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Analysis of Spawning Behaviour and Growth Indices of Zebrafish in Response to CO_2 Acidification

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Keywords

CO_2 acidification; zebrafish; growth performance; hatching rate; freshwater acidification

Abstract

The growth parameters and spawning behaviour of zebrafish in response to CO_2 acidification demonstrated differential results. The growth performance of zebrafish is determined by key indices, BWG, SGR, CF and CV. BWG shows subtle gain in 1500 µatm group (0.09 g) and a slight decrease in 2200 µatm group (0.056 g). SGR index showed similar pattern of results, whereas CF showed a gradual decrease. The other growth index CV again showed an increase in 1500 µatm group and slight decrease in 2200 µatm group in comparison to the control group. A significant decrease in the performance of spawning behaviour was observed. At 96 hpf, the survival rate of the embryos showed a significant hit and the number of dead embryos increased dose dependently. The embryos exposed to CO_2 showed a decrease in hatching rate with the increase in dose of CO_2. The CO_2 acidification causes notable changes in the growth and significant effect on reproductive behaviour.

1. Introduction

In addition to the devastating effect of increase in carbon dioxide resulting in acidic aquatic environment and the subsequent ecological catastrophe, the physiological impact caused by this acidification on organisms gets particular attention and needs to be studied elaborately. For years it was believed that the increase in CO_2 has minimal effect on marine water bodies because the marine lives can efficiently compensate the acid-base balance; however, many studies have surfaced indicating that there are many vulnerable species which can be threatened by the alarming rate of increase in acidic condition. It has been predicted that a decrease in pH of 0.77 will be documented by the end of the year 2300 (Caldeira and Wickett 2003; Meehl GA, Stocker TF, Collins WD, Friedlingstein P, Gaye AT, Gregory JM, Kitoh A, Knutti R, Murphy JM, Noda A, Raper SCB, Watterson IG, Weaver AJ 2007). Even though there are scarce reports on the effect of CO_2 acidification on freshwater fish, many studies have showed that different species of marine organisms such as corals, molluscs, crustaceans and fish show differential response against the increase in CO_2 (Talmage and Gobler 2009; Ries, Cohen, and McCorkle 2009; Kroeker et al. 2010). These responses vary from positive effects of elevated CO2 to negative or no effect on the growth of different marine fish. For instance, a finding by (Frommel et al. 2011) shows that although the Atlantic cod larvae underwent serious tissue damage due to high CO_2, the exposed larvae attained more weight than the unexposed control. Nevertheless, many researchers have reported the detrimental effect of elevated CO_2 on marine organisms (PARKER et al. 2009; Baumann et al. 2011; Frommel et al. 2011; Pimentel et al. 2014). Therefore, it becomes utmost important to understand similar effect on freshwater ecosystem in order to frame a coordinated plan to address the challenges on biodiversity, food webs, freshwater resources, tourism and economies.

In fish, two important parameters are need to be taken into account while studying the effect of an environmental hazards: growth and spawning. Both growth and spawning are affected by many physical, chemical and hormonal factors (Falahatkar, Bagheri,(i) and Efatpanah 2019; Abdollahpour et al. 2018). Zebrafish has been used by several researchers as a(ii) successful, efficient and convenient model for growth and nutrition (Tsukamoto et al. 2008; De-(iii) Santis and Jerry 2007) as well as for reproduction in the presence of toxic molecules (Chen et al. 2018).(iv) Regardless of growth that is less expected to be affected, it is speculated that spawning behaviour will have detrimental implication on the particular organism which survive as a species because it is documented that in acidic water, less number of embryos survive and hatch (Poppe 2020).

2. Materials and methods

Zebrafish collection and maintenance

Around 350 zebrafish were used for this experiment. Adult zebrafish of the short-fin wild-type zebrafish (*Danio rerio*) AB strain were purchased from a local aquarium farm (Sirago Aquafarm, Mettur, Tamil Nadu, India) and housed in mixed-sex groups in static 20-L glass tanks with airlift-driven filtration at 25°C on an ambient 14-hr/10-hr light–dark photoperiod. The diet for the adult zebrafish consisted of dry micropellets and brine shrimp (hatched from 25 g of eggs in 2 L salt water) daily along with rotifers every two days. The ratio of food per day was maintained at 4% of body weight of the adult fish.

