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Effects of Low pH and Low Salinity Induced by Meltwater Inflow on the Behavior and Physical Condition of the Antarctic Limpet, *Nacella concinna*

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Abstract: Seawater acidification and freshening in the intertidal zone of Marian Cove, Antarctica, which occurs by the freshwater inflow from snow fields and glaciers, could affect the physiology and behavior of intertidal marine organisms. In this study, we exposed Antarctic limpets, *Nacella concinna*, to two different pH (8.00 and 7.55) and salinity (34.0 and 27.0 psu) levels and measured their righting ability after being flipped over, mortality, condition factor, and shell dissolution. During the 35-day exposure, there was no significant difference in behavior and mortality between different treatments. However, the condition factor was negatively affected by low salinity. Both low pH and low salinity negatively influenced shell formation by decreasing the aragonite saturation state (Ω_{arg}) and enhancing shell dissolution. Our results suggest that, though limpets can tolerate short-term low pH and salinity conditions, intrusions of meltwater accompanied by the glacial retreat may act as a serious threat to the population of *N. concinna*.

Keywords: ocean acidification; glacial retreat; meltwater; *Nacella concinna*; Marian Cove; shell dissolution

1. Introduction

Increasing atmospheric CO_2 has induced ocean acidification with 0.1 unit reduction of pH over the last 200 years [1]. According to the climate change scenario RCP8.5 of 5th IPCC assessment, the global average surface pH is estimated to decline 0.3–0.32 by 2100 [2]. Moreover, the aragonite saturation state of the Southern Ocean is expected to become undersaturated by the year 2030, and no later than 2038, due to low seawater temperature of Southern Ocean [1,3,4]. In addition, aragonite undersaturation events are predicted to spread rapidly, affecting more than 70% of the Southern Ocean surface water this century [5]. Accordingly, marine calcifying organisms will have difficulty in maintaining calcium carbonate skeleton or shells. In addition to the increased atmospheric CO_2, glacial meltwater input due to shrinking ice sheets likely aggravates the acidification [6]. The fresh meltwater has a lower Total Alkalinity (TA) and concentration of Dissolved Inorganic Carbon (DIC) compared to seawater that contribute to carbonate undersaturation [7]. Therefore, the meltwater inflow could additionally reduce the pH in seawater. It was reported that freshwater dilution has facilitated low calcium carbonate saturation states in both the Amundsen Sea and Ross Sea [8].

The Western Antarctic Peninsula (WAP) is one of the regions on the earth where warming and glacial melting are proceeding quickly [9,10]. Recent studies revealed that the substantial reduction of marine-terminating glaciers in the WAP in recent years is the result of coastal-trapped waves and ocean

currents, which induce the warm seawater inflow [11,12]. There are few studies that determine the effect of meltwater inflow on Southern Ocean acidification on marine organisms. However, carbonate ion undersaturation was found to be a direct consequence of the recent extensive melting of sea ice in the Canada Basin; this phenomenon could affect both the planktonic and benthic ecosystems [6]. In the WAP, because of the complex coastline and fast glacial retreat, the study of the response of the ecosystem to ocean acidification and freshening should be addressed as an important issue.

Marian Cove, a small glacial fjord (~4.5 km long and ~1.5 km wide) is located in King George Island, the biggest island of the South Shetland Islands, which belongs to the WAP (Figure 1). In Marian Cove, the glacier has rapidly retreated around 1.9 km from 1956 to 2017 [13]. The glacier retreat occurs during most of the summer months and is being accompanied by massive intrusion of turbid melt water [14]. Freshwater streams and ponds are also developed from the surrounding snow field in summer, and a substantial amount of fresh water is introduced into the cove. The meltwater stream and pond water of Baton Peninsula and Weaver Peninsula, between which Marian Cove is located, has relatively lower pH ranges (meltwater: <4.38, pond water: <5.00), because King George Island is a volcanic island [15]. As a result, the surface water in Marian Cove has a distinct salinity gradient along the distance from the glacier during the austral summer months [16]. In addition, the pH and salinity of the seawater of Marian Cove fluctuates depending on the unexpected weather and tidal conditions in the austral summer (Supplementary Materials Figure S1). This suggests that local ocean acidification has been driven rapidly by the meltwater inflow from both the tidewater glacier and the snow fields, although their relative contribution was not quantified.

