °C thus occurred independent of CO_2 treatment. As data between 10 °C and 22 °C are the results from the 1st run and the ones between 25 °C and 31 °C from the 2nd run (see experimental protocol in Section 2.3) it seemed conceivable that the observed drop resulted from combining the two data sets. However, occasional measurements performed below 25 °C in the 2nd

Table 4
Haemolymph (HL) vs. extrapallial fluid (EPF) acid–base status in control (390 µatm) and CO_2 exposed (750 and 1120 µatm) blue mussels, *M. edulis* at control temperature (10 °C) and at the end of the warming protocol (31 °C). N = 3–7.

CO_2 exposure	T °C	Fluid	PO_2 Torr	PCO_2 Torr	pH_{NBS}	CCO_2 [mM]
390 µatm	10	HL	116 ± 5	0.93 ± 0.23	7.65 ± 0.06	1.53 ± 0.19
		EPF	128 ± 23	0.89 ± 0.18	7.55 ± 0.10	1.34 ± 0.07
750 µatm	10	HL	103 ± 12	1.80 ± 0.20	7.41 ± 0.12	1.83 ± 0.23
		EPF	113 ± 26	2.06 ± 0.57	7.35 ± 0.10	2.31 ± 0.25
1120 µatm	10	HL	100 ± 13	1.77 ± 0.52	7.48 ± 0.15	2.36 ± 0.06
		EPF	115 ± 17	1.35 ± 0.68	7.51 ± 0.07	2.37 ± 0.25
390 µatm	31	HL	85 ± 29	10.83 ± 4.57	7.02 ± 0.05	4.05 ± 1.20
		EPF	110 ± 14	8.88 ± 3.19	6.93 ± 0.13	3.75 ± 1.11
1120 µatm	31	HL	87 ± 23	7.79 ± 3.01	7.12 ± 0.11	2.51 ± 0.47
		EPF	103 ± 20	9.29 ± 3.08	7.02 ± 0.04	2.88 ± 0.47

