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Effects of elevated carbon dioxide and temperature on survival and morphology of Japanese whiting *Sillago japonica*

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Abstract
The objective of the present study was to examine the effect of elevated CO_2 and temperature on survival and morphology of *Sillago japonica* yolk sac larvae under the Institute for East China Sea Research (ECSER) Nagasaki, Japan. In this study, we examined hatching success, survival and morphology of the larvae of *Sillago japonica* under four conditions: control (C), seawater pCO_2 382µatm, temperature 27 °C; high CO_2 (HC), 915µatm, 27 °C; high temperature (HT), 385 µatm, 31 °C; and high CO_2+high temperature (HCT), 932µatm, 31 °C. Fertilized eggs were obtained from broodstock reared in natural seawater, and transferred to experimental containers in each treatment. Hatched larvae were observed until the completion of yolk sac absorptions on 3 days post hatching (dph). The experiment was repeated four times with 4 replicates for each treatment in each experiment. Also, the temperature appeared to have exerted a stronger influence on hatching success (Hs) and larval survival (Sv): Hs and Sv at 3 dph were both significantly (p>0.05) depressed in HT (Hs 52.5±1.25%, Sv 23.8±4.38%) and HCT (Hs 51.3±3.13%, Sv 20.0±0.63%) treatments than in C (Hs 98.1±0.94%, Sv 74.4±2.03%) and HC (Hs 95.0±2.5%, Sv 49.7±3.44%) treatments. In contrast, CO_2 was the predominant factor responsible for morphological abnormality: percentage morphological abnormality was significantly (p>0.05) higher in HC (15.8±2.72%) and HCT (41.0±10.86%) treatments than in C (0.4±0.65%) and HT (2.4±2.40%) treatments. Most individuals in HC and HCT treatments had body axis either curved or bent, with aberrant swimming behavior. These results indicate that projected future ocean environments will have significant negative impacts on hatching success, and larval survival and morphology of *S. japonica*, which might have serious ramifications for recruitment of the species. Comparative studies on other teleost and elasmobranch species are critically needed.

Keywords: Elevated Carbon dioxide, marine fish larvae, *Sillago japonica*, survival and morphology, temperature

1. Introduction
The anthropogenic activities like burning of fossil fuel are increasing atmospheric carbon dioxide (CO_2) concentration which is causing global warming and ocean acidification (Feely *et al.*, 2004 [1]; Orr *et al.*, 2005) [2]. The atmospheric carbon dioxide concentration that had been increasing at a rate of 1% per year during the 20th century is now increasing to approximately 3% per year (IPCC, 2007) [3]. The CO_2 concentration in the atmosphere is projected to reach 1000ppm by 2001 (SRES A1FI Scenario) and (IPCC, 2007) [3] while recent global mean CO_2 concentration is approximately to 390ppm (Conway and Tans, 2011) [4]. About one-third of the anthropogenic CO_2 produced in the past 200 years has been taken up by the oceans (Sabine *et al.*, 2004) [5] which alter ocean chemistry through acidification process. Modeling studies suggest that by 2100 surface ocean pH will decline by 0.3-0.41 units compared to current levels (Caldiera and Wickett, 2005 [6]. Doney *et al.*, 2009) [7]. The impacts of ocean acidification on invertebrate development have been broadly studied (Doney *et al.* 2009 [7]. Byrne, 2011 [8]. Dupont *et al.*, 2010 [9], Hendriks *et al.*, 2010 [10]; Kroeker *et al.*, 2010 [11]. Fabry *et al.*, 2008) [12]. Research suggested that ocean acidification affects the physiological processes of marine organisms (Pörtner, 2008) [13] and consequently, their ecological functions and interactions with other organisms (Widdicombe and Spicer, 2008) [14]. Comparatively few studies have investigated the effects of climate change on coral fish (Munday *et al.*, 2008[15]; Roesig *et al.*, 2004) [16]. The declining pH was reported to affect coral fish behavior

(Dixson *et al*, 2010 [17]; Munday *et al.,* 2010 [18]; Simposon *et al*., 2011 [19]; Ferrari *et al*., 2012 [20]; Domenici *et al*., 2012) [21], reduce hatching success or survival of finfish (Baumann *et al*., 2011 [22]; Chamber *et al.,* 2014) [23] and deform fish larvae (Baumann *et al*., 2011[22]).

