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Effects of ocean acidification on the larval growth of olive flounder (*Paralichthys olivaceus*)

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Abstract

Little is known about how marine fishes respond to the reduced pH condition caused by the increased CO_2 in the atmosphere. We investigated the effects of CO_2 concentration on the growth of olive flounder (*Paralichthys olivaceus*) larvae. Newly hatched larvae were reared in three different concentrations of CO_2 (574, 988 and 1297 µatm CO_2) in temperature-controlled water tanks until metamorphosis (4 weeks). Body lengths, weights, and the concentration of some chemical elements in larval tissue were measured at the completion of each experiment, and experiment was repeated three times in May, June, and July 2011. Results indicated that body length and weight of flounder larvae were significantly increased with increasing CO_2 concentration ($P < 0.05$). Daily growth rates of flounder larvae were higher (0.391 mm) from the high CO_2 concentration (1297 µatm) than those (0.361 mm and 0.360 mm) from the lower ones (988 and 574 µatm).The measurement on some chemical elements (Ca, Fe, Cu, Zn and Sr) in fish tissue also revealed the increasing tendency of element concentration with increasing CO_2 in seawater, although statistical significance cannot be tested due to the single measurement. It suggests that there are enrichment processes of these cations in larval tissue in the low pH condition.

1 Introduction

Since the industrial revolution of 18 century, steadily increasing combustion of fossil fuels has caused the enhanced carbon dioxide (CO_2) concentration in the atmosphere, and consequently global and ocean warming. Aside from global warming, ocean acidification due to the increase in atmospheric CO_2 became one of the crucial and central scientific issues in marine ecosystem research recently. It is anticipated that ocean acidification would be trouble in marine ecosystem near future, because the acidified seawater is likely affect organisms' individual performance (e.g. growth, survival, development, swimming ability, etc.), especially for many calcifying organisms such as

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corals and other shelled invertebrates that precipitate aragonite skeletons (Feely et al., 2004; Orr et al., 2005; Kleypas et al., 2006; Heogh-Guldberg et al., 2007; Gattuso and Hansson, 2011).

Ocean acidification seems to be related in phytoplankton growth, calcification capability of bivalves, strength of aragonite skeleton, etc. Very diverse results of acidification experiment have been reported since the early 2000s. Phytoplankton *Chrysochromulina* sp. reared under different CO_2 concentrations (400, 800, and 1200 µatm CO_2) indicated that cell number and chlorophyll volume were increased by increasing CO_2 concentration (Kawashima et al., 2010). Pacific oyster *Crassostrea gigas* and Blue mussels *Mytilus edulis* exposed to acidified seawater showed the decreased ability of calcification (Gazeau et al., 2010). The shipboard experiment on the growth of Arctic pteropods revealed that aragonite skeletons of pteropods formed in the high CO_2 condition were easily broken (Orr et al., 2005).

For fish, the experiment on olfactory sense of orange clownfish *Amphiprion percula* showed the decline of olfactory sensing capability at the high CO_2 concentration condition. Therefore, the fishes grown in higher CO_2 concentration were not easily escaped from their predators because they were not scent their predators (Dixson et al., 2010). This result suggested that their survival rate would be decreased by weakening of avoidance performance. Other several experiments carried out in the extremely high CO_2 concentration (1800 ~ 4200 µatm) condition showed the reduced growth and survival rates and severe tissue damage (Kikkawa et al., 2003; Frommel et al., 2011). The experiment with white sea bass reared in different CO_2 concentrations, however, showed that the otolith sizes of 7 to 8 day-old fishes grown under 993 and 2558 µatm CO_2 had 7 ~ 9 % and 15 ~ 17 % larger areas, respectively, than those of controlled fish grown under 380 µatm CO_2 (Checkley et al., 2009). Also, Munday et al. (2009) reared the eggs and larvae of the orange clownfish *Amphiprion percula* in seawater simulating a range of ocean acidification scenarios for the next 50–100 yr (390, 550, 750 and 1030 µatm CO_2), and demonstrated the increasing tendency in the growth rate of larvae at the enhanced acidification due to high CO_2 concentration

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after 11 day experiment. Larvae from some parental pairs were 15 ~ 18 % longer and 47 ~ 52 % heavier in acidified water compared with controls.