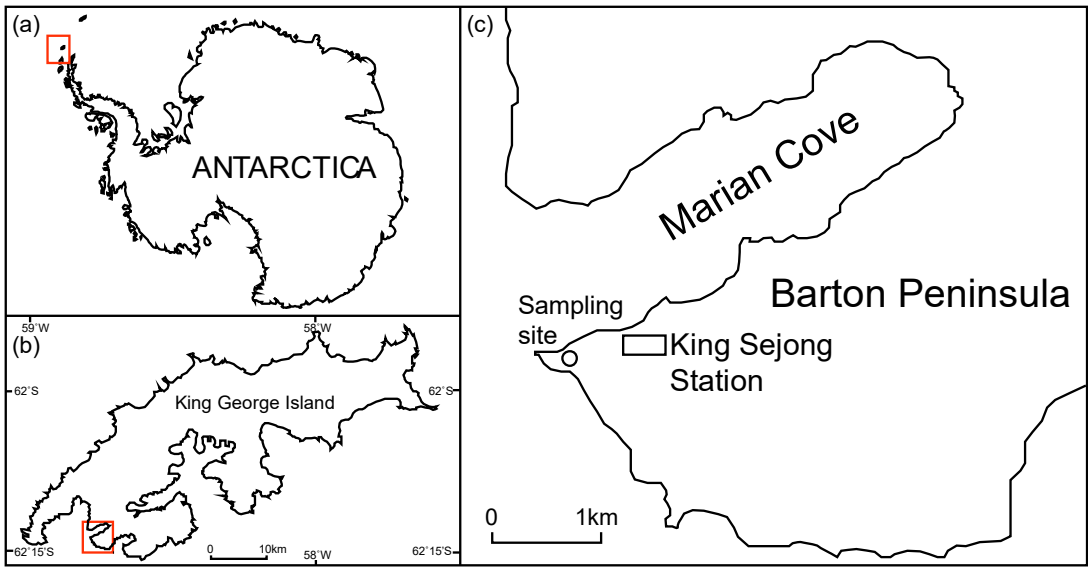

Figure 1. Study area: (**a**) the location of King George Island (red square), (**b**) the location of Marian Cove (red square), and (**c**) the sampling site (circle).

The retreat of marine-terminating glaciers, particularly those grounded on the sea floor (tidewater glaciers), has a profound influence on the nearshore coastal environment and inhabitants [17]. Antarctic benthic communities can be significantly impacted by a series of physical processes following glacier retreat, occurring mostly during the summer seasons (e.g., ice scouring, water column stratification, and nearshore turbidity) [14,18–20]. Not only physical impacts, but also large variations in the water chemistry, can occur because of meltwater inflow (e.g., pH, salinity, aragonite saturation, metal level, melting circulation, DIC, and TA changes) [21–23]. The Antarctic marine organisms inhabiting this area, particularly benthic fauna, are most likely vulnerable to climate changes, as they have low physiological flexibility, low growth rate, deferred maturity, and a constraining adaptation to short-term environmental changes [24–26]. Even pH and salinity are related, and these stressors

possibly occur at the same time due to the inflow of meltwater from glaciers and snow, in the polar research field, there are few studies that investigated the negative effect of the combined stressors of low pH and low salinity on Antarctic marine animals [7,27]. We need to consider the combined effect, because those stressors are related [28,29].

Nacella concinna (Strebel, 1908), occurring from intertidal to subtidal areas [30], is widely distributed in the Antarctic Peninsula and adjacent islands (including Subantarctic islands), where warming and glacial shrinking are fast proceeding in the recent decades [31,32]. *N. concinna* is the most prominent invertebrate at a depth of 8 m, with a density of more than 166 individuals per m^2, and the density increases as the depth decreases [33]. Moreover, it is the most common large-sized (>1 cm) faunal species to be observed by naked eyes in the intertidal zone, where environmental variation due to meltwater introduction is much greater than in the subtidal waters. *N. concinna* likely plays a key mediating role connecting benthic food webs with the predators at higher trophic levels such as the kelp gull (*Larus dominicanus*) [34]. Thus, ecology and physiology of *N. concinna* are relatively well-known [22,35], which could facilitate its utility as a sentinel species for assessing the climate-induced impacts on the shallow coastal ecosystem of the WAP.

Because *N. concinna* inhabits the intertidal areas, it may have an ability to adapt to the fluctuation of salinity and pH such as other intertidal animals [36]. However, it is known as a stenohaline organism, which cannot tolerate wide salinity changes [35,37]. The shell of *N. concinna* mainly consists of calcite, which is one of the forms of calcium carbonate and is often encrusted with coralline alga comprised of magnesium-calcite [38]. The calcium carbonate shell of *N. concinna* has been considered vulnerable to ocean acidification, even though it is more resistant than shells of other aragonitic mollusks [39]. Thus, *N. concinna* could be directly influenced by the low-pH and low-salinity meltwater in the intertidal zone. The Southern Ocean acidification has mostly been studied with the pteropod *Limacina helicina* and the bivalve *Laternula elliptica* [40,41]. However, the pH and salinity decrease in intertidal zones due to the increase in meltwater inflow from the snow fields and tidewater glaciers may impose serious restrictions on physiological process of *N. concinna*, which is distributed in the narrow region where the glaciers have rapidly retreated. Therefore, assessing the impact of pH and salinity levels lower than current natural variability caused by meltwater inflow on *N. concinna* should be prioritized.

Here we investigated the effects of low pH and low salinity on righting behavior and physical condition of the Antarctic limpet *N. concinna* which inhabit where the meltwater actively inflow due to the glacier runoff. We hypothesized that the low pH and low salinity would negatively affect behavior and physical condition of *N. concinna*. To test the hypothesis, we exposed *N. concinna* to two pH (8.00 and 7.55) and salinity (34.0 and 27.0 psu) levels for 35 days and observed the following response variables: righting behavior after being flipped over, mortality, condition factor, and shell surface dissolution. We predicted that the righting behavior may be negatively affected by the low pH, which inhibited the righting behavior of the Antarctic gastropod *Margarella antarctica* [42], and the physical condition may be worsened under the low salinity, which induces the physiological cost due to the osmotic stress. Furthermore, we predicted the shell dissolution would be increased by the low pH and low salinity, both of which not only decrease the aragonite saturation state but also can impair acid–base regulation system.

