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Simulated climate change causes immune suppression and protein damage in the crustacean *Nephrops norvegicus*

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ABSTRACT

Rising atmospheric carbon dioxide concentration is causing global warming, which affects oceans by elevating water temperature and reducing pH. Crustaceans have been considered tolerant to ocean acidification because of their retained capacity to calcify during subnormal pH. However, we report here that significant immune suppression of the Norway lobster, *Nephrops norvegicus*, occurs after a 4-month exposure to ocean acidification (OA) at a level predicted for the year 2100 (hypercapnic seawater with a pH lowered by 0.4 units). Experiments carried out at different temperatures (5, 10, 12, 14, 16, and 18 °C) demonstrated that the temperature within this range alone did not affect lobster immune responses. In the OA-treatment, hemocyte numbers were reduced by almost 50% and the phagocytic capacity of the remaining hemocytes was inhibited by 60%. The reduction in hemocyte numbers was not due to increased apoptosis in hematopoetic tissue. Cellular responses to stress were investigated through evaluating advanced glycation end products (AGE) and lipid oxidation in lobster hepatopancreata, and OA-treatment was shown to significantly increase AGEs', indicating stress-induced protein alterations. Furthermore, the extracellular pH of lobster hemolymph was reduced by approximately 0.2 units in the OA-treatment group, indicating either limited pH compensation or buffering capacity. The negative effects of OA-treatment on the nephropidae immune response and tissue homeostasis were more pronounced at higher temperatures (12–18 °C versus 5 °C), which may potentially affect disease severity and spread. Our results signify that ocean acidification may have adverse effects on the physiology of lobsters, which previously had been overlooked in studies of basic parameters such as lobster growth or calcification.

1. Introduction

The Intergovernmental Panel on Climate Change predicts that the global atmospheric temperature will rise by 1.8–4.0 °C by 2100 [1] due to increased levels of greenhouse gases. Use of fossil fuels, deforestation, and cement production are pointed out as the main sources of increased levels of the primary greenhouse gas, carbon dioxide (pCO_2) [2]. The oceans act as a sink for CO_2, and this so called ocean acidification, OA, alters the seawater chemistry shifting the equilibrium towards more dissolved CO_2 and hydrogen ions (H^+). It is estimated that the pH of the oceans has been decreased since the early 1900s, with 0.1 pH unit representing an increase in $[H^+]$ of about 30% [3,4]. Due to the slow vertical mixing of the ocean waters, the overload of H^+ is retained in the surface strata for a prolonged period of time and a further, approximately 0.4 unit decrease in pH is predicted by 2100, based on realistic scenarios for future CO_2 emissions [1]. This difference would be considerable, amounting to pH changes occurring 100 times faster than any change of this kind during the past 650,000 years [5].

A serious consequence of ocean acidification is the reduction of saturated calcium carbonate ($CaCO_3$), which in different forms such as calcite and aragonite, is a key building component for many photosynthetic organisms and animals that form shells and plates [6]. Shortage of calcium carbonate could directly affect organismal

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fitness, impacting factors such as growth rate, reproduction, and mortality [7,8]. Although research on the effects of ocean acidification on marine biota is a recent branch of science, with most papers published after 2007, an accumulating body of evidence suggests great differences in response to acidification between phyla as well as species, and also among calcifiers [7–9]. Marine crustaceans for example, appear to be capable of building solid shells at lower pH levels [9,10]; however, there is a lack of understanding on how decreased ocean pH would affect the more general organismal stress responses of crustaceans. Such studies have been conducted on the relationships between climate change and immune suppression in other marine species including bivalves [11,12] and the sea star *Asterias rubens* [13].

Pathogens constitute a constant threat for all organisms and survival requires good health status based on a robust immune defense. In general, invertebrates have an open or semi-open circulatory system making the rapid reaction of immune defenses and coagulation mechanisms critical [14]. In crustaceans, the main cell type responsible for these mechanisms are the hemocytes, developed from the hematopoietic tissue (Hpt) located on the dorsal side of the stomach [15]. Crustaceans have three subpopulations of hemocytes; the phagocytic hyalinocytes, granular cells, and semigranular cells. The main function of granular and semigranular cells is storage of the pro-phenoloxidase activating system (ProPO-AS), which can be released through degranulation and converted to the bactericidal enzyme phenoloxidase [16]. This activation cascade results in the generation of melanin, which physically shields the intruding organism and constrains the infection. The humoral response includes activation of intracellular signaling pathways that stimulate production of a range of defense proteins, such as antimicrobial peptides [17]. Aquatic invertebrates are constantly threatened by pathogens, and impairment of the immune response by environmental stressors has been shown to increase the susceptibility for infection [18,19].

During normal metabolism, and as a consequence of various environmental perturbations and pollutants, oxygen gives rise to several highly toxic and lethal intermediates that have to be neutralized by antioxidant defense mechanisms. Several antioxidants, such as superoxide dismutases (SODs) and peroxidases, are found in most biological systems. If the antioxidant system fails, reactive oxygen species (ROS) will react with macromolecules (e.g. DNA, proteins, lipids, etc) and may impair their function [20]. Several studies on benthic invertebrates have illustrated the relationship between environmentally induced failures of the antioxidant system and induction of oxidative damage of protein and lipids in tissues [21,22]. In addition, links between antioxidant system failures and immune suppression have been highlighted in a previous study [23]. As shown for many vertebrates, the cost for providing a stress-activated immunological defense creates trade-offs with other energy-demanding physiological processes such as growth and reproduction, and thus, may impact fitness and survival [24,25].