Increased level of dissolved CO_2 causes not only the acidification of theocean, but also the decrease in pH of animal tissue (Pörtner et al., 2004).The results from various groups of scientists suggest that the effects of ocean acidification and processes of calcification performance were different depending on species. Especially, invertebrate species having exoskeleton were affected by ocean acidification severely. When they were exposed to elevated seawater pCO_2 (hypercapnia), many organisms can regulate their acid–base balance by active ion transport and cellular bicarbonate buffering (Pörtner et al., 2005). However, the adaptive mechanism of organisms to a new environment usually requires a long period, and marine organisms under rapidly changing environment such as ocean acidification cannot get sufficient time to be adapted. Therefore, physiologically incomplete regulation of organisms on acid-base balance may cause differences in life history parameters such as growth and reproduction among species (Pörtner et al., 2005).

Olive flounder *Paralichythys olivaceus* is a temperate marine species, and is also important commercial fish species in Korean aquaculture industry. They reside in the coastal areas of the northwestern Pacific Ocean, and spawn floating eggs on February through June with the peak on March–May (NFRDI, 2010). At the end of the yolk sac stage, larvae start feeding and development. When they metamorphose, the symmetrical larvae (shaped like round fish) becoming flatfish. According to the fourth Assessment Report (AR4) of the Intergovernmental Panel on Climate Change (IPCC), the future ocean will be acidified seriously (IPCC, 2007). Marine ecosystem also will be modified due to the collapses of some organisms that have exoskeleton with calcium carbonate, and that were vulnerable to low pH environment. Reproduction and recruitment of fish species in local sea or coastal areas should be also influenced by the changes in ocean acidification. In this paper, we demonstrated the effects of CO_2-induced ocean acidification on the growth of the larval olive flounder *Paralichthys olivaceus*. Also, the concentrations of some chemical elements from larvae experimented were measuredto deduct the physiological reaction of marine fishes under changing environment.

2 Materials and methods

We designed the experiment system for making the artificial CO_2 concentration (Fig. 1). Firstly, CO_2 in the ambient air was removed by air filter filled soda lime. The CO_2-removed airand pure CO_2 gas were mixed in the mixing chamber. The control of CO_2 gas flow was accurately regulated by Mass Flow Controller (MFC, KOFIOC co., model 3660 series, Japan). The CO_2 concentration of mixed air in the mixing chamber was monitoredand measured continuously by sensor (SOHA Tech., SH-VT250, Korea). Using this system, we regulated the CO_2 concentration of the artificial air (400, 850 and 1550 µatm CO_2 as targets). This artificial air was supplied to the reservoirs and fish rearing tanks using vacuum pump (KnF co., N86KT.18, France). The seawater flowed from the reservoir to the fish rearing tanks continuously, and detritus and contaminated water was drained out from fish tanks. Fish tanks were painted by black color because the light is detrimental to the survival of fish larvae.

The experiment was carried out in University laboratory in Busan, Korea, and it was repeated three times: May, June and July 2011. Local commercial hatchery in Yeosu provided us the fertilized flounder eggs, and we brought them to the laboratory for hatching. Approximately 1200 fertilized eggs per fish tank (i.e., 30 ~ 40 eggs per liter) were allocated in six 30 L rearing tanks. Water temperature was maintained at 21 ± 0.5 °C using temperature sensor and electric heater. Dissolved oxygen (DO) was maintained at $8.0 \pm 1.0\,\mathrm{mg\,L^{-1}}$ and a photoperiod was controlled as 14L : 10D. The CO_2 concentration of each tank was maintained by aeration of mixed CO_2 gas using ceramic air stone. After 2 days from hatching, chlorella *C. pyrenoidosa* was added to the seawater for the practice of feeding, although the onset of first feeding was not started yet. Larvae were not fed during first 3 days from hatching because digestive system of larvae was incomplete. After 3 days from hatching, larvae fed on rotifers *Brachionus*

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rotundiformis with the concentration of 3 rotifers mL^{-1}, and it was gradually increased to 16 ind. mL^{-1}. After 13 days from hatching, prey organisms were changed to Artemia nauplii *Artemia salina* with the density of about 1 ind. mL^{-1}, and was increased gradually to 5 ind. mL^{-1} to the end of experiment. Experiment completed at the end of fourth week when larvae entered settling stage at bottom after metamorphosis.