2. Material and Methods

2.1. Sampling of Limpets and Preparation for the Experiments

Individuals of the Antarctic limpet *N. concinna* were collected from the intertidal zone at Cape Sejong (S62°12′40′′ W58°47′53.10′′) near King Sejong (KSJ) station from 31 December 2017 to 11 January 2018 (Figure 1). Collected limpets were transported to the laboratory of the station and acclimated for ~2 weeks before the experiment. The shell length (SL), width (SW), and height (SH) of each limpet was measured using a Vernier caliper to the nearest 0.01 mm and total (shell and soft tissue) wet weight was measured using an AE240 balance (Mettler Toledo, Columbus, OH, USA)

to nearest 0.0001 g. Then, 48 limpets with similar SLs (approximately 25 mm) were selected for the experiment. Each individual (N is 12 per each treatment) was placed into a transparent plastic cup (400 mL, 9.2 × 5.9 × 10.7 cm, upper Φ × bottom Φ × H) with 250 mL of filtered seawater, and the cups were placed in the large container (80 × 45 × 20 cm, W × L × H) containing sea ice and seawater to maintain the water temperature at approx. 1 °C (0.9 ± 1.0, mean ± SD °C). There were no significant differences in the total wet weight, shell size (SL, SW and SH) and sex ratio (1.53:1, f:m) between each group (Kruskal–Wallis test; total wet weight: $X^2 = 0.033$, $df = 3$, $p = 0.998$, shell length: $X^2 = 1.314$, $df = 3$, $p = 0.726$, shell width: $X^2 = 0.742$, $df = 3$, $p = 0.863$, shell height: $X^2 = 1.946$, $df = 3$, $p = 0.584$, sex ratio: $X^2 = 2.317$, $df = 3$, $p = 0.509$). During this acclimation, the pH of seawater was maintained at 7.93 ± 0.19 (mean ± SD) and the salinity was 34.0 ± 0.0 (mean ± SD) psu, close to the habitat environmental conditions of the experimental limpets. The seawater was replaced twice in every three days to maintain the seawater quality. The water temperature (1.2 ± 1.5, mean ± SD) and pH were regularly measured every day using a portable S8 pH meter (Mettler Toledo, USA), and the salinity and dissolved oxygen (DO) (10.32 ± 1.95, mean ± SD) were measured using a YSI Pro30 salt meter and YSI 5100 DO meter (YSI Inc., Yellow Springs, OH, USA), respectively, when the seawater was replaced. The photoperiod followed the same natural diurnal cycle (light from 3:30 a.m. to 10:30 p.m.) as that of where the limpets were captured, using LED mini light AMZ-L13 (amazon, Zhongshan, China).

2.2. Experimental Setup

Four treatment groups, crossing two levels of pH and salinity, were considered: control (C, pH 8.00 and 34 psu), low salinity (LS, pH 8.00 and 27 psu), low pH (LP, pH 7.55 and 34 psu), and low pH and low salinity (LPLS, pH 7.55 and 27 psu) (Figure 2). The control condition followed the data (34.0 psu) in the summer season at KSJ station [43]. The low pH (−0.45 units) was set at the lower level than natural variability given that pH level is expected to be much lower due to meltwater inflow of Marian Cove in near future [44]. The low salinity was set as the mean value of the lowest 25% of data collected in front of KSJ station from 1 January to 14 January 2018 (Supplemental Figure S1).

