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Interactive Effects of Rising Temperature, Elevated CO_2 and Herbivory on the Growth and Stoichiometry of a Submerged Macrophyte *Vallisneria natans*

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Abstract: Global climate changes are affecting organisms and their interactions in terrestrial and aquatic ecosystems, such as the increase in temperature and CO_2 concentration. Herbivory interaction is a very important part of nutrient cycle and energy flow in freshwater ecosystem, and climate changes may directly or indirectly affect aquatic plants, aquatic herbivores and their interactions. In this study, we explored the effects of the rising temperature, elevated CO_2 concentrations and herbivory by an herbivorous snail (*Radix auricularia* L.) on a submerged plant (*Vallisneria natans* L.). Our results showed that herbivory, temperature, and CO_2 had specific effects on snail and plant growth, statistically there was only one interaction-a reduction in leaf number. Under different experimental conditions, snail herbivory always has negative effects on biomass accumulation and growth of *V. natans*. Moreover, the increases in temperature also inhibited its growth. Snail herbivory reduced the content of total carbon and total nitrogen of *V. natans* in all treatments, while the total phenols content increased. Our findings indicate that the rising temperature, elevated CO_2 concentrations and herbivory have interactive effects on the growth and stoichiometry of submerged macrophytes, but further research is needed between aquatic plants and aquatic herbivores to aid prediction the impact of climate change on freshwater ecosystems.

Keywords: rising temperature; elevated CO_2; herbivory; submerged macrophyte; morphology; stoichiometry

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1. Introduction

Submerged plants play an important role in the stability of structure and function of aquatic ecosystem [1–4]. The community formation and dynamics of submerged plants in freshwater shallow lakes determine the state of the lake conversion between clear water and turbid water [5]. However, submerged plants in shallow lakes have experienced a decline in recent decades for many reasons, including climate change and eutrophication [6–8].

Climate changes are affecting submerged plants and mainly shown in the increase of temperature and atmospheric CO_2 concentration. Despite the increasing number of climate change mitigation policies, the average growth rate of anthropogenic CO_2 emissions per year is still increasing [9]. By the end of the century, it is predicted that atmospheric CO_2 concentration will increase up to 800 ppm [10]. Recent studies suggest that raising CO_2 concentration in atmosphere may increase CO_2 concentration in freshwater lakes [11]. Studies have shown that the CO_2 concentration of lake will rise to 1100 ppm if the atmospheric CO_2 concentration rises to 800 ppm [12]. In addition, studies have predicted that the temperatures would increase more than 2 °C due to greenhouse gas effects in 2100 [13].

These climate change factors are directly or indirectly affecting many aquatic plants, aquatic herbivores and their interactions.

Rising temperature may lead to distribution change and biomass increase of submerged plants in clear water systems [14], it may also exacerbate eutrophication and lead to the disappearance of submerged plants in eutrophic lakes [15]. At the same time, some studies suggest that higher temperature may inhibit the growth of eelgrass (*Zostera marina*) by reducing leaf life span [16] or have different effects on the growth and distribution of *Elodea canadensis* and *Callitriche cophocarpa* by affecting plant physiological characteristics. [17]. Rising temperature not only affects aquatic plants, but also may leads to some biological response of aquatic animals. Higher temperature may increase the consumption of aquatic plant *Potamogeton lucens* by snail *Lymnaea stagnalis* [18], and cause the average size of ectothermic aquatic organisms to decrease in aquatic systems [19–22]. These findings suggest that the effect of temperature on aquatic plants and animals may be species specific.

Elevated CO_2 concentration can improve photosynthetic capacity, growth rate and primary productivity of aquatic plants [23–26]. Studies have shown that aquatic plants may change its resource allocation pattern and allocated more resources to root growth when exposed to elevated CO_2 [23,24]. Increasing CO_2 may also affect the chemical content of aquatic plant tissues [27]. Elevated CO_2 concentration not only affects aquatic plants, but also directly or indirectly affects aquatic animals. Some studies suggested that elevated CO_2 restricts the distribution of macroinvertebrates in freshwater ecosystem [28,29], and indirectly influence the food preference and herbivory behavior of aquatic animals by affecting the nutrient content of algae and macrophytes. Previous studies have shown that elevated CO_2 concentration can increase the abundance and C/P ratio of green algae, the growth rate of *Daphnia pulicaria* also increased when fed with these green algae [30].

Herbivory has important effects on the growth rate, biomass, community structure, species diversity and distribution of aquatic plants [31–35]. In freshwater ecosystems, aquatic herbivores may reduce the median biomass of aquatic plants by 44–48% [31]. Grazed plants can resist herbivory by decreasing palatability (such as improving the C:N ratio of leaves) [36–38]. Herbivory has an effect on the synthesis of plant phenols, which may be species-specific. Some studies found that herbivory had no significant effect on the phenolic content of *Elodea canadensis* and *Elodea nuttallii*, while significantly increased the phenolic content of *Myriophyllum spicatum* [39].

Whether the responses of submerged macrophytes to climate changes, which include rising temperature and elevated CO_2 concentrations, and herbivory, which include herbivorous fish and snails, etc., remains unclear. In addition, the effects of climate change factors and herbivory on submerged macrophytes may be additive, multiplicative, synergistic, or antagonistic [40–43]. Addressing this will acquire a better understanding of the response of submerged macrophytes to climate changes and herbivory, as well as aid prediction the impact of climate change on freshwater ecosystems. Here, we studied the response of a submerged macrophytes (*Vallisneria natans* L.) to rising temperature, elevated CO_2 concentrations and herbivory by an herbivorous snail (*Radix auricularia* L.). Specifically, we (1) assessed the effects of rising temperature, elevated CO_2 concentrations and herbivory on the growth and stoichiometry of *V. natans*; (2) explored the effects of rising temperature, elevated CO_2 concentrations on the growth and stoichiometry of *R. auricularia*; and (3) generated new information that could be used to aid prediction the impact of climate change on freshwater ecosystems.

2. Materials and Methods

2.1. Experimental Design

In our experiment, we select a submerged plant (*Vallisneria natans* L.) and an herbivorous snail (*Radix auricularia* L.). *V. natans* and *R. auricularia* are widely distributed in various types of freshwater bodies in China and often co-occur in lakes, rivers and ponds during field surveys of aquatic organisms [44,45].

