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Physiological responses to ocean acidification and warming synergistically reduce condition of the common cockle *Cerastoderma edule*

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Abstract

The combined effect of ocean acidification and warming on the common cockle *Cerastoderma edule* was investigated in a fully crossed laboratory experiment. Survival of the examined adult organisms remained high and was not affected by elevated temperature (+3˚C) or lowered pH (-0.3 units). However, the morphometric condition index of the cockles incubated under high pCO_2 conditions (i.e. combined warming and acidification) was significantly reduced after six weeks of incubation. Respiration rates increased significantly under low pH, with highest rates measured under combined warm and low pH conditions. Calcification decreased significantly under low pH while clearance rates increased significantly under warm conditions and were generally lower in low pH treatments. The observed physiological responses suggest that the reduced food intake under hypercapnia is insufficient to support the higher energy requirements to compensate for the higher costs for basal maintenance and growth in future high pCO_2 waters.

Key words: Future ocean, ocean acidification, ocean warming, *Cerastoderma edule*, ecophysiology

1. Introduction

Estuaries are among the most productive marine ecosystems, supporting a high abundance and diversity of organisms (Beck et al., 2001). Other ecosystem services provided by these systems

include disturbance regulation (e.g. flood control, storm protection), nutrient cycling, biological control, habitat creation and others (e.g. Barbier et al., 2011; Meire et al., 2005), contributing to an estimated total annual monetary value of 4.1 trillion US$ (Costanza et al., 1997). However, these habitats are gradually degraded by increasing human activities compromising their function as feeding, nursery and breeding habitats (Seitz et al., 2014). In addition, coastal habitats like estuaries are in the frontline of current environmental change, including climate change (Scavia et al., 2002). The excess of CO_2 emissions produced by the burning of fossil fuels, cement production, and deforestation (Sabine et al., 2004) are interacting with the global climate and the ocean, causing warming and changes in ocean carbonate chemistry (Doney et al., 2009). To date, approximately 30% of the anthropogenic CO_2 in the atmosphere is being absorbed by oceans and is altering their chemistry, a process referred to as ocean acidification (OA) (Caldeira and Wickett, 2003; Sabine et al., 2004), with an estimated reduction in pH of 0.3 - 0.4 units by the end of the 21st century for the open ocean (Caldeira and Wickett, 2003; Feely et al., 2004; Orr et al., 2005). High temporal fluctuations in pH (Wootton et al., 2008) may mask the effect of rising pCO_2 in coastal habitats in the short-term; yet recent analyses show that rates of acidification are an order of magnitude higher in coastal habitats as compared to the open ocean (Provoost et al., 2010; Wootton et al., 2008), suggesting that these shallow water marine habitats might be particularly vulnerable to rising pCO_2 concentrations.

Over the last two decades, the consequences of changes in ocean carbonate chemistry on various life stages of calcifying marine organisms have been intensively investigated, including studies of OA effects on calcification, survival, acid-base regulation, metabolism, reproduction and immunity (reviewed in Gazeau et al., 2013; Kroeker et al., 2010; Parker et al., 2013). Calcifying organisms like corals, shellfish and crustaceans have been shown to be particularly vulnerable to OA (Kroeker et al., 2010). For example, the reduction in the success of fertilization and embryogenesis, and in the number, growth and survival of hatchlings in the Baltic tellin *Macoma balthica* at pH 7.5 (Van Colen et al., 2012) illustrates the adverse impacts OA can have on early life history processes. Another example is an 87-day study by Range et al. (2014) demonstrating that juvenile clam *Ruditapes decussatus* decreased their rates of respiration, clearance and ingestion, whereas excretion rates were increased under mimicked acidic conditions (e.g. 7.8 and 7.5). Furthermore, OA was shown to disrupt behavioural processes (e.g. finding shelter, ability to detect food, prey and predators), for example in adult hermit crabs (*Pagurus bernhardus)*, that might potentially affect the population fitness and their

effects on ecosystem functioning (Briffa et al., 2012). In short, OA does impact marine calcifying organisms in term of morphology, physiology and behaviour.

Ocean acidification does not occur in isolation (Byrne and Przeslawski, 2013). The Intergovernmental Panel on Climate Change (IPCC) has predicted an increase in global mean temperature ranging between 1.0 - 4.1˚C based on four CO_2 Representative Concentration Pathway (RCP) scenarios (Collins et al., 2013). While the separate effects of changes in seawater temperature and pH are well documented, comparatively few studies have hitherto focused on the combined effects of seawater temperature rise and acidification. Both stressors may act synergistically, antagonistically or additively on the condition and physiology of marine animals (Darling and Cote, 2008), and their combined effects can reduce the functional performance of species (e.g. foraging, growth, reproduction, competitiveness and behaviours) at ecosystem levels through narrowing of species' thermal windows (Pörtner, 2008; Pörtner and Farrell, 2008). Ocean acidification coupled with warming increased the energy metabolism of the adult Pacific oyster *Crassostrea gigas* (Lannig et al., 2010). Furthermore, Talmage and Gobler (2011) showed additive negative effects of both stressors on the mortality of bay scallops *Argopecten irradians* and hard clam *Mercenaria mercenaria* larvae, and larvae reared under high temperature and low pH conditions accumulated less lipids, were smaller and had an extended time to metamorphosis. Some studies on bivalves found that elevated temperatures had more pronounced impacts than lowered seawater pH on survival, immune response, growth and development (e.g. *Mytilus galloprovincialis* and *Mytilus edulis* (Gazeau et al., 2014; Mackenzie et al., 2014; Vihtakari et al., 2013)). On the other hand, Duarte et al. (2014) concluded that calcification rates and total weight of adult Chilean mussels (*Mytilus chilensis*) decreased more in response to lowered pH than to elevated temperature. In short, the effects of OA and warming are thus highly species-specific and can depend on the life stage, biogeography and environmental context.

To properly understand the impacts of future ocean scenario's on coastal ecosystems, it is crucial to assess the combined effects of temperature change and OA on species that play a key role in the ecosystem. The common cockle *Cerastoderma edule* is a sediment-dwelling filter-feeding bivalve that represents an important dietary component for shorebirds (e.g. the oystercatcher *Haematopus ostralegus* and the common eider *Somateria mollissima)* and crustaceans (e.g. the brown shrimp *Crangon crangon* and the shore crab *Carcinus maenas* (Beukema and Dekker, 2005; Sanchez-Salazar et al., 1987; Whitton et al., 2012)). Furthermore, the species' subsurface crawling and shaking behaviour causes disturbance of the upper