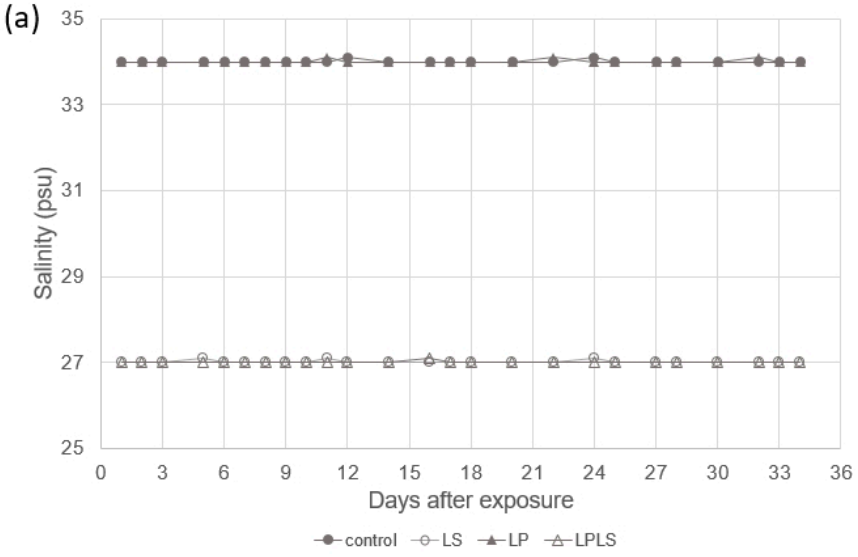

Figure 2. *Cont.*

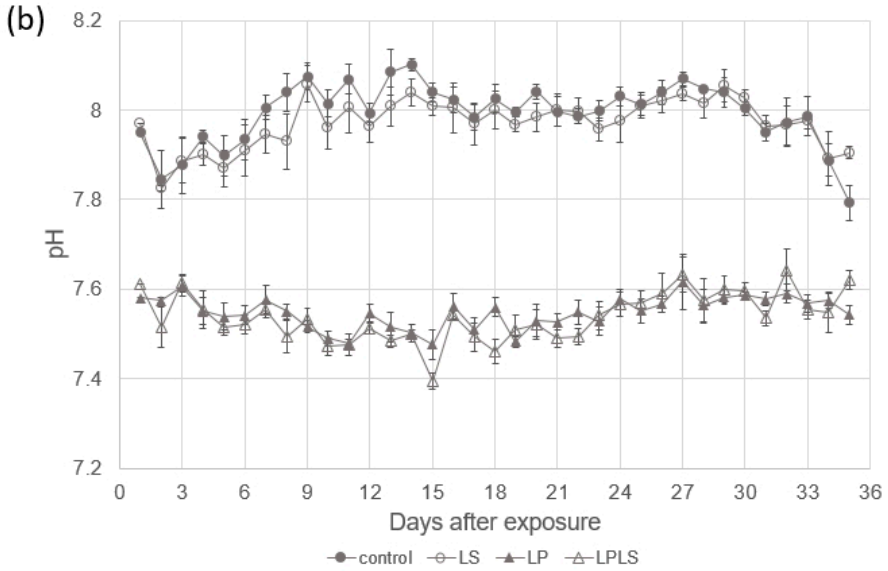

Figure 2. Salinity (**a**) and pH (**b**) data during this experiment in response to targeted exposure (Mean ± SE). The pH was measured every day and night and the salinity was measured when the experimental seawater was replaced.

Before being used in the experiment, seawater was filtered twice by two plastic filters (input 0.2 µm and output 0.07 µm, 8 × 25 cm, Φ × H; Sartorius, Germany). Low-salinity water was manufactured by freezing the filtered seawater (<−20 °C) for 12 h, allowing the separation of low-salinity ice from the remaining high-salinity seawater. The ice was then melted into low-salinity (<10 psu) seawater to manufacture the low salinity treatment [45]. The pH was regulated with very-low-pH seawater (<pH 4.00) made by dissolving a CO_2 tablet (SERA, Heinsberg, Germany) in seawater. This treated seawater was replaced twice every three days. The pH, salinity, and DO of seawater were not much different between just before and after replacement (Supplemental Table S2). The pH and temperature of experimental seawater were measured every day and night using a portable S8 pH meter (Mettler Toledo, USA). The pH meter was calibrated every week using Mettler Toledo™ electrolyte FRISCOLYT-B solution as buffers. The salinity and DO were measured when the seawater was replaced. Measured pH and salinity data are shown in Figure 2. A piece of red algae (1 × 1 cm), *Palmaria decipiens*, which is the preferred food of *N. concinna*, was provided as food for the limpets [42]. The collected *P. decipiens* was kept in a flow-through seawater container until the limpets were fed. The experiment was conducted for 48 days (13 days for acclimatizing and conducting the behavioral experiment before exposure to different treatments, and 35 days after exposure to the treatments).

2.3. Total Alkalinity (TA)/Dissolved Inorganic Carbon (DIC) Analysis

The TA, DIC, and aragonite saturation state (Ω_{arg}) in the experimental containers were determined three times (at the beginning, middle, and end of the experimental period) during the experiment, aliquots (300 mL) of seawater samples were taken from the experimental container just prior to the seawater replacement. The seawater samples were fixed with mercury chloride (Hg_2Cl_2). The TA was determined by an automated titrator AS-ALK2 (Apollo SciTech, Newark, DE, USA) and the DIC, pCO_2 and Ω values were calculated using the CO_2SYS Calc XLS program (v 2.1) [46].

2.4. Effects of Low pH and Low Salinity on the Behavioral Response and Physical Condition of the Limpets

Three indices were used to determine the behavioral response and physical condition of the limpets after the completion of the experiment: (i) righting after being flipped over, (ii) mortality, and (iii) condition factor.

Righting, defined in this study as turning back over after being turned onto their shell, is one of the stereotypic gastropod behaviors which represents the ability to return to a stable condition after being disturbed by predators or wave actions [42,47]. The righting response tends to slow down when an organism is under stress and the time taken to return to the original position would increase [42,48]. In this study, the righting responses of the limpets were quantified by turning them upside down and then counting the number of limpets that returned back to the initial position in 1 h [42]. This measurement of righting response was conducted prior to the exposure to each treatment, to ascertain if there was any significant difference in the behavior between groups. In the course of each treatment, the righting response was determined three times on Days 26, 31, and 34. The dead individuals during experimental period were not used for statistical analysis.

The number of dead individuals was counted every day during the experimental period, for 35 days (from Jan 14 to Feb 17). The mortality was calculated by counting the number of dead individuals every day. Limpets were determined as "dead" in the cases where they showed no movement of the tentacle or foot muscle, when the body was forced to detach from the surface of the plastic cup.

The Fulton's condition factor (CF) represents the general physical condition of fish (Nash et al., 2006). A modified condition factor (CF_m) appropriate to limpets was used in this study to assess the health condition of limpets (see the equation below) [47,49].

$$\text{Fulton's condition factor} = \text{mass} \times length^{-3}$$
$$\text{modified condition factor (CFm)} = \text{tissue mass} \times \text{shell volume}^{-1}$$
$$\text{tissue mass} = \text{whole body mass} - \text{shell mass}$$
$$\text{shell volume} = \text{shell length} \times \text{shell width} \times \text{shell height} \times \pi \div 12$$

The whole body mass of each limpet was measured after the mucus, residual water, and debris were removed carefully from the foot. Shell mass was measured by separating the shell from the flesh after finishing the experiment. The dead individuals during experimental period were not used for statistical analysis. The shell was dried in the open air, at room temperature (around 15 °C), and kept with silica gel for the shell dissolution analysis.