Global warming and ocean acidification are recognized as global problems (Doney *et al*., 2009) [7]. The global warming caused by additional CO_2 lead into an increase in mean sea-surface temperatures (SST) between 1.1 and 6.4 °C by 2100 with the best estimates ranging between 2 and 4.5 °C (IPCC, 2007) [3]. The increase in SST projected to occur will cause thermal stress to wider range of marine organism, in which most of them are already confined to their temperature tolerances or exceeded their limit (Hoffman and Todgham, 2010) [24]. Some studies on invertebrate showed that ocean warming has an effect on embryo development and caused embryo mortality as well as skeletal dissolution for juvenile invertebrate (Byrne 2011 [8]; Negri *et al*,. 2007 [25], Whalan *et al*., 2008) [26] Early findings reported that marine invertebrate larvae normally require a narrower temperature range for development compared to adults (Foster, 1971) [27]. High temperature under culture condition was reported to cause fish larvae morphology deformity for instance *Solea senegalensis* (Dionisio *et al.,* 2012) [28] *Pseudopleuronectes herzensteini* (Aritaki and Seikai, 2004) [29] halibut (Lewis-McCrea *et al*., 2004) [30] gilthead seabream (Georgakopoulou *et al*., 2010) [31], European seabass (Koumoundouros *et al*., 2001) [32] and wolfish (Pavlov and Moksness, 1996) [33]. Additionally, temperature was demonstrated to influence marine larval dispersal distance, with the implications for the understanding and effective management of marine populations, connectivity and ecosystem (O'Connor *et al.,* 2007) [34]

Very few studies have shown that future increases in CO_2 and SST will have synergistic effect in feeding behavior of coral fish (Nowicki *et al*., 2012) [35]. However, currently there is a limited evidence to support hypothesis that elevated CO_2 and temperature affect hatching, survival and morphology of marine fish larvae with yolk sac. The fish larva with yolk sac is a very crucial stage of fish development as related to predator escape, prey searching and resistance to starvation. Therefore understanding of these ability can help to provide information not only for an ecological understanding of marine fish-larvae, but also for conducting intensive larval rearing on a large scale and help to predict the future population.

Therefore, this study examined the effect of elevated CO_2 and temperature on survival and morphology of *Sillago japonica* yolk sac larvae. *S. japonica* was selected for the present study because they can hatch and reared easily under captivity; it is one of representative of coastal fish and is used for aquaculture.

2. Material and methods

2.1 Experimental animal and Egg Collection

Naturally `fertilized eggs of *S. japonica*) were obtained from Nagasaki Prefecture Fisheries Research (NPFR) around 08.30 am in the morning. Eggs were collected in 500ml beaker which was covered with plastic paper. The Nagasaki Prefecture Fisheries Research (NPFR) is about 100m from ECSER Institute, 32 °48'39"N, 129 °46'20"E), Nagasaki, Japan where research was conducted. The brood stock were reared under 3000L round plastic tank under ambient temperature (27.5 °C) and *p*CO2 (392 µatm) with continuous flow of sand filtered seawater. The Broodstock were fed twice a day 09h00 and 16h00 artificial feed according to their nutritional requirement. Sillago spawned around 12pm midnight. The eggs were examined under microscope and unfertilized eggs were removed.