sediment layer, which can induce erosion of the sediment bed (Ciutat et al., 2006) and alterations in the benthic community (Flach, 1996; Van Colen et al., 2013). Common cockles occur along the east Atlantic coast from Morocco to Norway, and along the Black, Mediterranean and Baltic Seas (Malham et al., 2012), where they are extensively harvested by fishermen. According to the Food and Agriculture Organization of the United Nations (FAO) (http://www.fao.org/fishery/statistics/global-aquaculture-production/en), the average annual harvest of common edible cockles was 17,073 tonnes (from 2008 till 2014). The shells of cockles are solely composed of aragonite, which is a more soluble calcium carbonate polymorph than calcite (Cubillas et al., 2005). Hence, cockle shell formation is expected to be particularly vulnerable to OA and low pH conditions could therefore also reduce the species' physiological tolerances to other environmental alterations such as ocean warming (Pörtner and Farrell, 2008; Widdicombe and Spicer, 2008). Here we experimentally investigated the physiology and condition of *C. edule* under current and future pCO_2 scenario's, i.e. warming and acidification under both single and combined stressor conditions. By exposing cockles to changes in pH and temperature we expected to find an enhanced energy demand related to the support cost of tissue protection systems; for example, damage repair mechanisms, the regulation of acid-base balance and ion transport (Sokolova et al., 2011). These compensatory metabolic strategies can facilitate organisms to cope with stress at the short term but may disrupt the energy budget balance at the longer term, ultimately affecting fitness of the population (Sokolova et al., 2011).

2. Material and methods

2.1 Collection and incubation of cockles

In June 2015, adult cockles were collected during low tide in the lower intertidal zone at the "Slikken van Viane", Oosterschelde estuary, The Netherlands (51º 37' N, 4º 2' E) and were transported within two hours to the laboratory. Forty-four randomly chosen cockles (average shell length of 27.7 ± 3.2mm (SD)) were cleaned and added to each of 12 aquaria (41cm x 31cm x 40cm) that had been filled with sediment (median grain size 224 ± 1.7µm) to a height of 15cm and allowed to acclimatize to preset laboratory conditions (15°C, salinity of 33 and pH of 8.1) for 3 days, reflecting average seawater surface temperature and pH in June at the sampling location (retrieved at location Zijpe (pH) and Bruinisse (temperature) from http://live.waterbase.nl and http://www.seatemperature.org/europe/netherlands/bruinisse-

june.htm). All cockles burrowed in the upper first centimetres of the sediment within one hour with their siphons flush or slightly extending from the sediment-water interface.

There were two factors in our fully orthogonal experiment; temperature (ambient or elevated) and pH (current or lowered). Seawater was stored in four barrels (250L) and pumped from each barrel to the three aquaria and circulated back to the barrel continuously. The seawater in the aquaria and barrels was aerated and the seawater pH was manipulated in the barrels. A stirrer was installed into each barrel in order to homogenise seawater. To maintain salinity a quarter of the seawater was renewed per week which took less than 20 minutes for the whole setup (i.e. 4 treatments, 12 aquaria). All aquaria were subjected to a 12:12h light:dark regime. Cockles were fed twice a week with 1ml of commercial Shellfish Diet 1800 (Reed Mariculture Inc., composed of 40% *Isochrysis*, 15% *Pavlova*, 25% *Tetraselmis* and 20% *Thalassiosira weissflogii*) diluted with 3 liter of seawater and distributed equally in the barrels. We acknowledge that the food quality and quantity used might not completely resemble to those present in the field. However, we deliberately chose to use a mixture of dead microalgae to avoid bias in our experiment related to temperature and pH effects on food quantity and quality (Hinga, 2002; Thomas et al., 2012) that we were unable to account for.

After three days of acclimatization under ambient conditions, the pH of the seawater was decreased by 0.1 pH unit and the temperature was increased by 1˚C per day over 3 days, until a pH value of -0.3 units and a temperature of +3˚C was achieved (see below). These conditions were maintained for 6 weeks (see Table 1 and Fig. A1). At the site of cockle collection field pH data for mid-June and July ranged between 8.0 and 8.2 (data retrieved at location Zijpe from 2010 to 2015 from http://live.waterbase.nl) and the daily average seawater temperature ranged from 15 to 18°C (https://weatherspark.com/y/51298/Average-Weather-in-Bruinisse-Netherlands). The manipulated combined pH and temperature conditions thus enable us to study realistic OA effects at two ambient temperatures, i.e. the average daily temperature and the highest temperature during the period of the experiment.

ProMinent Dulcometers coupled with Hamilton glass pH electrodes were used to control the seawater pH through the controlled bubbling of CO_2 in the lowered pH treatments. The pH values were logged every 10 minutes using pH electrodes and a Consort data logger (Model: C3040). All pH electrodes, including Hamilton (S/N: 16458 and 16451) and Consort electrodes (SP10B-50), were calibrated weekly using Hanna instruments NBS buffer solutions (pH 4.01, 7.01 and 9.18). All pH measurements were reported according the NBS scale. Meanwhile,

seawater temperature was regulated by temperature heater/chiller controllers (Teco Refrigeration technologies; Model: TK200H) and HOBO Pendant data loggers (Model: UA-002-08) were used to record seawater temperature every 10 minutes. Vertical profiles of sediment porewater pH (for a description on the profiling set up, see Braeckman et al., 2014) obtained during pilot tests with the used sediment demonstrated a strong gradient in pH with a steep and gradual decline from the sediment-water interface until 0.5 cm depth where the pH stabilises. Importantly, figures A2a and b indicate that the applied pH manipulation created similar differences (~0.3 pH units) between current and lowered pH treatments in both the water column and sediment.

30-ml seawater samples were collected from each tank on a weekly basis and filtered through GF/C filters for subsequent quantification of total alkalinity (TA). These samples were temporally stored at 4°C prior to subsequent Gran titration using a Mettler Toledo G20 compact titrator and a glass electrode calibrated using Hanna instruments buffer solutions (pH 4.01 and 7.01) and a TRIS buffer solution (pH 8.1 (MERCK Production Chemicals)). The obtained pH, TA, temperature and salinity values were used to determine parameters of the carbonate chemistry (e.g. partial pressure of carbon dioxide (pCO_2), saturation state of the seawater with respect to aragonite (Ωa), total inorganic carbon concentration (C_T) etc.) through CO_2SYS software (Pierrot et al., 2006) using the thermodynamic constants of Mehrbach (1973).

2.2 Cockle condition and physiology

Mortality of cockles was checked on a daily basis and dead cockles were removed. Cockle physiology was measured as respiration rates, clearance rates and calcification rates after 3 and 6 weeks of incubation. To this purpose, 6 - 10 cockles corresponding to a total wet weight of 61 -70g were removed from the sediment of each of the three aquaria per treatment, and placed inside their respective incubation chamber (Ø = 19cm, height = 30cm, volume = 8.2liter (Braeckman et al., 2014)) along with the seawater from their respective barrels. Subsequently, respiration, clearance and calcification rates were determined according to the methodologies described below. Temperature was maintained throughout all incubations by working in a climate room set at the desired temperature, i.e. 14.9 ± 0.5 and 17.9 ± 0.5°C. After the measurements, the soft tissues of individuals were separated from their shell. Individual shells and soft tissues were allowed to dry in an oven at 60°C for 2 days and weighed. The *condition indices* (CI) of cockles were determined using equation (1) from Lucas and Beninger (1985).