Experimental procedures

Three experimental tanks were set up to analyse growth parameters each containing around 100 fish, tank 1 is control with the CO_2 concentration set at ambient 400 µatm and tanks 2 and 3 received 1500 and 2200 µatm of CO_2 for a period of 60 days. CO_2 was infused for 1 hour continuously followed by an interval of 2 hour until 5 hours on daily basis. The initial mean weight of the fish in each tank was measured and documented. They were fed three times a day both with commercial feed and brine shrimp.

Growth performance

Fish were sampled for growth performance on day 60. The animals were fasted for 24 h before being anesthetized with ice water to measure the weight and the length from each tank. Growth performance was evaluated following the method of (Baloi et al. 2016) and the parameters taken into account were:

Body weight gain (BWG, in g) = mean final weight – mean initial weight

Specific growth rate (SGR, % d^{-1}) = [(In final weight – In initial weight)/duration in days] × 100

Condition factor (CF) = (body weight/total length^3) × 100

Coefficient of variation (CV) = (mean standard deviation of final weight/mean final weight of fish) × 100

Mortality

All the experimental tanks were monitored every day for mortality and recorded accordingly. Fish exhibiting symptoms of disease were separated and treated with recommended antifungal or antibacterial compound and rehabilitated on survival.

Courtship behaviour analysis

The effect of CO_2 on the reproductive performance was investigated. After 60 days, 3 pairs of healthy and active male and female fish were selected from each experimental tank. Each pair consisting of one male and one female were transferred to a separate opaque tank and allowed to stay overnight for acclimatization. In the morning, the lights were turned on and the behaviour of the fish were recorded for 10 minutes from the top of the tank. The courtship behaviour was analysed based on three responses: Undulation (chase), Escorting (touch) and encircle with quivering (Darrow and Harris 2004) as illustrated in Figure 1. Male fish swimming towards the female fish with tailfin flicking is considered chasing, while the male fish hitting the abdomen of female fish with its head is regarded as touch. When the male fish passes over and swims around a female fish thus completing a circle, it is called encircle. All the experiments were repeated three times are data are represented in mean of the three values obtained.

Embryo collection and determination of survivability and hatching rate

Healthy female and male fish from each experimental tank were selected for breeding following the standard protocol (https://wiki.zfin.org/display/prot/Breeding). After spawning, embryos were collected 3 hours post-fertilization (hpf) on a Petri dish and were washed with the freshly prepared E3 medium to clean the impurities and other debris (E3 medium: 5 mmol/l NaCl, 0.17 mmol/l KCl, 0.16 mmol/l $MgSO_4$ and 0.40 mmol/l $CaCl_2$). The embryos were monitored for up to 96 hpf and data were recorded for mortality and hatching rate for every 24 h.

3. Results

Growth performance

Mortality in the control (400 µatm), 1500 and 2200 µatm tanks were found to be 5, 15 and 23, respectively during the experimental period. The growth performance of the zebrafish under the presence of CO_2 in the environment determined using the key points BWG, SGR, CF and CV is given in Figs. 2 and 3. The BWG data shown in Fig. 2a show that there is a slight decrease in weight gain in the fish exposed with 2200 µatm of CO_2 when compared to unexposed control. Ironically, a subtle weight gain of 0.09 g was observed in the fish treated with 1500 µatm of CO_2, whereas for control it was 0.08 g; however, for the fish treated with 2200 µatm, the weight gain was documented to be 0.056 g.

Similar results were obtained for SGR and CF. The growth per day in the fish of control tank was 3.18 mg; for fish in the tanks treated with 1500 and 2200 µatm of CO_2, the weight gain per day was determined to be 3.28 and 2.84 mg, respectively (Fig. 2b). The condition factor was found to be 3.48, 3.38 and 2.33 for the experimental samples control, 1500 and 2200 µatm of CO_2 exposed zebrafish, respectively (Fig. 3a).

A small dip in coefficient of variation was observed in fish treated with 1500 µatm of CO_2 with a value of 3.92 when compared to control of 4.82 (Fig. 3b). However, for the group treated with 2200 µatm of CO_2, a near normal value of 4.71 was obtained. Similarly, the daily growth rate given in Fig. 3c shows a non-significant increase in the value of 0.36 with the fish exposed with 1500 µatm of CO_2 when compared to control with 0.3 and a sharp dip in the value of 0.2 with the samples treated with 2200 µatm of CO_2.

