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Sub-lethal and lethal toxicities of elevated CO_2 on embryonic, juvenile, and adult stages of marine medaka *Oryzias melastigma*

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ABSTRACT

The potential leakage from marine CO_2 storage sites is of increasing concern, but few studies have evaluated the probable adverse effects on marine organisms. Fish, one of the top predators in marine environments, should be an essential representative species used for water column toxicity testing in response to waterborne CO_2 exposure. In the present study, we conducted fish life cycle toxicity tests to fully elucidate CO_2 toxicity mechanism effects. We tested sub-lethal and lethal toxicities of elevated CO_2 concentrations on marine medaka (*Oryzias melastigma*) at different developmental stages. At each developmental stage, the test species was exposed to varying concentrations of gaseous CO_2 (control air, 5%, 10%, 20%, and 30%), with 96 h of exposure at 0–4 d (early stage), 4–8 d (middle stage), and 8–12 d (late stage). Sub-lethal and lethal effects, including early developmental delays, cardiac edema, tail abnormalities, abnormal pigmentation, and mortality were monitored daily during the 14 d exposure period. At the embryonic stage, significant sub-lethal and lethal effects were observed at pH < 6.30. Hypercapnia can cause long-term and/or delayed developmental embryonic problems, even after transfer back to clean seawater. At fish juvenile and adult stages, significant mortality was observed at pH < 5.70, indicating elevated CO_2 exposure might cause various adverse effects, even during short-term exposure periods. It should be noted the early embryonic stage was found more sensitive to CO_2 exposure than other developmental stages of the fish life cycle. Overall, the present study provided baseline information for potential adverse effects of high CO_2 concentration exposure on fish developmental processes at different life cycle stages in marine ecosystems.

1. Introduction

Carbon dioxide capture and storage (CCS) is an alternative technology used to control increasing anthropogenic carbon dioxide (CO_2) in the atmosphere (Pachauri et al., 2014). This technique captures atmospheric CO_2 compresses, and transports it to subseabed storage sites for (semi)-permanent long-term isolation. However, it is possible leakage from maritime CCS sites can elevate pCO_2 concentrations in the water column or in sediment layers (Carroll et al., 2014; De Vries et al., 2013). Consequently, marine organisms in accidental sites or even remote coastal areas might be at extreme risk for the adverse effects from CO_2 leakage.

The primary environmental impact of CO_2 leakage is reportedly a change in seawater chemistry associated with changing waterborne pH (Blackford et al., 2008). Phelps et al. (2015) suggested the change might be slow or negligible in the open ocean due to carbonate buffering and presumed a short-term event (over the course of a day). However, local acidification under pH as low as 5, might occur given certain environmental conditions (e.g. when seawater circulation is limited), which can lead to possible acute adverse effects on aquatic organisms (Auerbach et al., 1997; Caulfield et al., 1997; Payán et al., 2012). Indeed, several studies reported extreme elevated CO_2 concentrations on various marine animals, including fish, bivalves, and polychaetes (Basallote et al., 2012; Lee et al., 2003, 2016). O_2/CO_2 imbalance caused by a rapid increase of

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hydrogen ions in the organism is primarily associated with these physiological effects. This change in body chemistry can disturb acid-base regulation, blood circulation, respiration, and the nervous system of marine organisms, further leading to long-term impacts, including but not limited to reduced growth rates and reproductive problems (Frommel et al., 2013).

The relationship of fish to other organisms is complex viewed through the marine food web, i.e., decimation of fish populations also impacts the entire marine community consequently, it is vital to elucidate acute or chronic effects of elevated CO_2 concentrations on fish. High CO_2 tolerance of adult fish was extensively examined for decades (Basallote et al., 2012; Mu et al., 2015) and results suggested no measureable effects on mortality, even at pH < 6.0 (Lee et al., 2016). Furthermore, Baumann et al. (2012) did not sufficiently demonstrate fish susceptibility to elevated CO_2 concentrations during early life stages. Finally, studies reported fish have various ion exchangers, were more CO_2-tolerant than marine invertebrates which was inconsistent with research demonstrating fish were the most sensitive to elevated waterborne CO_2 concentrations during early developmental stages (Forsgren et al., 2013; Kikkawa et al., 2003).