191 $$CI(\%) = \frac{dry\ tissue\ weight}{dry\ shell\ weight} \times 100 \quad (1)$$

192 *Respiration rates* of batches of 6-10 cockles with a total wet weight of 61-70g were measured
193 using Pyroscience sensor technology. Cockles were not fed 7 days prior to respiration
194 measurements, however, they were not starved as cockles were observed feeding before
195 measurements took place. Sensor spots were glued on the inner wall of incubation chambers;
196 when the sensor spots are excited by red light, they show an oxygen-dependent infrared
197 emission. Lens Spot Adapters were placed on the outside of incubation chambers facing the
198 sensor spot and connected with spot fibers to a FireSting O_2 logger, which allowed continuous
199 display and recording of the O_2 concentration (µmol l^{-1}) of the seawater. Seawater oxygen
200 concentrations were logged continuously for 2 hours and 40 minutes and respiration rates were
201 calculated as in equation (2)

202 $$R = \frac{V(R_0 - R_1)}{g(t_1 - t_0)} \quad (2)$$

203 where t_0 and t_1 represent initial and final time (h) of measurement, respectively; R_0 and R_1
204 represent the oxygen concentrations (µmol l^{-1}) at time t_0 and t_1; g is the dry flesh weight (g) of
205 cockles and V is the seawater volume (l) of the incubation chamber after correction for the
206 biovolume of the cockles. An additional incubation chamber without cockles was used as a
207 blank to correct for bacterial respiration.

208 Following the respiration measurements, oxygenation of the seawater in each incubation
209 chamber was restored and 1 ml of Shellfish Diet 1800 was injected into each incubation
210 chamber. After an initial mixing period of 30 minutes, seawater samples were collected to
211 quantify the algal cell density over time. Samples were collected by removing 10 ml of seawater
212 from the incubation chamber using a syringe, while at the same time injecting the same volume
213 of seawater using a second syringe in order to keep the seawater volume in the chamber constant.
214 Samples were collected at t_0 (30 minutes after the injection of food into seawater in order to
215 homogenise the mixture before samples were taken to avoid bias) and at t_1 (after 150 minutes
216 of incubation) from each chamber.

217 In order to calculate the volume of seawater cleared by cockles, algal cell concentrations were
218 quantified using a Coulter Multisizer equipped with a 100-µm aperture tube. Hence, *clearance*
219 *rates* were calculated from the exponential decline in the cell concentration in the incubation
220 chamber following equation (3), modified from Widdows and Navarro (2007),

$$CR(L\ h^{-1}\ dry\ weight^{-1}) = \frac{V*(log_e C_1 - log_e C_2)}{t*g} \quad (3)$$

where V represents the volume of water in the incubation chamber, t is the time interval in hours, C_1 and C_2 represent the shellfish diet cell concentration at the start and end of the measurement, and g represents the dry flesh weight (g) of cockles present in an incubation chamber.

Calcification rates were estimated using the alkalinity anomaly-method according to the modification proposed by Gazeau et al. (2015) to correct for other processes (e.g. mineralisation and assimilation) that can affect TA independent from calcification. This method has been broadly used for short-term incubation as it is non-destructive and based on parameters that are easily collectable and accurately quantifiable. Two samples of 25ml seawater were collected at the beginning and end of a 2-hour incubation period. These seawater samples were analysed to determine the total alkalinity (TA), concentrations of nutrients (NH^{4+}, NO^{3-}+NO^{2-} and PO_4^{3-}) and *calcification rates* were calculated as shown in equation (4) modified from Gazeau et al. (2015).

$$G^*TA = \frac{\Delta {NH_4}^+ - \Delta TA - \Delta NO_x - \Delta {PO_4}^{3-}}{2*g*(t_1 - t_0)} \quad (4)$$

where t_1 and t_0 represent time (in hours) at the end and at the beginning of incubation, respectively; ΔNH_4^+, ΔTA, ΔNO_x and ΔPO_4^{3-} (in µmol g^{-1}DW h^{-1}) are the differences in concentrations of ammonium, total alkalinity, nitrate plus nitrite and phosphate between t_0 and t_1, and g is the dry flesh weight (in g) of the cockles present in the incubation chambers.

2.3 Statistical analysis

A 2 x 2 contingency analysis was used to examine the difference in survival proportion between pH and temperature levels at the end of the experiment (i.e. after 6 weeks of incubation). In order to test the effects of temperature (ambient, elevated), pH (current, lowered) and time (three weeks, six weeks) on the conditional and physiological response variables (condition index, respiration, clearance and calcification rate), we used a series of linear mixed effects models with appropriate error structures for each response variable. Since cockles were taken on two occasions (after weeks three and six) from each aquarium, we accounted for the non-independence of these data by allowing random intercepts for aquaria identity. Prior to analysis data were Log_{10} transformed to improve normality in case of non-normal data, and the model assumptions were checked using Q-Q plots. Data were modelled using a Gaussian error

distribution. For Gaussian models we calculated the *F*-statistic and associated *P*-values for each effect, using the Kenward-Roger method to estimate degrees of freedom. For non-Gaussian models we used log likelihood ratio tests. Analyses were conducted using the R software version 3.3.1 (Team, 2013) with packages lme4 (Bates et al., 2015), lmerTest and pbkrTest (Kuznetsova et al., 2015).

3. Results

Seawater carbonate chemistry, temperature, pH and salinity for each treatment throughout the experiment are shown in Table 1 and Figure A1. Temperature in the lowered pH-elevated temperature treatment and in the elevated temperature treatment were maintained at 2.57 ± 0.13 (SD) °C and 2.74 ± 0.15 °C, respectively, above the control treatment (15.4 ± 0.68 °C). pH treatments were kept constant throughout the experiment with average values of 8.10 ± 0.01 (SD) and 8.20 ± 0.01 for the control and elevated temperature treatment, respectively. Seawater pH was reduced by 0.37 ± 0.03 and 0.30 ± 0.02 pH units, respectively, in the lowered pH and lowered pH-elevated temperature treatments in comparison with the control (pH 8.11 ± 0.06). Throughout the experiment, total alkalinity (3568 ± 131 μmol kg^{-1}) and salinity (34.2 ± 1.0) did not differ between treatments (Table 1). Aragonite concentration remained favorable for calcification in all treatments, with average aragonite saturation states of 6.0, 3.7, 6.2, and 3.6 in control, lowered pH, elevated temperature and lowered pH-elevated temperature treatments, respectively.

Table 1 The seawater carbonate chemistry parameters in four treatments during the 6-week experiment: temperature (Temp, in ◦C), pH, salinity, total alkalinity (TA in μmol kg^{-1}), partial pressure of carbon dioxide (pCO_2,in μatm), total inorganic carbon concentration (C_T in μmol kg^{-1}), concentration of bicarbonate ion and carbonate ion (HCO_3^- and CO_3^{2-} in μmol kg^{-1}) and saturation state of the seawater with respect to aragonite (Ωa). Values between brackets represent maximum and minimum values during the experiment.