During the course of our experiment, we tried to keep stable supply of CO_2 to fish rearing tanks, because the maintaining of stable tank environment with the target CO_2 concentration is the key factor for the success of this experiment. Though $CO_2$with the target concentrationis flowed to the each tank, pH_{NBS} (NBS: National Bureau of standards, United States) of fish tank was also measured regularly in order to calculate actual dissolved CO_2 during the July experiment. We sampled the rearing water every week. The sampled water was kept in the 100 mL bottle and 0.05 mL HgCl2 was added and total alkalinity (TA) was measured in open-cell titration and calibrated with reference materials according to Dickson et al. (2007). Then, TA and other carbonate chemistry factors were used for calculation of dissolved carbon dioxide (pCO_2) using CO2SYS program (Pierrot and Wallace, 2006). We assume that the calculated pCO_2 concentration in July could be applied to May and June experiments because experimental environments had been the same through the 3 month experimental period.

After 4 weeks from hatching (i.e., the end of experiment), all live larvae were removed from rearing tanks, and anesthetized by ice. These larvae were photographed in an eye side under a stereomicroscope (Carl Zeiss co., Discovery V8, Germany) using digital microscope camera (Carl Zeiss co., ICC1, Germany). Standard length (SL) was measured for each larvae from the digital photograph using image analyser (Carl Zeiss co., Axiovision4.7, Germany). Wet weight of each fish was measured using the electronic scale for May samples (Mettler Toledo co., AB204-S, Switzerland). For measuring weights for June and July samples, larval weights of 5 larvae combined were measured due to too light weight of individual larva: number was counted and recorded on the cap of micro tube. Each tube with larvae was weighed, and we measured the weight of empty tube after we removed the larvae. The weight difference between with and without larvae was divided by larval number counted to calculate the mean individual larval weight.

In order to see the accumulation of some chemical elements to the larval tissue, concentrations of some chemical elements were measured. Larval specimens were measured for length and weight, and dried with vacuum freeze drying for 24 h. Dried larval weightwas weighed by electronic scale (Mettler Toledo co., AB204-S, Switzerland). Then, those samples weighed were treated with hot plate digestion method, and 13 chemical elements (Na, K, Ca, Mg, P, B, Zn, Si, Fe, Cu, Ba, Mn, and Sr) were measured using the Inductively Coupled Plasma atomic emission spectrometry (ICP-AES, HORIVA co., Japan). We compared the concentration differences of chemical element at lowest and highest CO_2 concentrations. Relative scale of each element between low and high CO_2 environments was made, therefore, the CO_2 concentration at 574 µatm was set to 1, and the relative ratios at 1297 µatm CO_2 for five elements were calculated.

3 Results

Dissolved carbon dioxide concentration (pCO_2) of rearing water was calculated from pH_{NBS} and TA, so that the target concentrations (control, 850 and 1550 µatm CO_2) were adjusted to new ones (i.e., 574 ± 0.35 µatm, 988 ± 0.11 µatm and 1297 ± 0.47 µatm, respectively) (Table 1). This result confirmed that our experimental tanks maintained the gradient of CO_2 concentration with a significantly different pCO_2.

Because eggs used in every month's experiment were from different batches, larval sizes experimented varied. Larval length after 4-week experiment, however, tended to be increased with respect to the increase in CO_2 concentration. For example, the larvae experimented in May were from the same batch, and its mean terminal length was about 14 mm in 574 µatm CO_2, but larval sizes reared in high CO_2 groups (988 and 1297 µatm CO_2) were slightly larger (i.e., 14.2 and 14.6 mm, respectively). The larval sizes in June and July, they were 11.5 mm and 12.2 mm in 574 µatm CO_2, but those in 988 µatm CO_2 were 12.1 mm and 12.6 mm, and those in 1297 µatm CO_2 were 12.9 mm

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and 13.2 mm, respectively. All three experiments demonstrated that larval lengths were increased with increasing CO_2 concentration (Fig. 2a), although statistical differences in length were found in June and July experiments, but not in May.

Though weight information indicated less clear evidence than length, mean body weights of larvae also tend to beincreased with increasing CO_2 concentration in general. In high CO_2 groups (988 and 1297 µatm CO_2), larvae were significantly heavier than low CO_2 group (574 µatm, $P < 0.05$). However, in May, the mean weight at 1297 µatm CO_2 was a little bit lower than 988 µatm CO_2 experiment (Fig. 2b).