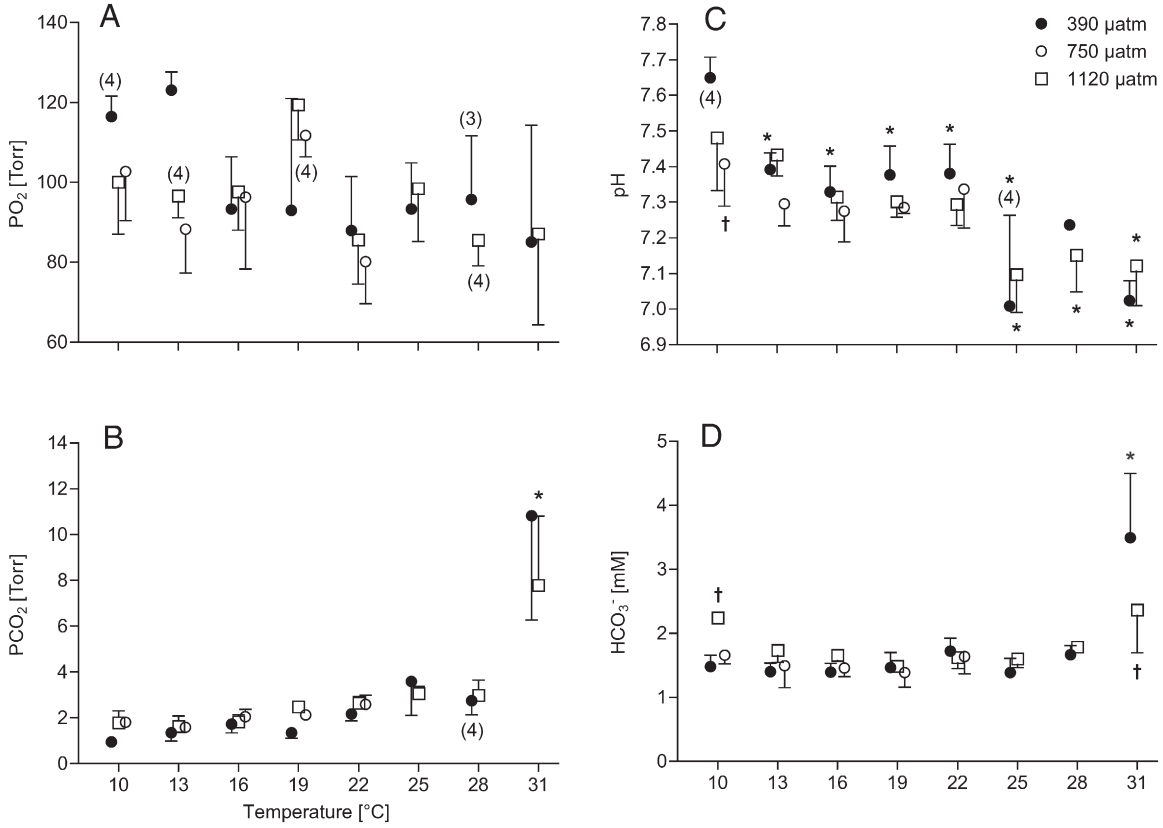

Fig. 4. Haemolymph partial pressure of oxygen (PO_2, A), partial pressure of carbon dioxide (PCO_2, B), pH (C), and bicarbonate content ($[HCO_3^-]$, D) of control (390 µatm) and CO_2 exposed (750 and 1120 µatm) blue mussels, *M. edulis* during acute warming (3 °C/night). The number of animals is given in parentheses if below 5. * = significantly different from the respective data at 10 °C, † = significantly different between control and CO_2 exposed mussels at the respective temperature.

run checked for the comparability of the data sets and these values confirmed the existence of the overproportional drop (data not shown).

At 10 °C haemolymph bicarbonate concentration ($[HCO_3^-]$) increased significantly from 1.48 ± 0.18 under normocapnia to 2.24 ± 0.07 mM at 1120 µatm ($P = 0.047$) (Fig. 4D). During acute warming from 10 °C to 31 °C further changes in haemolymph $[HCO_3^-]$ depended on the CO_2 treatment. In normocapnic mussels between 10 °C and 28 °C, haemolymph $[HCO_3^-]$ remained relatively constant around 1.48 ± 0.18 mM and increased significantly to a maximum of 3.50 ± 1.00 mM at 31 °C ($P < 0.001$). In contrast, haemolymph $[HCO_3^-]$ concentrations under 750 µatm varied slightly from 1.66 ± 0.14 at 10 °C to 1.39 ± 0.23 mM at 19 °C and 1.64 ± 0.27 mM at 22 °C. Haemolymph $[HCO_3^-]$ at 1120 µatm decreased progressively from 2.24 ± 0.07 at 10 °C to a minimum of 1.49 ± 0.07 mM at 19 °C and started to increase thereafter to 2.37 ± 0.67 mM at 31 °C, significantly higher than found at 19 °C, 22 °C and 25 °C ($P = 0.007$, $P = 0.037$, $P = 0.026$, respectively). Two-way ANOVA identified a significant interaction between temperature and CO_2 treatment ($P < 0.001$) and a main effect of temperature ($P < 0.001$).

At control temperature, haemolymph $[HCO_3^-]$ concentration was significantly higher under hypercapnia (1120 µatm, $P = 0.047$) than normocapnia, despite the lower pH value under hypercapnia. The difference was eliminated during acute warming until temperature rose from 28 °C to 31 °C, which resulted in a significantly higher $[HCO_3^-]$ level in normocapnic mussels than in hypercapnic ones ($P < 0.001$).