2.2 Experimental setup

Four (4) different treatment levels in four replicates were used in this study. (i) Control (C) i.e. temp = 27 °C; pCO_2 = 380 µatm (ii) Elevated CO_2 (HC) i.e. 27 °C; pCO_2 = 1000 µatm (iii) Elevated temperature (HT) i.e. temp = 31 °C; p CO_2 380 µatm (iv) Combination of elevated temperature and CO_2 (HCT) i.e. temp = 31 °C; pCO_2 = 1000 µatm. The experiment was repeated four during the month of July, 2013.

2.3 Sea water manipulation

Natural seawater was sand filtered before being pumped into 4 header plastic containers of capacity of 15liters. Pure CO_2 was bubbled into each tank in order to get the desired pH and pCO_2 for control 380ppm which was the current situation and 1000ppm according to year 2100 prediction by A1FI scenario (IPCC, 2001) [36]. The flow rate of 10L/minute CO_2 free air was used to obtain current situation and air: CO_2 was at a ratio of 10L/minute:6.20cc/minute respectively was used for to obtain the future pCO_2 and pH. The CO_2 blended gas was prepared with a gas blender (Kofloc, GB-2C, Japan). The electric heaters were used to adjust temperature of sea water, raised to 27 and 30.5 °C for current and future situation respectively. These temperatures correspond to current day July average SST at East China Sea and 3 °C increase beyond the July average SST.

The water pH and temperature of treatment tanks was monitored daily using digital probes and maintained at a desired level throughout the experimental time. Temperatures were measured using digital probe thermometer. The pH was measured using National Bureau of Standard (NBS) scale. Salinity was measured before the start of experiment by using portable refractometer (Atago, 100-S, Japan). Before starting the experiment the water sample was taken to estimate total alkalinity (AT) by using total alkalinity titrator (Kimoto, ATT-05, Japan) in duplicate. These data were used to determine partial pressure of CO_2 (pCO_2) in each treatment by using CO2Calc 1.2.0 (Robbins, 2010) [37], with dissociation constants K1 and K2 from Mehrbach *et al*., (1973) [38] refit by Dickson and Millero, (1987) [39]. Dissolve Oxygen (DO) concentration during the experiment was measured daily in each container using portable DO meter (Eyela, NCB-1200, Japan).

Table 1: The experimental seawater quality (mean±SD). The Dissolved Oxygen (DO), Temperature, PH and Total Alkalinity (TA) were measured but pCO_2 was calculated by using CO2calc 1.2.0

Treatments	Sea water parameters				
	DO (mg/l)	Temp ^{0}C	pH(NBS)	TA (µmol/kg SW)	pCO_2 (µatm)
C	6.65±0.24	26.8±0.25	8.17±0.01	2184.0±79.0	391.0±8.01
HC	6.55±0.16	27.0±0.32	7.85±0.01	2159.0±82.0	939.0±19.0
HT	6.36±0.11	30.8±0.32	8.16±0.01	2043.1±23.0	396.0±9.02
HCT	6.46±0.09	30.6±0.65	7.85±0.01	2144.0±28.0	947.0±18.0

2.4 Data Collection and Analysis

2.4.1 Hatching Percentage (HP)

Ten fertilized eggs were randomly assigned in 4 replicates in 500mls containers with a lid on it for each treatment to examine hatching percentage (HP) after 24 hour incubation. During incubation time the treated water was exchanged at a rate of 0.34ml/minutes. The fish larvae hatched were counted from each container then averaged percentage were calculated separately for each treatment

2.4.2 Larval survival and Morphology deformities

Fifty fertilized eggs were randomly assigned in 1000mls containers in 4 replicates for each treatment. Then 20 fish larvae were retained in each container with a lid on it to observe larval survival, morphology deformities with water exchanged at a rate of 2ml/minute. These parameters were examined at day three after incubation when the digestive system is completely developed and egg yolk is almost completely absorbed (Oozeki, *et al*., 1992) [40]. Fish larvae survived to day 3 in each container were counted and then used to compute mean percentage of survival for each treatment. The fish larvae were also observed, their morphological deformities from each container were recorded then percent of deformities was calculated for each container and then the mean percentage was calculated for each treatment.