In our experiment, we use heating rod (SUNSUN Aquariums, Zhoushan, China) to raise temperature and a CO_2 generator (Magic Aquarium, Huizhou, China) to elevate CO_2 concentration. The temperatures were set to 27 °C and 24 °C, respectively. The CO_2 generator produced pure CO_2 into the water from 08:00 am to 06:00 pm daily to ensure CO_2 concentration was 3–7.5 mg/L in the elevated group and 0–0.6mg/L in the control group. The daily variation of temperature and CO_2 concentration were shown in Figure 1. 64 plastic pots (d = 23 cm, h = 9 cm) were divided into 8 CO_2 and temperature treatments, with 8 replicates in each treatment: (i) 24 °C and control CO_2 (LTLC), (ii) snails, 24 °C and control CO_2 (S + LTLC), (iii) 24 °C and elevated CO_2 (LTHC), (iv) snails, 24 °C and elevated CO_2 (S + LTHC), (v) 27 °C and control CO_2 (HTLC), (vi) snails, 27 °C and control CO_2 (S + HTLC), (vii) 27 °C and elevated CO_2 (HTHC), (viii) snails, 27 °C and elevated CO_2 (S + HTHC).

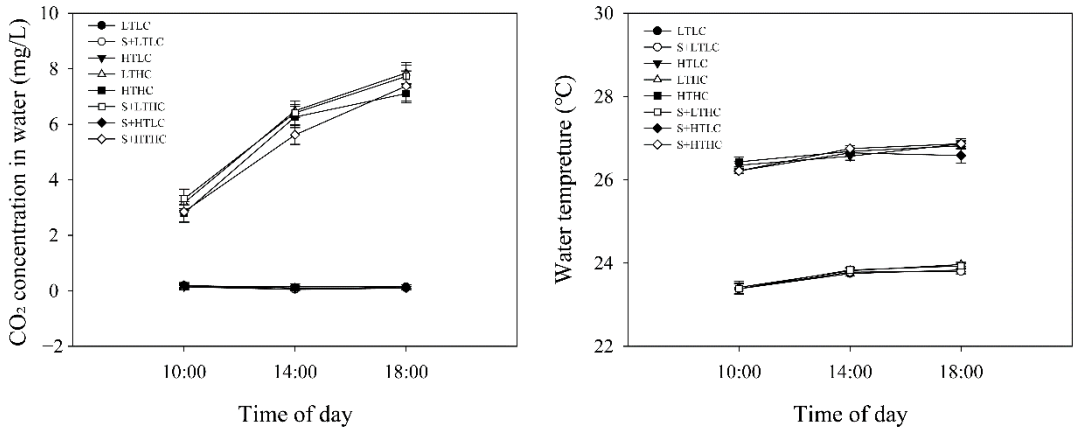

Figure 1. Diurnal changes of CO_2 concentration (**A**) and water temperature (**B**) in treatments (mean ± SE).

We collected *V. natans* and *R. auricularia* from a pond near Nanhu Lake in Huazhong Agricultural University. After one year of cultivation of *V. natans* in the aquarium, 192 no damage seedlings with similar leaf number (6.58 ± 0.06), plant height (18.75 ± 0.17 cm) and fresh weight (1.26 ± 0.01 g) were selected for the experiment (Mean ± SE). In our experiment, we used 64 plastic pots with the same mixed substrate (mud:sand = 1:1). Each pot was planted 3 plants. All plants were planted on 11 June 2018. Two weeks later, 96 snails of similar size (the shell length 1.29 ± 0.01 cm, shell width 0.77 ± 0.01 cm, aperture length 0.91 ± 0.01 cm, aperture width 0.53 ± 0.01 cm, body whorl length 1.14 ± 0.01 cm, and fresh weight 0.202 ± 0.003 g, respectively (Mean ± SE)) were selected. Next, put 3 snails (70 ind./m^2) in each pot (with herbivory) after carefully cleaning. All the snails were starved or 24 h before the experiment.

This experiment was performed from 25 June 2018 to 25 August 2018 at the experiment base at Huazhong Agricultural University (30°28′ N, 114°21′ E). Water quality indexes in this experimental system were shown in Table 1, including water temperature (T), conductivity (Cond), dissolved oxygen (DO), salinity (Sal), total dissolved solids (TDS) and pH were determined by portable meter (YSI Pro Plus, YSO, USA), and illumination was measured by photoelectric illuminometer.

Table 1. The water quality indexes in each group (mean ± SE).

	T (°C)	DO (mg/L)	Cond (μS/cm)	TDS (mg/L)	Sal (‰)	pH	Illumination (Lx)
LTLC	23.66 ± 0.07	4.57 ± 0.03	348.82 ± 1.69	233.53 ± 1.32	0.17	7.85 ± 0.03	451.2 ± 3.44
S + LTLC	23.66 ± 0.07	4.43 ± 0.03	357.07 ± 2.01	234.91 ± 0.69	0.17	7.80 ± 0.03	449.33 ± 2.11
LTHC	23.73 ± 0.08	4.46 ± 0.02	359.30 ± 2.67	234.36 ± 0.93	0.17	7.41 ± 0.02	449.17 ± 4.43
S + LTHC	23.72 ± 0.08	4.39 ± 0.02	359.40 ± 2.64	234.70 ± 0.86	0.17	7.39 ± 0.02	447.00 ± 4.14
HTLC	26.59 ± 0.08	4.35 ± 0.04	365.65 ± 2.81	236.36 ± 0.93	0.17	7.84 ± 0.02	445.00 ± 3.14
S + HTLC	26.48 ± 0.08	4.38 ± 0.03	361.96 ± 2.04	236.15 ± 1.03	0.17	7.75 ± 0.01	458.83 ± 3.50
HTHC	26.64 ± 0.06	4.38 ± 0.02	372.32 ± 4.02	239.76 ± 1.17	0.18	7.46 ± 0.02	446.17 ± 3.23
S + HTHC	26.61 ± 0.08	4.37 ± 0.03	371.59 ± 2.93	237.68 ± 1.24	0.18	7.46 ± 0.02	446.67 ± 2.30

LTLC: low temperature + low CO_2; S + LTLC: snails + low temperature + low CO_2; LTHC: low temperature + high CO_2; S + LTHC: snails + low temperature + high CO_2; HTLC: high temperature + low CO_2; S + HTLC: snails + high temperature + low CO_2; HTHC: high temperature + high CO_2; S + HTHC: snails + high temperature + high CO_2.