Spawning behaviour

Responses and behaviour pertaining to spawning or courtship include undulation – chasing by the male fish, escorting – touching the abdomen of the female fish with the head of male fish and encircling – the fish pair swim together in circular motion and the male continuously and actively flicks the tailfin with occasional quivering (Fig. 3). The fish from the control tank were highly active with significantly high number of undulation, escorting and encircling (Table 1) counted to be 263.67, 156 and 26.67, respectively. In contrast, the fish treated with CO_2 has significant reduction in the responses of undulation, escorting and encircling. The fish pair treated with 1500 µatm of CO_2 showed 109.33, 82 and 10 number of chases, touches and encircles, while for the fish pair exposed with 2200 µatm of CO_2 the count was 26, 28.33 and 2.33, respectively.

Survival of the embryos

The survival of the embryo of control and CO_2 exposed zebrafish was determined at specific time points. As shown in Fig. 4a about 68.76% of the embryos survived in the control sample 96 hpf. However, in the experimental tanks infused with

1500 and 2200 µatm of CO_2, there was a gradual but significant decline in the number of embryos in a time-dependent manner and after 96 h only 29.18 and 8.22% survivability of the embryos were observed.

Hatching rate

The hatching rate of the embryo from CO_2 unexposed and exposed parents is shown in Fig. 4c. Compared with the control group, there was a significant drop in the hatching rate of embryos collected from the fish exposed with 1500 and 2200 µatm of CO_2. The data show that the hatching rate of control fish is 96.86%, while for the experimental embryos from 1500 and 2200 µatm of CO_2 exposed fish reached a hatching rate of only 54.47 and 47.95%, respectively.

4. Discussion

The current study shows the effect of increased CO_2 on the growth performance and the spawning behaviour of adult zebrafish. Our data suggest that CO_2 exposed for 60 days has subtle effect on growth performance of the zebrafish exposed to 1500 µatm of CO2. A notable decrease in body weight of the zebrafish was observed when exposed to 2200 µatm of CO2. The reduction in weight may be due to the inability of the fish to regulate the acid-base equilibrium due to the presence of high CO2 in the environment (McCormick, and Nilsson 2012; Nowicki, Miller, and Munday 2012). (Olsson P.E., P. Kling 1998) has stated that CO2 can affect the physiology of the fish directly as well as can cause other damages by reducing the pH of the water. In this study, Figs. 1 and 2 show that BWG, SGR, CF and CV of the zebrafish demonstrated notable changes when compared to control. Similar conclusions were also drawn by previous researchers demonstrating the reduction in growth of the fish juveniles including yellowtail (*Seriola quinqueradiata*), silverside (*Menidia beryllina*) and Atlantic salmon (*Salmo salar* L.) (Fivelstad et al. 2015; H. Baumann, Talmage, and Gobler 2011; S. D. Baumann and Gillig 2012; Lee et al. 2003). During rise in CO_2, the fish utilizes most of the energy from the food for osmoregulation and as a result energy is deficit for growth and the weight of the fish is reduced (Thorarensen et al. 2018). In addition, probably stress could be also one of the important factors for the low weight of the zebrafish in the presence of high CO_2 (Martínez-Porchas, Rafael Martínez-Córdova, and Ramos-Enriquez 2009).

Reproduction is an essential and crucial process for the survival and success of a species population which may be adversely affected due to elevated CO2. In this study, the presence of CO_2 was hypothesized to cause adverse effect on the reproductive behaviour of the laboratory maintained freshwater organism, zebrafish. As proposed, there has been a drastic decrement in the spawning responses such as chasing, touching and encircling as well as hatching rate in the 1500 and 2200 µatm of CO_2 treated fish when compared to the control fish (Table 1). Literature also supports our findings on the negative correlation of reproduction and elevated CO_2 in different invertebrates (Havenhand et al. 2008; Fitzer et al. 2012). The mating behaviours are reported to be vulnerable by environmental changes such as hypoxia, agricultural contaminants and turbidity (Bertram et al. 2015; Wong and Candolin 2015; Pollock, Clarke, and Dubé 2007). Moreover, spawning events were notably lower in high CO2 environment in a pair of ocellated wrasse (*Symphodus ocellatus*) (Milazzo et al. 2016). An important indication towards the low male sexual behaviours could be the malfunction of androgen which plays a crucial role in male sexual and aggressive behaviours (Cunningham, Lumia, and McGinnis 2012) and it emerges as a critical area for further research.