2.5 Data analysis

Two-way ANOVA was used to compare effect of experimental time and treatments to assess the consistency of the results. The effect of treatments on hatching percentage (HP), survival percentage (SV) and Morphology deformity percentage (MD) were analyzed by using one-way ANOVA followed by post hoc Games Howells multiple comparison test to observe the differences between observed mean. Before analysis data were first tested for homogeneity of variance using Levene's test. If data did not show homogeneity of variance even after transformation into square root or log (X+1) then Games Howells multiple comparison test was used.

3. Results

3.1 Hatching percentage (n=40)

Results revealed that temperature has significant effect on hatching success of sillago fish. Each experiment showed consistently significant (p<0.001) decrease in hatching success at elevated temperature. The HT and HCT had significantly (p<0.001) lower hatching success for experiments 1, 2, 3 and 4. The control (c) and high CO_2 treatments showed no significant difference (p>0.05), for exp 1, 2, 3 and 4 treatments in hatching percentage. Likewise, the HT and HCT showed no significant effect (p>0.05) for all experiments) in hatching percentage (Figure 1).

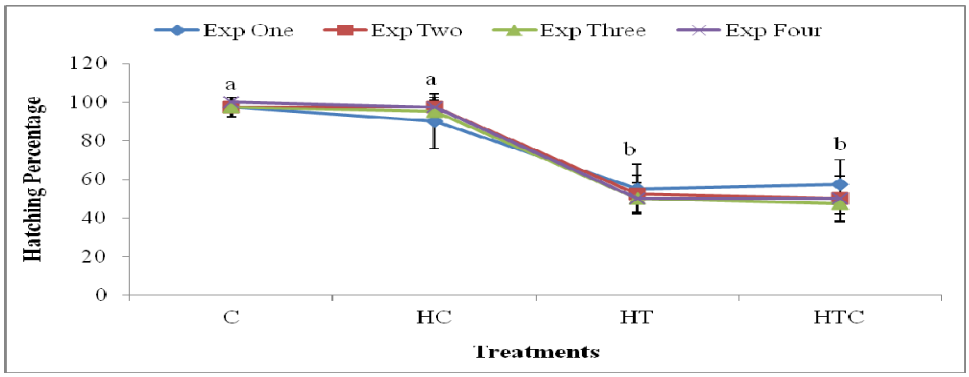

Fig 1: The average Hatching Percentage of *Sillago* eggs (n=10) at different treatment observed 24h after incubation for Each for experiments conducted at different time. Different letter on the error bar shows significant different at p<0.05.

Survival at day 3 (n=20)

The results revealed that elevated CO_2 and temperature (HC, HT and HCT) had significant (p<0.05) effect on survival of Sillago fish larvae (Figure 2). In all experiments, the control showed significant higher (p<0.01) larvae survival, three days post hatch followed by HC in all treatments. While treatments HT and HCT showed significant lowest ((p<0.01) survival in three days post hatch whereby HT and HCT showed no significant different (p>0.05).

