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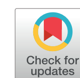
Cellular level response of the bivalve *Limecola balthica* to seawater acidification due to potential CO_2 leakage from a sub-seabed storage site in the southern Baltic Sea: TiTank experiment at representative hydrostatic pressure



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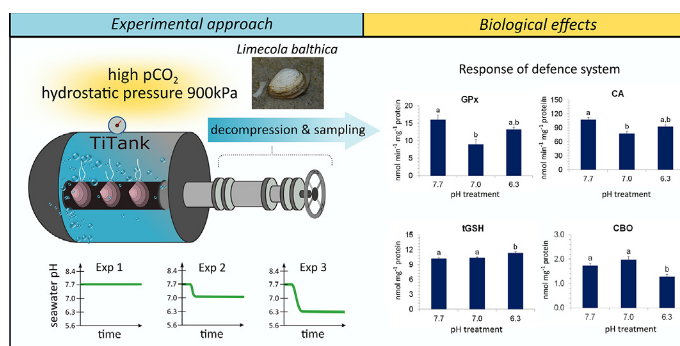
HIGHLIGHTS

- Cellular level responses of *L. balthica* to acidification caused by CO_2 was tested at 9 ATM pressure.
- The bivalve is tolerant to medium-term severe environmental hypercapnia.
- Seawater pH 7.0 induced effects on radical defence mechanisms (GPx, GST, CAT).
- pH 6.3 caused increased cellular oxidative stress (MDA) and detoxification (tGSH).

GRAPHICAL ABSTRACT



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ABSTRACT

Understanding of biological responses of marine fauna to seawater acidification due to potential CO_2 leakage from sub-seabed storage sites has improved recently, providing support to CCS environmental risk assessment. Physiological responses of benthic organisms to ambient hypercapnia have been previously investigated but rarely at the cellular level, particularly in areas of less common geochemical and ecological conditions such as brackish water and/or reduced oxygen levels. In this study, CO_2-related responses of oxygen-dependent, antioxidant and detoxification systems as well as markers of neurotoxicity and acid-base balance in the Baltic clam *Limecola balthica* from the Baltic Sea were quantified in 50-day experiments. Experimental conditions included CO_2 addition producing pH levels of 7.7, 7.0 and 6.3, respectively and hydrostatic pressure 900 kPa, simulating realistic seawater acidities following a CO_2 seepage accident at the potential CO_2-storage site in the Baltic. Reduced pH interfered with most biomarkers studied, and modifications to lactate dehydrogenase and malate dehydrogenase indicate that aerobiosis was a dominant energy production pathway. Hypercapnic stress was most evident in bivalves exposed to moderately acidic seawater environment (pH 7.0), showing a decrease of glutathione peroxidase activity, activation of catalase and suppression of glutathione S-transferase activity likely

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Limecola balthica
Representative hydrostatic pressure

in response to enhanced free radical production. The clams subjected to pH 7.0 also demonstrated acetylcholinesterase activation that might be linked to prolonged impact of contaminants released from sediment. The most acidified conditions (pH 6.3) stimulated glutathione and malondialdehyde concentration in the bivalve tissue suggesting potential cell damage. Temporal variations of most biomarkers imply that after a 10-to-15-day initial phase of an acute disturbance, the metabolic and antioxidant defence systems recovered their capacities.
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1. Introduction

In efforts to mitigate CO_2 release into the atmosphere and subsequently to prevent its dissolution and accumulation into the oceans, carbon capture and storage (CCS) has been proposed as a relatively safe technology with little likelihood of negative consequences to the marine ecosystem (Damen et al., 2006). Sub-seabed CCS systems are designed to capture point source carbon emissions and store them in geological rock formations below the sea floor such as saline aquifers and existing or abandoned hydrocarbon reservoirs (Reguera et al., 2009). Such reservoirs are generally roofed by rock layers of considerable thickness, generally considered leak-proof (Lessin et al., 2016), but geological processes or drilling activity may nevertheless open cracks or channels allowing CO_2 seeps to the seafloor (Queirós et al., 2015; Amaro et al., 2018).

Potential leaking of CO_2 from the storage reservoir into the surface seafloor sediments and the water column will lower pH in the vicinity of the leakage point, particularly in the interstitial and closely overlying waters (Amaro et al., 2018). Consequently, carbonate geochemistry in the bottom zone will change, and biological traits of sessile benthic organisms, which inhabit the surface sediments, will most likely be affected (Lessin et al., 2016).

Investigations of biological responses to severe seawater acidification over the recent years have expanded into a range of approaches i.e., laboratory and mesocosm experiments (e.g., Conradi et al., 2019), exposure experiments on organisms in situ (e.g., Molari et al., 2019) and studies at analogue sites (natural CO_2 seeps; e.g., Lucey et al., 2018). As a consequence, understanding of the physiological and ecological responses to increased CO_2 concentrations has improved considerably providing important support to environmental risk assessment in coastal and off-shore areas where CCS technology is considered (Carroll et al., 2014; Blackford et al., 2015; Sokołowski et al., 2018, 2020). While some biological endpoints such as mortality, behaviour, growth, shell erosion and physiological state were studied extensively in a number of invertebrate taxa (e.g., Basallote et al., 2012; Kurihara et al., 2013; Freitas et al., 2017; Vargas et al., 2017; Zhao et al., 2017; Liao et al., 2019), cellular level responses of infaunal bivalves to hypercapnic conditions (e.g., acetylcholinesterase, lipid peroxidation, catalase, glutathione S-transferase and reduced glutathione) have been addressed rarely in the context of potential CO_2 leakage from CCS storage sites (Jeeva Priya et al., 2017). Such research is thus required (Clements and Hunt, 2017), particularly in areas of less common but important environmental and ecological conditions such as the Baltic Sea (Sokołowski et al., 2018). Early-warning signals of occurring metabolic disruptions, internal acid-base imbalance and reactive oxygen species (ROS) overproduction due to hypercapnic stress, include low molecular weight scavengers and enzymes of the energetic metabolism such as lactate dehydrogenase (LDH), malate dehydrogenase (MDH), octopine dehydrogenase (ODH) and carbonic anhydrase (CA) as well as glutathione peroxidase (GPx), catalase (CAT) and superoxide dismutase (SOD) (Dahlhoff et al., 2002; Jeeva Priya et al., 2017; Freitas et al., 2017; Huang et al., 2018; Liao et al., 2019). Seawater acidification has also been shown to bring about indirect biological effects, for example through remobilisation of toxicants, which in turn affect detoxification systems (e.g., glutathione S-transferase, GST) and induce neurotoxic disruptions in marine invertebrates (e.g., acetylcholinesterase, AChE) (Matozzo et al., 2005).

As the solubility of CO_2 increases with hydrostatic pressure and declining temperature (Oldenburg and Lewicki, 2006) behaviour of carbon dioxide molecules and geochemical processes in seawater and sediment in deeper waters deviate from surface layers. For example, at a depth of 300 m and typical seafloor temperatures (3–7 °C) seeped CO_2 is expected to be dissolved and transferred by advective processes rather than escaping as gas bubbles (Oldenburg and Lewicki, 2006). As CO_2 leaking from sub-seabed storage sites will generally reach the seafloor at 100–300 m water depth, biological responses (particularly biochemical systems) of subsequent increased acidity in the bottom zone may therefore perform in a different way from what can be predicted from the laboratory exposures or in in situ shallow coastal experiments at atmospheric pressure 100 kPa (1 bar). Accordingly, the first laboratory research on impacts of possible CO_2 seepage on water chemistry at elevated hydrostatic pressure (3000 kPa) revealed weakening of the Fe/Mn shuttle, the release from sediment to water column of some metals such as Co, Ni and Ce and hence an increase of their bioavailability (Basallote et al., 2020). Elevated hydrostatic pressure was also proved to directly impact on biological responses such as enzymatic activities (e.g., LDH and MDH) and biochemical processes (ligand binding, protein catalysis) in marine organisms (Somero, 1990). Further scientific studies closely mimicking real environmental conditions, including high pressure and low temperature, are therefore needed (Ardelan et al., 2012; Basallote et al., 2020).

This study was set up to investigate responses of the infaunal bivalve, the Baltic clam *Limecola balthica* (formerly *Macoma balthica*), to seawater acidification induced by increased CO_2 levels. In the Baltic soft sediments, the bivalve can be found in large numbers both in inner semi-enclosed basins, e.g., the Gulf of Gdańsk (up to 990 ind. m^{-2}; Sokołowski, 2009), and in deeper open waters including the potential CO_2-storage site (B3 field) in the Polish Exclusive Economic Zone (several ind. m^{-2}; ECO2, 2014), often dominating the biomass of benthic communities (Jansson et al., 2013). The species has been shown to pose high resistance to environmental stress induced by low oxygen concentrations and the formation of hydrogen sulphide (e.g., Jahn et al., 1997), and changes in pH (e.g., Sokołowski et al., 2018). The study was performed as a laboratory simulation of potential leakage from sub-seabed storage site at hydrostatic pressure 900 kPa and seawater pHs of 7.7, 7.0 and 6.3. Modifications to cellular level responses in *L. balthica*, including oxygen-dependent system (LDH, MDH and ODH), antioxidant system (CAT, SOD, tGSH, GPx, MDA, CBO) and detoxification system (GST) were assessed. In addition, markers of neurotoxicity (AChE) and acid-base balance (shell calcification enzyme CA) were quantified.