2.2. Plant Measuring

Leaf number and fresh weight of 3 plants in each pot were measured carefully at the end of this experiment. Relative growth rate (RGR) of *V. natans* was calculated as: $RGR = \ln(W_f/W_i)/t$, where t is experiment days, W_i is the fresh weight of plants before the experiment, and W_f is the fresh weight of plants after the experiment. All plants were separated into shoots and roots, and then dried at 75 °C to constant weight, respectively. Total biomass, shoot biomass and root biomass were measured. After above measurement, ground the leaves into powder, respectively. Carbon content and nitrogen content of plant leaves were analyzed by a CHNS/O elemental analyzer (vario PYRO cube, Hanau, Germany). We extracted total phenols from leaves of *V. natans* (ca.5 mg) with 50% acetone (2.5 mL) for 2 h on the shaker, and then determined total phenols content with spectrophotometer (Unocal, UV2350) using a Total Phenols Kit method.

2.3. Snail Measuring

At the end of this experiment, we carefully cleaned the snails and measured their morphological indexes, including shell length, shell width, aperture length, aperture width and body whorl length. The snail body and shell were then separated and dried at 75 °C to constant weight. The dry weight of the snail without shell was measured, and the carbon content and nitrogen content were analyzed by a CHNS/O elemental analyzer (vario PYRO cube, Germany).

2.4. Statistical Analysis

We used three-way ANOVA to analyze the differences in plant morphological traits (i.e., leaf number, total biomass, shoot biomass, root biomass, root: shoot ratio, and relative growth rate) and plant chemical traits (i.e., C content, N content, C: N ratio, and total phenols content) among the three groups (temperature levels, CO_2 levels and snail herbivory) [46]. Next, we used two-way ANOVA to analyze the differences in plant morphological traits and plant chemical traits between factor 1 and factor 2 (i.e., temperature levels and CO_2 levels, CO_2 levels and snail herbivory, temperature levels and snail herbivory) with Bonferroni correction ($\alpha = 0.05$). Differences in snail morphological traits (i.e., shell length, shell width, aperture length, aperture width, body whorl length and dry weight) and snail elemental contents (i.e., C content, N content, C: N ratio) between the temperature levels and CO_2 levels were analyzed with two-way ANOVA with Bonferroni correction ($\alpha = 0.05$). Single-sample *t*-test was performed on all morphological traits of plants and snails before the experiment. If the data did not satisfy homogeneity of variances or normal distribution of residuals, they were log transformed before analysis. Data analyze was performed with IBM SPSS Statistics 19.0.

3. Results

3.1. Plant Growth

There were no significant interactions among temperature, CO_2 concentration and snail herbivory on the morphological traits of *V. natans* (except the leaf number) ($p > 0.05$). There was a significant interaction between snail herbivory and CO_2 concentrations on the leaf number, total biomass, shoot biomass, root: shoot ratio and relative growth rate of *V. natans* ($p < 0.05$), but there was no significant interaction on the root biomass ($p > 0.05$). There was a significant interaction between snail herbivory and temperature on the leaf number, total biomass, shoot biomass, root biomass and relative growth rate of *V. natans* ($p < 0.05$), but there was no significant interaction on root: shoot ratio ($p > 0.05$). There was a significant interaction between the temperature and CO_2 concentration on the measured morphological traits of *V. natans* ($p < 0.05$) (Table 2).

Table 2. *F* values and *p* values of snail herbivory, CO_2 concentration and temperature for leaves number, total biomass, shoot biomass, root biomass, root: shoot ratio, relative growth rate, total carbon, total nitrogen, C: N ratio and total phenols. Significant *p* values (<0.05) are in bold.

Source	*d.f.*	Snail Herbivory (s)		CO_2 Concentration (c)		Temperature (T)		S × C		S × T		C × T		S × C × T	
		F	*P*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
Leaves number	1.56	43.965	**<0.001**	5.495	**0.023**	73.494	**<0.001**	13.569	**0.001**	5.186	**0.027**	11.214	**0.001**	4.593	**0.036**
Total biomass	1.56	67.819	**<0.001**	0.066	0.798	54.247	**<0.001**	12.165	**0.001**	18.724	**<0.001**	4.817	**0.032**	2.412	0.126
Shoot biomass	1.56	62.419	**<0.001**	0.043	0.836	53.276	**<0.001**	13.199	**0.001**	16.323	**<0.001**	4.396	**0.041**	2.397	0.127
Root biomass	1.56	51.296	**<0.001**	0.400	0.530	15.331	**<0.001**	0.001	0.974	24.505	**<0.001**	4.025	**0.050**	0.527	0.471
Root: shoot ratio	1.56	36.481	**<0.001**	1.648	0.205	32.937	**<0.001**	23.387	**<0.001**	3.219	0.078	4.569	**0.037**	0.019	0.889
Relative growth rate	1.56	116.149	**<0.001**	3.300	0.075	65.411	**<0.001**	17.651	**<0.001**	8.184	**0.006**	13.879	**<0.001**	3.943	0.052
Total carbon	1.32	14.377	**0.001**	8.240	**0.007**	9.091	**0.005**	3.450	0.072	0.289	0.594	0.539	0.468	0.627	0.434
Total nitrogen	1.32	88.467	**<0.001**	6.365	**0.017**	1.437	0.239	0.106	0.747	6.240	**0.018**	10.404	**0.003**	0.265	0.611
C: N ratio	1.32	46.716	**<0.001**	0.704	0.408	0.001	0.982	0.764	0.389	4.983	**0.033**	7.353	**0.011**	0.167	0.685
Total phenols	1.32	132.402	**<0.001**	14.796	**0.001**	63.675	**<0.001**	6.997	**0.013**	41.857	**<0.001**	31.596	**<0.001**	2.918	0.097

Without herbivory damage, the leaf number was significantly greater at 24 °C than at 27 °C (Figure 2A). With herbivory damage, the leaf number of HTHC (27 °C and elevated CO_2), LTHC (24 °C and elevated CO_2) and HTLC (27 °C and control CO_2) was all significantly less than that of LTLC (24 °C and control CO_2) with herbivory damage ($p < 0.05$) (Figure 2A). Herbivory led to a significant reduction in the leaf number in both HTHC and LTHC ($p < 0.05$) (Figure 2A). In addition, herbivory led to a decrease in the leaf number in HTLC and LTLC, but it was not significant ($p > 0.05$) (Figure 2A).