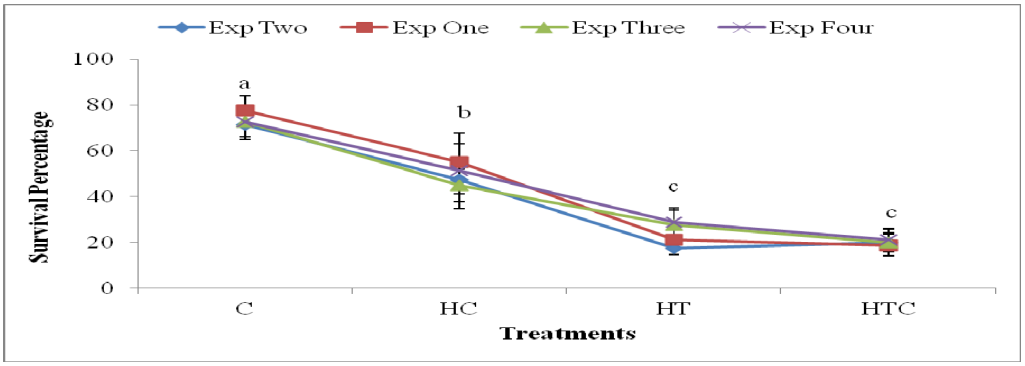

Fig 2: The average survival percentage (SV) of sillago at different treatment observed 3 days post hatch. n=20 for each treatment. Different letter on the error bar shows significant different at p<0.05.

Morphology deformity percentage as per number of survivors

Figures 3 and 4 showed that elevated CO_2 and temperature have great impact on sillago larvae morphology deformity (MD). In all experiments, HCT showed significant (p<0.05) synergistic effect of elevated CO_2 with temperature on impacts of morphology deformities of sillago fish larvae. The HC and HT showed significant (p<0.05) of sillago fish larvae however, the HC and HT showed no significant (p>0.05) difference on fish larvae morphology deformity. The control had significant (p<0.05) the lowest number of fish larvae with morphology deformity compared to other treatments (Figure 3).

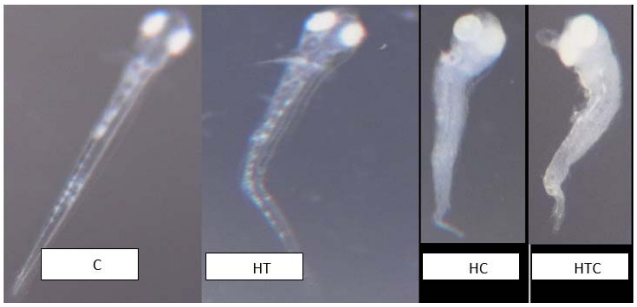

Fig 3: Effect of elevated CO_2 and temperature to early life stage of *Sillago japonica* survived 3 days post hatch

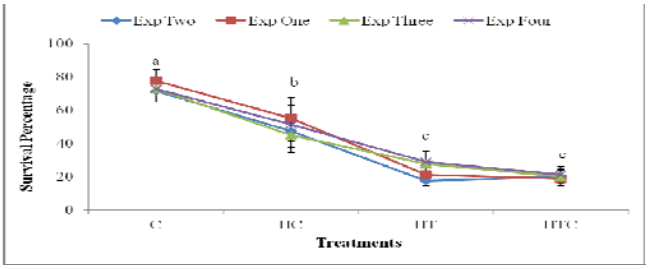

Fig 4: The average morphology deformity Percentage of sillago fish larvae survived 3 days post hatch at different treatment. Different letter on the error bar shows significant different at p<0.05.

