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The Multi-Generational Effect of Seawater Acidification on Larval Development, Reproduction, Ingestion Rate, and ATPase Activity of *Tigriopus japonicus* Mori, 1938

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Abstract: Ocean acidification threatens marine organisms continuously. To ascertain if adaptation of marine species to ocean acidification enhanced over multiple generations, we studied the transgenerational effects of ocean acidification on the development, reproduction, ingestion rate, and ATPase activity of a copepod *Tigriopus japonicus* Mori, 1938. In the first mode, individuals were exposed to either one of the pH levels (8.1 (control), 7.7, 7.3) for five successive generations. In the second mode, each successive generation was exposed to a lower pH level (pH levels: 8.1, 7.9, 7.7, 7.5, 7.3). After prolonged exposure to a constant seawater acidification level, the capacity to adapt to the stress increased. However, when exposed to seawater of descending pH, the detrimental effects gradually increased. Energy allocated to development and reproduction was reduced although the ingestion rate continued to improve in successive generations. Therefore, ongoing ocean acidification might lower the energy transfer of copepods to higher trophic levels.

Keywords: ocean acidification; *Tigriopus japonicus*; larval development; reproductive output; ingestion rate; ATPase; multi-generational effects

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1. Introduction

Atmospheric CO_2 concentration has increased over the last 200 years due to anthropogenic activities and is expected to increase further [1,2]. Dissolution of carbon dioxide in the oceans has resulted in a lowering of seawater pH, altering carbonate chemistry, increasing hydrogen ion (H^+) and bicarbonate ion (HCO_3^-), as well as dissolved inorganic carbon (DIC) concentrations [1,3], and decreasing carbonate ion (CO_3^{2-}) concentrations, a phenomenon called ocean acidification (OA) [1,3]. Since preindustrial times, ocean acidity has decreased by 0.1 pH units [3]. Based on the predictions of the Intergovernmental Panel on Climate Change [4], the future ocean pH will decline for a further 0.3–0.4 units by the end of the century and 0.77 units by the year 2300, with corresponding pCO_2 levels of 700–1000 ppm and 1900 ppm, respectively [1,3,5].

OA reduces calcification by depleting CO_3^{2-}, and therefore exerts a larger effect on calcifying organisms [3,6]. Declining seawater pH also has negative effects on survival, growth, and reproduction for marine organisms such as crustaceans, fish, and fleshy algae [7]. There has been a dramatic increase in studies on seawater acidification in recent years with most of them involving single species and single generations. In view of their short life cycle, high species diversity and ecological significance in the ocean, copepods are popular experimental animals in these studies. In many studies using projected future levels of pCO_2, limited effects on life history traits have been reported [8,9]. Early life stages have been found to be the most vulnerable period in copepods with their development being retarded under elevated pCO_2 [10–12]. Short-term (4–9 days) exposure to

low pH levels (pH 7.6–7.78) has no effect on hatching success, but hatching was significantly delayed, and overall hatching success possibly reduced at much lower pH levels (CO_2 = 9830 ppm; pH 6.71) [13]. Exposures to lower pHs (pH 7.5 and 7.0) of copepod *Tigriopus japonicus* resulted in the lengthening of the developmental time with decreased fecundity and body length [2,14].

Nevertheless, recent studies have demonstrated that the effects of seawater acidification can be alleviated by the transgenerational effect [15]. When a calanoid copepod *Pseudocalanus acuspes* was cultured under three pCO_2 levels (400, 900, and 1550 µatm) for two generations, effects on fecundity and metabolic stress at 1550 µatm were alleviated due to the transgenerational effect [16]. Several studies have revealed that parental conditioning to global change drivers leads to positive effects on the offspring's response to similar conditions [15,17]. As acclimation occurs, a species may also adapt to the new conditions by enhancing reproductive effort, and consequently increasing the probability of genotypes that are more suitable to thrive over subsequent generations [15,18,19]. In contrast, when various life stages of two consecutive generations of the copepod *Acartia tsuensis* were exposed to pCO_2 of either 380 µatm or 2380 µatm, higher CO_2 exposure did not significantly affect survival, body size, or developmental speed. Results were similar for both generations [8], indicating a high tolerance of *A. tsuensis* to ocean acidification, similar to earlier hatching of eggs from females with a high CO_2 selective history [2]. A long-term experiment (3.5 years) shows decreased copepod developmental rates and body size, and changed elemental body composition [20,21]. These results suggest marine environment changes due to ocean acidification may affect *Acartia* populations and cause overall fluctuations in copepods of the genus *Acartia* [22]. What is more, antagonistic interactions between warming and acidification in later generations decreased survival, thereby limiting full fitness recovery [23].

Seawater acidification can affect the physiology of marine organisms, particularly through increasing the concentration of Ca^{2+} ion in the cell [24] and disruption of extracellular acid-base balance [25,26]. Ca^{2+} is a very important cofactor for many enzymes, but it can be cytotoxic at high concentrations [27]. Therefore, Ca-ATPase is upregulated when dissolution occurs or when CO_3^{2-} concentration decreases with declining pH levels, possibly as a way to maintain calcite saturation state (Ω_{Ca}) in the calcification media [24]. Upon chronic exposure to predicted future levels of pCO_2, organisms rely on ion-exchange mechanisms and use protein carriers such as Na^+/K^+- and H^+ ATPase to maintain acid-base balance, processes which incur significant energetic costs [28,29]. To respond to and cope with the increased external acidity, copepods increased their food acquisition to compensate for the extra energy demand [28]. This helps explain the food-dependent effect on tolerance to elevated pCO_2 in copepods [30].

Copepods belong to Phylum Arthropoda, Class Crustacea, Subclass Copepoda. The copepod *Tigriopus japonicus* is a benthic harpacticoid species distributed widely along the west coast of the Pacific Ocean including China, Korea, and Japan, with high tolerance to temperature and salinity [31]. Its ease of culture in the laboratory, small body length (~1.0 mm), strong capability of regeneration, short life cycle (14 days), and clear sexual dimorphism [30,32] make it an excellent model organism for marine ecotoxicological studies. The larval development includes six naupliar stages and five copepodite stages before metamorphosing to the adult stage [30,32]. Both larva and adult are omnivorous and can be reared using diatoms, yeast, fish meal, bacteria, or macroalgae. The usual conditions for rearing *T. japonicus* are temperatures of 20–25 °C, salinity of 30–35, and a light/dark cycle of 16:8. In Qingdao, China, *T. japonicus* is commonly found in rock pools at high intertidal areas where pH varies between 7.7 and 9.6 in summer and 7.6 and 8.4 in winter [30].