Fish embryos and larvae are small and exhibit low locomotive capacity. Therefore, early life stages lack the ability to avoid CO_2 plumes. Mu et al. (2015) suggested this developmental period was more CO_2-sensitive than later developmental stages and also more suitable for study than other organisms; therefore fish represent viable test organisms. Furthermore, in fish early life stages, elevated CO_2 concentrations were shown to effect skeletal calcification due to a drop in carbonate availability (Munday et al., 2011).

Marine medaka (*Oryzias melastigma*) have been increasingly used as a model fish species for marine environmental risk assessments (Bo et al., 2011). Mu et al. (2015) was one of only a few studies that addressed sub-lethal and/or lethal effects of elevated CO_2 concentrations on marine medaka. Previously, we demonstrated marine medaka adults were unusually susceptible to relatively high CO_2 concentrations (Lee et al., 2016). Of note, in our previous study, marine medaka were exposed to very high CO_2 concentrations in a late developmental stage following organogenesis, including heart formation, considered a major developmental step (Lee et al., 2016). Since marine medaka take a considerable time to hatch (10–12 d), it has been difficult to determine the short-term effects of ocean acidification.

The objective of our study was to explore the data gaps in the current toxicological profiles related to marine medaka CO_2 exposure during various developmental stages under varying concentrations of exposed CO_2. Specific aims were to: 1) assess acute lethal and sub-lethal effects of elevated CO_2 on marine medaka at different life stages, i.e. embryo, juvenile, and adult; 2) scrutinize acute lethal effects at different embryonic stages of marine medaka, i.e. early–cleavage, segmentation, primary organogenesis, middle–blood circulation, and/or heart development, and late stages–hatching periods by a 4 d exposure to varying CO_2 concentrations under a short-term CO_2 exposure scenario; 3) build a toxicological database on pH levels (control: just seawater; treatments: 5%, 10%, 20%, and 30% CO_2 exposure) that effect various toxicities in marine fish species by compiling previous and present results, as part of a mini-review.

2. Materials and methods

2.1. Rearing of test organisms

Marine medaka (*O. melastigma*) was reared in the Laboratory of Marine Benthic Ecology at Seoul National University (Seoul, Republic of Korea) for over 12 generations, which were initially donated from NeoEnBiz Inc. (Bucheon, Republic of Korea). Continuously cultivated marine medaka were used for the CO_2 exposure tests. The organisms were placed in a glass tank at 26 °C with a light/dark photoperiod of 14 h/10 h, respectively and 35 psu of salinity. The fish were fed brine shrimp and dry flakes once a day until satiation. Collecting all eggs within 3 h following initiation of spawning and fertilization ensured developmental synchronization of embryos. Viable eggs were selected under a dissecting microscope and used for the series of experiments.

2.2. Experimental settings of CO_2 exposure

CO_2 exposure systems applied in the present study were developed in our previous work (Lee et al., 2016). The CO_2 exposure systems, particularly those targeting waterborne pH maintenance during CO_2 exposure with minimal physical disturbance to the test fish species were performed under strict quality assurance and control guidelines (Fig. S1 of Supplementary Materials (S)). The following two systems were used: i) an air-tight box system (indirect exposure) and ii) a glass chamber system (direct exposure), chosen based on the experimental design (Table 1). Under both systems, the target pH in the water column was successfully maintained with minimal variation (< ± 0.1) during CO_2 gas exposure. Dissolved oxygen (DO) was controlled > 6.0 mg L^{-1} and > 80% (ASTM, 2007, 2008) using O_2-balanced air, ensuring standard water quality throughout the exposure period.

The air-tight box system was designed for small-sized test organisms and used for fish embryo toxicity testing. This system employs indirect CO_2 exposure, which prevents direct bubbling of CO_2 gas in the 6-well plate (Fig. S1a). The CO_2 concentration in the air-tight box system was maintained by a continuous supply of air (control) or CO_2 gas mixture (5%, 10%, 20%, and 30% CO_2 balanced with 20% O_2). Two large beakers filled with seawater were placed inside the air-tight box. One beaker was bubbled with the direct injection of air or CO_2 gas mixture. The other beaker (without bubbling gas) was used to monitor pH and DO in the system. Preliminary tests confirmed pH in the test beaker was stabilized by continuously dissolving the gas, reaching targeted CO_2 concentrations within 48 h.