4. Discussion

The present study unambiguously revealed that the future projected elevated by both CO_2 and temperature of the oceans may affect hatching, survival and morphology of marine fish larvae. Regardless of marine fish being known to have well developed acid-base regulatory system, still they are susceptible to elevated CO_2 which leads to ocean acidification (Munday *et al*., 2010 [18]; Baumann *et al*., 2011 [22]; Ishimatsu *et al*., 2008 [41]; Frommel *et al*., 2011 [42]; Esbaugh *et al*., 2012[43]; Bignami *et al.*, 2013 [44]; Enzor *et al.*, 2013 [45]; Hurst *et al.*, 2013) [46]. Temperature is known as a main physical regulatory in marine life to have a noticeable power on metabolism and implications in development as well as induces ontogenetic plasticity in fish larvae (Dionisio *et al*., 2012 [28]; Pankhurst and Munday, 2011) [47]. The hatching percentage of sillago eggs were unaffected by elevated CO_2 when exposed to approximately 942 µatm CO_2 resulting into 7.85 pH during the present study. This could be due to short time of exposure to elevated CO_2 to make a significant changes as it took less than 24hrs to hatch also it could suggests that sillago eggs are resistant to elevated CO_2. Similar findings were reported elsewhere when eggs exposed to projected future elevated CO_2 (Munday *et al*., 2009 [48]; Franke and Clemmesen, 2011) [49]. However, findings from temperate Atlantic fish *Menidia beryllina* showed a considerable mortality of eggs when exposed to elevated CO_2 (Baumann *et al.*, 2011) [22] Also it was reported that the vulnerability of early developmental stage of fish like eggs, sperms, larvae and juveniles to elevated CO_2 levels is higher than for adults (Kikkawa *et al*., 2003 [50]; Ishimatsu *et al.*, 2004) [51]. The elevated temperature 31 °C has exerted stronger influence on hatching success in sillago fish. Failure of sillago eggs to hatch at elevated temperatures in all four successive experiments suggests that the elevated temperature is outside the tolerance limit for development of sillago eggs. It has also been reported that the hatching rate of sillago eggs has a tendency to decrease at temperatures over 28 °C (Hotta *et al*., 2001) [52]. It has also been reported that egg is the most sensitive life stages of fish species as small increases in temperature can severely increase egg mortality (Gagliano *et al*. 2007) [53]. These findings agree with the long time laboratory findings in sole fish egg mortality occurred as the temperature increases (Irvin, 1974 [54]; Pepnin, 1991) [55] and common carp (Richter *et al*., 1987) [56]. The same trend showed in the currently research that small increase in temperature increased mortality of northern rock sole larvae *Lepidopsetta polyxystra* (Laurel and Blood, 2011) [57] and common carp (El-Hakim and El-Gamal, 2009) [58]. Similar to this, temperature is known as the main environmental factor affecting development of fish eggs as well as certain morphological features and hatching rate (Nwosu and Holzlohnev, 2000 [59]; Bagenal and Braun, 1978) [60]. At temperature of 27 °C the hatching percentages of sillago fish was higher compared to future elevated temperature of 31 °C. It is being proposed that 27 ^{0}C is the optimum temperature for hatching of sillago eggs. This temperature corresponds to the environmental temperature during the period of natural reproduction. Therefore, the elevated temperature has greater implications for the future sillago stock abundance as fish hatching success predicts the next generation.

Our results also showed that elevated temperature exerts a stronger influence of survival of sillago fish larvae than CO_2 alone. This could be due to high metabolic rate beyond the tolerance limit of sillago fish larvae. The metabolic rate which is the fundamental measure of physiological activities mediates most of biotic effects of warming (Herbing and Boutilier, 1996 [61]; McLeod *et al*., 2013) [62]. The similar findings were reported on *Amphiprion percula*, coral fish larvae that high temperature reduces their survival (McLeod *et al*., 2013) [62] and cardinal fish species (Munday *et al*., 2009) [48]. All organisms are known to have lethal limits to their temperature range and within that range organisms have optimal temperature for development of physiological activities (Hokanson, 1977 [63]; Rombough, 1997) [64]. The elevated CO_2 and reduced pH alone also showed impacts on survival of sillago fish larvae. This was also reported to exert severe effect on survival of silver sea bream *Pagrus major* eggs and larvae (Ishimatsu *et al*., 2004) [51]. This suggested that the acid-base regulatory system was not well developed at this young stage and thus compromising their physiological compensatory mechanisms. Also their small size could be increasing cost of homeostasis hence more vulnerable to environmental elevated CO_2 predicted to occur in near future (Brauner, 2009 [65]; Melzner *et al.*, 2009) [66]. However, contrary to what was reported that the elevated CO_2 had no effect of on growth and survival of juveniles of common coral reef fish, the spiny damselfish *Acanthochromis polyacanthus* (Munday *et al.*, 2011) [67] that was consistent with some cold water fish showed to tolerate elevated CO_2 levels above those

predicted to occur in the shallow ocean due to increasing anthropogenic CO_2 emissions (Kikkawa *et al*., 2003[50]; Ishimatsu *et al*., 2004 [51]). This showed that the ability of fish to tolerate low pH due to elevated CO_2 is species specific.