2.5. SEM Image Analysis on Shell Dissolution

Scanning electron microscope (SEM) analysis was conducted to investigate the shell dissolution. The dried shells were transported to the Korea Polar Research Institute (KOPRI) and prepared for SEM imaging. The samples were coated with platinum for 120 s (Q150TS; Quorum) [50]. The shell surface images were taken with an SEM (S-4300SE; Hitachi), at 150× magnification, at the Research Institute of Standards and Analysis, Inha University. The voltage used was 15 kV. To better compare each sample, the growth line of the shell was aligned in the "one o'clock" direction.

Because the ruggedness of an SEM image is relative to the brightness, the ruggedness could affect the proportion of light pixels in SEM pictures; the more rugged the image, the higher the proportion of light pixels will be on the image [51]. To determine the degree of ruggedness, the program Image J® was used. This program can count the number of pixels and represent the pixel's lightness/darkness as a numerical value, from 0 to 255. The light pixel level was considered as 20% of the highest rank (level: 206–255). The proportion of light pixels was calculated, and used as a ruggedness index. The dead individuals during experimental period were not used for statistical analysis.

2.6. Statistical Analyses

Data from 48 limpets were used for the analysis. When the data satisfied the assumption of normality (Kolmogorov–Smirnov or Shapiro–Wilk tests, $p > 0.05$), we used a parametric test to analyze them. If not, we used a non-parametric test. To determine if there was no difference in the total wet weight, size, and behavior between groups before exposure to different treatments, the Kruskal–Wallis test was used. A one-way analysis of variance (ANOVA) was used to determine

if there was no difference in the water temperature and to compare pH and salinity values between groups. A two-way repeated measures ANOVA was applied to determine if the behavioral responses (righting) were different between treatments. When sphericity (equality of the variance of the data among the experimental exposure days) was violated (Mauchly's test, $p < 0.05$), we used Huynh–Feldt corrections. One-way ANOVA was used to determine if there was a difference in the condition factor between groups before exposure, and two-way ANOVA was used to determine that after exposure and mortality. The difference in shell surface ruggedness between the groups was determined with two-way ANOVA after removing the outlier using Tukey's method [52].

3. Results

3.1. Experimental Treatment

The water-chemistry data are summarized in Table 1. There was no significant difference in seawater temperature between groups (one-way ANOVA; $F_{3, 765} = 0.682$, $p = 0.563$) during the experiment. There was significant difference in pH and salinity between the different treatment groups (one-way ANOVA; pH: $F_{3, 766} = 1695.370$, $p < 0.0001$, salinity: $F_{3, 100} = 523573.894$, $p < 0.0001$), but there was no significant difference in pH between the same pH groups (Bonferroni test, pH: $p = 0.130$ between C and LS, $p = 1.000$ between LP and LPLS) or in salinity between the same salinity groups (Salinity: $p = 1.000$ between C and LP, $p = 1.000$ between LS and LPLS). Salinity was well maintained with small standard error, and pH was also well maintained throughout the whole experimental period (Figure 2). The DO values of all groups were maintained at more than 8.5 mg/L during the experimental period. The water chemistry of the sampled experimental seawater is shown in Table 1. The C group had the highest Ω_{arg} (1.32 ± 0.08, mean ± SE) and the LPLS group had the lowest Ω_{arg} (0.36 ± 0.01, mean ± SE).

Table 1. The water quality of sampled experimental seawater (mean ± SE).

Group	C	LS	LP	LPLS
pH(NBS)	8.01 ± 0.01	7.99 ± 0.01	7.61 ± 0.02	7.59 ± 0.01
Salinity (psu)	34.0 ± 0.0	27.0 ± 0.0	34.0 ± 0.0	27.0 ± 0.0
Water temp. (°C)	1.0 ± 0.3	0.9 ± 0.5	1.0 ± 0.4	0.8 ± 0.6
TA (µmol/kg)	2498.1 ± 47.7	2052.5 ± 95.2	2569.5 ± 101.5	2135.7 ± 96.0
DIC (µmol/kg)	2401.5 ± 47.1	2003.9 ± 95.8	2606.3 ± 108.5	2187.9 ± 102.9
pCO_2 (µatm)	594.2 ± 13.8	552.5 ± 30.4	1595.9 ± 118.5	1496.3 ± 97.8
Ω_{cal}	2.09 ± 0.12	1.33 ± 0.05	0.87 ± 0.02	0.59 ± 0.02
Ω_{arg}	1.32 ± 0.08	0.82 ± 0.03	0.55 ± 0.02	0.36 ± 0.01
N	7	4	5	4

The experimental seawater was sampled for the carbon chemistry analysis. The group exposed to control condition, the group exposed to low salinity, the group exposed to low pH, and the group exposed to low pH and low salinity are labeled as C, LS, LP, and LPLS, respectively. TA is Total Alkalinity. DIC is Dissolved Inorganic Carbon. Ω_{cal} and Ω_{arg} are the saturation states of calcite and aragonite, respectively.