Fig. 5 (normocapnia, 390 µatm) and Fig. 6 (hypercapnia, 1120 µatm) depict the comparison between haemolymph pH on the one hand and heart rate (A) or oxygen consumption (B), on the other hand. It should be noted that heart rates above 22 °C were only available from N = 1–2 animals per treatment (see figures and Section 2.4), such that statistical analysis was only possible for data from 10 °C to 22 °C. Hypercapnia at 750 (not shown) and 1120 µatm had no significant effect on heart rate of *M. edulis* between 10 °C and 22 °C, when compared to normocapnia. Regardless of CO_2 treatment, acute warming between 10 °C and 22 °C caused heart rates to rise progressively to significantly higher values at 19 °C than at acclimation temperature ($P = 0.001$ at 390, $P = 0.011$ at 750, $P < 0.001$ at 1120 µatm). Two-way ANOVA suggested a significant effect of temperature ($P < 0.001$) and CO_2 ($P < 0.001$) but the post hoc test did not confirm any differences between the three CO_2 treatments. During further warming, the increase in heart rate became limited above or at 25 °C under both normocapnia and hypercapnia (1120 µatm). While heart rate under hypercapnia levelled off above 25 °C, rates under normocapnia levelled off and started to decrease at 31 °C. The limitation to a further increase in heart rate occurred when haemolymph pH had suddenly dropped below 7.3 regardless of CO_2 treatment (Figs. 5A and 6A). In normocapnic mussels the same pattern was found for oxygen consumption: when haemolymph pH had dropped the limitation to a further rise in MO_2 became effective (Fig. 5B). In contrast, hypercapnic mussels showed a further exponential increase in MO_2 until 28 °C regardless of acidosis (Fig. 6B).

4. Discussion

The aim of this study was to investigate the interacting effects of temperature and CO_2 levels according to near future ocean acidification scenarios, and to interpret the data by use of the OCLTT concept.

4.1. Thermal limits under normocapnia

Oxygen consumption rate (MO_2) of *M. edulis* at acclimation temperature (10 °C) ranged between 4.4 and 9.8 µmol O_2 h^{-1} g DW^{-1} under normocapnia (390 µatm). Rates were found well in the range of previously published data for *M. edulis* (Okumus and Stirling, 1994; Schlüter and Johansen, 1994; Sukhotin and Pörtner, 2001). Acute

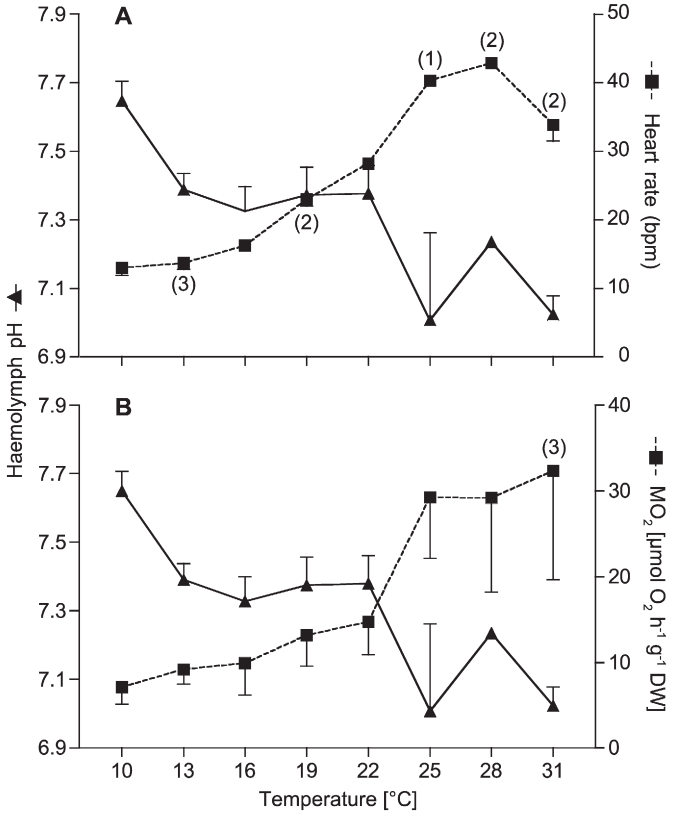

Fig. 5. Combined depiction of haemolymph pH and (A) heart rate or (B) MO_2 of *Mytilus edulis* during acute warming (3 °C/night) under normocapnia (390 µatm). The number of animals is given in parentheses if below 4.