Total biomass of HTHC, LTHC and LTLC was all significantly reduced when herbivory damage occurred ($p < 0.05$), and HTLC was also reduced by snail herbivory, but not significant ($p > 0.05$). Without snails, the total biomass of *V. natans* in the low temperature group was always higher than that in the high temperature group regardless of the concentration of CO_2 (Figure 2B). With herbivory damage, the total biomass of HTHC, HTLC and LTHC was all significantly less than that of LTLC with herbivory damage ($p < 0.05$) (Figure 2B).

There was no significant difference in root biomass of *V. natans* in each group when there was herbivory damage ($p > 0.05$) (Figure 2C). Root biomass of LTHC and LTLC both reduced significantly when herbivory damage occurred ($p < 0.05$) (Figure 2C). Snail herbivory significantly decreased the root biomass of LTHC and LTLC ($p < 0.05$), and reduced the root biomass of HTHC and HTLC, but not significant ($p > 0.05$) (Figure 2C). When there was no herbivory damage, regardless of the concentration of CO_2, the root biomass of *V. natans* in the low temperature group was always higher than that of in the high temperature group (Figure 2C).

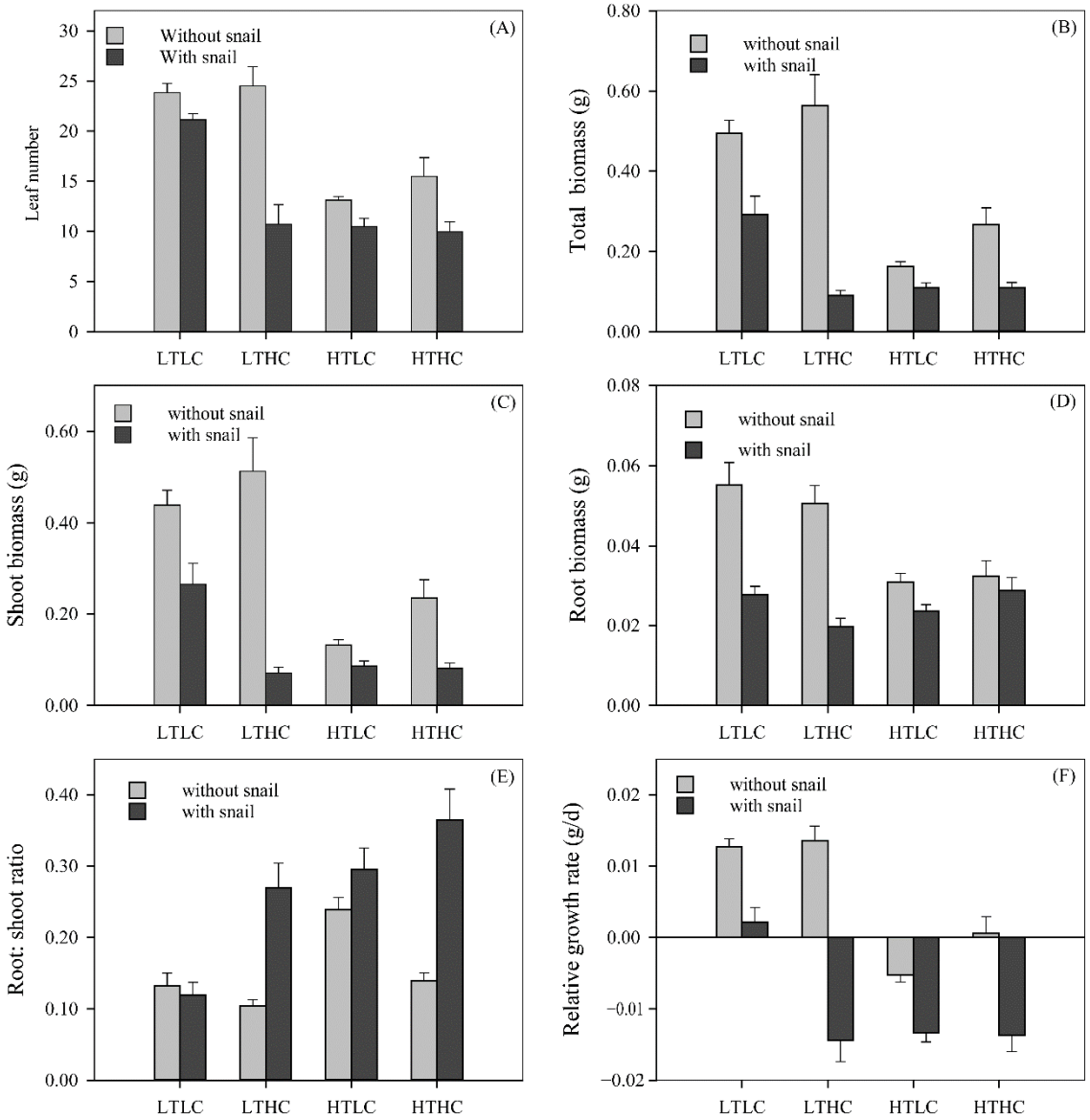

Figure 2. The differences in leaf number (**A**), total biomass (**B**), shoot biomass (**C**), root biomass (**D**), root: shoot ratio (**E**), and relative growth rates (**F**) of *V. natans* in all groups. Data are presented as mean ± SE (n = 8).