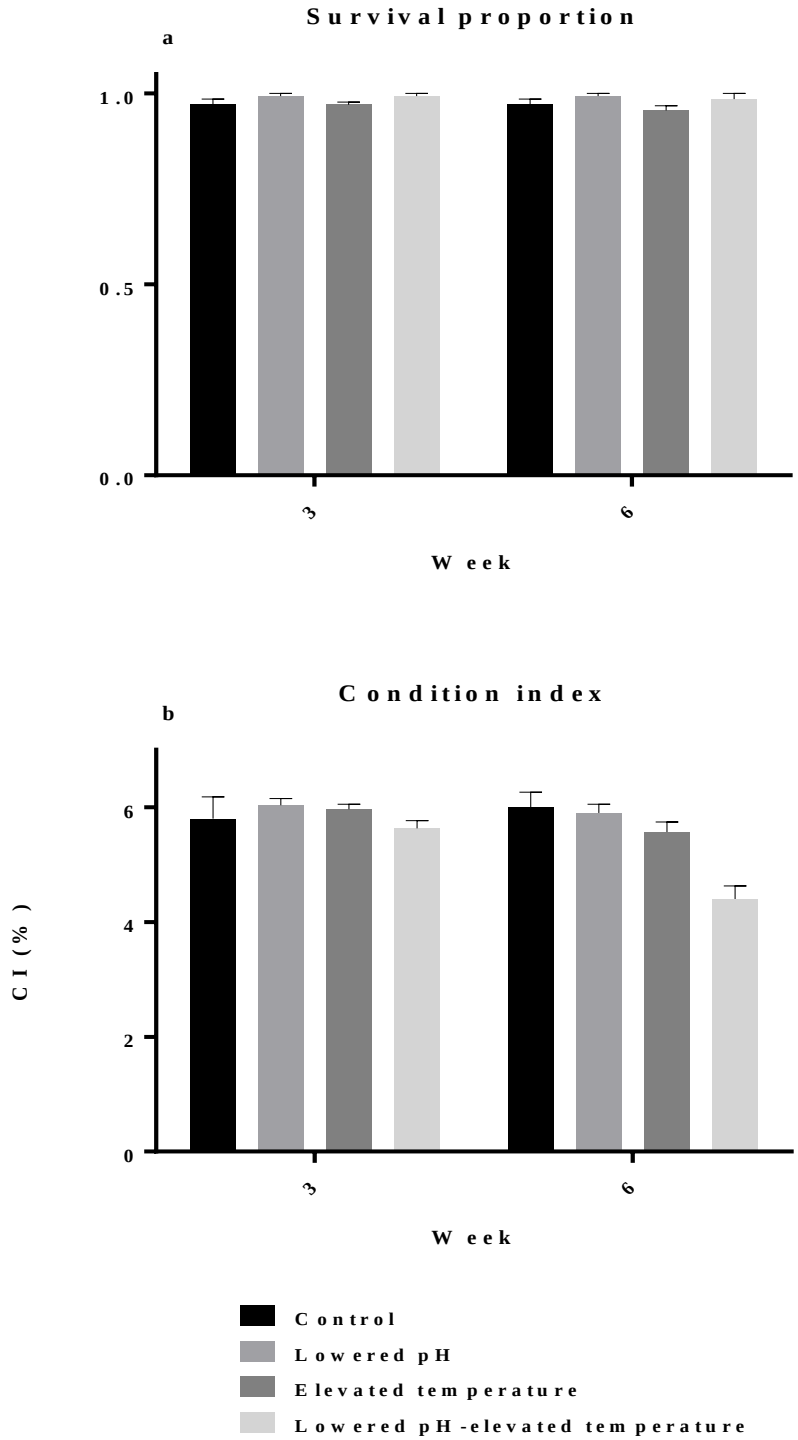
Treatment	Control	Lowered pH	Elevated temperature	Lowered pH-elevated temperature
Temperature	15.4 (17.3; 13.1)	15.3 (16.3; 15.6)	18.2 (19.2; 15.6)	18 (19.0; 15.5)
pH	8.11 (8.20; 8.00)	7.75 (7.98; 7.50)	8.2 (8.38; 8.08)	7.82 (8.06; 7.53)
Salinity	34.4 (33; 36)	33.8 (33; 35)	34.5 (33; 36)	34.2 (33; 37)
TA (μmol/kg^{-1})	3568 (3372; 3667)	3563 (3415; 3766)	3546 (3471; 3696)	3600 (3525; 3768)

pCO2 out (µatm)	325 (286; 367)	746 (288; 1065)	335 (280; 385)	870 (322; 1055)
C_T (µmol/kg^{-1})	3069 (2917, 3507)	3283 (2965, 3557)	3039 (2924, 3142)	3332 (3211, 3406)
HCO_3^- (µmol/kg^{-1})	2622 (2681; 2553)	3099 (3317; 2963)	2621 (2703; 2470)	3131 (3167; 3087)
CO_3^{2-} (µmol/kg^{-1})	386	207	402	198
Ωa	6 (5.4; 7.2)	3.7 (2.5; 6.1)	6.17 (5.6; 6.8)	3.6 (2.8; 6.6)

During the experiment, survival remained high (> 93%) and did not differ significantly among treatments (Fig. 1a). Dead cockles laying on top of the sediment were removed immediately during the daily observations. There was no difference in survival between current and lowered pH treatments (χ^2_1 = 2.4, P = 0.12) or between ambient and elevated temperature treatments (χ^2_1 = 2.4, P = 0.12) at the end of the 6-week incubation. There was no three-way interaction on *condition index* (CI) of cockles between temperature, pH and time ($F_{1,8}$ = 0.69, P = 0.43 (Fig 1b)). Similarly, there was no two-way interaction between pH and time ($F_{1,9}$ = 3.8, P = 0.082). However, there was a significant two-way interaction between temperature and pH ($F_{1,8}$ = 7.5, P = 0.025) indicating that reduced pH led to a decrease in CI in cockles held at 18°C but had no effect on CI for cockles held at 15°C (Fig. A3a). Furthermore, there was a significant two-way interaction between temperature and time ($F_{1,9}$ = 8.2, P = 0.020), indicating that there was no difference in CI between the two temperatures at week 3, but that by week 6 cockles held at 18°C had a lower CI than those held at 15°C (Fig. A3b). There were no main effects of time ($F_{1,11}$ = 3.8, P = 0.077) and pH ($F_{1,9}$ = 2.9, P = 0.12), but there was a significant effect of temperature ($F_{1,9}$ = 7.3, P = 0.025).