3.2. Behavioral Response—Righting

The righting response was not significantly different between groups before (Kruskal–Wallis test; $X^2 = 2.405$, $df = 3$, $p = 0.493$) and after exposure to different treatments (two-way repeated measures ANOVA; pH: $F_{1, 39} = 0.194$, $p = 0.662$, salinity: $F_{1, 39} = 1.440$, $p = 0.237$, pH × salinity: $F_{1, 39} = 0.397$, $p = 0.532$, time × pH: $F_{1.928, 75.195} = 0.229$, $p = 0.788$, time × salinity: $F_{1.928, 75.195} = 0.092$, $p = 0.906$, time × pH × salinity: $F_{1.928, 75.195} = 0.73$, $p = 0.924$) (Figure 3).

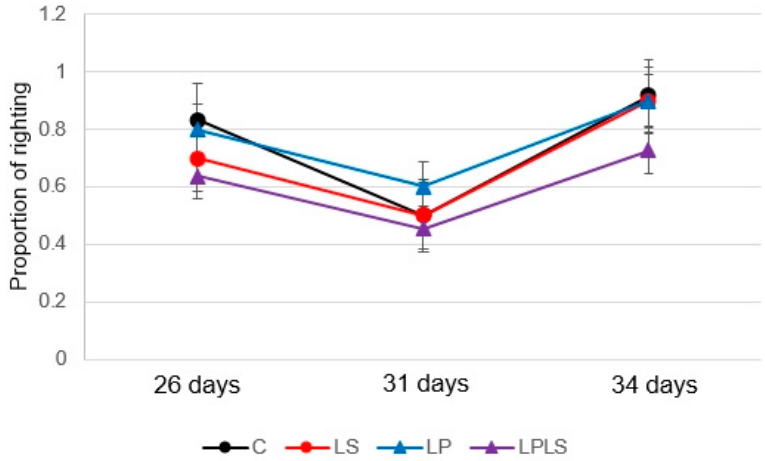

Figure 3. Proportion of righting for each group with exposed days (Mean ± SE). The control pH and salinity, low salinity, low pH, and low salinity and pH groups are represented by black, red, blue, and purple, respectively, and N = 12, 10, 10, and 11, respectively.

3.3. Mortality and Condition Factor

The individuals of the control group (C) were all alive, but there were two dead individuals in both the LS and LP groups, and one dead individual in the LPLS group. The mortality was not significantly different between groups (two-way ANOVA; pH: $F_{1, 44} = 0.216$, $p = 0.645$, salinity: $F_{1, 44} = 0.216$, $p = 0.645$, pH × salinity: $F_{1, 44} = 1.941$, $p = 0.171$).

The modified condition factors (CF_m) between groups showed no significant difference before exposure to different treatments (one-way ANOVA, $F_{3, 39} = 1.764$, $p = 0.170$). After 35 days of exposure, however, the CF_m was significantly reduced by low salinity (LS, LPLS) (Figure 4; two-way ANOVA; pH: $F_{1, 39} = 0.874$, $p = 0.356$, salinity: $F_{1, 39} = 7.888$, $p = 0.008$, pH × salinity: $F_{1, 39} = 0.278$, $p = 0.601$).

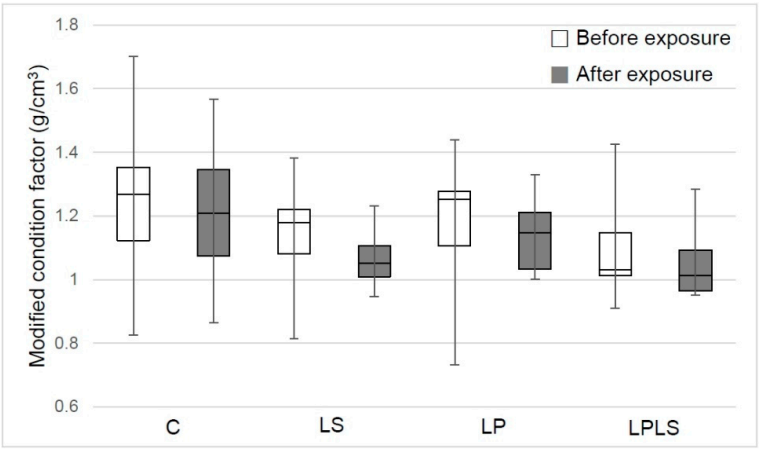

Figure 4. Modified condition factor (CF_m) of each group. The low salinity significantly affected the condition factor). The condition factors before and after treatment are represented by white and gray boxes, respectively, and N are 12, 10, 10, is 11 respectively.

3.4. Shell Dissolution

The height of the growth line on the shell surface appeared to become lower as the Ω_{arg} decreased (Figure 5). The dissolution of the growth line was affected by the pH, salinity, and the combined effect of pH and salinity even in the short-term exposure (Figure 6, two-way ANOVA, pH: $F_{1, 28} = 32.050$, $p < 0.001$, salinity: $F_{1, 28} = 13.228$, $p = 0.001$, pH × salinity: $F_{1, 28} = 11.231$, $p = 0.002$). In the low-pH (7.55) groups, the ruggedness was not significantly different (unpaired t-test: $t_{14} = 0.483$, $p = 0.636$)

between different salinity treatments (LP and LPLS), but in the pH 8.00 groups, it was significantly different ($t_{14} = 3.658$, $p = 0.003$) between C and LS.