The glass chamber system was used for toxicity testing of marine medaka at juvenile and adult life stages. This spacious chamber system represents direct exposure conditions, which were more suitable for exposure of large-sized fish and the individuals did not appear effected by the vigorous gas flow (Fig. S1b). In this system, the CO_2 gas mixture (5%, 10%, 20%, and 30% CO_2 balanced with 20% O_2) was directly injected into each test chamber containing 2.5 L of filtered seawater. Of note, the preliminary tests confirmed CO_2 and DO in the glass chamber system (i.e. direct exposure) were saturated faster (< 3 h) than the same gases in the air-tight box system (i.e. indirect exposure). The exposure experiments were initiated with a decreasing gas flow to maintain saturation and to minimize possible physical stress on the fish.

Water quality was monitored daily for pH and DO using a pH meter (Orion Star, Thermo Scientific, Waltham, MA) and a YSI multi-parameter meter (Yellow Springs, OH), respectively. Total alkalinity was measured applying the five-pH point titration method at the same time daily (Moosbrugger et al., 1993). Other parameters (total inorganic carbon content, HCO_3^-, CO_3^{2-}, CO_2, pCO_2, saturation state of calcite, and saturation state of aragonite) were calculated using the CO2SYS program (Pierrot et al., 2006) with the dissociation constant reported by Mehrbach et al. (1973), refit following Dickson and Millero (1987), and KSO_4 as described by Dickson (1990) (data refer to Table S1).

The experimental design might influence results, e.g. tank effect on pH values. Therefore, a two-way analysis of variance (2-way

Table 1
Experimental design showing the three toxicity tests in embryo, juvenile, and adult developmental stages of marine medaka (*Oryzias melastigma*), including detailed descriptions of CO_2 exposures, experimental conditions, and endpoints. CO_2 exposure systems are given in Fig. S1.

Conditions	Embryo toxicity test	Juvenile toxicity test	Adult toxicity test
Test organism			
Species	*Oryzias melastigma*	*Oryzias melastigma*	*Oryzias melastigma*
Life stage	Embryo (> 1.5 mm)	Juvenile (> 11 mm)	Adult (> 40 mm)
CO_2 exposure			
Exposure route	Indirect exposure (seawater)	Direct exposure (seawater)	Direct exposure (seawater)
Exposure duration (h)	96	96	96
Control	Air	Air	Air
% $CO_{2\ gas}$	5, 10, 20, 30	5, 10, 20, 30	5, 10, 20, 30
pH range	5.5–8.2	5.5–8.2	5.5–8.2
Experimental condition			
Water system	Static-renewal (100% every 1 d)	Static-renewal (80% every 2 d)	Static-renewal (80% every 2 d)
Experimental duration (d)	14	4	4
CO_2 exposure at Day	0 (early), 4 (middle), 8 (late)	0	0
Monitoring interval	daily	daily	daily
Temperature (°C)	25	25	25
Salinity (psu)	35	35	35
Water volume	5 mL	2.5 L	2.5 L
Number of organisms	12	10	10
Number of replicates	3	3	3
Endpoints			
Sub-lethal toxicity	Cardiac edema Tail abnormality Abnormal pigmentation	–	–
Lethal toxicity	Mortality	Mortality	Mortality

ANOVA) was applied to test for potential artificial effects on pH levels when the CO_2 gas was injected (Table S2). The following factors were included in the analysis: supplied CO_2 gas concentrations, measured pH values, and the two tank system types (Figs. S1a and S1b). CO_2 gas concentrations significantly contributed to pH values, supporting the delivery of target CO_2 concentrations given to treatments throughout the experimental period (Table S2). Finally, target CO_2 concentrations, namely pH (5%, 10%, 20%, 30% CO_2 on pH 6.3, pH 6.1, pH 5.7, and pH 5.5, respectively), did not significantly differ between the two tank system types among the given treatments, which enabled us to directly compare mortality toxicity data between the embryo test (well plate) and the juvenile and adult fish tests (glass chamber) (Table S2).