We observed no three-way interaction between temperature, pH and time on *respiration rates* ($F_{1,8}$ = 0.096, P = 0.76 (Fig. 2a)) and no two-way interactions between temperature and time ($F_{1,9}$ = 0.96, P = 0.35) or temperature and pH ($F_{1,8}$ = 0.020, P = 0.89). However, a significant interaction between pH and time ($F_{1,9}$ = 6.4, P = 0.033) reflected that respiration rates decreased between weeks three and six for the current pH treatment but increased between weeks three and six for the lowered pH treatment (Fig. A4). There were no main effects of time ($F_{1,11}$ = 0.18, P = 0.68), pH ($F_{1,9}$ = 2.9, P = 0.12) or temperature ($F_{1,9}$ = 1.4, P = 0.28). In terms of *clearance*

rates, there was no three-way interaction ($F_{1,8}$ = 4.9, P = 0.057) and no two-way interactions between temperature and pH ($F_{1,8}$ = 0.061, P = 0.81) or pH and time ($F_{1,9}$ = 3.1, P = 0.11). There was, however, a borderline non-significant trend for an interaction between temperature and time ($F_{1,9}$ = 5.0, P = 0.053), illustrating that there was no difference in clearance rate between the two temperatures at week 3 but that by week 6 clearance rates were greater for cockles held at 18°C compared to those held at 15°C (Fig. 2b). There were no main effects of time ($F_{1,11}$ = 3.9, P = 0.074) and pH ($F_{1,9}$ = 2.2, P = 0.17). Finally, a significant effect of temperature ($F_{1,9}$ = 6.8, P = 0.028) indicated that clearance rates increased with elevated temperature treatment as compared to the ambient temperature treatment at weeks three and six (Fig. A5). No three-way interaction between temperature, pH and time ($F_{1,8}$ = 1.09, P = 0.33), nor two-way interactions between temperature and pH ($F_{1,8}$ = 0.016, P = 0.90), temperature and time ($F_{1,9}$ = 0.0074, P = 0.93) or pH and time ($F_{1,9}$ = 0.22, P = 0.65) were found on *calcification rates* (Fig. 2c). There was no main effect of temperature ($F_{1,9}$ = 0.97, P = 0.35) on calcification rate but a significant effect of pH ($F_{1,9}$ = 5.9, P = 0.038) demonstrated a higher calcification rate under current pH as compared to low pH conditions (Fig. A6a). Furthermore, there was a significant effect of time ($F_{1,11}$ = 5.1, P = 0.045) indicating a lower calcification rate at week 3 compared to week 6 (Figure. A6b).



316

317 **Fig. 1. Effects of temperature and pH on survival (1a) and condition index (1b) after 3 and 6**
318 **weeks of incubation in the four treatments. Error bars represent standard errors (±SE).**

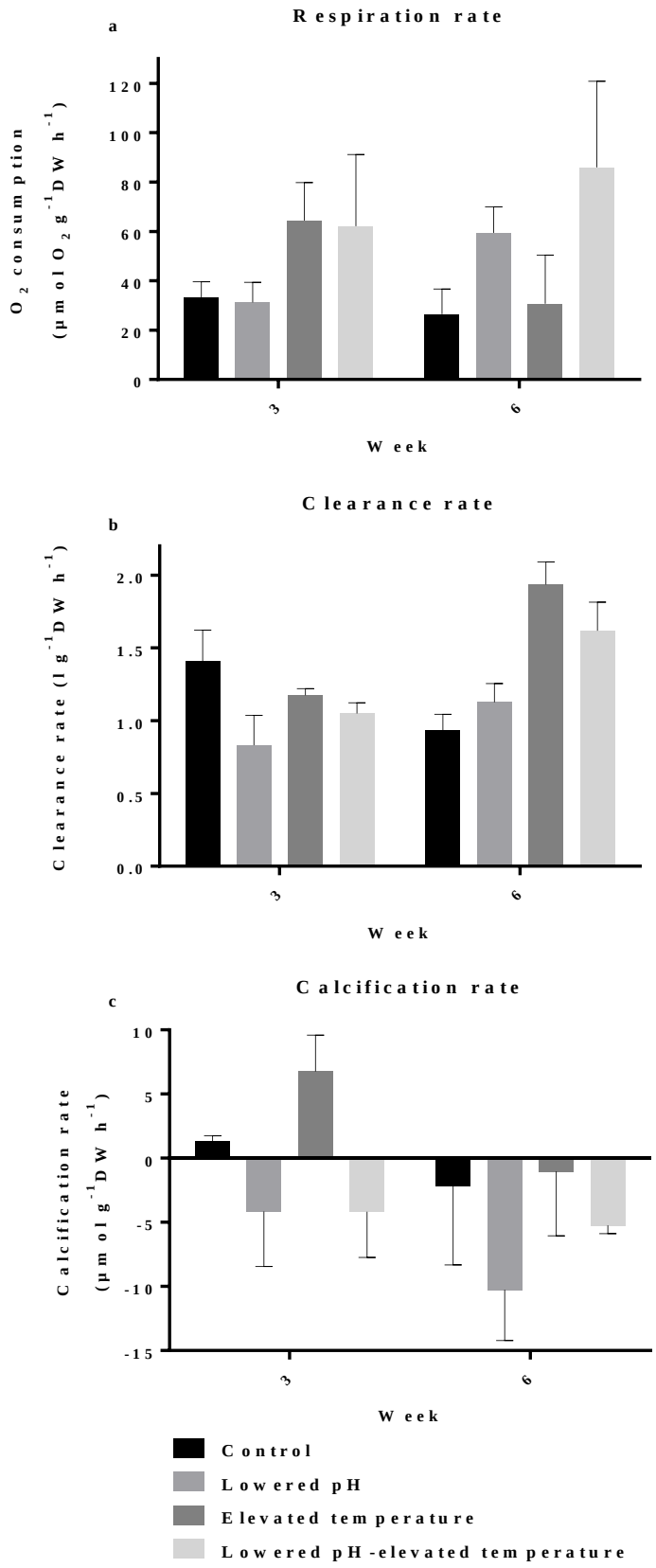


Fig. 2. Physiological responses measurement of *C. edule* (respiration, clearance and calcification rates) at week 3 and week 6 incubated in four treatments. (Error bars represent standard errors (±SE), n = 3 replicates per treatment).

4. Discussion

According to Pörtner and Farrell (2008) and Pörtner (2012), OA may narrow the thermal window of aquatic animals, probably through a reduction in functional capacity of tissue caused by the accumulation of CO_2. Our study, which mimicked the conditions projected for a future high pCO_2 ocean, i.e. warming and acidification, showed no effect of both applied stressors, alone or in combination, on the survival of cockles. Schade et al. (2016) demonstrated equally low mortality rates of the same species collected from the Baltic Sea in seawater with a pH of 7.4 to 7.8 (the latter being the ambient pH for the studied population). The lack of a mortality effect under increased temperature and lowered pH might be due to temperatures used which are well within the thermal window of the studied species (4 - 38°C (Compton et al., 2007; Rygg, 1970)). Furthermore, the 6 week duration of our experiment might have been too short to demonstrate mortality and could therefore rather represent a moderate level of environmental stress (Sokolova et al., 2012).