2. Material and methods

2.1. Sampling and sample treatment

Model organisms (i.e., the bivalve *L. balthica*) and sediments were collected from the same geographic location. Sampling campaigns were made only in the cold seasons (April and October 2016, and January 2017 corresponding to early spring, autumn and winter, Table 1) in order to collect sediments of reduced presence of freshly deposited organic matter, to avoid variation in the gametogenetic stage of bivalves and to minimise the effect of environmental conditions

Table 1
Sampling dates and hydrological variables of the overlying bottom water at the sampling site (MW) on different sampling occasions. Transportation period refers to departure day from Gdynia (Poland) and arrival day to Trondheim (Norway).

Treatment			
	pH = 7.7	pH = 7.0	pH = 6.3
Sampling date	02.01.2017	20.10.2016	25.04.2016
T (°C)	4.9	9.7	5.9
S	7.3	7.3	7.4
Dissolved O_2 (mg dm^{-3})	no data	8.40	14.9
pH	8.03	7.89	8.50
Transportation period	8–9.01.2017	28–29.10.2016	2–3.05.2016
Temperature during transportation (°C)	5.6–9.0	9.9–12.7	8.6–9.5

(e.g., food availability and quality) on their physiological performance (Honkoop and van der Meer, 1997; Cardoso et al., 2007).

The infaunal Baltic clams *L. balthica* (Linnaeus, 1758) and sediments were collected during a series of cruises onboard r/v Oceanograf at one coastal site MW (φ 54°37′30.6″N, λ 18°37′25.8″E, water depth 33 m) that is located in the external part of Puck Bay, the Gulf of Gdańsk, southern Baltic Sea. The sampling site was selected to represent sedimentary and geo-chemical conditions similar to those in the bottom zone of the B3 field (water depth 80 m and a depth of storage reservoir under the seabed 1450 m) (ECO2, 2014). It was therefore assumed that the bivalves from the sampling site and those inhabiting the sea floor in the B3 field present similar physiological traits. Basic hydrological variables in the overlying bottom water in the vicinity of the MW site change seasonally within a range 0.0–>10.0 mg dm^{-3}, 1.6–21.0 °C, 6.8–7.2 and 7.4–8.5 for dissolved oxygen, temperature, salinity and pH, respectively (Sokołowski, 2009).

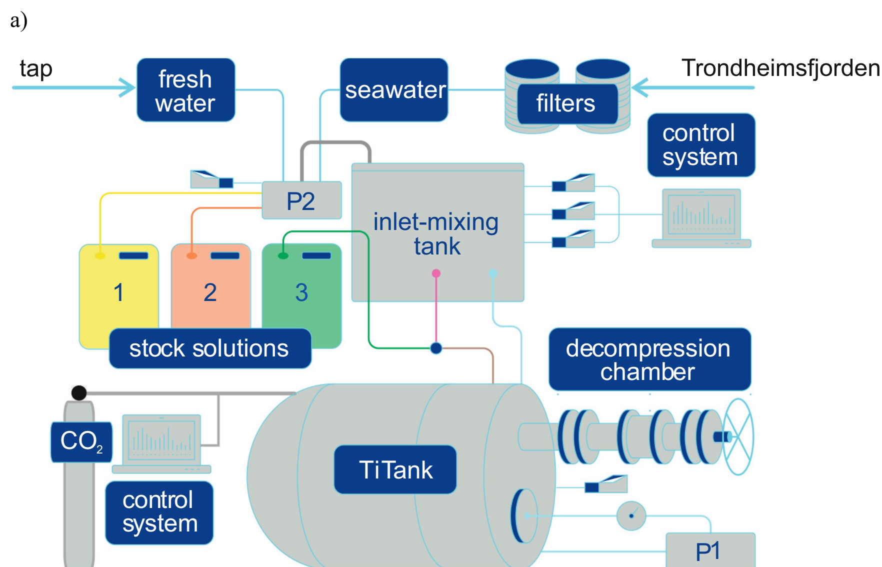
The bivalves were collected using a standard benthic dredge. Living individuals were sorted from the dredge samples according to shell length to fall in a range between 11.44 and 15.00 mm which represent the most numerous fraction of the population (Jansen et al., 2007). After collection, the bivalves were kept in containers filled with aerated seawater taken in situ. Surface sediments were sampled with a van Veen grab (catch area 0.1 m^2). The topmost layer (0–1 cm) was scooped out to a polypropylene container (24 dm^3) while the next 3 cm thick layer was transferred to separate containers (24 dm^3). These fractions served to form the top 1 cm thick and underlying 3 cm thick sediment layers in the experimental trays. Sediments in containers were covered with the overlying bottom seawater taken at the sampling site to prevent from desiccation and reduce temperature change. In addition, the overlying bottom water (ca. 0.2 m above the sea floor) was collected with a 5 dm^3 GoFlo Niskin water sampler to record basic hydrological parameters with a portable WTW Multiset 340i meter (Table 1). On each sampling occasion in the period of from April 2016 to January 2017, a total of 500 clams were collected to provide a sufficient number of individuals for the laboratory experiments. Immediately after sampling, materials were transported to the laboratory where they were pre-acclimated in temperature-controlled laboratory to experimental thermal conditions ($T = 10$ °C) by increasing seawater temperature at a rate 1 °C 24 h^{-1} for maximum 8 days before transportation to Norway for the exposure studies. Seawater temperature in containers with sediments and animals was raised by increasing air temperature using the automatic air-conditioning system. For transportation from the Institute of Oceanography, University of Gdańsk (Gdynia, Poland) to NTNU SeaLab (Trondheim, Norway) a car equipped with a temperature control unit was used. During transportation, the clams were wrapped in wet paper tissue and kept in thermo-isolated polystyrene boxes while sediments were covered with seawater and kept in polypropylene containers. Water temperature inside the boxes and containers was monitored continuously with autonomous automatic loggers HOBO UX100-01 M, and varied within a range 5.6–12.0 °C (Table 1). Pressure inside of the transportation containers was maintained at atmospheric conditions. Total time of shipment did not exceed 48 h in any case.

2.2. Experimental set-up

All experiments were conducted in a hyperbaric tank (Karl Erik TiTank; Fig. 1) operated at the NTNU SeaLab (Trondheim, Norway). The TiTank is constructed from titanium minimising the risk of contamination during the experiments. The tank is a flow-through high-pressure chamber of a total volume of 1.4 m^3 that can attain a pressure of up to 3000 kPa (30 bars). For the needs of the present study, the tank was equipped with a rotating custom-designed cylindrical sample-holder (so called carousel) made of titanium and polyvinyl chloride components (PVC) which can hold up to 51 individual PVC trays and an internal mechanism to transfer samples to a decompression chamber. Such a construction has been already used with success for other studies on seawater acidification at increased hydrostatic pressure (Borrero-Santiago et al., 2020; Bonnail et al., 2021). The water quality used in the experiments corresponds to pH, salinity and total alkalinity recorded in the southern Baltic (Müller et al., 2016). Brackish water (named later as stock brackish water; mean ± SE; final salinity 7.0 ± 0.2 $n = 2506$) was produced by a continuous mixture of marine water from the Trondheimsfjorden (range of salinity 32–33, pH 8.0–8.1 and total alkalinity = 2240–2300) and tap water from the Jonsvatnet reservoir (salinity = 0, range of pH 8.1–8.4 and total alkalinity 1200–1350) (Ardelan, pers. comm.). To adjust and stabilise pH and total alkalinity to 7.7 and 1900 μmol kg^{-1}, respectively, stock solutions of $NaHCO_3$ (0.095 M) and HCl (0.013 M) were continuously added by a peristaltic pump. The peristaltic pump also supplied phytoplankton as food to the bivalves during the experiments (Fig. 1). Temperature and salinity of the prepared water were continuously monitored in the inlet-mixing tank with a Thermo Scientific Orion 4 Star Muliparameter meter.

All experiments were conducted at constant flow-through water rate of 0.452 ± 0.002 dm^3 min^{-1}. Basic hydrological parameters (temperature, pH and redox potential) were monitored every 2 h in a measurement tube at the outlet from the tank with the Mettler Toledo M300 meter while salinity and oxygen saturation were recorded every 1.5 h with a Hach Sension 6 and Hach HQ20 meter, respectively. The values from the M300 and Hach meters were crosschecked weekly against values measured by regularly calibrated laboratory meters. Because the electrochemical potential measured by pH glass electrodes is dependent upon seawater variables (e.g., salinity, temperature), calibration was performed to ensure that the electrodes were not exhibiting non-Nernstian behaviour. The electrodes were calibrated with the reference TRIS buffers within a range of pH and ionic strength expected in each experiment. Hydrostatic pressure was measured automatically in a continuous way with high-pressure sensor, recorded once a day and crosschecked manually with a standard manometer mounted to the top of the tank. In addition, triplicate water samples from the TiTank outflow were collected in polyethylene bottles to measure total alkalinity (TA; Wolf-Gladrow et al., 2007) on each sampling occasion. Acidification of the seawater in the experimental system was induced by injecting gaseous carbon dioxide directly into the water inflow stream leading to the TiTank. The amount of CO_2 added was controlled automatically by a mass-flow controller (Bronkhorst High-Tech B.V.) based on water total alkalinity and regular pH measurements with the use of software Flowcode 8 (Matrix TSL) for microcontrollers. Due to the one-chamber construction of the experimental system (the hyperbaric TiTank) exposure experiments were conducted separately for each seawater pH. Three separate experiments were run at different seawater pHs: pH 7.7 - ambient seawater, 7.0 and 6.3 corresponding to pCO_2 1080 μatm, 5536 μatm and 27,877 μatm, respectively (Robbins et al., 2010) for a duration of 50 days each. The partial pressure of carbon dioxide was calculated using the CO2calc 1.2.0 calculator (https://pubs.usgs.gov) where mean physical parameters and total alkalinity for the exposure period were used as input constants with default settings for

a)



b)

Fig. 1. a) Schematic diagram of the flow-through high pressure experimental system with the hyperbaric Karl Erik TiTank. 1-$NaHCO_3$ solution, 2-HCl solution, 3 – mixed algae suspension, P1-hydrostatic pressure pump, P2-peristaltic pump; b) view from rear sight with decompression chamber and sluice.