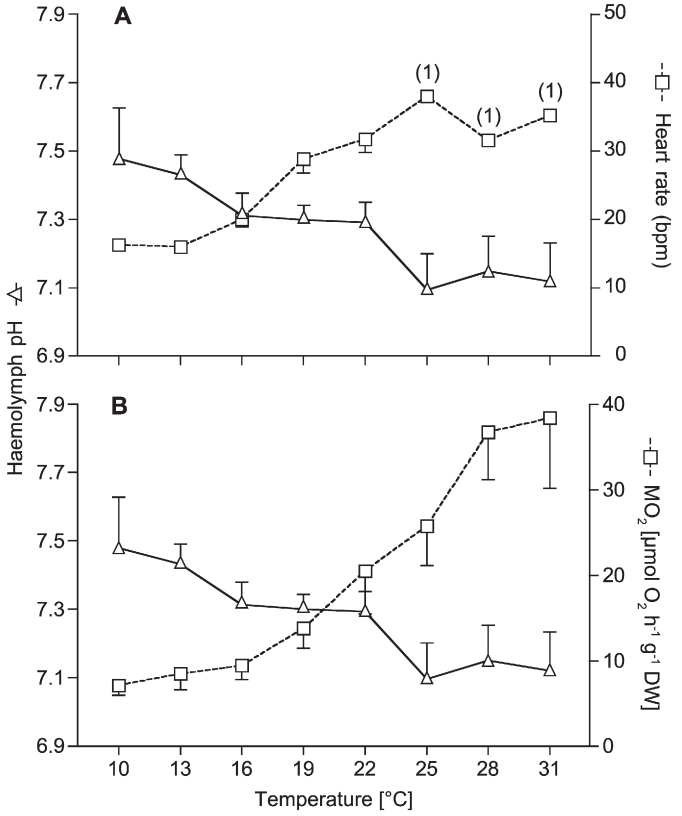

Fig. 6. Combined depiction of haemolymph pH and (A) heart rate or (B) MO_2 of *Mytilus edulis* during acute warming (3 °C/night) under hypercapnia (1120 µatm). The number of animals is given in parentheses if below 4.

warming from 10 °C to 31 °C resulted in a progressive rise in MO_2 following a Q_{10}-value (10–28 °C) of 2.19. During the last temperature rise from 28 °C to 31 °C, 43% (3 of 7) of the animals were able to further increase their MO_2 while the others showed a decrease. This suggests that about half of the animals had exceeded their critical temperature limit, likely due to lower individual performance capacity. These findings of anaerobic metabolite accumulation and haemolymph PCO_2 confirmed that the critical temperature (T_C) was reached above 28 °C (discussed below).