The Norway lobster, *Nephrops norvegicus,* is a stationary and abundant inhabitant of soft bottom sediments at 30–800 m depth in Atlantic and Mediterranean waters. Ecologically, it is regarded as a key marine species, and it is also of great economic importance. Here, we aimed to investigate the immune and stress responses of lobster from the Skagerrak (NE Atlantic) after long-term exposure (4 months) to CO_2-acidified seawater at varied temperatures (5, 10, 12, 14, 16 and 18 °C). We predicted that acidified sea water (ΔpH −0.4) at high temperatures (i.e. >12 °C, which are rarely exceeded at 30 m in the Skagerrak; data from Swedish Meteorological and Hydrological Institute) will cause synergistic disruption of homeostasis, affecting the number of hemocytes and their immune response in terms of phagocytic capacity and the pro-phenoloxidase activating system. Previous studies by our research group have indicated such parameters as sensitive targets when studying the effects of environmental changes on marine invertebrates [13,26–29]. In addition, we examined lipid and protein damage in hepatopancreatic tissue as endpoints of oxidative stress and homeostatic imbalance.

2. Materials and methods

2.1. Experimental conditions

N. norvegicus were caught (August–September 2010) by using baited creels at approximately 40 m depth at the mouth of the Gullmarsfjord, situated on the west coast of Sweden. This depth is below the halocline where seasonal fluctuation of temperature goes from approximately 4–12 °C and occasionally reaches up to 16 °C in September (data from Swedish Meteorological and Hydrological Institute). The lobsters were kept wet when transported to Sven Lovén Centre for Marine Sciences, Kristineberg, positioned close to the sampling site. On arrival, the lobsters were immediately transferred to large holding tanks supplied with a flow-through system of natural seawater ($S = 32$ PSU, $T = 14$ °C). The mean carapace length of the males was 47.0 ± 3.7 mm and for the females it was 45.1 ± 4.4 mm.

In the beginning of October lobsters were individually placed in 15 L basins equipped with a PVC tube refuge in thermo constant room and connected to the flow-through system with water passing a header tank of 200 L (in total 24 header tanks were used). The temperatures of the water entering the header tanks were set to 5, 10, 12, 14, 16, and 18 °C, respectively, to which the lobsters were gradually adapted over a 2-week time. At each temperature there were four header tanks, each supplying six basins (equal number of males and females out of a total of 144 lobsters). In two of the header tanks at each temperature, the pH was not regulated (controls). In the other two header tanks, experimental ocean acidification was induced by reducing pH by 0.4 units, which corresponds to the predicted future level of change by the year 2100 [1] (OA-treatment). The reduction in pH was obtained by bubbling seawater with CO_2 (bottled gas with 100% CO_2), controlled by pH probes and connected to computers that regulated the gas flow (Aqua Medic, Bissendorf, Germany). Environmental stability (temperature, salinity, and pH) was monitored daily and, in addition, twice a week total alkalinity (A_T, μmol/kg; Eppendorf BioPhotometer, Hamburg, Germany) was analyzed according to Sarazin et al. [30], and temperature and pH controlled by using WTW pH 3310 with a SenTix 41 electrode (Weilheim, Germany). $pH_{total\ scale}$ was determined by using TRIS- (2-amino-2-hydroxy-1,3 propanediol) and AMP- (2-aminopyridine) buffers [31]. *p*CO2 (μatm) was calculated using CO2sys software [32] (Table 1). Temperature dependence of seawater $pH_{total\ scale}$ corresponded both with that calculated from data measured in the Gullmarsfjord

Table 1
Mean (±SEM) of temperature and $pH_{total\ scale}$ of the experimental waters measured twice a week. Mean (±SEM) of $pCO_{2(\mu atm)}$ were calculated by using CO2sys software [32].

Temperature, °C		$pH_{total\ scale}$		$pCO_{2(\mu atm)}$	
Control	OA	Control	OA	Control	OA
5.2 ± 0.5	5.1 ± 0.5	8.1 ± 0.01	7.7 ± 0.03	319 ± 10	857 ± 0
10.0 ± 0.1	10.0 ± 0.1	8.1 ± 0.01	7.6 ± 0.02	416 ± 11	1060 ± 136
11.7 ± 0.1	11.7 ± 0.1	8.0 ± 0.01	7.6 ± 0.02	455 ± 3	1231 ± 86
13.6 ± 0.4	13.6 ± 0.4	8.0 ± 0.01	7.6 ± 0.02	510 ± 15	1358 ± 119
15.8 ± 0.2	15.7 ± 0.2	7.9 ± 0.01	7.5 ± 0.03	567 ± 3	1437 ± 15
17.9 ± 0.1	17.9 ± 0.3	7.9 ± 0.01	7.5 ± 0.02	622 ± 0	1725 ± 34