CO_2 constants, i.e. K1, K2 from Mehrbach et al. (1973) and refit by Dickson and Millero (1987), $KHSO_4$ (Dickson, 1990), total boron (Uppstrom, 1974) and air-sea flux (Wanninkhof, 1992). The CO2calc 1.2.0 calculator was also used to calculate other CO_2 system parameters of the stock brackish water in all pH experiments (Table A in *Appendix I*). Experimental pH conditions were selected to mimic changes in seawater acidity in the bottom layer following a scenario of CO_2 plume distribution after a potential leakage from a CCS sub-seabed site in the southern Baltic Sea (Grimstad et al., 2009; ECO2, 2014; Schade et al., 2016).

For each experiment (experiments at seawater pH 7.7, 7.0 and 6.3), 51 polyvinylchloride trays of cylindrical shape (40 cm × 7 cm) were filled with Baltic sediments in such a way that a deeper 3 cm thick layer was moulded from deeper sediments and a 1 cm cover layer was formed from top sediments, reflecting the observed sediment lamination in the field. The sediments were then left to stabilise submerged in stock brackish water at temperature 10 °C for 24 h in large containers. During this time, the clams were kept in aerated Baltic Sea water that was sampled at MW in the Gulf of Gdańsk and brought in polypropylene containers from Poland. Only individuals showing locomotory activity, i.e., extended either their siphons and/or foot and not having any morphological damage of shell, were selected for the experiments. 14 clams were assigned randomly to each of 24 trays and allowed to bury with each experiment containing a total of 336 individuals. The final density of the bivalves was 700 ind. m^{-2}, which corresponded to their densities in the organic-rich bottom of the Gulf of Gdańsk (Warzocha et al., 2018). Ragworms *Hediste diversicolor* from the Baltic Sea were put in the remaining 15 trays and 12 trays remained without animals; those containers were a subject of another investigation. The experimental trays were then placed onto the carousel that was positioned within the high-pressure chamber, which was subsequently filled with stock brackish water. A high-performance pump increased

hydrostatic pressure in the TiTank to 900 kPa (9 bars) which simulated the pressure conditions at the potential CO_2 sub-seabed storage site (B3 field) in the southern Baltic Sea (water depth 80 m).

During the experiment, the bivalves were fed by dosing an algal solution at a rate 4 cm^{-3} min^{-1} directly to the water inlet of the high-pressure chamber. The stock solution contained live algae of three phytoplankton species *Dunaliella tertiolecta*, *Rhodomonas baltica* and *Isochrysis galbana* measuring from 3 μm to 15 μm cell size. The algal stock solutions were mixed de novo every two days in a proportion of 1:2:4 (v/v/v) to a total volume of 10 dm^3 that yielded the final concentration of 2×10^5 cells cm^{-3} and a dose of 2.3×10^6 cell $ind.^{-1}$ per day. In order to maintain a similar food supply per individual throughout the experiment, the feeding dose was reduced proportionally to the decreasing number of the clams in the TiTank following sampling.

2.3. Sampling protocol

In each experiment, before acidification started, the clams were acclimated to experimental conditions at ambient pH (7.7), constant temperature (10 °C) and hydrostatic pressure (900 kPa) in the TiTank over 10 days (Thompson et al., 2012). Then, in the pH-reduced experiments, injection of CO_2 commenced but since seawater pH in the TiTank was reduced gradually and reached a target level within 48 h, the experiment commenced on the day CO_2 injection began and ran for 40 days. The bivalves were sampled in triplicate (three trays, 14 individuals each) at different time intervals corresponding to the following CO_2-exposure days: acclimatisation end and start of acidification (0 d), and then three days (3 d), 10 d, 15 d, 20 d, 30 d and 40 d after acidification began. Each tray selected for sampling was positioned by rotating the sample holder in front of an open valve (diameter 10 cm) leading into the pressurized decompression chamber. The tray was pushed through the valve into the decompression chamber by an internal linear actuator system, whereafter the valve connection to the main tank was closed. The sample was then manually decompressed over several minutes in such a way that the pressure inside the tank did not change. After decreasing pressure to 100 kPa, the decompression chamber was drained, leaving only a small amount of water above the sediment in the sample tray. The chamber was then opened so that the tray could be collected for sampling of animals. Out of the 42 individuals collected at each time point, 12 clams (four individuals from each tray) were deshelled, and the soft tissue of each specimen was frozen individually at −80 °C.

2.4. Biomarker analyses

Frozen soft tissues of the bivalves were homogenized individually to obtain 20% (w/v) homogenates with ice-cold Tris sulphate buffer (50 mM Tris-H_2SO_4, 0.1 mM EDTA, 1 mM PMFS, 2 mM DTT, TritonX-100, pH 7.8) in a glass tissue grinder. The homogenates were then centrifuged at 14000 RCF and 4 °C for 30 min. The supernatants containing a suspended cytosolic fraction were collected in clean tubes and kept in ice prior to bioassays. The following components of cellular defence systems were selected for analysis to assess the response of *L. balthica* to reduction of seawater pH: oxygen-dependent system (lactate dehydrogenase, EC 1.1.1.27 (LDH); malate dehydrogenase, EC 1.1. 1.37 (MDH) and octopine dehydrogenase, EC 1.5.1.11 (ODH); shell calcification system (carbonic anhydrase, EC 4.2.1.1 (CA); antioxidant and detoxification system (glutathione (tGSH = GSH + GSSH); glutathione peroxidase, EC 1.11.1.9 (GPx); glutathione S-transferase, EC 2.5.1.18 (GST); catalase, EC 1.11.1.6 (CAT) and superoxide dismutase, EC 1.15.1.1 (SOD); neurotoxicity (acetylcholinesterase, EC 3.1.1.7 (AChE), lipid peroxidation (malondialdehyde, MDA) and protein peroxidation (carbonyl groups, CBO). The cytosolic enzyme activity was measured spectrophotometrically (UV-VIS Beckman Coulter Spectrophotometer) based on methods adopted for standard macro polystyrene cuvettes (3 cm^3) and microplate reads (Synergy HTX, BioTek plate reader).

The cytosolic protein content was measured according to the method described by Lowry et al. (1951) with modifications of Peterson (1977) at a wavelength of 750 nm using bovine serum albumin (BSA) as a standard.

Activities of the enzymes and concentrations of total glutathione, MDA and CBO were measured using standard bioassay methods and commercial kits, description of the methods used were presented in detail in *Appendix II*.

2.5. Statistical analysis

Due to high inter-individual variability of enzyme activity in soft tissue of marine organisms (Wheelock et al., 2005; Kurman et al., 2017) univariate variance test (based on the mean and the standard deviation (SD) multiplied by two) was used post-hoc to detect and remove outlining data from the dataset. Analyses of normality (the Shapiro-Wilk test for goodness of fit) and homogeneity of variances (Levene's test) were performed on raw data as prerequisites to the parametric approach. Due to intended change of seawater acidity in two treatments after acclimatisation, parametric test assumptions for pH data were verified only for the exposure period. Analyses of differences in the initial activity of enzymes or concentrations at the beginning of the exposure period (ANOVA, data not shown) revealed significant variations for most biomarkers, the exceptions were LDH activity and tGSH and MDA levels, which did not differ among treatments on 0 d. Thus, it was necessary to employ adjustment to allow for this effect for nine biomarkers, and, for consistency, adjustment was also applied in the case of the remaining three. The adjustment procedure was performed on average data individually for each biomarker by dividing a value obtained on a given sampling occasion by an adjustment coefficient (AC). The adjustment coefficient was calculated as the ratio of biomarker value in a given treatment on 0 d and the least value of the same biomarker out of three treatments that occurred on 0 d. In addition, to track relative temporal variation and regulation patterns of biomarkers normalisation was employed by dividing the mean value obtained on a given sampling occasion by the respective value on 0 d times 100 within the same treatment. The significance of differences between variables for main effects (seawater pH and exposure time) and interactions between them was estimated on 0d-ajusted data by analysis of variance (ANOVA) followed by the Bonferroni post-hoc test (α/n) when F was significant, and between two variables by paired *t*-test. The functional relation between pairs of variables was described with Pearson correlation analysis. When assumption of normal distribution of data was not fulfilled, non-parametric tests of Kruskal-Wallis ANOVA and Mann-Whitney *U* were employed for more than two and two variables, respectively. The level of significance for all tests was $p < 0.05$. All results, including hydrological and biochemical data, were expressed as mean ± standard error (SE) for a given day unless otherwise specified. To assess variation of the data, coefficient of variation (CV, %) was calculated as the ratio of the standard deviation σ to the mean μ times 100%. The software package STATISTICA (version 13.1, StatSoft Inc.) was used for the statistical analyses.

3. Results

3.1. Hydrological variables

Out of seven hydrological variables measured (temperature, pH, redox potential, salinity, oxygen saturation, total alkalinity and hydrostatic pressure), only data for total alkalinity did not violate assumptions for the use of parametric analyses. Monitoring of basic physic-chemical variables of seawater in the hyperbaric TiTank indicated stable experimental conditions during all experiments with CV ranging from 0% to 3.9% (Table 2; Fig. 2). Such low divergence in relation to the mean

Table 2
Physic-chemical properties of seawater (mean ± SE) at the outlet from the hyperbaric TiTank during bivalve acclimatisation and exposure periods in different pH treatments. Coefficient of variation (CV) is given in parentheses.