In addition to reduction in spawning behaviour, a dramatic decrement in hatching rate was documented laid by the fish treated with 1500 and 2200 µatm of CO_2 when compared to the untreated control (Fig. 3b). Contrastingly, (Welch and Munday 2015) has reported an increased egg survival and hatching success in *Acanthochromis polyacanthus* when subjected to elevated CO_2 and inferred that fish reproduction varies from species to species in the presence of CO_2. It is noteworthy to consider that ocean acidification causes varying affects in marine organisms including negative, positive and neutral effects as well. In line with our finding on reduced hatching rate, (PARKER et al. 2009) has reported reduced fertilization success in Sydney rock oyster. The reduced egg number and clutch size may be attributed to the fact that the energy stored for the reproduction activity has been incurred for acid-base regulation, which costs additional energy in an elevated CO_2 surroundings (Ishimatsu et al. 2008; Barry and Widdicombe 2011; Strobel et al. 2012; Aze et al. 2014). Besides, reproduction in fish is also linked to $GABA_A$ receptors which is the major inhibitory neurotransmitter receptor in vertebrate brain and also plays a role in mediating hormone secretions during normal reproductions (Zohar et al. 2010; Di Yorio et al. 2019). Surprisingly, it is reported that the influence of GABA on reproductive hormone vary among species, it may be stimulatory in some and inhibitory in others (Trudeau et al. 2011). The latter could be another factor in lower egg production due to inhibitory effect of GABA in zebrafish exposed to high CO_2 in the present study.

5. Conclusion

The performance markers with respect to growth and reproduction showed considerable effect on the zebrafish in response to CO_2 exposure. The response to high concentration of acute CO_2 environment is not unilateral in zebrafish. However, high concentrations of CO_2 can affect the growth and spawning behaviour of zebrafish.

Conflict of interests

The authors declare no conflict of interests.

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Author contribution

Both SA and AT conceptualized the work; AT and BK performed laboratory and data analyses; AT and SA wrote the manuscript; AS and BK edited the manuscript.

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Tables

Table 1 Spawning behaviour parameters for zebrafish under different CO_2 doses

Spawning parameters	Control (400 µatm pCO_2)	1500 µatm pCO_2	2200 µatm pCO_2
Undulation	263 ± 15	109 ± 17	26 ± 4
Escorting	156 ± 11	82 ± 9	28 ± 7
Encircling	26 ±6	10 ± 2	2 ± 0.3

Figures

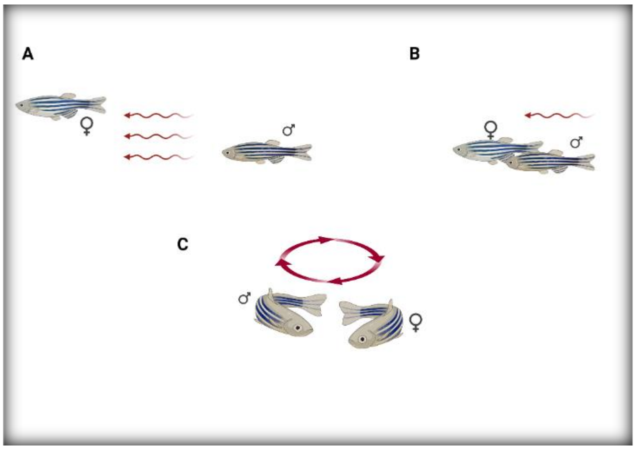
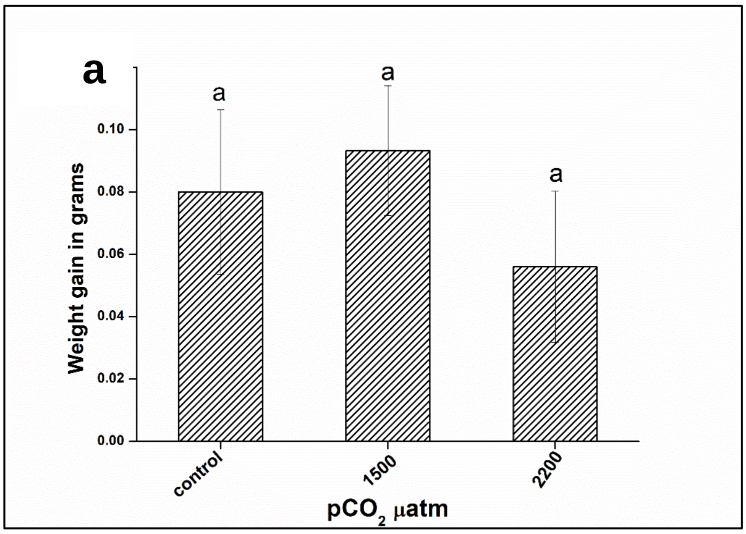