The shoot biomass of HTHC, LTHC and LTLC were all significantly reduced by snail herbivory, but the reduction in shoot biomass in HTLC was not significant ($p > 0.05$) (Figure 2D). When there was no herbivory damage, regardless of the concentration of CO_2, the shoot biomass in the low temperature group was always higher than that in the high temperature group (Figure 2D). Shoot biomass of HTHC, HTLC and LTHC was significantly lower than that in LTLC ($p < 0.05$) with herbivory damage (Figure 2D).

Snail herbivory significantly increase the root: shoot ratio of HTHC and LTHC ($p < 0.05$) but had not significantly increase in the root: shoot ratio of HTLC ($p > 0.05$). However, herbivory caused a slightly decreased in root: shoot ratio of LTLC ($p > 0.05$) (Figure 2E). With herbivory damage, the root: shoot ratio of HTHC was significantly higher than that of LTHC and LTLC ($p < 0.05$), but there was no significant difference between HTHC and HTLC ($p > 0.05$) (Figure 2E). Root: shoot ratio of HTLC and LTHC was significantly higher than LTLC ($p < 0.05$) (Figure 2E). When there was no herbivory damage, the root: shoot ratio of HTHC, LTHC and LTLC was significantly lower than that of HTLC ($p < 0.05$) (Figure 2E).

Without herbivory damage, the relative growth rate of *V. natans* in HTLC was significantly lower than that in other three groups ($p < 0.05$). The relative growth rate in HTHC was significantly lower than that in LTHC and LTLC ($p < 0.05$) (Figure 2F). The relative growth rate of each group was significantly decreased by snail herbivory ($p < 0.05$), and even HTHC, HTLC and LTHC showed a negative growth rate (Figure 2F). With herbivory damage, the relative growth rates in HTHC, HTLC and LTHC were significantly lower than that in LTLC ($p < 0.05$), but there was no significant difference among HTHC, HTLC and LTHC ($p > 0.05$) (Figure 2F).

3.2. Plant Chemical Traits

There was no significant interaction among temperature, CO_2 concentration and snail herbivory on total carbon content, total nitrogen content, C: N ratio and total phenol content of *V. natans* ($p > 0.05$). There was a significant interaction between snail herbivory and CO_2 concentration on the total phenols content ($p < 0.05$), but there was no significant interaction on total carbon content, total nitrogen content and C: N ratio ($p > 0.05$). There was no significant interaction between snail herbivory and temperature on total carbon content ($p > 0.05$), but there was a significant interaction on total nitrogen content, C: N ratio and total phenols content ($p < 0.05$). There was no significant interaction between temperature and CO_2 concentration on total carbon content ($p > 0.05$), but there was a significant interaction on total nitrogen content, C: N ratio and total phenols content ($p < 0.05$) (Table 2).

When there was herbivory damage, the total carbon content of *V. natans* in each group showed a downward trend, especially the total carbon content in HTHC and LTHC decreased significantly ($p < 0.05$) (Figure 3A). With herbivory damage, the total carbon content in HTHC was significantly lower than that in HTLC ($p < 0.05$), and LTHC was significantly lower than that in LTLC ($p < 0.05$). There was not significantly different in total carbon content between HTHC and LTHC, and also not significantly different between HTLC and the LTLC with herbivory damage ($p > 0.05$) (Figure 3A). When there was no herbivory damage, there was not significantly different in total carbon content between HTHC and HTLC, and also not significantly different between HTHC and LTHC ($p > 0.05$) (Figure 3A). There was not significantly different between HTLC and LTHC without snail herbivory, and not significantly different between HTLC and LTLC without snail herbivory ($p > 0.05$) (Figure 3A).

With herbivory damage, the total nitrogen content of the leaves of *V. natans* in each group significantly decreased ($p < 0.05$) (Figure 3B). With herbivory damage, the total nitrogen content of HTLC and LTHC was significantly higher than that of HTHC ($p < 0.05$). There was not significantly different in total nitrogen content between LTLC group and HTLC, and also not significantly different between LTLC and LTHC ($p > 0.05$) (Figure 3B). The total nitrogen content of both HTHC and LTLC was lower significantly than that in HTLC ($p < 0.05$) when there was no herbivory damage (Figure 3B).

Herbivory led to a decrease in C: N ratio of the leaves of *V. natans* in each group, especially C: N ratio of HTHC, HTLC and LTLC decreased significantly ($p < 0.05$) (Figure 3C). Without herbivory damage, C: N ratio in HTLC was lower significantly than that in LTLC ($p < 0.05$). With herbivory damage, C: N ratio in HTHC was higher significantly than that in LTHC ($p < 0.05$) (Figure 3C).

Total phenols content of *V. natans* in HTHC, HTLC and LTLC was significantly increased by snail herbivory ($p < 0.05$), which increased the total phenols content of LTHC slightly, but not significant ($p > 0.05$) (Figure 3D). Without herbivory damage, there was not significantly different in total phenols content of *V. natans* in each group ($p > 0.05$) (Figure 3D). With herbivory damage, total phenols content in HTHC, HTLC and LTLC was significantly higher than that in LTHC ($p < 0.05$), and total phenols content in LTLC was significantly lower than that in HTHC and HTLC ($p < 0.05$), but there was not significantly different between HTHC and HTLC ($p > 0.05$) (Figure 3D).