Studies on transgenerational effects on marine organisms over two or more generations are very limited. This study investigated the transgenerational effects of seawater acidification on larval development and reproductive output of *T. japonicus* over five generations using two exposure modes. To our knowledge, all previous studies on copepods exposed them to either a lower pH level for the whole duration of the experiment or a short-term (100 h) stepwise decrease in pH (Widdicom et al., 2008; Sato et al., 2005). In the first exposure mode, we exposed *T. japonicus* to one of the pH levels (8.1 (control), 7.7, and 7.3) for five successive generations (Generation 0–4) whereas in the second exposure mode, each successive generation was exposed to a pH level lower than the one experienced by their parents. The first exposure mode allowed us to understand how a species is adapted to an abrupt change in the pH environment, whereas the second mode reflects what is happening in the real world where pH decreases with successive generations. For the second exposure mode, we also studied the feeding rate and the activity of Na^+/K^+- and Ca^{2+} ATPase over five generations. We hypothesized that both larval development and reproductive output would be negatively affected under both exposure modes. Since a longer time was available for *T. japonicus* to adapt to a new pH in the first exposure mode, the effects of acidification should be smaller as compared with the second exposure mode. Counteracting the effect of acidification through maintaining a stable intracellular pH requires an increase in energy input and more efficient ionic transports. Therefore, we predicted that both the feeding rate and ATPase activities would increase under progressive acidification stress.

2. Materials and Methods

2.1. Maintenance of Animals and Experimental Exposure

Individual specimens of *T. japonicus* were collected from an intertidal rock pool at Huiquan Bay (120.339° N, 36.061° E), Qingdao, China [30]. Upon transportation to the laboratory, they were maintained for 24 generations in aerated 0.45 µm filtered seawater (pH 8.1) at 22 ± 1 °C and a 16:8 light/dark cycle (light intensity: 33 µmol photons m^{-2} s^{-1}). Unicellular algae *Phaeodactylum tricornutum* and *Platymonas helgolandica* and baker's yeast (*Saccharomyces cerevisiae*; Anqi®, Yichang, China) were offered as food every other day in a ratio of 2:2:1 (3.0×10^5 cells mL^{-1} in total). At the beginning of the experiment, five experimental pH levels, 8.1, 7.9, 7.7, 7.5, and 7.3, were set up by pre-mixed bubbling pure CO_2 (99.99%) and air into the filtered seawater. During the experiment, the pH levels were maintained by keeping the set ups in carbon dioxide climate incubators (Wuhan Ruihua Instrument Equipment®, HP-250G-D, Wuhan, China) equipped with CO_2 probes and the feedback control system [30].

The pH was measured with a pH meter (Mettler Toledo®, DELTA 320, Greifensee, Switzerland) that was calibrated with buffer solutions of pH 4.01, 6.86, and 9.18 (Mettler Toledo®) before use. The salinity was measured with a refractometer (Zhengzhou Nanbei Instrument Equipment®, HB-212ATC, Zhengzhou, China). Total alkalinity was measured with a total alkalinity mini-titrator (Hanna®, HI 84431, Padova, Italy), whereas dissolved oxygen was measured with a dissolved oxygen meter (Leici®, JPBJ-608, Shanghai, China) [30]. The partial pressure of CO_2, the total dissolved inorganic carbon, and the calcium carbonate saturation state for calcite (Ω_{Ca}) were calculated from the pH and total alkalinity using the software CO2Sys (http://cdiac.esd.ornl.gov/oceans/co2rprt.html, accessed on 10 February 2023) [33]. All seawater quality parameters were measured immediately before and after the seawater renewal (Tables 1 and 2).

Table 1. Environmental parameters of the control group before and after water renewal during the experiment. The pH (NBS scale), temperature (T), salinity (S), dissolved oxygen (DO), and total alkalinity (AT) were measured. All other values (partial pressure of carbon dioxide: pCO_2; total dissolved inorganic carbon: C_T; and calcium carbonate saturation state for calcite: Ω_{Ca} were calculated using CO2Sys from pH and total alkalinity. The pH values represent means [n = 30 for the treatments with constant pH] (maximum value − minimum value). The values of salinity, dissolved oxygen, total alkalinity, and other calculated factors represent means [±standard deviation (SD); n = 30] for the first three treatments with constant pH levels.

		Treatments with Constant pH		
		pH 8.1	**pH 7.7**	**pH 7.3**
pH	before	8.09 (8.07–8.10)	7.70 (7.69–7.72)	7.31 (7.30–7.33)
	after	8.12 (8.10–8.14)	7.72 (7.71–7.74)	7.32 (7.30–7.34)
S	before	29 (±0.3)	29 (±0.4)	29 (±0.4)
	after	30 (±0.5)	30 (±0.3)	30 (±0.5)
DO (mg/L)	before	6.2 (±0.4)	5.9 (±0.2)	5.6 (±0.3)
	after	5.9 (±0.8)	5.7 (±0.6)	5.3 (±0.6)
TA (mg/L)	before	141 (±1.7)	136 (±1.4)	127 (±1.5)
	after	137 (±1.3)	132 (±1.3)	125 (±1.3)
pCO_2 (µatm)	before	413 (±8)	997 (±14)	2555 (±40)
	after	391 (±9)	942 (±20)	2515 (±43)
$C_{T(mM/kg)}$	before	1933 (±42)	2026 (±34)	2159 (±38)
	after	1917 (±39)	1989 (±30)	2126 (±30)
Ω_{Ca}	before	2.45 (±0.03)	1.65 (±0.02)	0.58 (±0.01)
	after	2.41 (±0.04)	1.59 (±0.05)	0.53 (±0.01)

Table 2. Environmental parameters of the treatment groups before and after water renewal during the experiment. The pH (NBS scale), temperature (T), salinity (S), dissolved oxygen (DO), and total alkalinity (AT) were measured. All other values (partial pressure of carbon dioxide: pCO_2; total dissolved inorganic carbon: C_T; and calcium carbonate saturation state for calcite: Ω_{Ca} were calculated using CO2Sys from pH and total alkalinity. The pH values represent means [n = 30 for the treatments with constant pH; n = 10 for the treatment with descending pH] (maximum value − minimum value). The values of salinity, dissolved oxygen, total alkalinity, and other calculated factors represent means [±standard deviation (SD); n = 10] for the treatment with descending pH.