Treatment (pH level)	pH	Temp (°C)	O_2 (%)	Salinity	P (kPA)	TA[a] (μmol kg^{-1})	E_h (mV)
Acclimatisation							
Control (7.7)	7.67 ± 0.00 (0.6)	10.0 ± 0.0 (0.6)	90.2 ± 0.1 (0.9)	7.13 ± 0.01 (1.0)	900 ± 0 (0.0)		260 ± 6 (14.1)
7.0	7.76 ± 0.01 (0.6)	10.0 ± 0.0 (1.0)	97.1 ± 0.2 (1.3)	6.92 ± 0.00 (0.5)	914 ± 6 (1.7)		258 ± 16 (31.2)
6.3	7.74 ± 0.00 (0.4)	9.9 ± 0.0 (1.6)	95.4 ± 0.1 (0.6)	7.25 ± 0.01 (1.0)	912 ± 4 (1.1)		230 ± 3 (9.1)
Exposure							
Control (7.7)	7.72 ± 0.00 (0.3)	9.9 ± 0.0 (0.9)	95.2 ± 0.1 (2.4)	7.13 ± 0.00 (0.6)	902 ± 1 (0.4)	2014 ± 23 (5.2)	265 ± 3 (14.3)
7.0	7.07 ± 0.02 (3.5)	10.0 ± 0.0 (0.6)	96.7 ± 0.1 (2.0)	6.78 ± 0.00 (1.7)	925 ± 1 (1.6)	1818 ± 12 (3.1)	339 ± 4 (12.7)
6.3	6.26 ± 0.03 (3.9)	9.7 ± 0.0 (0.7)	95.0 ± 0.1 (2.1)	7.31 ± 0.00 (0.6)	926 ± 3 (1.9)	1880 ± 89 (21.7)	254 ± 3 (9.0)

[a] Total alkalinity (TA) was measured only during the exposure period.

(Table 2) contributed likely to differences in the variables measured among different pH treatments (Table 3) despite a narrow span of means for most variables (range of data for the entire experiment; temperature 9.7–10.0 °C; oxygen content 90.2–97.1%; salinity 6.8–7.3 and pressure 990–926 kPa; pH range under control conditions for the exposure period pH 7.67–7.76). Exceptions were redox potential and total alkalinity which differed markedly among treatments (range of means; E_h 230–339 mV and TA 1818–2014 μmol kg^{-1}) and their CVs varied from 9.0% to 31.2% and from 3.1% to 21.7%, respectively. After introduction of gaseous CO_2 into the TiTank, only a small decrease in temperature at seawater pH 6.3 and a slight improvement of redox potential consistently in the 6.3 and 7.0 treatments were noticed (Table 3, Fig. 2). Other variables remained at a similar level during the acclimatisation and exposure periods although statistical tests revealed significant effects of seawater acidification on all hydrological parameters (Table 3).

3.2. Biomarkers of cellular level response

3.2.1. Differences among pH treatments

Since the vast majority of biomarkers showed normal distribution of data (241 out of 252 individual cases, i.e. 12 markers analysed in three pH treatments on seven sampling occasions), the variances among groups across sampling occasions were homogenous (24 out of 36 cases, i.e. 12 markers measured in three pH treatments), and data sets from different groups for a given biomarker were balanced in most cases (i.e. contained equal number of data), the parametric approach was employed in the statistical analyses.

Five markers (CA, tGSH, GPx, GST and CBO) varied significantly among pH treatments (Table 4). LDH, MDH, CA, GPx, GST and MDA all exhibited similar V-shaped variation patterns with a relatively marked decline in the pH 7.0 treatment (Fig. 3). In the case of LDH this pattern was symmetric, i.e., similar values were denoted under control and the lowest pH conditions, but for most biomarkers (CA, GPx and GST) higher values were observed at seawater pH 7.7. Only MDH and MDA demonstrated increased levels in the bivalves kept in the pH 6.3 treatment. A reverse pattern with elevated activity at seawater pH 7.0 and low values in the 7.7 and 6.3 treatments was observed for ODH and AChE. Concentration of tGSH tended to rise with reduced seawater pH while SOD activity showed a tendency to decline with increasing acidity.

3.2.2. Temporal variation

The 40-day exposure period and regular sampling intervals permitted tracking of the development of bivalve response to seawater acidification and assessing medium-term effects of potential CO_2 leakage from a sub-seabed storage site (Fig. 4). No biomarker varied significantly over time (Table 4), but several temporal trends were detected in different pH treatments (Fig. 4).

Activities of the oxygen-dependent enzymes, LDH and MDH, in the clam tissues showed similar temporal variations under control conditions (correlation analysis on average data; $r = 0.852$ $p = 0.015$ $n = 7$). After the initial alternating activations and suppressions until 15 d of exposure, both enzymes decreased their activities on 20 d and elevated thereafter. In the most acidic environment, the lowered activities of LDH and MDH during the first 15 days of bivalve exposure were followed by a substantial increase of their activities until the end of the experiment (correlation analysis on average data; $r = 0.756$ $p = 0.049$ $n = 7$). The treatment with seawater pH 7.0 induced, in turn, a gradual inhibition of the LDH activity while that of MDH activity increased until 10 d and then declined substantially. The ODH activity decreased over most of the course of the clam exposure in the pH 7.7 and 6.3 treatments while it grew until 20 d and then reduced at seawater pH 7.0.

Temporal variations of the CA activity in the soft tissue of *L. balthica* followed inconsistent patterns with maximum in the middle of the exposure period (15 d) in the control treatment. Under the moderate and most acidic conditions, CA was markedly inhibited, except at 20 d in the pH 6.3 environment when the enzyme was activated by nearly 21% relative to the initial value on 0 d (Figs. 4 and 5).

In the antioxidant and detoxification system, concentration of total glutathione (tGSH) and the CAT activity also changed in clam tissues under control conditions (correlation analysis on average data; $r = 0.751$ $p = 0.050$ $n = 7$) when, after a clear decrease during the first 10 days of exposure, the level of tGSH and the CAT activity increased to a maximum on 30 d (Figs. 4 and 5). In the pH 6.3 treatment, the concentration of tGSH and the CAT activity increased until 20 d and declined later (Fig. 5). At moderate pH, tGSH level and CAT activity in the bivalves showed a declining trend until 5 d and 10 d, respectively and then increased gradually until 30 d. The GST and SOD activities showed a similar temporal pattern in the most acidic environment (correlation analysis on average data; $r = 0.762$ $p = 0.047$ $n = 7$) and tended to follow a similar course with seawater at pH 7.0 with peak activities on 20 d. Under control conditions, GST and SOD activities in the bivalves reached maximum on 10 d and 15 d, respectively, and decreased thereafter. The GPx activity demonstrated a downward trend until 20 d and 30 d in the pH 7.0 and 6.3 treatments, respectively and then rose over the remaining time of the experiment. At ambient seawater pH, alternating activation and inhibition of GPx were observed during the first half of exposure while its activity augmented linearly thereafter.

The activity of acetylcholinesterase in the *L. balthica* tissues tended to follow an akin temporal trend in the pH 7.0 and 6.3 treatments with increasing activity until 20 d and a gradual decline in the final phase of the exposure period (Fig. 4). Reduction of the enzymatic activity was more pronounced at the lowest seawater pH where its value at the end of experiment was similar to that on 0 d. The most inconsistent pattern in the AChE activity occurred under control conditions where alternately increasing and decreasing activities until the termination of the experiment were observed (Fig. 4).

The concentration of lipid peroxidation products in the bivalve tissue lowered in both acidic environments over 30 days of the exposure, particularly at seawater pH 6.3 where the MDA level decreased by as