2.3. CO_2 exposure of marine medaka

2.3.1. Embryo toxicity test

Twelve marine medaka embryos were randomly placed into 6-well plates containing 5-mL of seawater (control) or acidified seawater (treatments), with 3-replicates per treatment (total n = 36 embryos per CO_2 treatment) and maintained at 26 °C and 35 psu (Table 1 and Fig. S1a). Throughout CO_2 exposure, 100% of seawater was daily replaced with fresh seawater. CO_2 exposure of marine medaka at early embryonic stage was initiated shortly after the fertilization (3–4 h post fertilization; hpf) and terminated following the end of organogenesis at 4 d post fertilization (dpf), just prior to hatching; early embryonic CO_2 exposure treatment lasted 0–4 d. We also conducted CO_2 exposure experiments on marine medaka at middle (4 dpf; CO_2 exposure from 4 to 8 d) and late (8 dpf; CO_2 exposure from 8 to 12 d) embryonic stages, prior to hatching. After CO_2 exposure completion for each embryonic experimental stage, each embryo was transferred to clean seawater until the end of the experiment. Over the experimental period of 14 d, marine medaka mortality was measured daily as an endpoint of lethality and dead embryos were removed. Sub-lethal endpoints, identified under a light microscope (Zeiss Stemi 2000C), were monitored daily and included the following attributes; cardiac edema, tail abnormality, and abnormal pigmentation for all individuals (Fig. S3). First, the deformities were calculated based on the proportions of abnormal embryos and counted as 'deformity' if at least one of the sub-lethal endpoints was observed; namely development delay, pigmentation, tail abnormality, and cardiac edema. Second, after observing a deformity, embryos were continuously monitored without removing (or replacing) from the experimental system. Third, the survival and hatching data simply refers to the all the individuals excluding the dead ones, thus it would be redundant and not necessary to show at this moment. To reflect the variations cross the treatments, the values given as Mean ± SD.

2.3.2. Juvenile and adult toxicity tests

For CO_2 exposure experiments of juvenile and adult marine medaka, 10 individuals were placed into each glass chamber containing 2.5 L of filtered seawater for three control replicates (i.e. ambient seawater, total 30 individuals) and three treatment replicates (i.e. acidified seawater; 5%, 10%, 20%, and 30% CO_2 exposure, total 30 individuals in each of the CO_2 treatments). The test chambers were placed in a temperature-controlled room maintained at 26 °C during the experimental periods (Table 1 and Fig. S1b). For juvenile and adult toxicity tests, only daily mortality was monitored as the CO_2 lethal effect endpoint during the experimental period of 4 d.

2.4. Data analysis and statistics

SPSS 22.0 (IBM, Armonk, NY) was used to perform statistical analyses. Non-parametric statistics were used to investigate the relationships across the measured endpoints in the CO_2 exposure

experiments because the data did not meet the assumptions of normality. Regression statistics (n = 65; each end-point) was used to determine the relationship between mortality rate and other sub-lethal endpoints at each embryonic stage of marine medaka examined (Table 2). Spearman rank correlation coefficient analysis (r, n = 65; each end-point) was applied to examine cross-association of sub-lethal and lethal endpoint effects in each medaka embryo stage (Table S4). Scheffe's one-way ANOVA was employed as follows: 1) to compare elevated CO_2 concentration sensitivity among embryonic, juvenile, and adult stages of marine medaka individuals; 2) to determine lethal effect pH ranges under early-, mid-, and late-stages of marine medaka embryo mortality; 3) to generate the cumulative probability distribution of sub-lethal and lethal effect pH ranges cross various test organisms or developmental stages of marine fish; and 4) to compare the CO_2 sensitivities among the behavioral responses, sub-lethal, and lethal toxicities on various fish species (Tables S5, S6, S8, and S9).

We calculated mean H^+ concentration causing lethal toxicity for medaka samples from selected metadata, including the present datasets (Table 3 and Table S7). $L[H^+]_{50}$ was defined as the H^+ concentration that caused a lethal effect in 50% of the exposed test samples. This parameter was estimated from calculated deformities and/or mortality that caused death in 50% of the exposed population. Probit analysis was used for the calculation and expressed as the LpH_{50} value. Proton (H^+) concentrations were expressed as moles per kilogram H_2O on the NBS scale (Basallote et al., 2012). No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) were expressed in pH units across the sub-lethal endpoints for medaka embryos. A one-way ANOVA was applied for each treatment to determine if significant differences existed between NOEC and LOEC endpoint values. Finally, effective pH values from 85 total metadata sets from CO_2 toxicity tests (Table S7), including three datasets from the present study were compiled and plotted. This analysis served to obtain a cumulative probability distribution of a viable effective pH range across the different medaka test samples at various life stages (Tables S8 and S9 and Fig. 4).