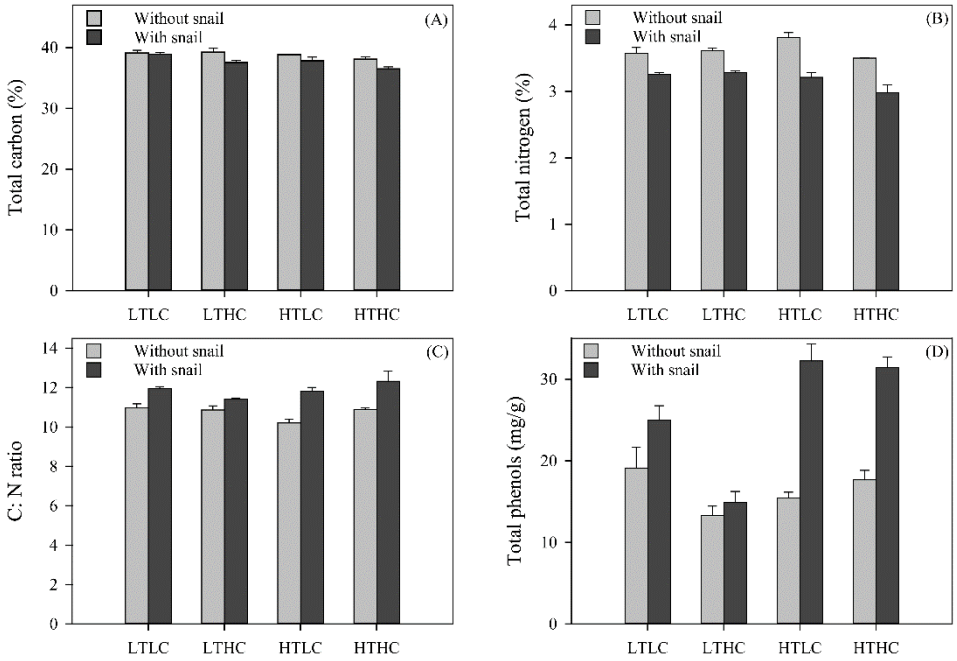

Figure 3. The differences in total carbon content (**A**), total nitrogen content (**B**), C: N ratio (**C**) and total phenols content (**D**) of *V. natans* in all treatments. Data are presented as mean ± SE (n = 5).

3.3. Snail Growth

There was no significant interaction between temperature and CO_2 concentration on snail morphological traits (shell length, shell width, aperture length, aperture width, body whorl length and dry weight) (Table 3). Under high CO_2 conditions, shell length and shell width of snails were significantly smaller at 27 °C than at 24 °C ($p < 0.05$), while the aperture length, aperture width, body whorl length, fresh weight, dry weight with shell and dry weight without shell of snails were not significantly different at two different temperatures conditions ($p > 0.05$) (Figure 4). Under low CO_2 conditions, the shell width of the snail is significantly wider at 24 °C ($p < 0.05$), while the other measured morphological traits of snails were not significantly different at two different temperatures conditions ($p > 0.05$) (Figure 4). At 27 °C, the shell length of snails was significantly longer under low CO_2 conditions ($p < 0.05$), while the shell width, aperture length, aperture width, body whorl length, fresh weight and dry weight with shell of snails were larger under low CO_2 conditions, but not significant ($p > 0.05$) (Figure 4). However, at 24 °C, all of the measured morphological traits of snails were not significantly different under two different CO_2 conditions ($p > 0.05$) (Figure 4).

Table 3. *F* values and *p* values of CO_2 concentration and temperature for the shell length, shell width, aperture length, aperture width, body whorl length, fresh weight, dry weight with shell, dry weight without shell, total carbon, total nitrogen and C: N ratio. Significant *p* values (<0.05) are in bold.

Source	*d.f.*	Temperature (T)		CO_2 Concentration (C)		T × C	
		F	*p*	*F*	*p*	*F*	*p*
Shell length	1.36	4.371	**0.044**	2.524	0.121	3.084	0.088
Shell width	1.36	20.763	**<0.001**	0.831	0.368	0.017	0.897
Aperture length	1.36	1.866	0.180	0.207	0.652	1.004	0.323
Aperture width	1.36	3.938	0.055	0.723	0.401	0.723	0.401
Body whorl length	1.36	4.524	**0.040**	0.785	0.381	0.126	0.725
Fresh weight	1.36	0.404	0.529	0.331	0.569	1.855	0.182
Dry weight with shell	1.36	0.831	0.368	2.804	0.157	0.624	0.435

Table 3. *Cont.*

Source	*d.f.*	Temperature (T)		CO_2 Concentration (C)		T × C	
		F	*p*	*F*	*p*	*F*	*p*
Dry weight without shell	1.36	0.933	0.341	0.639	0.429	0.459	0.503
Total carbon	1.36	0.107	0.745	0.005	0.944	0.253	0.618
Total nitrogen	1.36	0.034	0.855	0.110	0.742	1.412	0.243
C: N ratio	1.36	2.891	0.098	2.023	0.164	4.020	0.053