		Treatments with Descending pH				
		pH 8.1	**pH 7.9**	**pH 7.7**	**pH 7.5**	**pH 7.3**
S	before	8.10 (8.09–8.11)	7.91 (7.90 ± 7.93)	7.69 (7.69 ± 7.71)	7.50 (7.49 ± 7.52)	7.30 (7.29 ± 7.33)
	after	8.13 (8.11–8.15)	7.92 (7.91 ± 7.94)	7.71 (7.7 ± 7.74)	7.51 (7.51 ± 7.54)	7.31 (7.31 ± 7.35)
DO(mg/L)	before	29 (±0.5)	29 (±0.4)	29 (±0.3)	29 (±0.5)	29 (±0.6)
	after	30 (±0.7)	30 (±0.5)	30 (±0.4)	30 (±0.6)	30 (±0.6)
TA (mg/L)	before	6.1 (±0.4)	6.0 (±0.4)	5.8 (±0.3)	5.8 (±0.4)	5.5 (±0.3)
	after	5.8 (±0.9)	5.9 (±0.9)	5.7 (±0.8)	5.7 (±0.9)	5.3 (±0.5)
pCO_2 (µatm)	before	143 (±1.9)	140 (±1.8)	135 (±1.5)	133 (±1.4)	125 (±1.6)
	after	139 (±1.1)	138 (±1.2)	131 (±1.3)	130 (±1.5)	124 (±1.5)
C_T (mM/kg)	before	403 (±5)	588 (±8)	987 (±15)	1537 (±17)	2556 (±40)
	after	387 (±9)	562 (±9)	939 (±21)	1466 (±20)	2510 (±45)
Ω_{Ca}	before	1930 (±44)	2002 (±24)	2026 (±34)	2096 (±22)	2159 (±38)
	after	1910 (±40)	1995 (±20)	1998 (±35)	2045 (±24)	2120 (±21)
pH	before	2.45 (±0.03)	2.17 (±0.02)	1.65 (±0.02)	0.89 (±0.01)	0.58 (±0.01)
	after	2.41 (±0.04)	2.15 (±0.02)	1.59 (±0.05)	0.88 (±0.02)	0.53 (±0.01)

2.2. Experimental Set Up

Two experiments were conducted. In Experiment 1, individuals of *T. japonicus* were maintained in the same experimental pH level for five consecutive generations (generations 0–4). Three pH levels, 8.1, 7.7, and 7.3, with corresponding CO_2 concentration of 380 ppm, 950 ppm, and 1900 ppm, respectively, were set up. They represented the present-day scenario, and the levels predicted to occur for the years 2100 and 2300, respectively [1,3,8]. One night before the start of the experiment, adult *T. japonicus* carrying eyed eggs were collected from the cultivation stock at pH 8.1 and transferred to a petri dish with sufficient food and filtered seawater [30]. Nauplii were collected randomly, less than 24 h after hatching. For each experimental pH level, 180 nauplii were divided equally into three groups as replicates, with each group being transferred to a well of a 6-well plate (15 mL) and referred to as the first generation (Generation 0). The control group (pH 8.1) was placed in a climate chamber (Ningbo Jiangnan Instrument®, GXZ, Ningbo, China) [30] while the other two groups (pH 7.7 and 7.3) were placed in separate carbon dioxide climate chambers (Wuhan Ruihua Instrument Equipment ®, HP-250G-D, Wuhan, China). The culture conditions were the same as the cultivation stock. Seawater was renewed every 2 days before feeding [30]. All water quality parameters were measured immediately before and after water renewal [30] (Tables 1 and 2). The nauplii were cultured until they developed into adults. After the gravid females spawned, 60 newly hatched nauplii (<24 h after hatching) were collected from each replicate and kept in the same experimental conditions [30]. The process was repeated for another four generations (Generations 1–4).

In Experiment 2, five pH levels (pH 8.1, 7.9, 7.7, 7.5, and 7.3) were set up, with each successive generation of *T. japonicus* exposed to a lower pH than their parents. Five generations were studied, with the first generation (Generation 0) exposed to pH 8.1, the second generation to pH 7.9, the third generation to 7.7 and so on. Three replicates with 60 nauplii each were prepared for each pH level. The parental generation (Generation 0) was cultured in a climate chamber (Ningbo Jiangnan Instrument®, GXZ, China) [30], whereas the next four generations in the carbon dioxide climate chambers (Wuhan Ruihua Instrument Equipment®, HP-250G-D, China) corresponding to CO_2 concentrations of 550 ppm (pH 7.9), 950 ppm (pH 7.7), 1250 ppm (pH 7.5), and 1900 ppm (pH 7.3), respectively. The culturing conditions were the same as those in the first experiment.

2.3. Larval Development and Reproduction

In both Experiment 1 and Experiment 2, the developmental time of 60 nauplii (days required for 50% of the nauplii to molt into copepodites) and 60 copepodites (days required for 50% of the copepodites to molt into adults) per replicate at each pH level were observed under a stereomicroscope [30]. To understand the influence of pH on reproductive output, two females bearing egg sacs from each replicate were picked randomly and transferred to separate wells of six-well plates and cultured at corresponding pH levels. The number of hatching egg sacs and average number of nauplii hatched from each egg sac were recorded for each female every day for 10 days, with counting started on the first day the female spawned. Three replicates were prepared for each of the generations for both the larval development and reproductive output study.