Bivalve condition indices represent a good reflection of the energy available for growth of bivalves (Lucas and Beninger, 1985; Nilin et al., 2012). Consequently, such indices give a realistic indication of bivalve fitness at longer time scales than can usually be studied under laboratory conditions and are therefore used to assess the condition of bivalves under variable environmental conditions (e.g. Dove and Sammut, 2007; Nilin et al., 2012; Norkko et al., 2005). We found a temperature and time effect on the CI of cockles with lower CI at the high temperature treatments after six weeks of incubation. The temperature effect corroborates Hiebenthal et al. (2013) who reported that CI of *M. edulis* and *Arctica islandica* decreased with increasing temperature. Furthermore, we also observed a synergistic effect of ocean warming and acidification on cockle condition with a pronounced additive negative effect on CI related to low pH in the combined treatment. This finding illustrates that physiological responses to low pH (see below) aggravate the negative effect of ocean warming on cockle condition

This study shows that the respiration rates of cockles were significantly higher under acidified conditions after 6 weeks of incubation, while such effect was absent after three weeks. We hypothesise that different compensatory mechanisms for energy homeostasis under low pH can explain this time-dependent effect. At the short term cockles may have compensated the enhanced costs for basal maintenance through the allocation of energy from storage tissue (lipid and glycogen), whereas at the longer term (e.g. after 6 weeks) when the storage material has been depleted, increased respiration was required to maintain homeostasis (Sokolova et al.,

2012) and support the need for a higher expression in biomineralization-related enzymes and acid-base regulation (Beniash et al., 2010). Similar upregulation of the aerobic respiration after two months of incubation at a pH 7.70 was reported by Thomsen and Melzner (2010) for blue mussels (*M. edulis*) and after an 11-weeks of incubation at pH 7.5 by Beniash et al., (2010) for *C. virginica*. Furthermore, increased oxygen uptake with decreasing pH is found in other marine taxa as well, e.g. ophiuroid brittlestar (*Amphiura filiformis*) and arctic pteropods (*Limacina helicina* (Comeau et al., 2010; Wood et al., 2008)). Opposite effects have also been demonstrated, e.g. for *M. chilensis* (Navarro et al., 2013) and juvenile and adult Mediterranean mussels (*M. galloprovincialis*) (Michaelidis et al., 2005), but Gazeau et al. (2014) and Fernández-Reiriz et al. (2012) found no effect of pH on respiration in the latter species.

Since no pseudofaeces production was observed during the measurements the rate of food ingestion equals the rate of clearance multiplied by the cell concentration of the diet (Iglesias et al., 1992; Lu and Blake, 1997). In this study, clearance rates were predominantly affected by temperature with higher clearance rates in the elevated temperature treatments. Similarly, Smaal et al. (1997) found a positive relation between cockle clearance rates and elevated temperature in a field setting, which indicates that the cockles incubated under laboratory conditions in this study reacted similarly to those from the field. Walne (1972) observed that clearance rates of *Ostrea edulis* scaled down by 45%, and of *C. gigas* and *M. edulis* by 25% when temperature was lowered from 20 to 10°C. In general, clearance rates in our study were lower under low pH conditions but this effect was not statistically significant ($p = 0.17$). However, both short and long-term observations on adult mussels (*M. chilensis*), juvenile clams (*R. decussatus*), adult noble scallops (*Chlamys nobilis*) and adult green-lipped mussels (*Perna viridis*) have demonstrated negative clearance rate responses to low pH (Fernández-Reiriz et al., 2011; Liu and He, 2012; Navarro et al., 2013)). In general, marine bivalves thus seem to reduce costs related to filtration in low-pH waters rather than to ingest more food to cope with hypercapnia.

Shell dissolution rates of bivalves are often associated with the properties of a protective external organic layer or periostracum that separates the shell from the ambient seawater (Ries et al., 2009). As cockle has a thin periostracum of around 2µm (Hall-Spencer et al., 2008) and its shell is solely composed of aragonite - the most soluble polymorph of carbonate (Cubillas et al., 2005; Taylor, 1973), cockles may be particularly vulnerable to OA. We observed a reduction in calcification rates at lowered pH despite the fact that the seawater remained oversaturated with respect to aragonite ($\Omega_{aragonite} = 3.7$ and 3.6 in lowered pH and lowered pH-

elevated temperature treatments, respectively). Similarly, e.g. Beniash et al. (2010) and Ries et al. (2009) found a reduction of calcification of juvenile and adult oysters (*C. virginica*) in low-pH seawater that remained oversaturated with respect to calcite and aragonite. Clearly, the reduction of calcification or dissolution of bivalves does not solely depend on the calcite and aragonite saturation state. According to Cyronak et al. (2016), elevated proton concentrations (H^+), rather than the concentration of carbonate ions (CO_3^{2-}), is likely to be responsible for the reduction of calcification rates of marine calcifying organisms. Elevated [H^+] in acidic seawater alters the proton gradient between intracellular fluid and external seawater, thus, hampering calcifying organisms from maintaining pH homeostasis (Cyronak et al., 2016). A recent study on the blue mussel *M. edulis* indicated that seawater [HCO_3^-]/[H^+] ratio is crucial in regulating calcification rates of the mussel (Thomsen et al., 2015). Our result shows that calcification rates were lower at week 3 (1st July 2015) than that of week 6 (28th July 2015 (Fig. A6b)). We hypothesise that this reduction in calcification over the course of the experiment results from a shift in energy allocation. Cockles start to spawn at the site of collection around July (Rueda et al., 2005) and spawning cockles were observed in the last week of the experiment. It therefore seems likely that cockles have allocated relatively more energy towards gonad production than to calcification by the end of the 6-week incubation.

In summary, our results indicate synergistic effects of ocean warming and acidification on the condition index of the common cockle *C. edule*. Since synergistic effects were not found for the separate physiological responses addressed in this study, the interactive effect of OA and warming on cockle condition must be explained by the cumulative impact of different responses. We hypothesize in *C. edule* that the reduced food intake under low pH conditions is insufficient to support the higher energy requirements in future high pCO_2 oceans to compensate for (1) higher basal maintenance in warmer and more acidic waters, and (2) growth in low-pH waters. Furthermore, cockles may allocate additional energy from energy storage pools to cover the increasing maintenance demand (e.g. tissue repair and maintenance) but this mechanism will leave less energy available for reproduction and growth, which will in turn likely have repercussions on population of cockles and their roles in ecosystem functioning, e.g. their influence on recruitment of other benthic species (Flach, 1996; Van Colen et al., 2013), the mediation of benthic primary production (Swanberg, 1991), and as a food source for higher trophic levels ((Beukema and Cadée, 1996)). In order to disentangle the different mechanisms that will determine future population stability of bivalves, and of benthic invertebrates in

general, future research needs to address the different components that govern energy allocation under multiple, combined stressor scenarios.