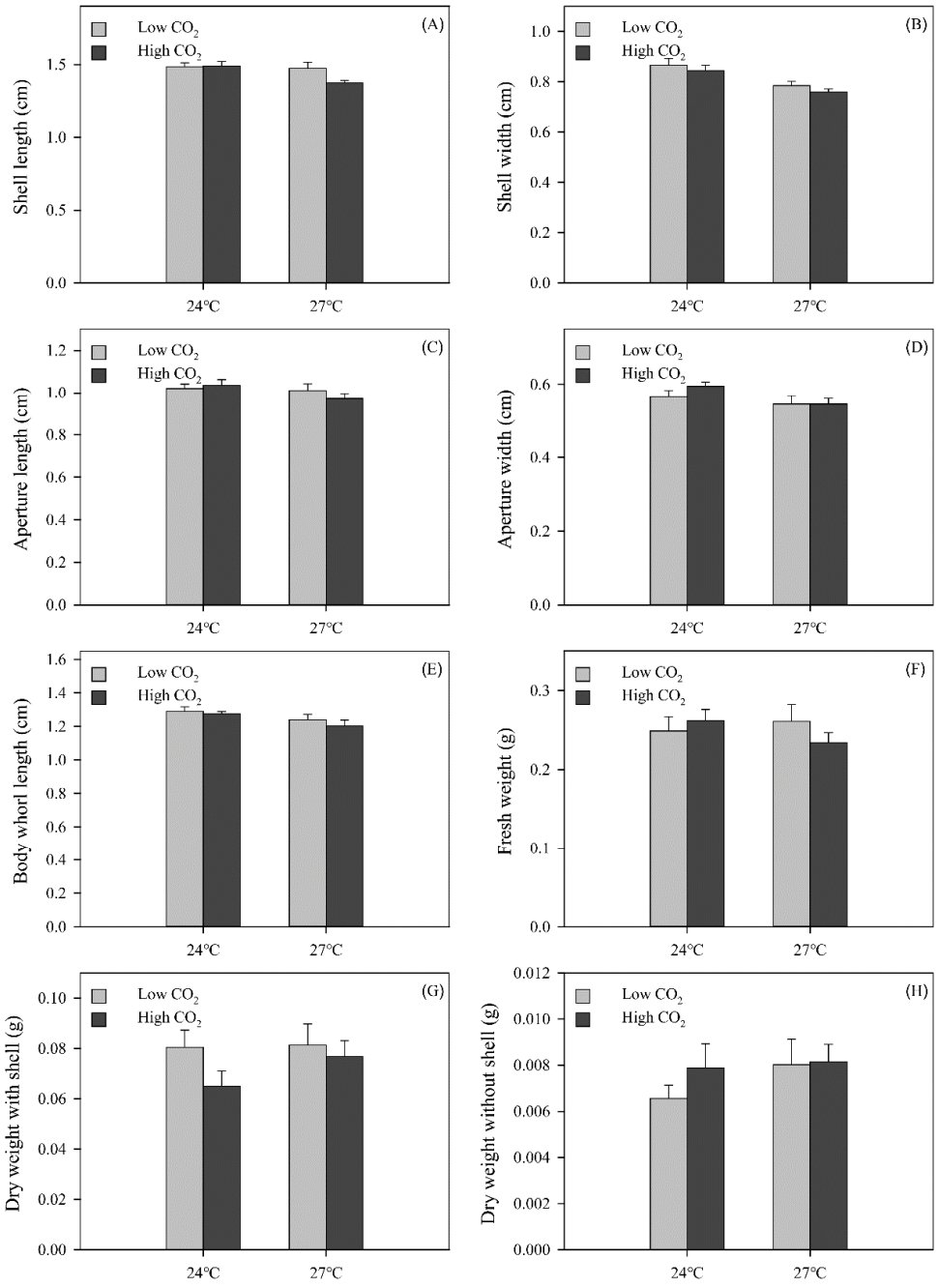

Figure 4. The differences in shell length (**A**), shell width (**B**), aperture length (**C**), aperture width (**D**), body whorl length (**E**), fresh weight (**F**), dry weight with shell (**G**) and dry weight without shell (**H**) of *R. auricularia* between the temperature and CO_2 levels treatments. Data are presented as mean ± SE (n = 10).

3.4. Snail Chemical Traits

There was no significant interaction on total carbon content, total nitrogen content and C: N ratio of snails between temperature and CO_2 concentration (Table 3). Regardless of the CO_2 concentration, total carbon content and total nitrogen content of snails were not significantly different at two different temperature levels ($p > 0.05$) (Figure 5). At 24 °C, C: N ratio of snails under low CO_2 condition was significantly lower than that under high CO_2 conditions ($p < 0.05$). However, at 27 °C, C: N ratio of the snails was not significantly different at two different CO_2 concentrations ($p > 0.05$) (Figure 5). Under high CO_2 conditions, C: N ratio of the snails was significantly higher at 24 °C ($p < 0.05$). However, under low CO_2 conditions, C: N ratio of the snails was not significantly different between at two temperature levels ($p > 0.05$) (Figure 5).

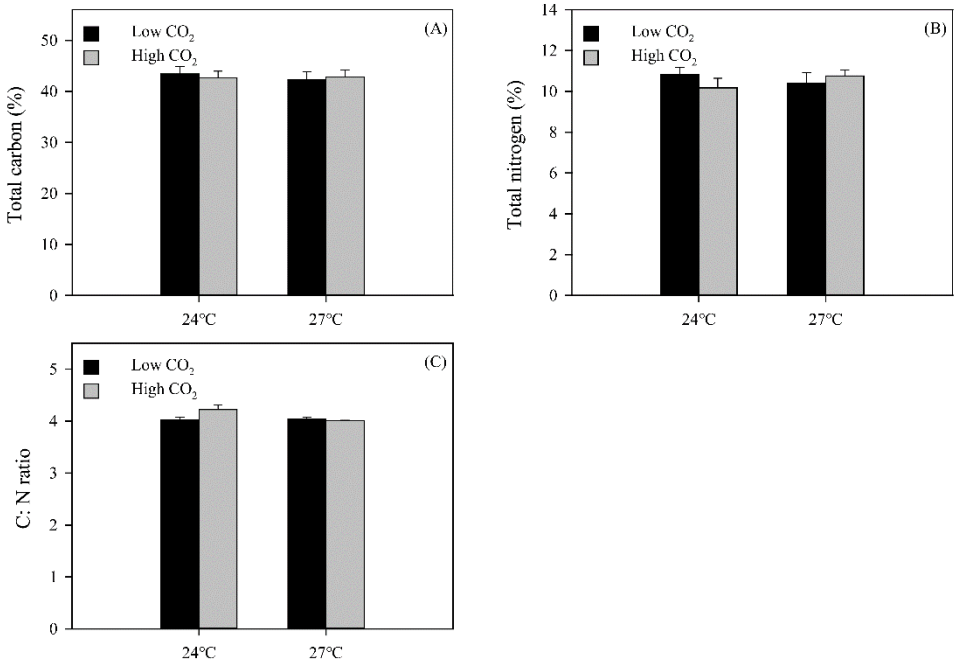

Figure 5. The differences in total carbon content (**A**), total nitrogen content (**B**) and C: N ratio (**C**) of *R. auricularia* between the temperature and CO_2 levels treatments. Data are presented as mean ± SE ($n = 10$).

4. Discussion

Our findings suggested that herbivory had significantly negative effect on the leaf number, total biomass, shoot biomass and relative growth rate of *V. natans* in different treatments. Similar to other studies, that is, herbivory has a significant inhibitory effect on the growth of aquatic plants and the accumulation of biomass [18,47–49]. A certain range of warming (increased 2.5–3 °C) had a positive effect on the growth of shoots of *Equisetum fluviatile*, and the biomass accumulation was also positively affected by temperature [50]. Higher temperature conditions led to good conditions for *Hydrilla verticillata* to compete with other aquatic plants (mainly: *Egeria najas*, *Egeria densa*, *Cerathophyllum demersum*) [51]. However, our findings suggested that a certain range (approximately increased 3 °C) of temperature raised had an inhibitory effect on the growth and the biomass accumulation of *V. natans*. This may be caused by species-specificity of aquatic plants in response to raised temperature.