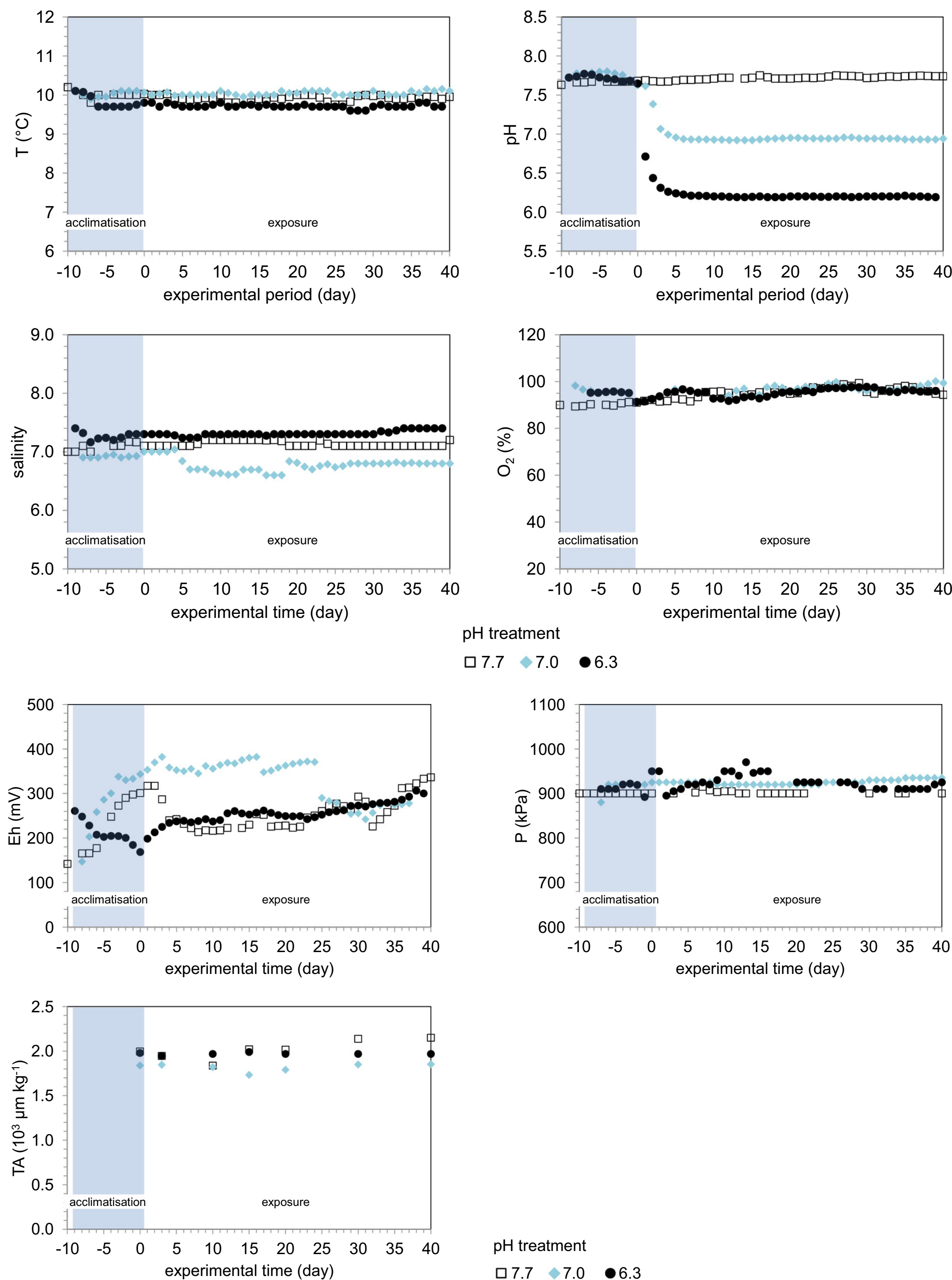


Fig. 2. Hydrological variables of seawater at the outlet from the hyperbaric TiTank during bivalve acclimatisation and exposure periods in different seawater pH treatments. Data are presented as mean for one day except hydrostatic pressure for which single measurement data (every day) are showed.

much as 86%. In contrast, in the pH 7.7 treatment the concentration of MDA in the clams declined until 15 d and remained stable thereafter (Fig. 4).

The level of protein oxidation products in the soft tissue of the clams showed similar temporal variations in the pH 7.7 and 7.0 experiments. After acidification had started, the concentration of carbonyl groups increased during the first 10 days and then declined over the next 20 days of exposure. In the most acidic environment, concentration of CBO declined just after the beginning of the bivalve exposure to CO_2, remaining stable between 3 d and 15 d to reach a minimum on 30 d (Fig. 4).

4. Discussion

4.1. Enzymatic activity and non-enzymatic markers

This study is the first to assess the impact of seawater acidification on a key Baltic species *L. balthica* using a biochemical approach that allows exploration of potential sub-lethal effects of CO_2 enrichment on various important cellular processes. To our knowledge no research so far has investigated enzymatic responses of infaunal bivalves from the Baltic Sea to elevated seawater acidity in the combination with increased hydrostatic pressure. Laboratory experiments in the hyperbaric

Table 3
Results of statistical analyses for testing the significance of seawater pH (Kruskal-Wallis ANOVA test and parametric ANOVA test for TA) and experimental period (acclimatisation vs. exposure; *U* Mann-Whitney test) on hydrological parameters. For seawater pH only data from the exposure period were included in the analyses. Due to a significant effect of pH treatment on most parameters measured differences between experimental periods were tested separately for each seawater pH. Significant values are **in bold**.

	pH	Temp	O_2	Salinity	P	TA[a]	E_h
Seawater pH							
H	269.7	154.3	426.4	2201.5	2201.5	(F) 11.9	142.8
df	2	2	2	2	2	2	
p	**<0.001**	**<0.001**	**<0.001**	**<0.001**	**<0.001**	0.003	**<0.001**
Experimental period							
pH 7.7							
Z	−8.7	5.3	−20.1	−0.5	−1.2		0.6
df	1	1	1	1	1		1
p	**<0.001**	**<0.001**	**<0.001**	0.621	0.213		0.569
pH 7.0							
Z	7.0	0.1	1.2	12.5	−2.9		−5.4
df	1	1	1	1	1		1
p	**<0.001**	0.957	0.219	**<0.001**	**0.004**		**<0.001**
pH 6.3							
Z	9.5	6.5	−0.4	−7.6	−2.0		−5.3
df	1	1	1	1	1		1
p	**<0.001**	**<0.001**	0.716	**<0.001**	**0.043**		**<0.001**

[a] Total alkalinity (TA) was measured only during the exposure period.

TiTank produced, therefore, unique empirical data on cellular level response in the bivalves at environmental hypercapnia. Enzymatic activities and concentrations of tGSH, MDA and CBO measured in the cytosolic fraction provided important insights into the current knowledge on cellular processes that respond to direct and indirect effects of CO_2-induced seawater acidification (Velez et al., 2016; Freitas et al., 2017; Liao et al., 2019). It is noteworthy that although the Baltic clam has been shown to pose a certain level of pre-adaptation to long-term increases in carbon dioxide levels in the Baltic sediments (e.g., Sokołowski et al., 2018), pH conditions, which were set up in this study, might trigger some unmeasured physiological changes that could generate non-typical responses within the chain of cellular level responses.

Reduced seawater pH significantly affected five biomarkers measured indicating hypercapnic stress and possible adverse implication for the physiological status of *L. balthica*. Increased CO_2 concentration tended to induce a decrease of LDH activity and increase of ODH activity in the clams in the pH 7.0 treatment whereas under control and pH 6.3 conditions activities of the enzymes remained at a similar level (Table 4, Fig. 3). LDH is an important enzyme that accelerates ATP production through anaerobic processes to maintain energy homeostasis (Strahl et al., 2011). Lowering of LDH activity in the bivalves during moderate pH exposure implies that maintaining acid-base status under these conditions did not modify the glycolytic pathway. Elevated energy requirements for counteracting internal hypercapnia were covered primarily from assimilated food while high-energy reserves were utilized only to a limited extent (Sokołowski et al., 2018). A similar effect was reported for the Manila clam *Ruditapes philippinarum* which maintained/regulated physiological status and biochemical performance without mobilisation of glycogen stores at reduced seawater pH 7.3 (Velez et al., 2016). Cao et al. (2018) found that during a 28-day exposure of the Pacific oyster *Crassostrea gigas* to seawater pH 7.6 (0.5 unit below control conditions) energy expenditure was modulated by inhibition of aerobic energy metabolism, stimulation of anaerobic metabolism and increasing activity of glycolytic enzymes, i.e., hexokinase and pyruvate kinase. ODH plays a crucial role in maintaining redox balance under functional anaerobiosis (e.g., cell anaerobic state generated by elevated muscular activity) and is considered an indicator of glycolytic mechanisms with higher activity when opines are anabolised in energy production pathway (Gäde, 1980; Dando et al., 1981). Given good oxygen conditions and similar temperature across different experiments, an increase of ODH activity in the clams at seawater pH 7.0 by 30.0% and 88.8% relative to the bivalves kept in the pH 7.7 and pH 6.3 treatments, respectively suggest higher oxygen demand within the former (Harcet et al., 2013). Another enzyme of the oxygen-dependent system, malate dehydrogenase, contributes to the citric acid cycle and catalyses a number of metabolic processes including the malate-aspartate shuttle, lipogenesis, amino acid synthesis and gluconeogenesis (Fields et al., 2006). This enzyme is involved in malate production from glucose and its activity was reported to be positively correlated with oxygen consumption rate in two mollusc species, the mussel *Mytilus californianus* and the dogwhelk *Nucella ostrina* (Dahlhoff et al., 2002). The activity of MDH in molluscs can thus be a good proxy of their metabolic level and an alternative measurement of rates of various life processes such as respiration and clearance. In this study, the overall MDH activity was high but did not differ among organisms exposed to different seawater pHs (Fig. 3). Relatively high MDH activity and only a slight reduction of LDH activity in *L. balthica* suggest that aerobiosis was a dominant energy production pathway in the clams under all tested pH conditions. Interestingly, LDH and ODH activities in the bivalves, which were kept under control and exposed to the most acidic conditions, responded differently than in the animals exposed to moderate hypercapnia. This mode of behaviour suggests a supportive role of MDH activity in glycolysis of the clams at pH 7.0 (Mansouri et al., 2017). It can also highlight specific defence/compensatory metabolic mechanisms (e.g., related to the presence of chemoautotrophic endosymbiotic bacteria), which enable *L. balthica* to cope efficiently with acute CO_2 stress.

Decreased seawater pH did impact SOD activity in the soft tissue of *L. balthica* (Table 4, Fig. 3) suggesting effective neutralization of the O_2^- radicals in bivalves in all experiments, presumably owing to sufficient reactive oxygen species (ROS) scavenging capacity (Świeżak, 2020). In contrast, GPx activity was markedly elevated in the bivalves under control conditions and lowered in the pH 7.0 treatment while CAT activity tended to be higher at moderate seawater pH. This collective group of antioxidant enzymes acts synergistically to prevent organisms from ROS overproduction. Superoxide dismutases (CuZnSOD + MnSOD) catalyse neutralization of superoxide anion radical (O_2^-) to H_2O_2, whereas CAT decomposes hydrogen peroxide to H_2O and O_2. GSH-mediated reduction of H_2O_2 to water and oxygen as well as the reduction of peroxide radicals (i.e., lipid peroxides) to alcohols, ketones and oxygen are catalysed by GPx (Tabet and Touyz, 2007). In this study, the presence of elevated ROS levels in the clams' cellular system

Table 4
Results of two-way ANOVA analyses for testing the significance of seawater pH and exposure time on different biomarkers of the Baltic clam L. *balthica*. Significant *p*-values are **in bold**.