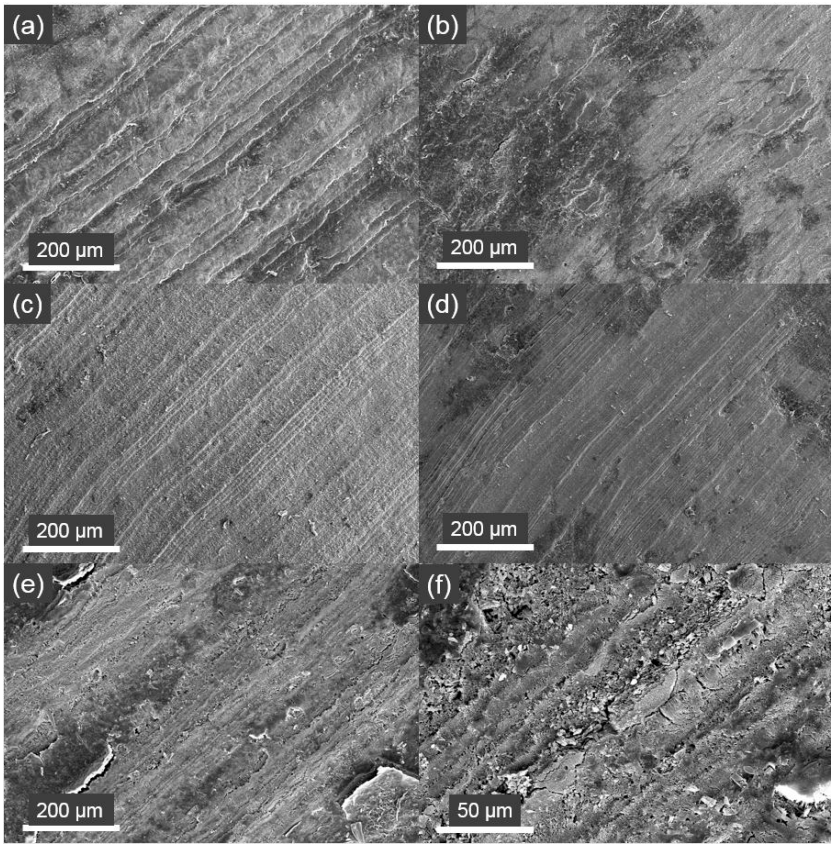

Figure 5. SEM image of the shell surface of *Nacella concinna* in each group. It showed the height of the growth decreased (flatness) when the aragonite saturation state was lower. Individuals in the LPLS group showed both flatness (**d**) and ruggedness (**e,f**) due to the dissolution of the shell surface. (**a**) An example of group C, (**b**) an example of group LS, (**c**) an example of group LP, (**d,e**) examples of group LPLS, and (**f**) magnified picture of (**e**).

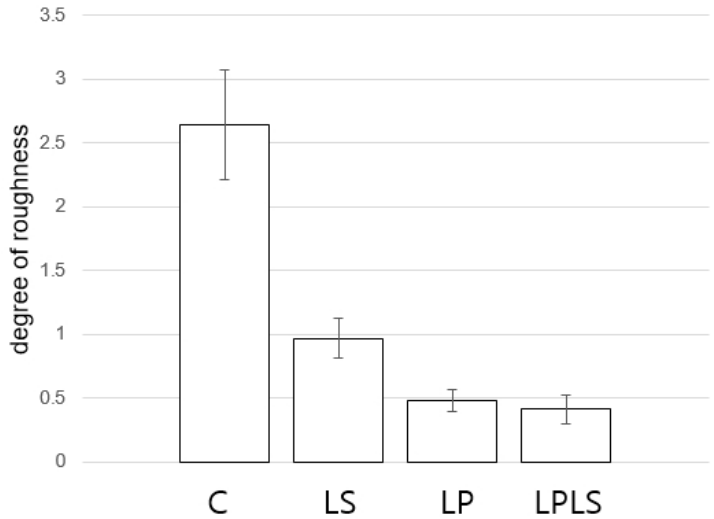

Figure 6. The degree of shell ruggedness in each group (Mean ± SE). The number of replicates for each group was eight. The degree of ruggedness decreased when the pH and salinity was low and significantly affected by those factors.

4. Discussions

When *N. concinna* was exposed to different pH or salinity treatments for 35 days, there was no difference in righting behavior and mortality between treatments. However, low salinity negatively affected condition factor and the combined condition of both stressors most severely affected shell dissolution. The results support the hypothesis that *N. concinna* is vulnerable to low-pH and low-salinity environments, which could be induced by meltwater inflow due to glacial retreat.

The righting behavior showed no difference between different treatments (Days 26, 31, and 34). It was reported that *N. concinna* that inhabit intertidal areas have taller and steeper morphotypes, which make it easier for them to right themselves [53,54]. This suggests that the limpets in the intertidal zone used in this study might have a better righting ability than those in the subtidal areas. It is likely that, because intertidal limpets are vulnerable to environmental stressors, such as physical impacts (e.g., wave exposure, rolling rocks, and ice scouring) and variations in environmental conditions (e.g., light, pH, and desiccation), they might have the morphological strategy to deal with environmentally harsh conditions [55].