Interestingly, a phase change in the MO_2 rise during acute warming was found at a calculated breakpoint temperature of 25.2 °C (Fig. 2A, vertical line). This breakpoint indicates the onset of a limitation in oxygen supply, and might involve metabolic depression. According to the OCLTT concept the progressive reduction in the scope for oxygen supply may be caused by a rise in baseline energy demand, paralleled by a capacity limit of cardiac performance reached at the pejus temperature (T_P) (Pörtner, 2001). In accordance, a limitation in heart rate was also observed above 25 °C (see below). The present findings allow to put changes in extracellular pH into this context. The correlation between haemolymph pH on the one hand and oxygen consumption or heart rate, on the other hand (see Fig. 5) indicates that a sudden drop in haemolymph pH between 22 °C and 25 °C might have influenced the subsequent course of the other parameters. Beyond 25 °C the Q_{10} of MO_2 fell close to 1 possibly due to metabolic depression induced by falling extracellular pH as reported by Reipschläger and Pörtner (1996) and Michaelidis et al. (2005). *Mytilus*, as an inhabitant of sub- and intertidal zones, is adapted to daily temperature fluctuations. Consequently, it is most likely that *M. edulis* in the North Sea is exploiting mechanisms such as metabolic depression to passively withstand short-term acute temperature extremes (e.g. Connor and Gracey, 2012). A putative T_P of around 25 °C is also supported by earlier studies on *M. edulis* from the North Sea, where several physiological parameters such as ventilation rate, filtration rate and the scope for growth and activity were reduced at around this temperature (Bayne et al., 1973; Widdows, 1973a, 1973b, 1976).

These findings indicate a relationship between metabolic rate transitions at thermal limits and underlying modes of metabolism quite different from those observed in a cephalopod mollusc and in fishes (Mark, 2001; Melzner, 2005; Melzner et al., 2006). In those groups the phase change in MO_2 was suggested to be an indicator of T_C, whereas mussels may respond by metabolic depression already at T_P. Fish and cephalopods possess a closed circulatory system and strong ion regulation capacity. These characters may delay the onset of metabolic depression during environmental challenges. Also, they may not exploit or express any capacity to depress energy demand due to their permanently submerged mode of life.

A heart rate of 13 ± 2 bpm at acclimation temperature (10 °C) is in good agreement with data published previously for *M. edulis* at similar salinities and temperatures (Braby and Somero, 2006; Widdows, 1973b). Upon acute warming, heart rate increased progressively up to 25 °C and levelled off thereafter (indicating T_P, see discussion above) before a decrease was found at 31 °C. The onset of a drop in heart rate indicates the progressive break down of the circulatory system and thus T_C (>28 °C) (Frederich and Pörtner, 2000) supporting the conclusions from the MO_2 data. The temperature-dependent heart rates of the present study are in line with findings by Widdows (1973b) in *M. edulis* acclimated at 15 °C. These animals displayed a progressive increase in heart rate up to 25 °C followed by a drastic drop at 30 °C.

The critical temperature is the physiological limit where oxygen supply becomes insufficient resulting in a transition to anaerobic metabolism (for review, see Pörtner, 2010). The onset of anaerobic metabolism is indicated by the accumulation of succinate, a key anaerobic metabolite of bivalve mitochondria (Zurburg and Kluytmans, 1980; Sukhotin and Pörtner, 1999; Hines et al., 2007). In fact, a sharp

increase in succinate concentration occurred in mantle tissue above 28 °C (Fig. 3A). The involvement of anaerobic metabolism can be explained by a fall in blood PO_2 to levels too low for sustained oxygen diffusion to all mitochondria, as demonstrated for several marine taxa (Frederich and Pörtner, 2000; Lannig et al., 2004). Peck et al. (2002) showed that warming in the Antarctic bivalve *Laternula elliptica* first leads to a slight decrease in haemolymph PO_2 until the critical temperature was reached due to a subsequent drop by >70% to a level of 20.3 Torr. Haemolymph PO_2 of mussels in the present study indicated a decreasing trend with warming but values remained quite high even above 28 °C (~85 Torr) when succinate increased and T_C was reached. The same pattern was found in a comparative study on *M. edulis* from the White Sea (Zittier et al., in prep.). In the present study, haemolymph samples were collected from the posterior adductor muscle, whereas Peck et al. (2002) took samples directly from the pericardium. Mussels have an open circulatory system where blood is pumped into a number of open sinuses within tissue and organs, which are supposed to contain a mixture of pre- and postbranchial haemolymph (Booth et al., 1984; Walsh et al., 1984), before it is returned via main vessels into the heart. Therefore, haemolymph sampled directly from the pericardium might be a more suitable indicator of thermal limitation in oxygen supply. However, a drastic increase in haemolymph PCO_2 at critical temperatures was found, potentially emphasized by the acidification and uncompensated by gas exchange above 28 °C, which is in line with the beginning limitation of respiratory and cardiovascular capacity (see above). Therefore, in this study the PCO_2 more than PO_2 of haemolymph may be a suitable indicator for characterizing critical limits.