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685

686 **Appendices**

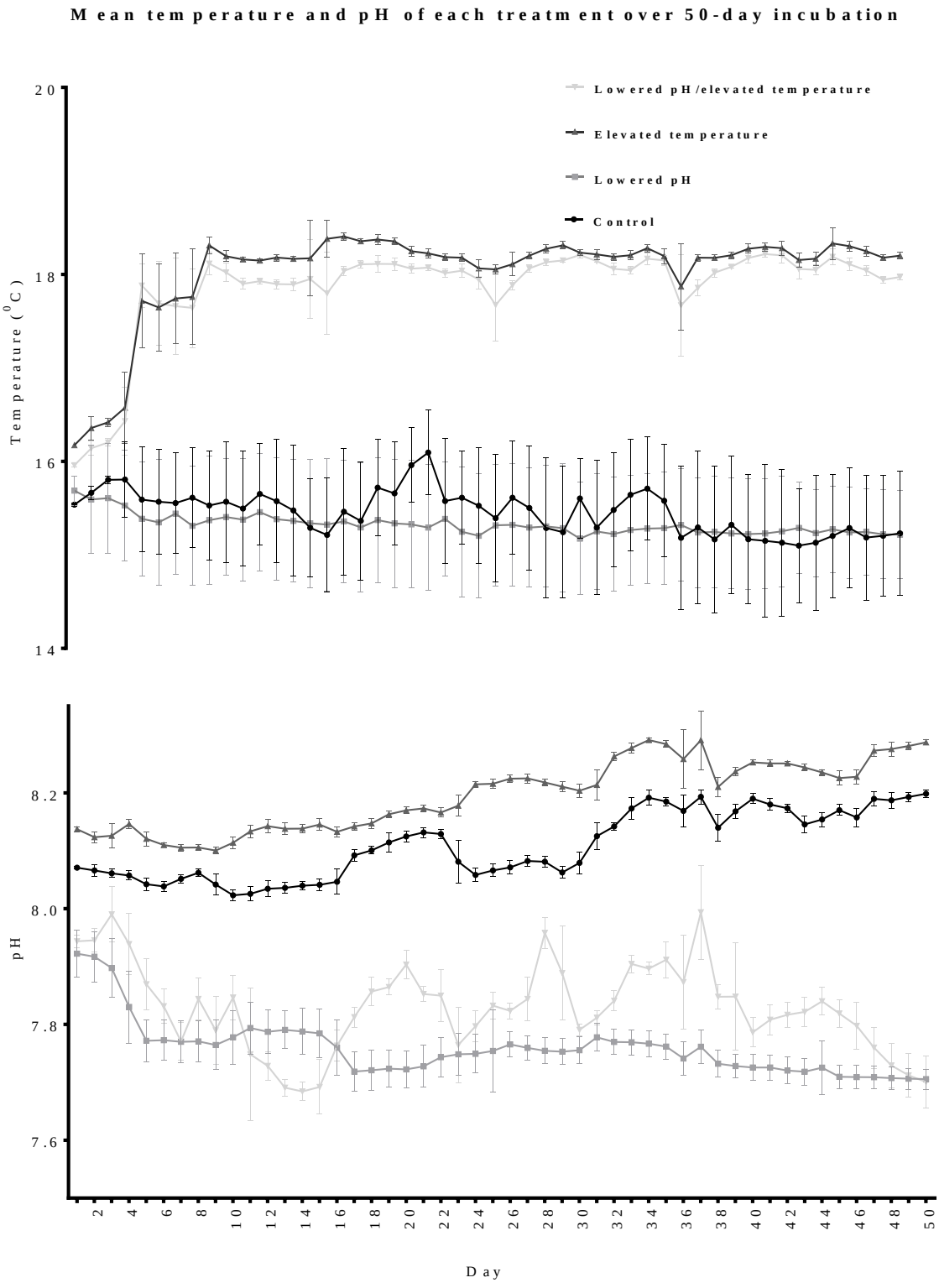
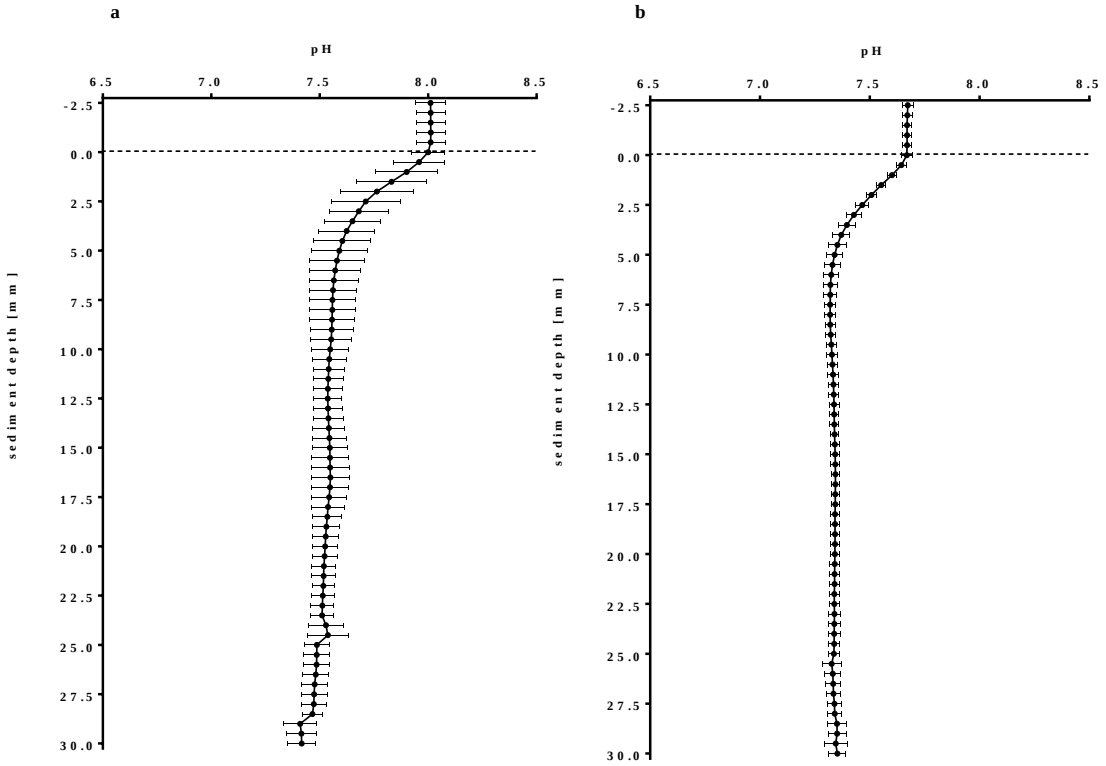


Fig. A1. Variations of daily temperature and pH in four treatments for 50-day incubat*ion.* The experiment started at 6th day of the incubation. Two conditional and physiological responses measurements were conducted at day-26 and day-47. Error bars represent standard deviation (±SD).