Our results also showed that the plants biomass of HTLC was lower than LTLC with or without herbivory, which may be mainly due to the high temperature limited the growth of plants. With herbivory damage, the leaf number, total biomass, shoot biomass and relative growth rate of plants in HTHC were significantly lower than those in LTLC, and also these characteristics in LTHC were significantly lower than those in LTLC, while there was not significantly different between HTHC and HTLC. The effects of elevated CO_2 on

herbivories in different temperature treatments may be different. Increasing temperatures enhanced the consumption of aquatic plants *Potamogeton lucens* by snail *L. stagnalis* [18], and elevated CO_2 caused a decline in plants consumed [52]. Therefore, we deduced that elevated CO_2 and rising temperature temperatures had the important interactions between aquatic plants, aquatic herbivore and their interaction.

Herbivory damage to the aboveground tissues may cause submerged plants to change their resource allocation patterns, which means that plants will allocate more resources to the underground tissues for storage when they are being in a state of herbivory, and then use these resources for reproduction or compensatory growth in the future [53,54]. However, herbivory was associated with the decrease in root biomass of *Cirsium arvense* during the growing season reported by Cripps et al. [55]. This is similar to the results of our experiment, that is, snail herbivory significantly reduced plant root biomass at 24 °C, while not significantly at 27 °C. This may be the result of temperature and herbivory interactions, or it may be due to different strategies used by plants to cope with herbivory damage under different conditions, because there are many strategies for plants to resist herbivory, such as compensatory growth [53,56], changes in resource allocation patterns [57], changes in tissue nutrient content [58,59], synthesis of secondary metabolites and more [60,61].

Without herbivory damage, there was no significant difference in total carbon content, total nitrogen content, and C: N ratio between HTHC and LHHC group, but total nitrogen content was significantly higher and C: N ratio was lower in HTLC than LTLC group. With herbivory damage, there was not significantly different in total carbon content, total nitrogen content, and C: N ratio between HTLC and LHLC group, but the total nitrogen content was significantly lower and the C: N ratio was higher in HTHC than LTHC group. This may be caused rising temperature promoted plant nitrogen synthesis in the control CO_2 treatment [62], and this effect may be offset by the abnormal plant metabolism caused by the acidification in the elevated CO_2 treatment and herbivory [63]. Fornoff and Gross (2014) found that plants reduced their palatability (decreased nitrogen content or increased C: N ratio) to resist herbivory, and may be also use most of their growth resources to synthesize secondary metabolites to resist herbivory, thereby reducing resources for self-growth [38].

Phenols are a common and important secondary metabolite [60]. They are recognized as chemical defense substances widely found in aquatic plants and have a certain resistance to the damage of herbivory animals [64]. Our results showed that total phenols content of *V. natans* in HTHC, HTLC and LTLC were all significantly increased, and total phenols content in LTHC was also increased, but not significantly. This is probably a defensive strategy for plants. Bryant et al. (1983) found that the nitrogen-based defenses of plants in high-nutrient environments will become more important [65], while total carbon content and total nitrogen content of the sediment used in this experiment are 25.803 ± 0.103 mg/g and 2.480 ± 0.012 mg/g, respectively, indicating that the nutrients required for *V. natans* were sufficient, so they synthesized more nitrogen-based defense substances-total phenols. There was not significantly different in total phenols content of plant in different treatments without snail herbivory, while total phenols content of plant in LTHC was significantly lower than that in HTHC, HTLC and LTLC, respectively. These may be the results of interaction between herbivory, temperature, and CO_2 concentration.

This study showed that, at 27 °C, the shell length of snails under high CO_2 conditions was significantly lower than that under low CO_2 conditions. Under high CO_2 conditions, the shell length of snails at high temperature group (27 °C) was significantly lower than that at 24 °C. Therefore, it can be inferred that the warming due to CO_2 emissions may cause the size of snail to become smaller. Some studies have shown that climate warming may cause the size of aquatic animals to become smaller [19,20]. Another result in this study may also be the verification of this statement, that is, the shell width of snail at high temperature (27 °C) was always less than that at low temperature (24 °C) (Figure 4). Studies have shown that acidification of water caused by high CO_2 may affect some calcified aquatic animals [66]. This study also had similar finding that, regardless of temperature, the fresh weight and dry weight with shell of snails under high CO_2 conditions were generally

slightly less than that of snails under low CO_2 conditions, while the dry weight without shell of snails under high CO_2 conditions was slightly higher than that of snails under low CO_2 conditions. These results indicated that the weight of snail shell under high CO_2 conditions was less than that of snail shell under low CO_2 conditions. This may be due to the increase in CO_2 concentration in the water, which cause pH decrease in the water environment, which in turn had an inhibitory effect on the calcification of the shell.

At 27 °C, total carbon content and total nitrogen content of snails under high CO_2 conditions both always higher than that of snails under low CO_2 conditions. However, at 24 °C, total carbon content and total nitrogen content of snails under high CO_2 conditions both always lower than that of snails under low CO_2 conditions. These results may be due to the differences in total carbon content and total nitrogen content of their unique food *V. natans* under different conditions.

5. Conclusions

Climate change may directly or indirectly affect the freshwater ecosystem, including aquatic macrophytes, aquatic herbivores and plant-herbivore interactions. Here, we studied the response of submerged macrophytes to rising temperature, elevated CO_2 concentrations and herbivory in order to acquire a better understanding of the impact of climate change on freshwater ecosystems. Herbivory had significant effects on growth and chemical traits of *V. natans*, CO_2 concentration significantly affect leaf number, total carbon, total nitrogen and total phenols, and temperature significantly affect growth, total carbon, and total phenols. However, only temperature had significant effects on snail growth traits (shell length, shell width and body whorl length). Snail herbivory had negative effects on the growth and biomass accumulation and caused the total carbon content and total nitrogen content decrease, while total phenols content increased. Our findings indicate that the rising temperature, elevated CO_2 concentrations and herbivory have interactive effects of rising temperature, elevated CO_2 and herbivory on the growth and stoichiometry of submerged macrophytes, but further research is needed between aquatic plants and aquatic herbivores to aid prediction the impact of climate change on freshwater ecosystems.

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