Parameter	LDH	MDH	ODH	CA	tGSH	GPx	GST	CAT	SOD	AChE	MDA	CBO
Seawater pH												
F	1.77	0.08	2.44	9.17	6.92	9.62	15.51	1.77	0.73	2.96	1.34	10.60
df	2	2	2	2	2	2	2	2	2	2	2	2
p	0.212	0.919	0.129	**0.004**	**0.010**	**0.003**	**<0.001**	0.212	0.502	0.076	0.297	**0.002**
Exposure time												
F	0.45	0.26	1.88	0.40	0.69	0.86	1.16	1.18	1.45	2.59	2.69	0.85
df	6	6	6	6	6	6	6	6	6	6	6	6
p	0.829	0.946	0.166	0.865	0.663	0.552	0.389	0.378	0.275	0.076	0.068	0.556

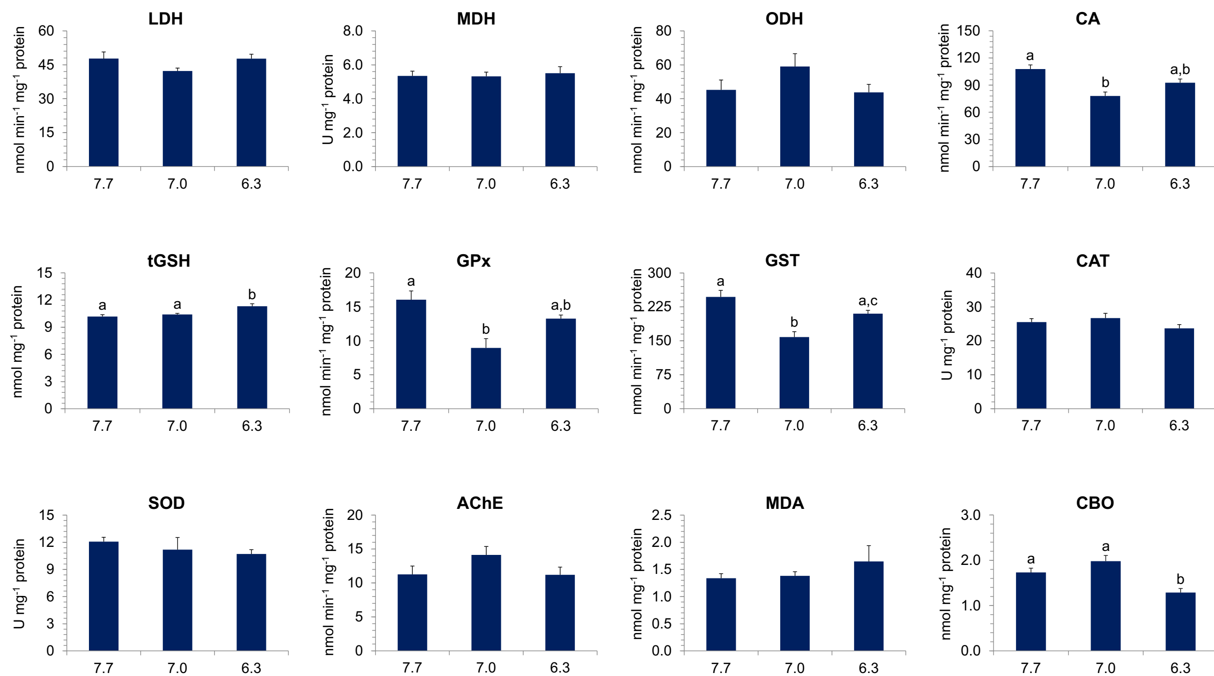


Fig. 3. 0d-adjusted cellular level markers of the Baltic clam *Limecola balthica* exposed to different seawater pH conditions. Data are presented as mean ± SE for all sampling occasions ($N = 7$). Significant differences (Bonferroni post-hoc test (α/n), $p \leq 0.05$) among conditions are represented with different letters.

is supported by an inhibition of GPx activity and by elevated CAT activity that was observed in the bivalves at moderate hypercapnia (pH 7.0). Such a response of catalyse to seawater acidification was also reported in the cuneate wedge shell *Donax cuneatus* by Jeeva Priya et al. (2017) and in the thick shell mussel *Mytilus coruscus* by Huang et al. (2018). In addition, Liao et al. (2019) documented higher CAT activity in the adductor muscle of the Yesso scallop *Patinopecten yessoensis* after exposure to pH reduction by 0.5 units, whereas SOD was activated in hepatopancreas, mantle and adductor muscle compared to the individuals from natural seawater pH 8.0.

Antioxidant status of the *L. balthica* during exposure to different seawater pHs was complementarily assessed by measurement of the concentration of total glutathione (tGSH) and glutathione S-transferase (GST) activity which both play an important role in regulation of oxidative stress and detoxication process (Meister and Anderson, 1983; Kelly et al., 1998). The average tGSH concentration remained similar in the bivalves kept at seawater pH 7.7 and 7.0 and increased considerably in organisms in the pH 6.3 treatment (Table 4, Fig. 3) implying acidification-induced glutathione-related oxidative stress. Oxidative stress is suggested to be involved in the toxic mechanism of seawater acidification (Tomanek et al., 2011). Reduced glutathione and GST were shown to take part in the phase II biotransformation of xenobiotics. Glutathione S-transferase in conjunction with tGSH detoxifies lipid hydroperoxides from the cellular system and transforms toxic electrophiles to more soluble forms (Hellou et al., 2012). Elevated concentration of tGSH and higher GST activity were also observed in tissues of marine bivalve *D. cuneatus* and indicated a sub-lethal effect of acute environmental hypercapnia (pH 6.5–5.5) (Jeeva Priya et al., 2017). It is noteworthy that exposure to seawater pH 7.0 brought about an apparent decrease of GST activity in *L. balthica* indicating that moderate acidification inhibits the antioxidant system response (Sun et al., 2017). This inhibition was probably caused by the insufficient supply of GST induced by environmental stress, which enhanced formation of intracellular ROS (Turrens, 2003).

Acetylcholinesterase, a neurotransmission enzyme involved in the synaptic transmission of nerve impulses, is also considered a useful biomarker of neurophysiological stress due to environmental pollution (e.g., Leiniö and Lehtonen, 2005; Turja et al., 2013; Carvalho et al., 2017). In our study, a potential toxic effect was demonstrated by increased AChE activity in only the bivalves exposed to seawater pH 7.0 (Table 4, Fig. 3). AChE activation can therefore imply elevated contamination level in the water at moderate hypercapnia but not in the most acidic environment. A plausible explanation of this pattern provides kinetics of sediment-bound metal mobilisation under acidic conditions. Prolonged (up to 9 days) CO_2 seepage (1300–20,000 ppm CO_2) was shown to mobilise labile Ni forms from sediment into the water column with intense fluxes in the upper section of the sediment (0–9 cm) under increased hydrostatic pressure (3000 kPa). In addition, increased metal mobility was observed to be more intense during the first few days of a seawater pH decline to 6.8 (Ardelan and Steinnes, 2010; Ardelan et al., 2012; Basallote et al., 2020). Surface sediments in the Gulf of Gdańsk contain relatively large amounts of bioavailable (mobile) trace and heavy metals (e.g., mean concentrations of 1 M HCl extractable sediment-bound fraction: 5493 μg Fe g^{-1} dry wt, 118.0 μg Mn g^{-1} dry wt, 11.7 μg Cu g^{-1} dry wt and 53.9 μg Zn g^{-1} dry wt; Sokolowski et al., 2007; Sokołowski, 2009) which can be easily released into the overlying water under acidic conditions. Since at low seawater pH metal toxicants leach into water at a higher rate and over shorter time, biological effects might have been acute but short in the pH 6.3 treatment in this study. Whereas moderate acidification might have induced slower metal mobilisation (and likely also of other contaminants) and thus prolonged exposure of the bivalves to lower concentrations of toxicants released from the sediment (Ardelan and Steinnes, 2010; Ardelan et al., 2012). Tomlinson et al. (1981) proved activation of acetylcholinesterase by Mg^{2+}, Ca^{2+}, Mn^{2+} and Na^{+} solutions of low ionic strength. AChE is capable of increasing in activity at the low concentrations of metal ions such as iron caused by up-regulated of cholinesterase (ChE) gene to produce more ChE to degrade the accumulation of acetylthiocholine in the synaptic cleft (Bainy et al., 2006; Sant'Anna et al., 2011). The increasing activity of AChE after exposure to sublethal metal concentrations was also proved for several fish species, e.g., *Leporinus obtusidens* (Gioda et al., 2013), *Oreochromis niloticus* (Şen and Karaytuğ, 2017), *Sparus auritus* (Romani et al., 2003), and the bullfrog tadpoles *Lithobates catesbeianus* (Carvalho et al., 2017). Such a pattern is consistent with the previous findings of Bainy et al. (2006) who reported an increase in AChE synthesis in the brown mussel

Perna perna digestive gland under acute exposure to metals that interact with acetylcholine receptors, thereby affecting their binding efficiency. However, exposure to acidification-induced elevated concentration of metals (e.g., Fe, Mn, Cu and Zn) did not cause necessarily increased tissue accumulation of the elements, presumably due to impairment of tissue functions as shown for *R. philippinarum* by Rodríguez-Romero et al. (2014).