Extrapallial fluid and haemolymph yielded similar oxygenation and acid–base variables in all experimental groups. This supports the observations by Thomsen et al. (2010) that both fluids are characterized by a similar carbonate system, also recently described in *M. galloprovincialis* (Gazeau et al., 2014). Therefore, only haemolymph data are discussed here. All haemolymph parameters determined in this study under control conditions (normocapnia, 10 °C) are in good agreement with values previously reported for *M. edulis* (e.g. Booth et al., 1984). During the first temperature rise from 10 °C to 13 °C haemolymph pH dropped significantly and remained relatively stable thereafter until a second significant drop from 7.38 ± 0.08 to 7.01 ± 0.25 occurred between 22 °C and 25 °C. A reduced extracellular pH can induce metabolic depression in marine invertebrates (e.g. Reipschläger and Pörtner, 1996). Accordingly, the second drop in pH was accompanied by stagnating MO_2 rates, which might reflect the above mentioned metabolic depression at or above 25 °C. These findings might suggest that an extracellular pH lower than 7.3 induces metabolic depression in *M. edulis* from the North Sea. In *M. edulis* from the Baltic Sea CO_2 exposure to 4000 μatm at constant temperatures led to haemolymph pH of 7.1 (Thomsen et al., 2010), which had no impact on the metabolic rate determined in a parallel study under the same conditions (Thomsen and Melzner, 2010). In contrast, in *M. galloprovincialis* from the Mediterranean Sea exposed to ~5000 μatm aerobic metabolism was already depressed at a haemolymph pH of 7.4 (Michaelidis et al., 2005). These findings suggest that the pH threshold inducing metabolic depression is species and possibly population specific, and may as well be influenced by temperature and subject to adaptation to high CO_2 environments. Sensitivity to hypercapnia induced extracellular acidosis may correlate with the PCO_2 fluctuations a species encounters in its natural environment. In contrast to relative stable CO_2 levels in the northern North Sea (see Section 2.1) seawater PCO_2 in the Western Baltic Sea is elevated for most of the year (average ~ 700 μatm) reaching peak values of >2300 μatm during summer and autumn (Thomsen et al., 2010).

Lower invertebrates like bivalves and echinoderms are considered weak acid–base regulators that can tolerate an extracellular acidosis up to a certain point. Interestingly, mussels allowed extracellular pH to fluctuate passively during short-term warming on timescales longer than occurring in the intertidal. Only when a strong and sudden increase in haemolymph PCO_2 occurred at the critical temperature *M. edulis* started to defend its internal milieu against further acidification by $[HCO_3^-]$ accumulation. In mussels, $[HCO_3^-]$ can partially derive from dissolution of the $CaCO_3$ shell (Lindinger et al., 1984, >34,200 μatm; Michaelidis et al., 2005, ~5000 μatm). As this process occurs in longer term under moderate hypercapnia (Michaelidis et al., 2005; Melzner et al., 2011, 4000 μatm), the sudden increase in $[HCO_3^-]$ observed in the present study likely results from active pH regulation through proton equivalent ion exchange potentially stimulated by reaching the upper thermal limit provoking a last effort on defence.