The synergistic interactions between temperature and CO_2 consequently have great implications on survival of unfed sillago larvae and therefore ecological implication to marine fish abundance and distribution. The findings showed that in marine organisms, low pH is thought to enhance ion regulatory costs and thus baseline energy demand whereas elevated temperature increases baseline metabolic rate (Kreiss *et al*., 2015)[68]. This synergistic interaction effect was reported to increase mortality of *Ostorhinchus doederleini* which suggested that the physiological condition of individuals was severely compromised at elevated CO_2 and temperature was sufficient to cause increased mortality (Munday *et al*., 2009)[48]. Such differences suggest that the composition of marine fish communities may shift due to changes in relative rates of mortality among species in global warming world. Sillago fish feeds in low food chain and therefore reduction of their number will have an impact on their predators' abundance and distribution as well as changes in eating habits.

From the findings, it can be revealed that elevated CO_2 was the predominant factor responsible for morphological deformity of sillago fish larvae. This suggests that early life stages can be negatively impacted by elevated CO_2, but how does the elevated CO_2 affect fish morphology is currently unknown. Recent findings reported that elevated CO_2 caused considerable higher percentage of malformations in newly hatched temperate Atlantic fish *Menidia beryllina* larvae (Baumann *et al*., 2011) [22]. It was proposed that, sensitivity to elevated CO_2 at the earliest life stages could concomitantly reflect poor development of acid-base regulation and cardio-respiratory control mechanisms. These mechanisms are possibly linked to increased gill function and muscle activity due to swimming activity (Perry and Gilmour, 2006) [72] as well as tissue damage and necrosis in fish larvae (Frommel *et al*., 2011) [42]. Likewise, such mechanism could also be due to high metabolic cost incurred to maintain physiological activities at elevated CO_2 and ultimately reduces temperature energy available for tissue synthesis (Ishimatsu *et al*., 2004 [51]; Kurihara *et al*., 2008 [69]; Shirayama, 2002 [70]) and therefore affect energy requirements for activity among ontogenetic stages (Baumann *et al.,* 2011) [22]. HT which also showed significant sillago larvae morphology deformity, was also similar to those reported in *Solea senegalensis* (Dionisio *et al*., 2012) [28], *Pseudopleuronectes herzensteini* (Aritaki and Seikai, 2004) [29], halibut (Lewis-McCrea *et al.,* 2004) [30], gilthead seabream (Georgakopoulou *et al.,* 2010) [31], European seabass (Koumoundouros *et al*., 2001) [32] and wolfish (Pavlov and Moksness, 1996) [33]. It was also suggested that the skeleton deformity at high temperature could be due to effect of stress caused by altered developmental timing (Das *et al.,* 2006) [71].

5. Conclusion

This study highlights the potential effects of future elevated CO_2 and temperature on hatching, survival and morphology of unfed sillago fish larvae. Elevated temperature showed high impacts on hatching and survival of sillago fish larvae. While the elevated CO_2 exerted strong effect on fish larvae morphology deformity, however, the additive effect of elevated temperature worsen the situation. The synergetic impacts of elevated CO_2 and temperature have great impacts on the early life and reproductive stages will have consequences on aquatic ecology. Most individuals in HC and HCT treatments had body axis either curved or bent, with aberrant swimming. These results indicate that projected future ocean environments will have significant negative impacts on hatching success, and larval survival and morphology of *S. japonica*, which might have serious ramifications for recruitment of the species. Comparative studies on other teleost and elasmobranch species are critically needed and such effort can eventually help fisheries managers and policy-makers take proactive measures targeting most vulnerable species.

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