Adverse impact of seawater acidification on the clams was indicated also by MDA, which demonstrated elevated concentrations in the bivalves kept in the most acidic environment. Malondialdehyde is a product of lipid peroxidation by a variety of enzymatic and non-enzymatic oxygen radicals that attack double carbon-carbon bonds of lipids (Ayala et al., 2014). The presence of pollutants in the external environment has been documented to induce an increase of MDA levels in marine invertebrates (Pellerin-Massicotte, 1994; Freitas et al., 2017). Lower concentration of MDA in the clams exposed to pH 7.0 and 7.7 has showed, in turn, little toxicological effect and sufficient defence mechanisms of *L. balthica* to prevent cellular damage. In contrast, the concentration of CBO was highest in the bivalves at seawater pH 7.0, suggesting oxidative stress. Accumulation of carbonylated proteins of the mitochondrial fraction of the cell was also reported in the bay scallop *Argopecten irradians* after post-anoxic and post-hypoxic recovery (Ivanina and Sokolova, 2016).

4.1.1. Temporal variation in defence system responses

Analysis of biomarkers at different time intervals during the 40-day exposure period allows for tracking of variation in responses of *L. balthica* to CO_2-induced seawater acidification over time. In contrast

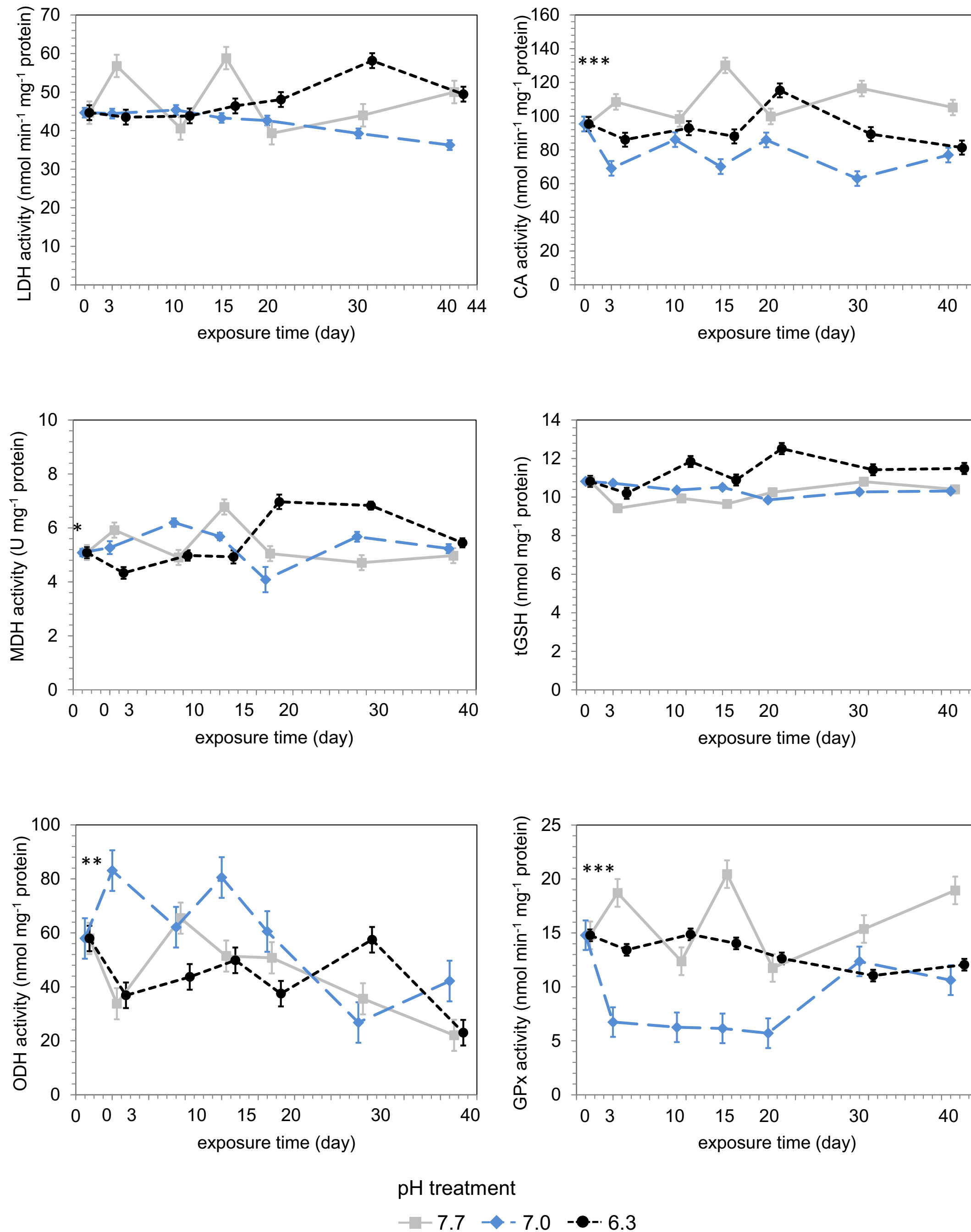


Fig. 4. Temporal variations of 0d-adjusted cellular level markers of the Baltic clam *Limecola balthica* exposed to different seawater pH conditions over 40 days. Data are presented as means ± SE for a given sampling occasion ($N = 8 \div 12$).

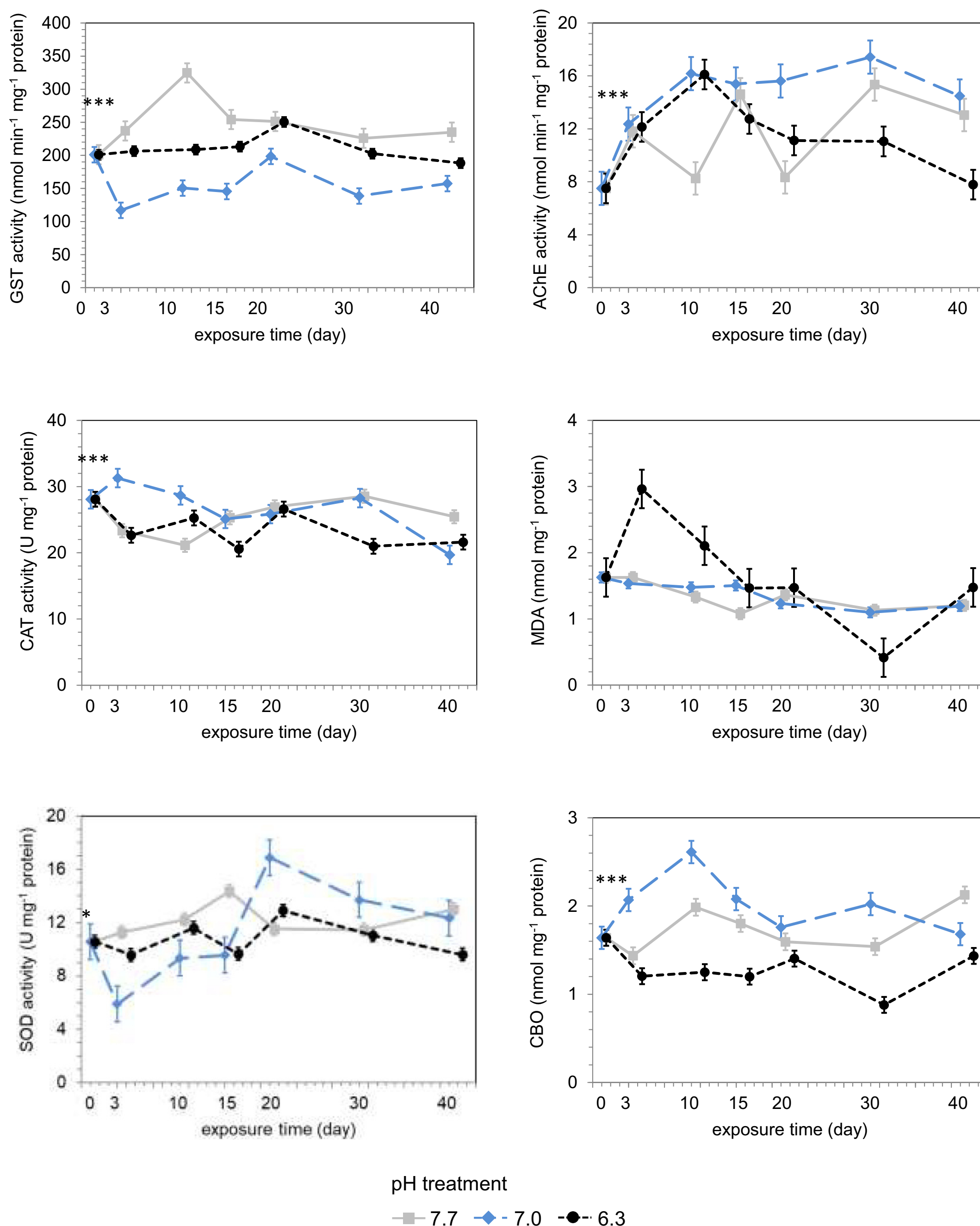


Fig. 4 (*continued*).

to many other single-species studies, which relied on two-point response curve, multi-step monitoring of biomarkers provided a timeline of organism's response to environmental hypercapnia. Following the initial 10–15-day period, LDH and MDH activities were reduced in bivalves exposed to seawater pH 7.7 and 7.0 (the latter observed only for LDH) indicative of the lack of modification to the glycolytic pathway. In contrast, explicit activation of these enzymes occurred in clams kept in the most acidic environment suggesting acceleration of glycolysis to cover increased energy demands but anaerobic pathways were not mobilised as shown by decreased activity of ODH (Fig. 5). Temporal changes of carbonate anhydrase activity, a biomineralisation-related enzyme involved in shell calcification, did not follow any consistent pattern, however, relative data (Fig. 5) showed apparent suppression of CA in bivalves exposed to seawater pH 7.0. Moderate acidification disrupted likely acid-base balance in these clams during an increased alkali buffer demand with possible effect on shell formation (Pörtner et al., 2004). Vidal-Dupiol et al. (2013) observed that genes coding CAs responsible for calcification were upregulated at moderate seawater pHs 7.8 and 7.4 but downregulated at reduced pH 7.2 in the cauliflower coral *Pocillopora damicornis* during a three-week exposure. It can therefore be hypothesized that the reduction of CA activity in *L. balthica* derived from a lowered expression level of gene coding CA under pH 7.0 conditions, but occurred to a limited degree in the most acidic environment only during the first two weeks. Acidic conditions also induced inhibition of GPx, most evident at seawater pH 7.0, over the entire exposure period presumably due to elevated levels of hydrogen peroxide radicals in the cellular system (Świeżak, 2020). After an initial decrease of SOD activity at the very beginning of exposure, which might be related to the increase of energetic metabolism and enhanced ROS production in the bivalve tissue, SOD activity increased in