4.2. CO_2 effects on thermal limits

Hypercapnia (750 and 1120 μatm) had no effect on oxygen consumption (MO_2) of *M. edulis* at acclimation temperature (10 °C) when compared to normocapnia (390 μatm) indicating neither elevated energy demand nor metabolic depression in mildly hypercapnic animals. Consistent with these results, recent studies imply that short- and long-term moderate hypercapnia exposures (≤2000 μatm) have almost no significant impact on aerobic metabolic rates of bivalves (Lannig et al., 2010; Thomsen and Melzner, 2010; Schalkhausser et al., 2012). However, findings by Michaelidis et al. (2005) and Thomsen and Melzner (2010) indicate that more extreme PCO_2 levels (>4000 μatm) will likely cause a downregulation of energy demand and metabolic rates in *Mytilus* species.

Acute warming (750 μatm: 10–22 °C, 1120 μatm: 10–31 °C; 3 °C/night) caused a progressive rise in MO_2, which became significantly elevated above 22 °C. Metabolic stimulation was somewhat stronger under hypercapnia (1120 μatm) leading to a slightly but not significantly higher Q_{10}-value (10–28 °C) than in control animals (2.49 vs. 2.19, respectively). For comparison, oysters (*Crassostrea gigas*) showed (significantly) higher Q_{10}-values after acute warming (5–25 °C, 5 °C/48 h) under hypercapnia (PCO_2 = 1500 μatm) compared to normocapnia (Lannig et al., 2010). In their study animals were exposed to long-term hypercapnia (26–55 days vs. 14 days in the present study) before experimentation, which had no impact on oyster metabolic rates but resulted in a decreased body condition index. This suggests reduced growth efficiency and, hence, indicating shifts in energy allocation, possibly exacerbated by starvation. Similarly, long-term moderate hypercapnia at constant temperatures had no impact on metabolic rates in blue mussels but reduced shell growth rates (*M. edulis*: Berge et al., 2006, ~2600 μatm; Thomsen and Melzner, 2010, 1000–4000 μatm; *M. galloprovincialis*: Michaelidis et al., 2005, ~5000 μatm). Hence, longer exposure times to hypercapnia would likely exacerbate the effect of warming on energy demand resulting in a significantly increased Q_{10} than under normocapnia as seen in oysters.

During the last temperature step from 28 °C to 31 °C, only 63% (5 out of 8; 1120 μatm) of the mussels were able to increase their MO_2 further, while others showed a decrease as seen under normocapnia. These findings suggest that the hypercapnic T_C was reached at the same temperature as under normocapnia, confirmed by anaerobic metabolite and haemolymph PCO_2 data (see below). However, the MO_2 course under hypercapnia showed a somewhat higher mean slope and consequently does not reflect metabolic depression in all animals, at least up to 28 °C. The data indicate an increase in variability in the response. The discontinuity in MO_2 under hypercapnia occurred at a calculated breakpoint temperature of 28.84 °C and thus, close to the T_C. In contrast, the discontinuity in normocapnic animals occurred at lower temperature (25.19 °C), was attributed to metabolic depression and interpreted to mark onset of the pejus range. The T_P of hypercapnic mussels seems to be similar as haemolymph pH dropped during the temperature rise from 22 °C to 25 °C followed by a restriction of cardiac performance beyond 25 °C (see Fig. 6) as seen in normocapnic mussels. Overall, the data indicate that during warming, CO_2 exposed animals remained in a somewhat more active state than those under normocapnia.

Extrapolating from the discussion above small increments in CO_2 may prevent early metabolic depression during warming.

The question arises how mild hypercapnia in *M. edulis* can prevent metabolic depression and thereby support tolerance to extreme warming. Other studies in fact report a CO_2 dependent stimulation of mechanisms strengthening resilience. For example, hypercapnia reportedly induces a release of adenosine into the haemolymph of crustaceans and thereby stimulates cardiac performance (e.g. Stegen and Grieshaber, 2001). The fact that heart rate did not increase indicates that this hypothesis does not apply to the blue mussel (see Fig. 6). Findings in mammals show that CO_2 can stimulate neuronal function (Dulla et al., 2005). There are no such findings reported for mussels, leaving the exact mechanism behind putative CO_2 induced metabolic stimulation obscure.