3. Results and discussion

3.1. Sub-lethal effects of elevated CO_2 exposure

The sub-lethal effects of CO_2 exposure on marine medaka embryos showed concentration (or pH) dependent responses over the three treatments of early, middle, and late stage exposure. More importantly, sub-lethal responses exhibited substantial variation in magnitude of response and temporal trends, depending on the time of initial CO_2 exposure. In the early stages of CO_2 exposure, medaka embryo tail abnormalities were significantly higher than mid- and late-stages at pH = 6.3 (0–4 d) (n = 65, $p < 0.05$, F = 23.9) (Fig. 1a). However, during mid- and late-stages of CO_2 exposures, tail abnormalities were not significantly different from other embryonic stages, suggesting medaka embryos were less sensitive to elevated CO_2 after the first 4 d of development (Fig. 1b and c). The presence of embryonic cardiac edema observed at pH < 6.1 in early stage exposure treatments was also higher (up to 97%) than that of the control ($p < 0.05$), comparable to %-tail abnormalities under the corresponding at pH < 6.3 (up to 100%, $p < 0.05$) (Fig. 1a). In the late stage exposure treatments, embryonic cardiac edema showed significant decrease compared with embryos exposed at the early- and mid-developmental stages at pH < 5.7 (n = 116, $p < 0.05$, F = 26) (Fig. 1b–c). Abnormal pigmentation was significantly different between early- and mid-, and late-stages of CO_2 exposures (5–27%; n = 65, $p < 0.05$, F = 11.4) (Fig. 1a), however, mid- and late-stages exhibited no significant differences in exposure treatments (Fig. 1b and c).

Marine medaka deformation rate tended to decrease as CO_2 initial exposure time was delayed, namely in the order of early, middle, and late stage exposure treatments, indicating increasing tolerance in maturing embryos against elevated CO_2 exposure. Therefore, it is noteworthy that medaka embryos generally showed continuing deformations (particularly for cardiac edema), despite the termination of CO_2 exposure for all three-exposure scenarios. Overall, results suggested hypercapnia caused long-term, time-lagged problems to medaka embryos, even after the samples were transferred to clean seawater and CO_2 exposure was terminated.

Furthermore, the degree of embryonic developmental delay seemed to be associated with CO_2 exposure level, i.e. in a concentration-dependent manner. Of note, any delayed time in embryonic development showed an increase over sequential organ formation. For example, in early stage CO_2 exposure, the earliest organ formation, i.e. eyes were delayed up to ~30 h at pH = 5.5 and 5.7, but the final organogenesis, i.e. spleen was delayed up to ~108 h at pH = 5.5 (Fig. 2). It was also noteworthy exposure time (or duration) was one key factor controlling the delayed effect of embryonic development, e.g. delayed effect for middle stage exposure developing organs, such as cerebrum, heart, and spleen was decreased compared to those of early stage exposure organs (Fig. 2). The delayed effect time for late stage exposure treatment was very small, within 4 h at the lowest pH (pH = 5.5).

Overall, our results indicated the elevated CO_2 concentrations adversely effected fish embryos in a time- and concentration-dependent manner relating to the variations in sensitivity and/or tolerance throughout *O. melastigma* embryonic developmental stages. Mu et al. (2015) explained similar observation of adverse effect, linked to apoptosis, by elevated CO_2 concentrations, which

Table 2
Regression statistics for lethal and other sub-lethal endpoints on each developmental stage of marine medaka embryos. Higher beta values in bold indicated stronger statistical significance between mortality rate and other sub-lethal toxicities.

Embryonic stage	Endpoint	Standardized Coefficients (Beta)	r^2	Durbin-Watsoon	SS	DF	*F* value	*P* value
Early	Cardiac edema	**0.887**	0.788	0.730	58764.919	64	233.650	< **0.01**
	Tail abnormality	**0.927**	0.948	0.702	70728.845	64	1148.115	< **0.01**
	Intracranial hemorrhage	**0.869**	0.869	0.657	56319.550	64	193.989	< **0.01**
	Abnormal pigmentation	**0.723**	0.523	0.311	39034.980	64	69.127	< **0.01**
Middle	Cardiac edema	**0.939**	0.883	1.018	71329.531	64	473.819	< **0.01**
	Tail abnormality	**0.886**	0.785	0.804	63406.770	64	229.485	< **0.01**
	Intracranial hemorrhage	**0.965**	0.930	0.731	75193.424	64	842.881	< **0.01**
	Abnormal pigmentation	**0.687**	0.472	0.466	38146.816	64	56.326	< **0.01**
Late	Cardiac edema	**0.851**	0.725	0.939	11250.543	64	165.771	< **0.01**
	Tail abnormality	**0.925**	0.856	1.063	13287.251	64	373.876	< **0.01**
	Intracranial hemorrhage	**0.916**	0.839	1.597	13022.448	64	327.671	< **0.01**
	Abnormal pigmentation	−0.002	<0.01	0.517	0.093	64	0.000	0.985

Table 3
CO_2 exposure concentrations exhibiting sub-lethal and lethal toxicities in marine medaka; toxicities shown in embryo, juvenile, and adult fish developmental stages.