In order to see the growth pattern of larvae, we measured the 5 ~ 8 individual larval lengths every 2 ~ 4 days during the June experiment. Larval size at 8 days after hatching were about 4 mm, and its terminal sizes after experiment ranged 11.5 ~ 12.6 mm (Fig. 3). Growth was usually slow in the early stages, but seemed to be increased after 20 days of experiment. Linear regression indicated that the growth rates of larvae experimented in different CO_2 concentrations were different each other (ANCOVA, $F = 2.10$, df = 1.14, $p < 0.05$). Higher growth rate appeared in fish larvae in higher concentration of $p\text{CO}_2$. The larvae at the lowest CO_2 concentration (i.e., 574 µatm) showed daily growth of 0.3603 mm, while those in the highest CO_2 concentration (1297 µatm) 0.3908 mm. The larvae at intermediate concentration (988 µatm) showed a growth rate of 0.3613 mm day^{-1}.

Concentrations of some chemical elements were measured using ICP-AES to detect the difference in chemical constitutes of larval tissue reared in normal and extreme environments. Among 13 chemical elements we measured, only five elements (Sr, Zn, Fe, Cu, and Ca) were chosen to demonstrate because their proportions to body weight were much higher compare to others. We compared the concentration difference of chemical elements at two different CO_2 concentrations using relative scale. In general, the concentration of chemical elements in larval body was increased as CO_2 concentration of rearing water was increased (Fig. 4). The similar results were repeated in all three months, although the changing rate of each element was not always the same through the experiment.

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4 Discussion

The pH and CO_2 concentration of seawater were always changing in wild ocean, and the reason of fluctuation was various: respiration of organism, photosynthesis of phytoplankton and sea plant, and change of temperature, etc. Sometimes, marine organisms were experienced a great change of environment and this experience may increase their tolerance to mild CO_2 acidification. Generally, some invertebrates are unable to compensate fully for disturbances in their acid–base balance when exposed to elevated CO_2 and this can lead to metabolic depression and reduced growth (Kurihara and Shirayama, 2004; Pötner et al., 2004; Michaelidis et al., 2005, 2007; Miles et al., 2007; Frommel et al., 2011). In contrast, most shallow-water fish tested to date appear to compensate fully their acid–base balance within several days of exposure to mild hypercapnia (Michaelidis et al., 2007; Ishimatsu et al., 2008). The well-developed capacity for acid–base regulation in fishes may explain why exposure to high CO_2 condition had little obvious effect on the early life history of fishes.

Our experiment showed the enhanced growth of flounder larvae at the higher CO_2 concentration than normal environment. Enhanced growth performance in acidified water could be achieved either by increased energy intake or reduced energy expenditure. Acid-exposed rainbow trout exhibited increased appetite to compensate for greater branchial ion loss at low pH and the associated increased energy intake increased growth rate (Morgan et al., 2001). Increased flux of ions is less likely to be a problem for marine fishes. However, it is still possible that elevated CO_2 and low pH stimulated appetite and dietary intake by larvae, because they must actively excrete excess ions to maintain their osmotic balance. Munday et al. (2009) demonstrated that exposure to approximately 1030 µatm CO_2 increased the attraction of larval fishes to a range of olfactory stimuli. If gustatory senses are similarly stimulated by elevated CO_2, it is possible that feeding activity of some larvae could be enhanced in acidified water (Munday et al., 2009).

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Ca^{2+} occurs 98 % in skeleton of fish (Mugiya and Watabe, 1977), and we performed the analysis of calcium (Ca^{2+}) and some minor and trace elements using whole larval fish to find out physiological reaction of larval fish under stressful environments. Increased concentration of chemical elements appeared with increasing CO_2 concentration in rearing water. Exposure to elevated seawater pCO_2 results in elevated internal pCO_2 and requires the net excretion of acid to compensate for the respiratory acidosis in body fluids. Similar to cephalopods or crustaceans (Wheatly and Henry, 1992), the gills are the primary sites of acid-base regulation processes in fishes (Perry and Gilmour, 2006). On the other hand, the resulting enrichment of Ca^{2+} in the gut fluid leads to the precipitation of calcium carbonates. The bicarbonate required for this process is secreted by the intestine, thereby leading to an acidification of the blood plasma (Cooper et al., 2010). We thought that increasing of trace element was caused by these processes, because the Ca and other 4 elements (Fe, Cu, Zn and Sr) are all divalent cations that have similar features and characteristics.