The results of the present study imply that the circulatory system of *M. edulis* reached its temperature-induced capacity limit above 25 °C in both groups, indicating the onset of the pejus range at this temperature. The T_C of *M. edulis* was also unaffected by moderate hypercapnia and found above 28 °C when succinate concentration rose sharply and regardless of CO_2 treatment. In crustaceans, it could recently be shown that hypercapnia led to a downward shift of the upper critical temperature (Metzger et al., 2007: *Cancer pagurus* at 10,000 μatm; Walther et al., 2009: *Hyas araneus* at 710 and 3000 μatm). No such shift could be observed in the present study, which might again be explained by the different habitats and pre-adaptation of *Mytilus* to life in the sub- and intertidal zone with highly fluctuating CO_2 concentrations and temperatures. Similar to permanently submersed cephalopods and fish, crustaceans regulate their internal milieu rather well (for review, see Whiteley, 2011). A higher energetic effort in acid–base regulation may lead to thermal tolerance shifts responding more strongly to hypercapnia in crustaceans than in the bivalve.

Hypercapnic exposure caused a reduced haemolymph pH in *M. edulis*. The extracellular acidosis remained uncompensated despite some compensation visible in a significantly increased [HCO_3^-] level. The same patterns were reported for other bivalves (Michaelidis et al., 2005; Lannig et al., 2010) and lower marine invertebrates (Pörtner et al., 1998).

Although no significant impact of increasing CO_2 level was detected on haemolymph PCO_2 (neither at acclimation temperature nor during warming), values were increased under hypercapnia at control temperature and match the ones found by Thomsen et al. (2010) under similar conditions. During warming, levels increased suddenly above 28 °C at the critical temperatures in all treatments. Nevertheless, heat stress under normocapnia resulted in higher internal CO_2 accumulation above 28 °C, due to acidosis and less gas exchange. A stronger respiratory acidosis in normocapnic animals was prevented by significant [HCO_3^-] accumulation. This indicates potential use of residual acid–base regulation capacity under normocapnia, which was not available to hypercapnic mussels in the warmth. Future studies have to show whether metabolically depressed animals have the ability to sustain critical conditions longer than mussels e.g. under hypercapnia, when metabolic depression is prevented.

5. Conclusion

Overall, physiological transitions observed during warming of North Sea blue mussels *M. edulis* mirror the tolerance thresholds as defined by the OCLTT concept. The warming induced fall in haemolymph pH may be involved in setting the pejus temperature (T_P, onset of falling aerobic scope) at 25 °C by dampening heart rate regardless of CO_2 treatment. The critical temperature found above 28 °C (T_C, onset of anaerobic metabolism) also remained unaffected by moderate hypercapnia indicating that the population studied may be resilient to CO_2 oscillations, possibly related to its pre-adaptation to life in the intertidal zone. While normocapnic mussels showed a somewhat earlier limitation in temperature-dependent oxygen consumption rate (breakpoint temperature 25.2 °C) and actively defended their internal milieu when reaching critical limits most hypercapnic mussels remained in an active state (breakpoint temperature 28.8 °C) and failed to significantly increase their haemolymph [HCO_3^-] level in the warmth. Thus, CO_2 exposure modulated the response to warming by somewhat reducing the degree of metabolic depression in the heat. It remains to be explored whether this effect is adaptive or reduces the capacity to sustain fitness in a highly variable environment. Especially during longer periods of exposure the latter may be harmful for sustained ecological performance of *M. edulis*.

Author contributions

Conceived and designed the experiments: ZMCZ and HOP.
Performed the experiments: ZMCZ, with the help of CB.
Analysed and interpreted the data: ZMCZ, with the help of HOP, CB and GL.
Contributed reagents/materials/analysis tools: ZMCZ, CB, and HOP.
Wrote the paper: ZMCZ, CB, and HOP.
Revision of the paper: ZMCZ, GL, and HOP.

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