The summer season of Antarctica exhibits an increase in the temporal and spatial variability of water temperature, salinity, and pH relative to the spring and early winter [43,56]. Therefore, Antarctic marine species have a physiological tolerance to temporal environmental change [56]. *N. concinna* is distributed only in the Western Antarctic Peninsula and adjacent islands, where distinct variations in seawater properties possibly occurs due to glacial melt water inflow [14,16,22]. Not only seasonal, but also fluctuating variation in water properties, which is prominent in the intertidal zone, has affected *N. concinna* for a long time [37]. Especially the meltwater which inflows to Marian Cove is acidic, because the King George Island is volcanic Island [15]. Therefore, the meltwater in Marian Cove has a low pH level in summer season.

Though short-term exposure to low salinity was not fatal, the condition factor of *N. concinna* was decreased by low salinity. The decrease in condition factor even after short-term exposure to low salinity indicates that *N. concinna* is sensitive to salinity changes and responds promptly. *N. concinna* are basically stenohaline osmoconformers with a median lower lethal salinity of 20.9 psu for four days and a median lethal time for freshwater exposure of 2 h 18 min [37]. Thus, decreases in salinity due to the meltwater inflow from the land snow and tidewater glaciers may impose severe restrictions on physiology of *N. concinna*. Intertidal limpets could experience incessant fluctuations in salinity, but the limpets in this experiment were kept under the consistent low salinity. In particular, when the limpets are exposed to air for a prolonged time during ebb tides, receiving fresh meltwater, they could experience salinity lower than the lethal limit. In this experiment, however, the limpets could not recover their osmotic condition, and they could accumulate physiological stress during the exposure period. Osmotic stressors (desiccation and rainfall) could lead to cellular energy stress [57], and energy expenditure might have reduced the body content. Even though the exposure period was short (35 days), the prompt and significant impact of low salinity on the condition factor could emphasize the negative impact of ocean freshening by meltwater inflow. Moreover, this result suggests that continuous osmotic stress could become fatal.

The shell dissolution was also significantly increased by both low-pH and low-salinity treatment, as predicted. The Ω_{arg} decreased due to both low-pH and low-salinity treatment. The ruggedness under low pH (7.55) treatment was not significantly different between salinity treatments, whereas, under the ambient pH (8.00), it was significantly different between salinity treatments. The reason that there was no difference between salinity treatments under the low pH treatment could be that some individuals of the LPLS group showed severe dissolution and erosion of both the growth line and shell surface. When the shell was exposed to low pH conditions, it showed a flat shape due to the erosion of the growth line. In the LPLS group, however, the shells showed the dissolution of both the growth line and surface, which caused an increase in ruggedness. This phenomenon has also been shown in other studies that investigated the dissolution of the Antarctic bivalve, *Laternula elliptica*, which has a lower and thinner growth line than that of *N. concinna* (Cho et al., unpublished data).

The combined stressors (pH × salinity) induced the severe undersaturation of Ω_{arg}, and probably impaired the acid–base regulation system to maintain shell formation [58,59]. This might have caused the critical dissolution of the shell.

In another study, when the *N. concinna* was exposed to a pH of 7.4, its shell surface also exhibited deterioration by five weeks, and there was exposure of the calcitic prisms within the shell architecture by eight weeks [39]. In this study, we did not expose the limpets to conditions as harsh as those of other previous studies, which aimed to investigate the effects of low pH on the calcified shell [39,60,61]. However, this study also exhibits the significant dissolution of shells due to the pH treatment.

Even though *N. concinna* can repair their shell, this is just on the inner part and the edge of the shell [34]. The erosion of the growth line on the outer part of the shell surface is irreversible when Ω_{arg} is undersaturated. Although treatment exposure was short (35 days), accumulated dissolution during the period showed significant difference between treatments. In addition, the Ω_{arg} was severely reduced by the combined stressors; therefore, the degree of dissolution followed the Ω_{arg} trend. In the upper littoral zone, the heavier shells of *N. concinna* were likely naturally selected by the physical impacts of ice abrasion [62]. However, the results of this study indicate that even the heavier shells of the limpets could be impaired by meltwater inflow, which is apparently accelerated by anthropogenic climate change. In addition, the weakened shell could be easily fragmented, and the limpets can be targeted by predators (e.g., kelp gull, sheathbill, starfish, etc.). Because *N. concinna* occurs along the WAP and the island of the Scotia Arc, which is the hot spot of glacier runoff, *N. concinna* could be a sentinel species for assessment of the impact of climate change [32].

In conclusion, there would not be devastating instant effects of acidification and freshening on survival and behavior of the Antarctic limpet *N. concinna* because it might have adapted to a large variation in pH and salinity. However, given that the consistent low salinity negatively affected the physical condition and undersaturated Ω_{arg} caused by both low pH and low salinity increased the shell dissolution, increasing meltwater inflow due to rapid Antarctic warming could be a serious problem for *N. concinna* in the long term. As a consequence, the Antarctic ecosystems, including *N. concinna*, which play an important role in the ocean food chain, could be impacted directly or indirectly. Therefore, further study should be conducted on the interaction between *N. concinna* and other species which have a relationship between predator and prey, in response to future climate changes in Antarctica.

Supplementary Materials: The following are available online at http://www.mdpi.com/2077-1312/8/10/822/s1. Figure S1: Variation in the salinity and pH of sea water collected from the intertidal zone in front of King Sejong Station. Table S1: pH and salinity of the sea water and meltwater stream of inside and outside of Marian Cove (mean ± SD). Table S2: pH, salinity and DO of the experimental sea water before and after replacement (mean ± SD).

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