2.4. The Ingestion Rate

In Experiment 2, the effect of seawater acidification on ingestion rate was studied for successive generations of adult *T. japonicus* exposed to descending pH. Twenty individuals (male:female ratio = 1:1) in each replicate were starved for 24 h and then pipetted into one well of a 6-well plate containing filtered seawater of corresponding pH levels. The same number of wells containing filtered seawater, but without *T. japonicus* served as the control group. A green alga *Platymonas helgolandica* was added to each well as food at a concentration of 3×10^5 cell mL^{-1}. The 6-well plates were sealed and cultured in an incubator (Tianjin City Taisite Instrument®, Tianjin, 101-2AB, China) at 22 °C in darkness with the plates shaken gently once an hour. After incubation for four hours, a l mL water

sample was collected from each well after sufficient shaking. The concentration of algal cells in the water sample was measured by a hemocytometer. Ingestion rate was calculated as [34]:

$$I = \frac{V}{N} \times \frac{lnC_t - lnC_{tf}}{t} \times \frac{C_{tf} - C_0}{lnC_{tf} - lnC_0}$$

where I is ingestion rate (cells ind^{-1} h^{-1}), the number of algal cells ingested by each *T. japonicus* per hour; V the total volume of water (mL); N the number of adult *T. japonicus* (ind); C_0 the initial concentration of algal cells (10^5 mL^{-1}); C_t the final concentration of algal cells in the control (10^5 mL^{-1}); C_{tf} the final concentration of algal cells in the experimental group (10^5 mL^{-1}) and the feeding time (h) [35].

2.5. ATPase Activity

The effect of descending pH on ATPase activity of successive generations of *T. japonicus* was studied. For each generation, 20 mature individuals (male:female = 1:1) from each replicate were put in each of the three centrifuge tubes (1.5 mL) and stored at −80 °C. Copepods were homogenized in tris-buffer (1 mM EDTA, 0.25 mol L^{-1} sucrose, 0.15 mol L^{-1} NaCl, 1 mM dithiothreitol) at pH 7.6 with a Teflon homogenizer. The homogenate was centrifuged at 3500× *g* for 10 min at 4 °C [30]. ATPase activity of the supernatant was measured using an ATP assay kit (Nanjing Jiancheng®, A016-2, Nanjing, China) [30] at 660 nm of an ultraviolet spectrophotometer (Agilent®, Cary60, Palo Alto, CA, USA) [30]. The ATPase activity measurements were normalized by total protein and represented as percentage of the control. Total protein was measured using a total protein kit (Nanjing Jiancheng®, Nanjing, China) at 550 nm of an enzyme mark instrument (Thermo Fisher Scientific®, Multiskan FC, Waltham, MA, USA) [30].

ATPase activity was calculated as:

$$ATPase\ activity = \frac{OD(value\ of experimental\ group) - OD\ (value\ of\ control\ group)}{Standard\ OD\ value} \times Standard\ concentration \times Sample\ Dilution\ Ratio \times 6 \div Sample\ Protein\ Concentration$$

Standard concentration unit is 1 μmol mL^{-1},
Sample protein concentration unit is mgprot mL^{-1}.

2.6. Statistical Analysis

Statistical analyses were computed using the SPSS software version 17.0 (Chicago, IL, USA) [30]. Normality and homogeneity of the data were checked by the Kolmogorov-Smirnov test and Levene's test, respectively [30]. Data were subjected to transformation if the test for normality or homogeneity of variance failed [30]. The combined effects of elevated pCO_2 and generation on the larval developmental time and reproduction were analyzed by two-way ANOVA followed by pairwise multiple comparisons. In Experiment 1 and Experiment 2, individuals exposed to the same pH levels have different exposure history and their larval development and reproductive output were compared by independent t-test. Statistical significance was accepted when $p < 0.05$ [30].

3. Results

3.1. Larval Development and Production of Nauplii at Constant pH Conditions

In Experiment 1, naupliar development (Figure 1) was significantly affected by pH ($F = 19.60$, $p < 0.05$), generation ($F = 6.10$, $p < 0.05$), and pH–generation interaction ($F = 9.85$, $p < 0.05$). At pH 7.7, the naupliar development of Generation 3 was significantly delayed compared to Generations 0–2 (Tukey HSD, $p < 0.05$) whereas at pH 7.3, the naupliar development of Generations 0 and 4 were significantly delayed compared to Generations 1–3 (Tukey HSD, $p < 0.05$). When the developmental time was compared among different pHs for each generation, a longer developmental time was observed at pH 7.7 than at pH 8.1 and pH 7.3 for Generation 3 (Tukey HSD, $p < 0.05$). For both Generation 0

and Generation 4, the development time at pH 7.3 was significantly longer than at pH 8.1 (Tukey HSD, $p < 0.05$).

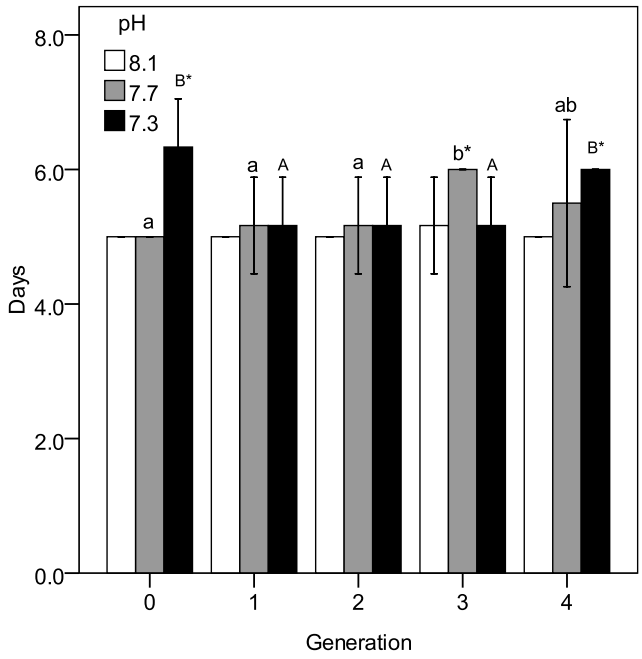

Figure 1. Effect of seawater acidification on naupliar developmental time (mean ± standard deviation, n = 3) of *Tigriopus japonicus*. All of the five generations were exposed to the same pH levels. Statistical differences among generations ($p < 0.05$) are represented by different letters. Asterisks (*) indicate significant differences ($p < 0.05$) in the developmental rate between pH 8.1 and pH 7.7, as well as between pH 8.1 and pH 7.3 for each generation.

Copepodite development (Figure 2) was significantly affected by pH (F = 31.20, $p < 0.05$), generation (F = 21.70, $p < 0.05$), and pH–generation interaction (F = 8.20, $p < 0.05$). The developmental time of Generation 3 was the longest and followed by Generation 4. Both generations had a developmental time significantly longer than Generations 0 and 1 (Tukey HSD, $p < 0.05$); Generation 2 also had a longer developmental time than Generation 4 (Tukey HSD, $p < 0.05$). For individual generations, both Generations 2 and 3 had a significantly longer developmental time at lower pH (Tukey HSD, $p < 0.05$).