Figure 1. Illustration of spawning behaviour in male (♂) and female (♀) zebrafish. A) Undulation – pursuit of adult male zebrafish on the female adult zebrafish. B) Escorting – Male fish follows closely and touches the female fish. C) Encircling – the fish pair swims together in circular motion and the male shows quivering occasionally.



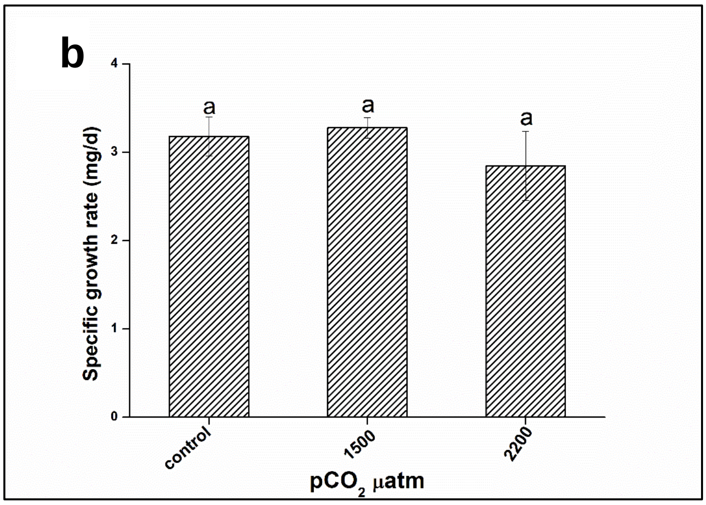
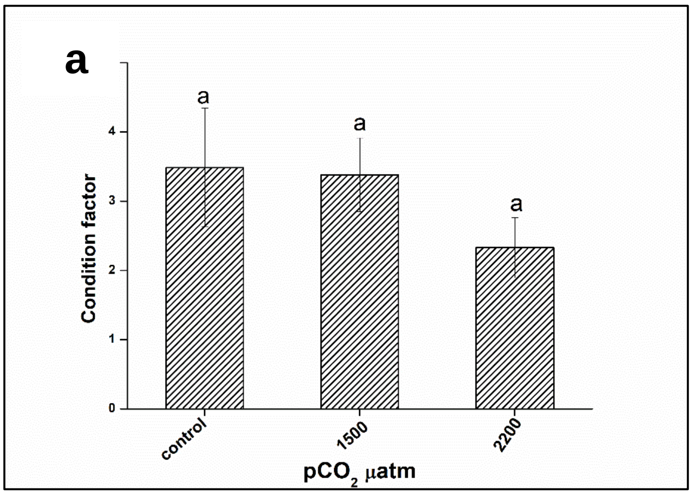
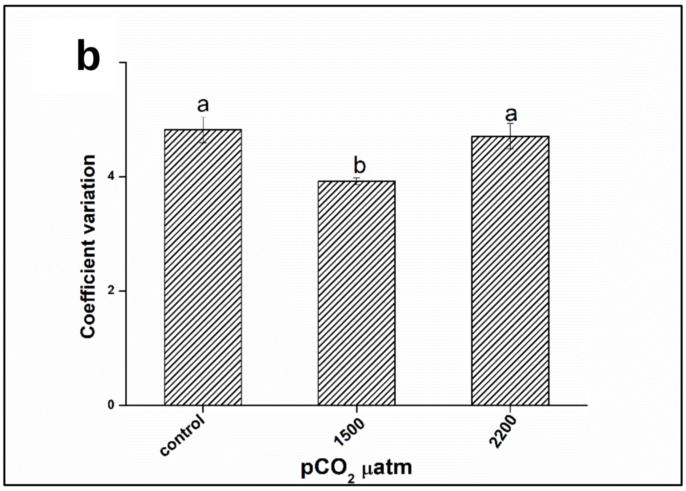


Figure 2. Growth performance parameters, body weight gain (BWG) (a) and specific growth rate (SGR) (b) of zebrafish exposed to 1500 and 2200 µatm of CO_2 for 60 days. Values with different letter are significantly different between groups ($p \leq 0.05$).