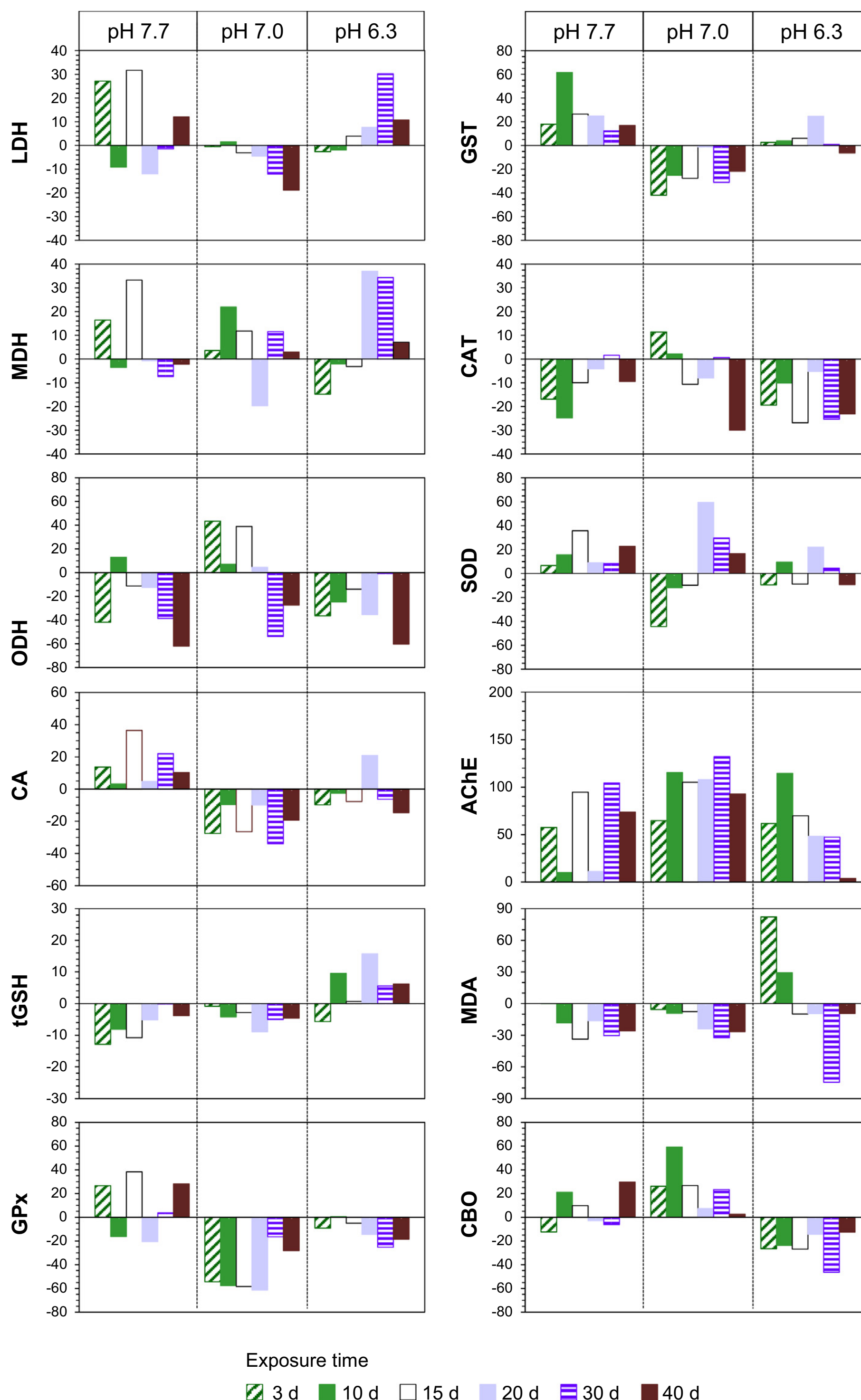


Fig. 5. Relative temporal variations (based on normalised data) of cellular level markers of the Baltic clam *Limecola balthica* exposed to different seawater pH conditions over 40 days. Positive values indicate upregulation and negative values downregulation of a given biomarker.

the clams at seawater pH 7.0 after 15 days of exposure which signals acclimatisation of animals to moderate environmental hypercapnia (Fig. 4). Following the 10-day increase at seawater pH 7.0, CAT activity was dominantly reduced under both acidic conditions, most pronounced at moderate hypercapnia (Fig. 5). SOD catalyses the dismutation of superoxide radical to H_2O_2 that is further degraded by CAT or the GPx–reduced glutathione (GR) cycle (Turja et al., 2013). An increased CAT activity has been reported in many marine invertebrate species in response to high H_2O_2 levels while the GPx–GR pathway is preferred in lower intracellular H_2O_2 concentrations (Orbea and Cajaraville, 2006). Since GPx activity reduced markedly and CAT activity showed slight reduction or even an increase at seawater pH 7.0, this could indicate higher ROS production in this exposure experiment. Apparent increase after 15 day-exposure was observed in the pH 6.3 treatment for total glutathione, which is considered the most abundant antioxidant among living cells (Kelly et al., 1998). Elevated tGSH concentrations in the clams reflects likely activation of compensatory mechanisms that indicate large antioxidant capacity and ability of the

bivalves to cope with cellular stress imposed by reduced seawater pH (Świeżak, 2020). A pattern of temporal variations for AChE activity was prevailed by a parallel increase over the first 10 days in both acidic environments to remain high or decrease thereafter in the pH 7.0 and pH 6.3 treatments (Fig. 4), respectively. Malondialdehyde accumulated in the tissues over the first 10 days in the pH 6.3 treatment suggesting higher metabolic rate of organisms in defensive response to increased energy demands for regulation of internal pH (del Rio et al., 2005). Later reduction of the MDA level implies metabolic depression in the bivalves and changes in metabolic pathways, which contribute to maintaining the cellular acid-base balance under hypercapnic conditions. In bivalves exposed to seawater pH 7.0, a drop of MDA concentration indicative of metabolic slowdown occurred as late as 15 days after acidification started. CO_2 addition to the hyperbaric tank also induced production of carbonyl groups until 10 d, which decreased thereafter, demonstrating oxidative stress in the initial phase of the exposure period and subsequent recovery of the antioxidative capacity of the cells. Under pH 6.3 conditions, CBO concentration in the clam tissues remained at a lower level over the entire experiment relative to the initial value on 0 d, which shows activation of physiological mechanisms of carbonyl detoxification. This is consistent with temporal dynamics of activity of antioxidant enzymes (GST, GPx, SOD) that indeed showed mostly strong suppression over the first few days of bivalve exposure to pH 7.0 (Fig. 5).

5. Conclusions

Seawater acidification in a range simulating the likely modifications in acidity in the bottom zone following a CO_2 leakage from sub-seabed CCS storage site in the southern Baltic Sea interfered with cellular level responses in the Baltic clam *Limecola balthica*. Changes in oxygen-dependent enzymes (LDH and MDH) indicate that aerobiosis was a dominant energy production metabolism and elevated energy requirements for counteracting internal hypercapnia did not mobilise high-energy reserves in the organisms. Hypercapnic stress was most evident in bivalves exposed to pH 7.0, which induced a decrease of GPx activity and activation of CAT in response to enhanced free radical production. Formation of intracellular ROS also caused inhibition of antioxidant system (suppression of GST) presumably due to insufficient supply of this enzyme. In addition, the clams subjected to a moderately acidic environment (pH 7.0) also demonstrated AChE activation that might be linked to prolonged impact of contaminants that were released from sediment. The most acidified conditions (pH 6.3) stimulated tGSH elevation and increased MDA concentration in the bivalve tissue reflecting potential cell damage. Temporal patterns of changes of most biomarkers (except activities of GPx and AChE) imply that after a 10-to-15-day initial phase of an acute and strong disturbance, the metabolic and antioxidant defence systems recovered their capacities. Our results documented that *L. balthica* from the southern Baltic is able to tolerate medium-term severe environmental hypercapnia that makes the species resilient to episodes of acidification.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.148593.

Ethics statement

No specific permits were required for the study, which complied with all relevant regulations. The species collected in this study are not endangered or protected.

CRediT authorship contribution statement

Adam Sokołowski: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. **Justyna Świeżak:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Anna Hallmann:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. **Anders J. Olsen:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Marcelina Ziółkowska:** Conceptualization, Formal analysis, Writing – review & editing. **Ida Beathe Øverjordet:** Conceptualization, Funding acquisition, Investigation, Writing – review & editing. **Trond Nordtug:** Conceptualization, Data curation, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Dag Altin:** Conceptualization, Methodology, Writing – review & editing. **Daniel Franklin Krause:** Conceptualization, Data curation, Methodology, Writing – review & editing. **Iurgi Salaberria:** Conceptualization, Investigation, Methodology, Writing – review & editing. **Katarzyna Smolarz:** Conceptualization, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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