The reproductive output (Figure 3) was significantly affected by pH (F = 46.57, $p < 0.05$), generation (F = 11.84, $p < 0.05$), and pH–generation interaction (F = 9.27, $p < 0.05$). For the number of nauplii per egg sac (Figure 3A), the lower the pH, the lower the number was with the largest difference among different pHs observed in Generation 0. The difference, however, diminished with successive generations, resulting in the number of nauplii per egg sac being independent of pH for Generation 4. At the lowest pH (pH 7.3), the total number of nauplii produced by Generation 0 was significantly lower (Tukey HSD, $p < 0.05$) than other generations except Generation 2 (Figure 3B). At pH 7.7, Generation 3 produced the highest number of nauplii, which was significantly higher (Tukey HSD, $p < 0.05$) than that produced by Generations 0 and 1. For each generation, the lowest pH (pH 7.3) produced a significantly higher number (Tukey HSD, $p < 0.05$) of nauplii than the control (pH 8.1) for Generations 1, 3, and 4. At pH 7.3, the number of egg sacs produced (Figure 3C) was significantly lower (Tukey HSD, $p < 0.05$) than at pH 8.1 for all five generations, whereas at pH 7.7, only Generations 2 and 3 produced less egg sacs (Tukey HSD, $p < 0.05$) than other generations. When different generations were compared for each pH, Generations 0 and 1 produced less egg sacs (Tukey HSD, $p < 0.05$) than other generations at pH 7.3, whereas at pH 7.7, less egg sacs (Tukey HSD, $p < 0.05$) were produced in Generations 2 and 3.

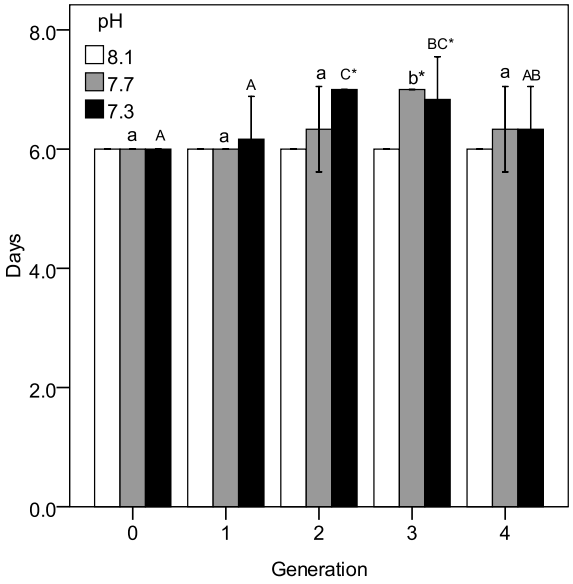

Figure 2. Effect of seawater acidification on copepodite developmental time (mean ± standard deviation, n = 3) of *Tigriopus japonicus*. All of the generations were exposed to the same pH levels. Statistical differences among generations ($p < 0.05$) are represented by different letters. Asterisks (*) indicate significant differences ($p < 0.05$) in the developmental rate between pH 8.1 and pH 7.7, as well as between pH 8.1 and pH 7.3 for each generation.

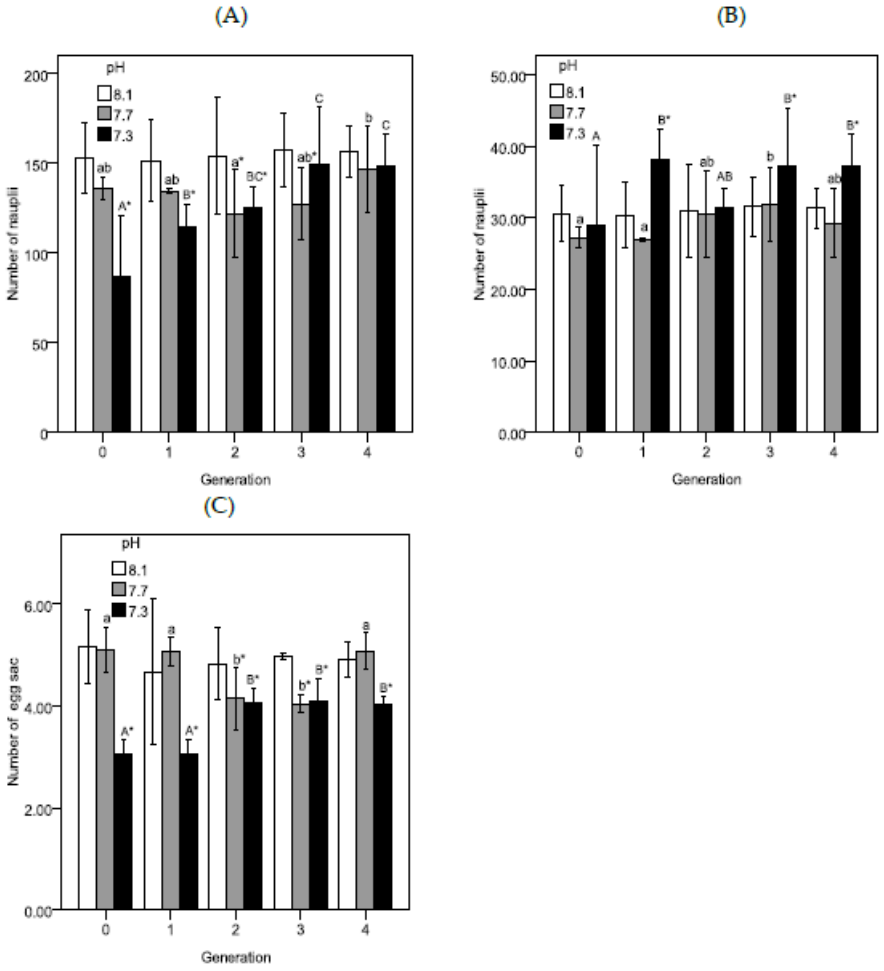

Figure 3. Effect of seawater acidification on the fecundity (mean ± standard deviation, n = 6) of *T. japonicus* in five generations. All of the generations were exposed to the same pH. (**A**) The average

number of nauplii per egg sac, (**B**) the total number of nauplii hatched in 10 days, and (**C**) the number of egg sacs. Statistical differences among generations at the same pH ($p < 0.05$) are represented by different letters. Asterisks (*) indicate significant differences ($p < 0.05$) in the fecundity index between pH 8.1 and pH 7.7, as well as between pH 8.1 and pH 7.3 for each generation.