Life stage	End point	NOEC [a]			LOEC [b]			EpH_{50} or LpH_{50} on *O.mela.* [a]		
		O.mela [a]	*O.mela*[b]	*O.lati*[c]	*O.mela*[a]	*O.mela*[b]	*O.lati*[c]	*Early stage*	*Middle stage*	*Late stage*
Embryo	***Sub-lethal***									
	Development delay	8.2	n.a.[c]	8.1	6.3	n.a.	7.1	n.c.[d]	n.c.	n.c.
	Tail abnormality	8.2	n.a.	n.a.	6.3	n.a.	n.a.	6.04 (5.69–6.40)	n.c.	n.c.
	Cardiac edema	8.2	7.6	n.a.	6.3	7.2	n.a.	5.99	6.27 (6.09–6.52)	n.c.
	Pigmentation	6.3	n.a.	n.a.	5.7	n.a.	n.a.	n.c.	n.c.	n.c.
	Deformity	8.2	7.6	8.1	6.3	7.2	7.1	6.36 (6.01–6.76)	n.c.	n.c.
	Lethal–Mortality	8.2	7.2	8.1	6.3	n.a.	7.6	6.42 (6.01–7.19)	7.03 (6.81–7.31)	6.23 (5.83–6.78)
Juvenile	***Lethal***–Mortality	6.1	n.a.	7.1	5.7	n.a.	n.a.	6.00 (5.89–6.05)		
Adult	***Lethal***–Mortality	6.1	n.a.	7.1	5.7	n.a.	n.a.	5.91 (5.57–6.01)		

Data for NOEC, LOEC, and LpH_{50} values obtained from the studies of [a] this study, [b] Mu et al., 2015, and [c] Tseng et al., 2013.
[a] NOEC: no observed effect concentration.
[b] LOEC: lowest observed effect concentration.
[c] n.a. not available.
[d] n.c.: not calculated due to data limitation.

might subsequently delay organ development. However, even if late stage exposure embryos showed minor tolerance to increased CO_2 concentrations, Mu et al. (2015) demonstrated metabolic costs might reduce the energy required for organogenesis or additional post-hatching survival of attenuated embryos, therefore potential toxicities remain concerns. Although late stage fish embryos, including marine medaka appeared tolerant to low-pH conditions, high CO_2 concentrations and/or associated changes in carbonate chemistry might remain critical to fish larval development (Baumann et al., 2012; Mu et al., 2015).

3.2. Lethal effects of elevated CO_2 exposure

In general, increased *O. melastigma* embryo mortality was detected at early- and mid-stage exposure compared with late-stage exposure treatments (Fig. 3). We found it unusual in that the middle stage exposure marine medaka embryos exhibited higher mortality than the early stage CO_2 exposed embryos at $pH < 6.3$ (Table S5 and Fig. 3). The vascular systems of many embryos (early-stage; up to 0–97%, middle-stage; up-to 41–81%) troubled to develop between 2.5 and 8 dpf (when the cardiac system develops). These results strongly supported our hypothesis that elevated CO_2 concentrations impaired cardiac development, which was a significant cause of embryo mortality. Regression statistics indicated the %-cardiac edema and intracranial hemorrhage showed a very similar pattern to embryo mortality in the middle stage exposure treatments (Table 2). However, early stage CO_2 exposed embryo mortality was consistent with tail abnormality, compared with other sub-lethal evidence, such as cardiac edema, intracranial hemorrhage, and abnormal pigmentation. Following completed cardiac developmental status changes in late embryonic stage, results showed significantly reduced cardiac edema and/or mortality sensitivity (cardiac edema; $n = 117$, $p < 0.05$, $F = 2.76$; mortality; $n = 117$, $p = 0.15$, $F = 1.71$) relative to other embryonic stages at $pH > 6.1$, but significant differences were not detected at $pH < 5.7$ (cardiac edema; $n = 78$, $p = 0.82$, $F = 0.20$, mortality; $n = 78$, $p = 0.84$, $F = 0.18$). It should be noted that pH directly affected the embryo (i.e. hypercapnia), rather than lethal effects caused by cardiac edema at $pH < 5.7$. Of note, previous study showed hypercapnia impacted respiration, circulation, and metabolism imbalance in fish (Ishimatsu et al., 2005).