Whatever the mechanisms responsible for increased growth in acidified water are, large size and rapid growth appears to be advantageous for fish larvae (Searcy and Sponaugle, 2000; Meekan et al., 2003). Mean sea surface temperatures will increase due to global warming, and at the same time ocean pH will be declined because of the ocean's increased absorption of CO_2. Temperature is known to influence growth duration and survival of some fishes such as flounder, especially during embryonic and larval stages (Fonds et al., 1992; Munday et al., 2009). In general, increased temperature tends to decrease embryonic and larval duration and increase larval growth rate (Green and Fisher, 2004; Sponaugle et al., 2007; Munday et al., 2009). Small increases in temperature, however, may also reduce the survival rate of embryos (Gagliano et al., 2007). This means that complex interactions between the effects of temperature and acidification will ultimately determine the consequences of climate change for the early life histories of marine fishes. The Korean waters are experiencing severe warming and potentially acidification in the coastal areas near the industrial complexes. On the other hand, the capture fishery of olive flounder in Korea was about 3000 tons annually in

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the 2000s, while the production of olive flounder aquaculture was approximately 80 000 tons in 2010 (KSIS, 2010). Therefore it is important to figure out the relationship between larval survival of olive flounder and ongoing acidification, because olive flounder is one of the most important aquaculture species in Korea.

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Table 1. Measurements of pH, temperature, and salinity of experimental tank water in July, and calculated pCO_2 using chemical information.

Target pCO_2 (µatm)	pH	Temperature (°C)	Salinity (psu)	Total Alkalinity ($\mu mol\,kg^{-1}$)	Calculated pCO_2 (µatm)
400	8.05	22.0	32.4	2280.2	574.0
850	7.84	22.0	32.4	2275.7	988.0
1550	7.73	22.0	32.4	2266.6	1297.2

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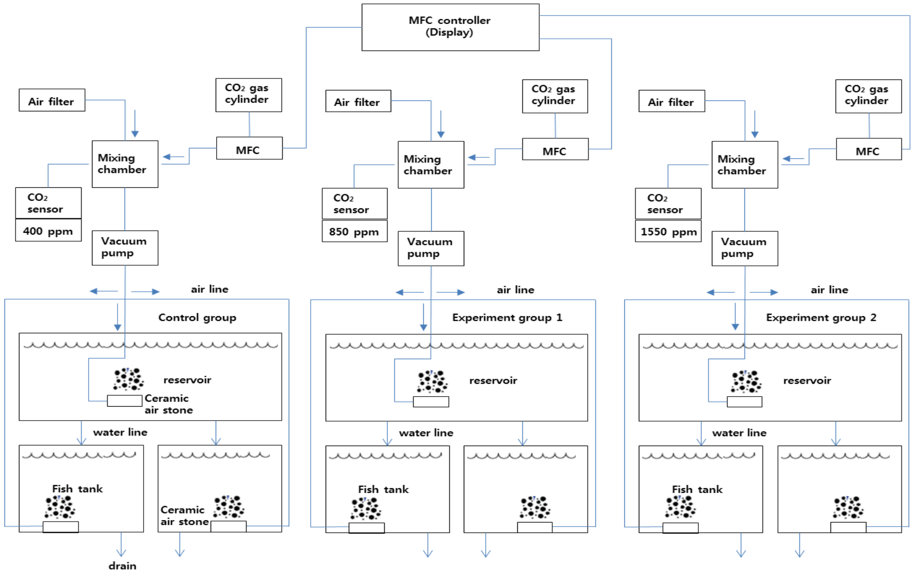

Fig. 1. Schematic illustration of the experimental system used for rearing larval fishes. MFC denotes Mass Flow Controller.