3.2. Larval Development and Production of Nauplii at Descending pH Conditions

For Experiment 2 in which successive generations were exposed to lower pHs, the developmental time of both nauplii (F = 9.30, $p < 0.01$) and copepodite (F = 8.30, $p < 0.01$) was delayed for younger generations (Figure 4). For nauplii, the developmental time increased significantly (Tukey HSD, $p < 0.05$) for Generations 2 (pH 7.7) and 3 (pH 7.5) as compared with Generations 0 (pH 8.1) and 1 (pH 7.9). For copepodite, a significantly longer developmental time (Tukey HSD, $p < 0.05$) was observed for Generation 4 (pH 7.3) than Generations 0 (pH 8.1) and 1 (pH 7.9).

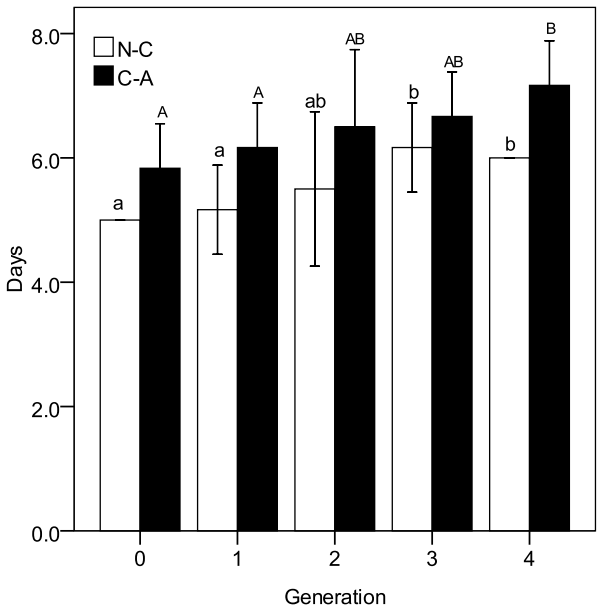

Figure 4. Effect of seawater acidification on the developmental time (mean ± standard deviation, n = 3) of larval *T. japonicus* in five generations (N-C developmental time of nauplii, C-A developmental time of copepodites). Successive generations were exposed to a lower pH than their parents. Statistical differences among generations ($p < 0.05$) are represented by different letters.

The reproductive output, in terms of the total number of nauplii (F = 13.10, $p < 0.05$) and number of nauplii per egg sac (F = 12.98, $p < 0.05$), was reduced when successive generations were exposed to lower pH levels (Figure 5A,B) with Generations 2–4 (pH 7.7–7.3) producing less than Generations 0 (pH 8.1) and 1 (pH 7.9) (Tukey HSD, $p < 0.05$). The number of egg sacs, however, was independent of pH (F = 0.30, $p = 0.871$) (Figure 5C).

The larval development and reproductive output were compared between Experiment 1 and Experiment 2 for each pH. This allowed us to compare generational differences in their responses to pH because individuals in the two experiments had different exposure history. For example, at pH 7.7, individuals in Experiment 1 were transferred directly from pH 8.1 to that pH, whereas in Experiment 2 their parents and grandparents were exposed to pH 7.9 and 8.1, respectively. At pH 7.7, the exposure history did not affect the developmental time of both the nauplii (t = 1.00, $p > 0.05$) and copepodite (t = 2.12, $p > 0.05$) as well as reproductive output (t = 1.88, $p > 0.05$). But at pH 7.3, the copepodite developmental time in Experiment 2 with descending pH gradient was significantly longer than that in Experiment 1 (t = 3.536, $p < 0.05$). The total number of nauplii in Experiment 2 was also significantly lower than in Experiment 1 (t = 3.736, $p < 0.05$).

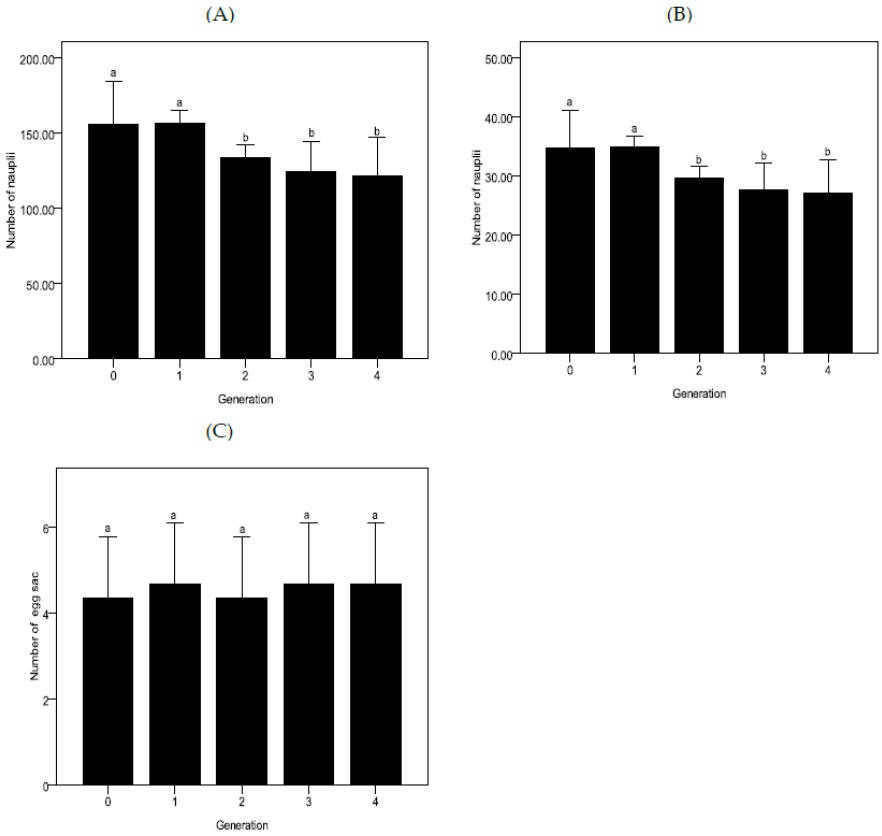

Figure 5. Effect of seawater acidification on the fecundity (mean ± standard deviation, n = 6) of *T. japonicus* in five generations. Successive generations were exposed to a lower pH than their parents. (**A**) The total number of nauplii produced in 10 days, (**B**) the average number of nauplii per egg sac, and (**C**) the number of egg sacs. Statistical differences among generations ($p < 0.05$) are represented by different letters.