Results further showed late stage exposed embryo mortality was consistent with other sub-lethal effects, but analyses overestimated mortality rate by the low levels and other toxicological endpoints (Fig. 3); and the juvenile marine medaka stage appeared more sensitive to elevated CO_2 conditions than the adult stage ($n = 69$, $p < 0.01$, $F = 6.33$) (Table S6). As expected, adult *O. melastigma* were relatively tolerant to elevated CO_2 concentrations (lower surface area to volume to ratio), compared with other developmental stages. This result indicated marine medaka at the juvenile stage exhibited a relatively low degree of CO_2 tolerance. However, the gap between juvenile and adult stage mortality was not as large as expected and no significant difference was found at $pH \geq 6.1$.

Grosell et al. (2001) demonstrated more than 90% of all acid-base regulation in fish occurred via ion transport processes across the branchial epithelium and the intestine and kidney were also involved in ion regulation. In addition, Pörtner et al. (2004) found marine fish possessed various ion exchanger isoforms for osmoregulation; therefore, marine fish are well adapted to regulate acid-base balance. It was proposed acidified waters have negligible effects on fish, as fish calcify internal skeletal elements rather than external elements (i.e. shell) (Baumann et al., 2012). Alternatively, fish might avoid the effects of elevated CO_2 exposure through migratory avoidance behavior (Briffa et al., 2012; Nilsson et al., 2012). Mu et al. (2015) showed the otolith area of larval marine medaka exposed to intermediate CO_2 concentrations (pH 7.6) was smaller than control samples. Any alteration in otolith size, shape, or symmetry of juvenile or adult fish could result in serious problems (i.e. as balance, movement, directional indication of gravity, and sound detection) for survival and/or individual breeding performance (Munday et al., 2011).

3.3. EpH_{50} and LpH_{50} values

The EpH_{50} (or LpH_{50}) values for sub-lethal and lethal toxicities caused by CO_2 exposure on test organisms were calculated using the data generated from the present study. NOEC and LOEC values were collected from the toxicological metadata available from the previous literature as well as the present study (Table 3). Results indicated a mean $pH = 6.04$ (EpH_{50}) caused tail abnormalities in 50% of the early embryonic stage marine medaka samples. Cardiac edema EpH_{50} values at early- and mid-embryonic stages were 5.99 and 6.27, respectively, which indicated embryos were more sensitive to cardiac edema at CO_2 mid-compared to early-stage endpoints (Fig. 3a–b). LpH_{50} values at early-, mid-, and late-embryonic stages were 6.42, 7.03, and 6.23, respectively, with mean LpH_{50} of 6.56. The lethal toxicity of adult marine medaka was apparently smaller ($LpH_{50} = 5.91$) compared to those of juveniles ($LpH_{50} = 6.00$) and embryos ($LpH_{50} = 6.42$; early stage exposure). Despite the uncertainty factors (e.g. experimental conditions; CO_2 concentrations, exposure duration, and species, among others)