692

693 **Fig. A2. pH sediment profile of muddy sand sediment at current pH (a) and lowered pH (b).**

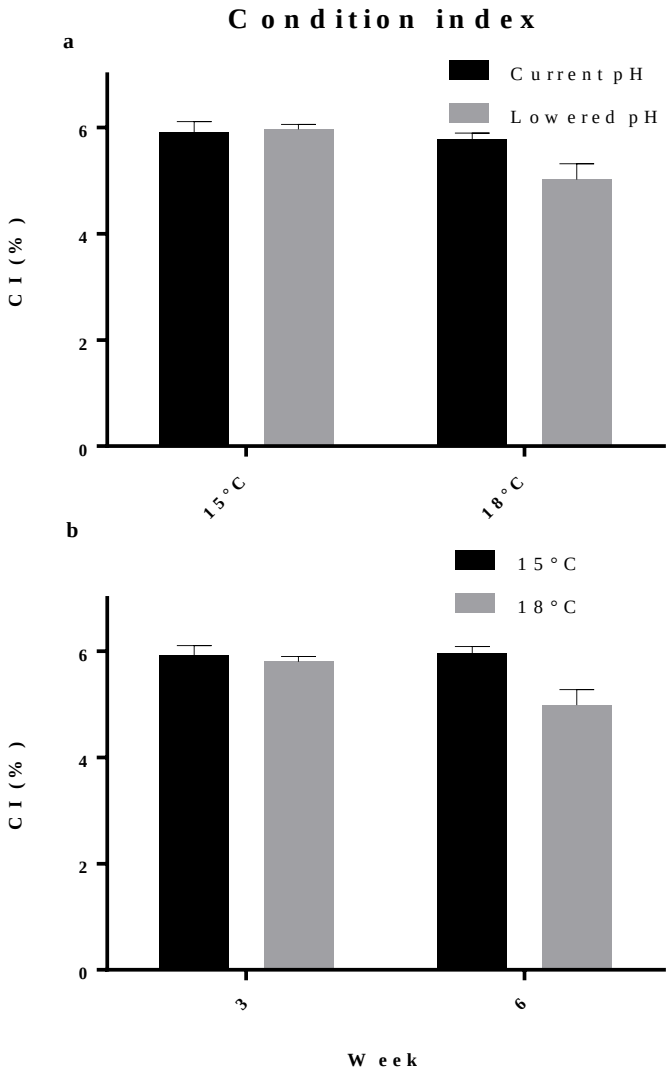
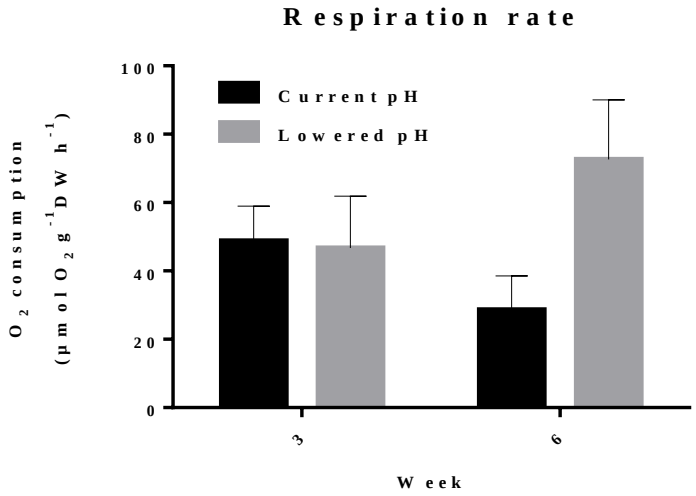
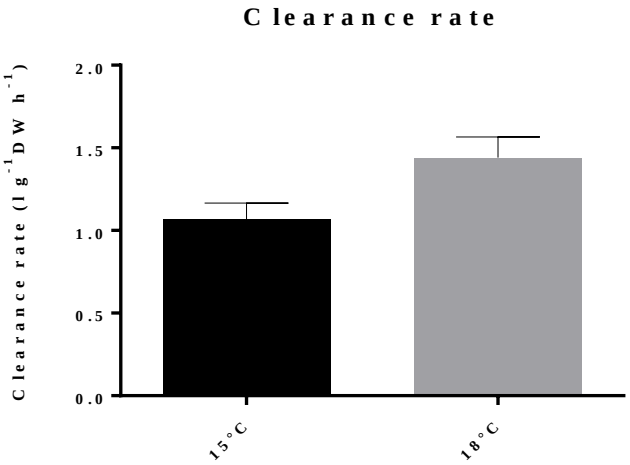


Fig. A3. Condition indices of cockles. a) The interaction effect between temperature and pH and b) the interaction effect between temperature and time. Error bars show standard errors.



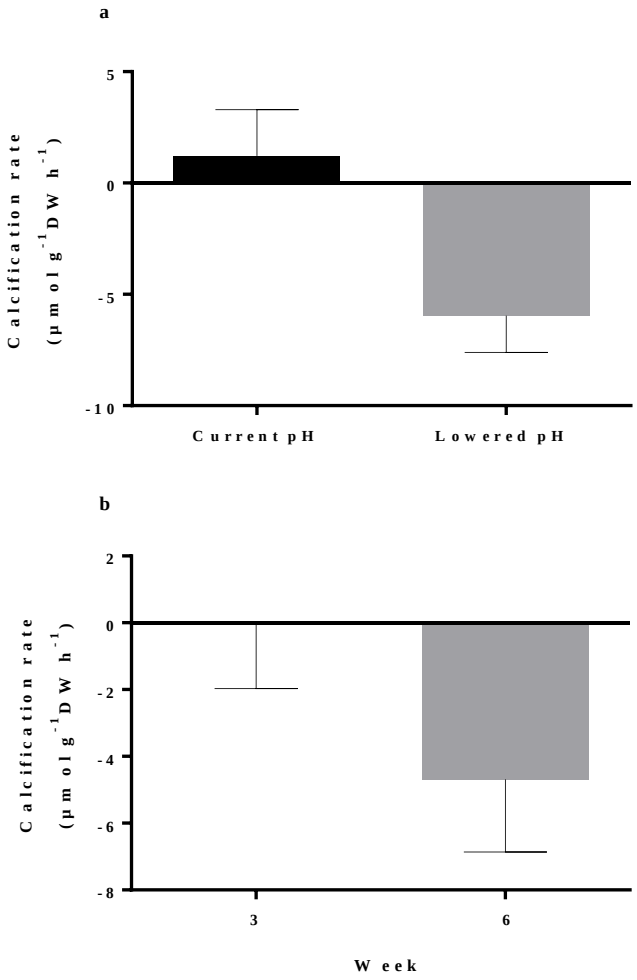
697

698 **Fig. A4. The interaction effect between time and pH. Analysis was conducted on Log_{10}**
699 **transformed data but raw data are plotted here. Error bars show standard errors.**



700

701 **Fig. A5. The main effect of temperature. Analysis was conducted on Log_{10} transformed data but**
702 **raw data are plotted here. Error bars show standard errors.**



703

704 **Fig. A6. Calcification of cockles. a) The main effect between pH and b) the main effect of time.**
705 **Error bars show standard errors.**