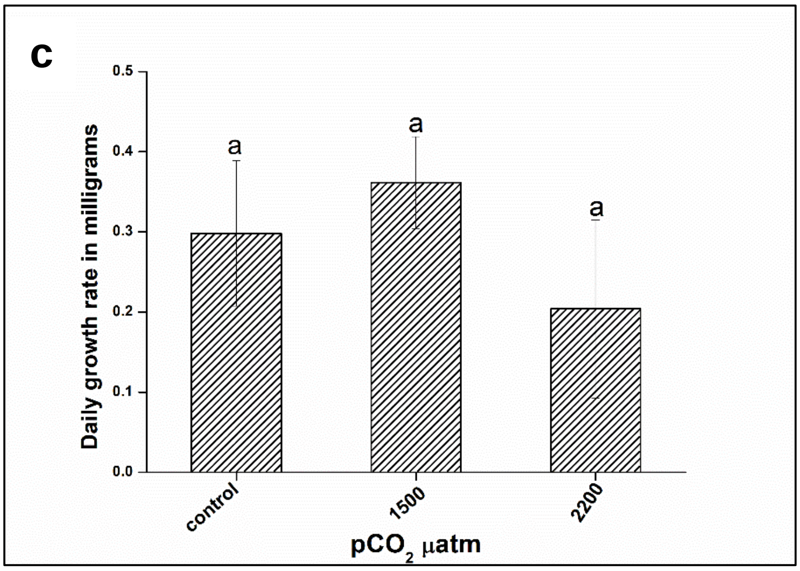


Figure 3. Values represent condition factor (a) of the zebrafish exposed to 1500 and 2200 µatm of CO_2 for 60 days calculated based on weight gain and standard length. Coefficient of variation (b) and daily growth rate (c) of zebrafish exposed with or without CO2. Values with different letter are significantly different between groups ($p \leq 0.05$).

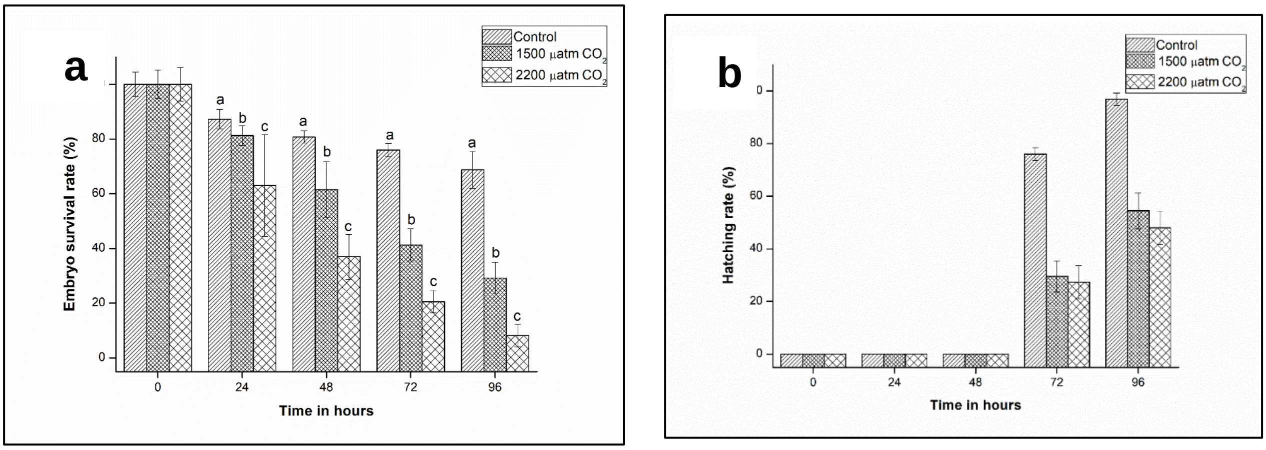


Figure 4. Reproductive characteristics of breeding pairs of zebrafish fish treated with or without CO_2. Survivability (a) and Hatching rate (b) of the embryos observed from 0 to 96 hpf. Values are the mean and standard error of three independent experiment and the significance is at the level of $p \leq 0.05$.