3.3. The Ingestion Rate and ATPase Activity at Descending pH Condition

The pH level has a significant effect on ingestion rate (F = 13.100, $p < 0.05$; Figure 6), Na^+/K^+—ATPase activity (F = 67.655, $p < 0.05$; Figure 7), and Ca^{2+}—ATPase activity (F = 169.167, $p < 0.05$; Figure 7). The lower the pH, the higher the feeding rate as well as ATPases' activities (Tukey HSD, $p < 0.05$).

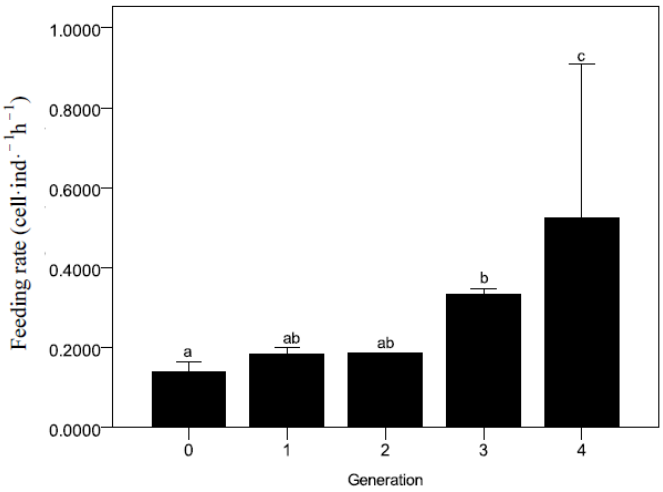

Figure 6. Effect of seawater acidification on the ingestion rate (mean ± standard deviation, n = 3) of *T. japonicus* in five generations. Successive generations were exposed to a lower pH than their parents. Statistical differences among generations ($p < 0.05$) are represented by different letters.

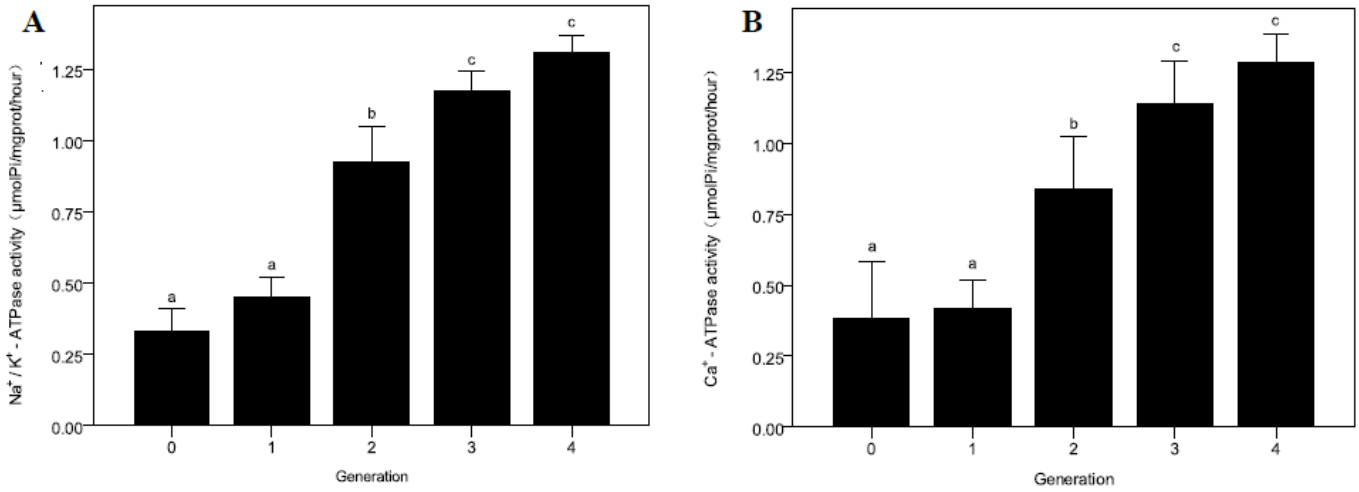

Figure 7. Effect of seawater acidification on ATPase activity (mean ± standard, n = 3) of *T. japonicus* in five generations. Successive generations were exposed to a lower pH than their parents. (**A**) Na^+/K^+-ATPase activity. (**B**) Ca^{2+}-ATPase activity. Statistical differences among generations ($p < 0.05$) are represented by different letters.

4. Discussion

The exposure of multiple successive generations of *T. japonicus* to lower pH levels prolonged the developmental time of both larval stages, i.e., nauplii and copepodite, and the lower the pH, the greater was the retardation of the development. The effect became insignificant in Generation 4 for copepodite but still persisted in nauplii, indicating that the naupliar stage is more sensitive to elevated pH. A higher susceptibility of nauplii to elevated pCO_2 was reported in other studies such as Cripps et al. (2014) [36]. Decrease in pH also affected reproductive output negatively with less egg sacs and less nauplii in each sac produced. The effect persisted in Generation 4 at pH 7.3 (for number of egg sacs) but not at pH 7.7. Therefore, the pH 7.7 predicted to occur in 2100 might not have any long-term effects on both the larval development and reproductive output in *T. japonicus* in this study. This is understandable, as *T. japonicus* lives in rock pools which exhibit wide diurnal and seasonal fluctuations in important ecological factors such as pH and salinity [37], so they are expected to be less sensitive to lower pH. At pH 7.3, although the effect of lower pH still persisted after four generations, both the number of egg sacs and number of nauplii per egg sac increased with subsequent generations [33], indicating an enhancement of tolerance. Pedersen et al. (2014) also demonstrated that calanoid copepods possess considerable adaptive capacity through phenotypic plasticity and/or adaptive selection to counteract the potentially negative impact of seawater acidification scenarios predicted for the year 2300 [38,39]. Such adaptive selection, however, may have a constraint in *T. japonicus* as the increase in the number of egg sacs was halted from Generation 2 onwards, regardless of an increase in the total number of nauplii and number of nauplii per egg sac. In contrast to gradual adaptations to ocean acidification in successive generations, Griffith and Gobler (2016) found that the impacts of acidification on northern quahog, *Mercenaria mercenaria* larvae may become more severe over multiple generations under ocean acidification, as larvae originating from adults undergoing reproductive conditioning in acidified environments were more sensitive to low pH environments themselves [40].