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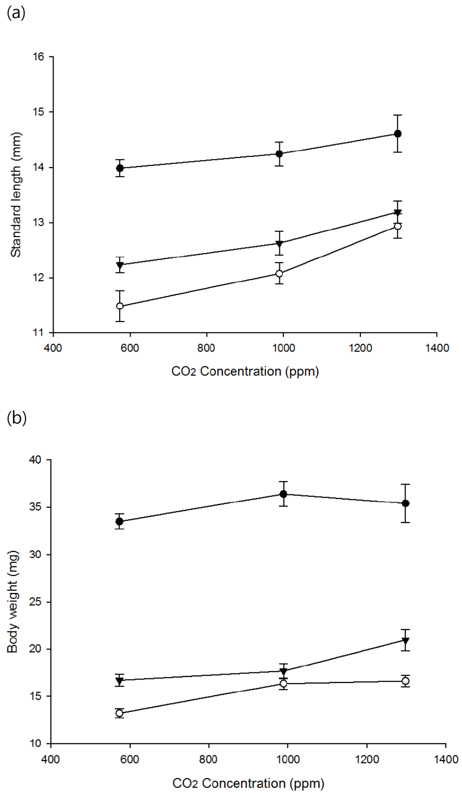

Fig. 2. Measurement of larval length and weight in different dissolved CO_2 after 4-week experiment. **(a)** Mean ± s.e. SL and **(b)** Mean ± s.e. weight of flounder larvae reared in approximately 574, 988, and 1297 µatm CO_2. Each experiment was coded with a different symbol (May – black circle, June – white circle, July – black inverted triangle).

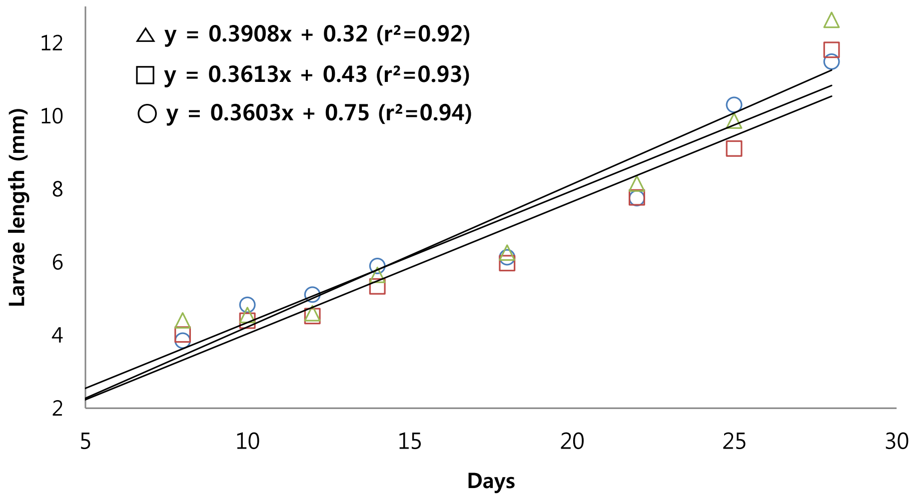

Fig. 3. Growth rate of flounder larvae from 3 different CO_2 concentrations. Each group was coded with a different symbol (574 µatm CO_2 – circle, 988 µatm CO_2 – quadrangle, and 1297 µatm CO_2 – triangle).

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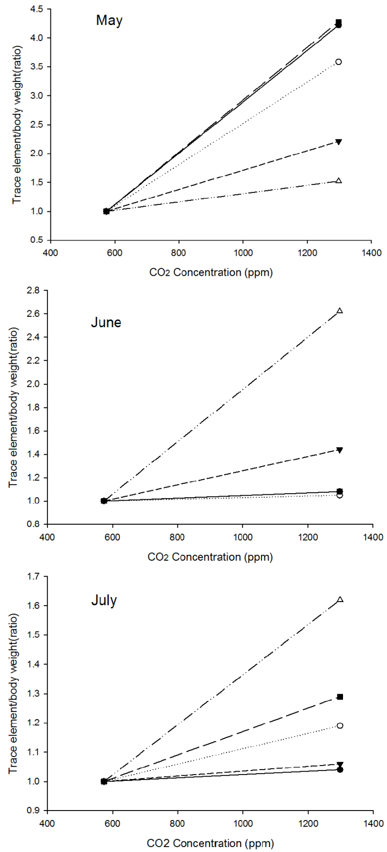

Fig. 4. Concentration of major and trace elements in the tissue of olive flounder larvae rearing 4 weeks in the different conditions (574 and 1297 µatm CO_2). Each elements were coded with a different symbol (Ca – black circle, Zn – white circle, Fe – black inverted triangle, Cu – white triangle, Sr – black square).

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