When successive generations were exposed to lower pH levels than their parents, both larval development and reproductive output were negatively affected. The effects of lower pH, however, did not intensify in subsequent generations starting at Generation 2. Although the pH decreased progressively from pH 7.7 in Generation 2 to pH 7.3 in Generation 4. This means that adaptations developed could counteract the impacts of further decrease in pH, at least down to pH 7.3. However, when individuals of the two experiments exposed to pH 7.3 but with different exposure history were compared, the effect

on both larval development and reproductive output was greater for the group with parents/grandparents exposed to successive decrease in pH. Although Generation 0 in Experiment 1 were exposed to a larger decrease in pH at the beginning of the experiment, subsequent generations experienced the same degree of stress as Generation 0, in contrast to Experiment 2 in which every generation experienced a lower pH than its parents. Therefore, individuals in Experiment 1 had a longer time, or more appropriately, more generations for adaptations to develop against lower pH. In most of the previous studies on marine copepods, the approach used was similar to Experiment 1 in this study. In view of a gradual and continual decrease in pH in the future, this approach may underestimate the impact of ocean acidification.

Tolerance to CO_2-induced stress is probably associated with energetic constraints as compensatory responses against stress incur energetic costs, leaving less energy to support key biological processes such as growth and development [30], resulting in elevated metabolic rates and developmental delay in sea urchin larvae *Hemicentrotus pulcherrimus* and *Echinometra mathaei* [41]. In the present study, Na^+/K^+-ATPase and Ca^{2+}-ATPase activity increased with successive generations exposed to decreasing pH. Similar observations have been reported in Atlantic cod (*Gadus morhua*) and was considered as an enzymatic adjustment to cope with the CO_2 induced acid-base load [34]. Elevated seawater pCO_2 can lead to hypercapnia and acidosis [38,39,42], which in turn may result in a reallocation of resources away from growth and reproduction, due to mobilization of energy demanding acid−base regulatory processes to counteract internal pH reduction [25,38,39]. Acid–base adjustments made by crustaceans are likely to be metabolically expensive, due to the dependence on HCO_3^- uptake from the seawater via electroneutral ion exchange. The electroneutral exchange of HCO_3^- for Cl^- and H^+ for Na^+ is, in turn, dependent on the presence of ion gradients across transport epithelia that are maintained by active ion transporting pumps, i.e., Na^+/K^+- and H^+-ATPases [25,43]. Most of the previous studies have shown that the calcification rate in crustaceans remains unchanged or is even enhanced under elevated pCO_2 [44,45]. Seawater acidification can increase the concentration of Ca^{2+} ion in the cell, and Ca-ATPase appears to play an important role in actively regulating intracellular Ca^{2+} balance [24]. It has been suggested that as a consequence of maintaining in vivo acid-base balance, ocean acidification may cause alterations in the concentration of in vivo ions such as H^+, HCO_3^-, and Ca^{2+} in marine invertebrates. Some amorphous calcium carbonate is found in the exoskeleton of crustaceans [46,47], and this form of calcium carbonate dissolves easily when ocean acidification occurs [29]. When barnacles *Semibalanus balanoides* and *Amphibalanus amphitrite* were exposed to acidified seawater caused by high pCO_2, they had high calcification rate and their shell was found to be soluble [44]. Conversely, at lower pH conditions, crustaceans such as crabs could maintain an elevated pH and CO_3^{2-} concentration at their sites of calcification through a specific proton-regulating mechanism. This mechanism converts much of the increased inorganic carbon (DIC), occurring primarily as HCO_3^-, to CO_3^{2-} [43,45]. The enhancement of calcification may promote the transport of the two ions depending upon the efficiency of Na^+/K^+-ATPase and Ca^{2+}-ATPase [43]. Much less is known about the mechanisms to answer calcification processes in crustaceans. The physiological processes, i.e., ionoregulation, acid–base balance and calcification, could be linked via the mobilization of Ca^{2+} and HCO_3^- from the exoskeleton [29]. The ion transport mechanism eventually increases the energy consumption.

Additional energy is required for acid–base regulation and Ca^{2+} transport in cells under acidic perturbation. This can be achieved by increasing food acquisition as demonstrated by *T. japonicus* in this study and in the copepod *Centropages tenuiremis* [48]. Considering the dominance of copepods throughout the world's oceans [49], an enhanced food consumption will eventually increase feeding pressure on the phytoplankton [50,51]. Copepods may have the capacity to adapt the over generations, of about one year, using the standing genetic variation that exists in natural populations. Nonetheless, reciprocal transplant and food challenges revealed the huge costs of this adaptation; populations lost

physiological plasticity, the ability to tolerate food limitation, and final adaptive genetic variation for acidification conditions [50].

In conclusion, the larval developmental time was prolonged and reproductive output decreased in *T. japonicus* when successive generations were exposed to pH 7.3 and the effects persisted in Generation 4. When successive generations were exposed to decreasing pH, the effects of reduced pH were more pronounced. To cope with seawater acidification, both the ingestion rate and ATPase activity increased simultaneously. Increasing the activity of both Na^+/K^+-ATPase and Ca^{2+}-ATPase enhanced ionic transportation for calcification and maintaining stable intracellular pH. An increase in the energy demand associated with increased enzymatic activities was met by an increase in food consumption. In this study, we found that multi-generational exposure to CO_2-induced seawater acidification could lead to negative biological consequences at both constant pH condition and at descending pH condition, and a successive decrease in pH resulted in greater detrimental effects in successive generations. However, five generations might still be too short a time frame to observe adaptative evolution in *T. japonicus*. The predicted outcome, however, is subjected to uncertainty considering the rate of decrease in pH in nature is slower as compared with the present study. A longer time for successive generations to adapt to decreasing pH may allow the species to cope with the stress more effectively through natural selection.

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