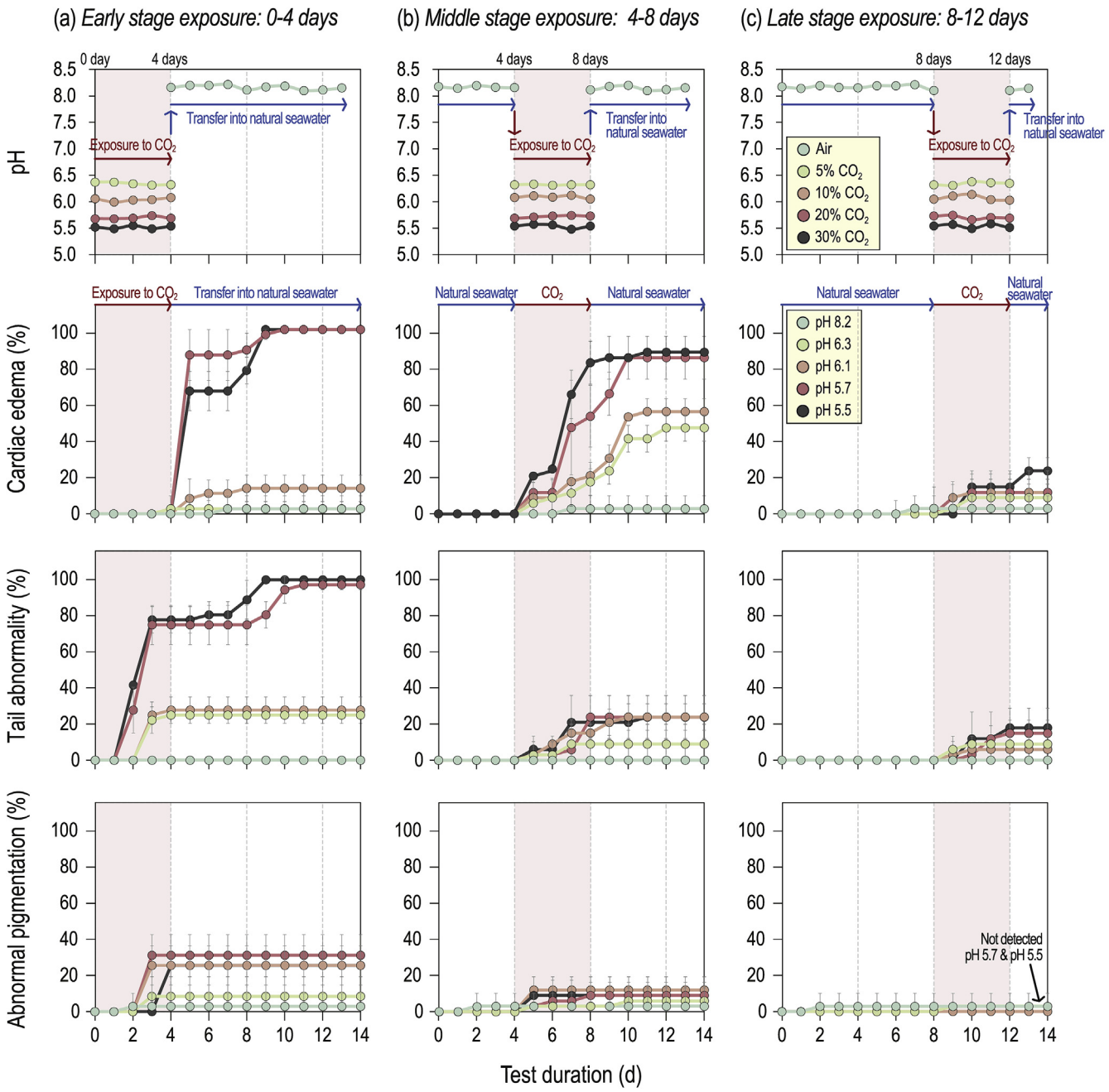

Fig. 1. Sub-lethal toxicities for varying CO_2 exposure concentrations (control air, 5%, 10%, 20%, and 30% CO_2; 5-CO_2 levels x 3-developmental stages x 3-replicates) on marine medaka given at early- (CO_2 exposed at 0–4 d), mid- (4–8 d), and late- (8–12 d) developmental stages (n = 12 individuals per replicate; total n = 36 embryos per CO_2 treatment). Following termination of CO_2 exposure treatments, medaka embryos were transferred to clean seawater for the remaining 14 d experimental period. The pH values were measured in each CO_2 exposure treatment over the entire experimental period of 14 d. Sub-lethal endpoints included tail abnormality, cardiac edema, and abnormal pigmentation. The detailed experimental conditions are provided in Table 1.

influencing toxicological effects, approximately 63% of juvenile marine medaka survived at pH 7.2 for 21 d, which back-supported the previous findings of Mu et al. (2015) (Table 3). In general, previous studies reported fish at early life developmental stages were more sensitive to toxicants than those at the adult stage (Chambers et al., 2014; Hutchinson et al., 1998). Our results clearly indicated toxicological sensitivity to elevated CO_2 concentrations varied among *O. melastigma* embryonic developmental stages. Embryos at the middle developmental stage showed a significantly greater LpH_{50} (7.03) value than samples at early (6.42) and late (6.23) embryonic stages (Table 3). This result suggested the middle embryonic stage (4–8 d) was the most sensitive to elevated CO_2 exposure; we therefore hypothesized this a critical period in fish development. Furthermore, the present data provided evidence the mortality risk due to CO_2 exposure was directly influenced by the developmental period of some important organs, such as the heart.

3.4. Toxicological effects of CO_2 on various marine fishes: A mini review

We collected toxicological metadata to examine sub-lethal and lethal effects of CO_2 exposure on various marine fish species and

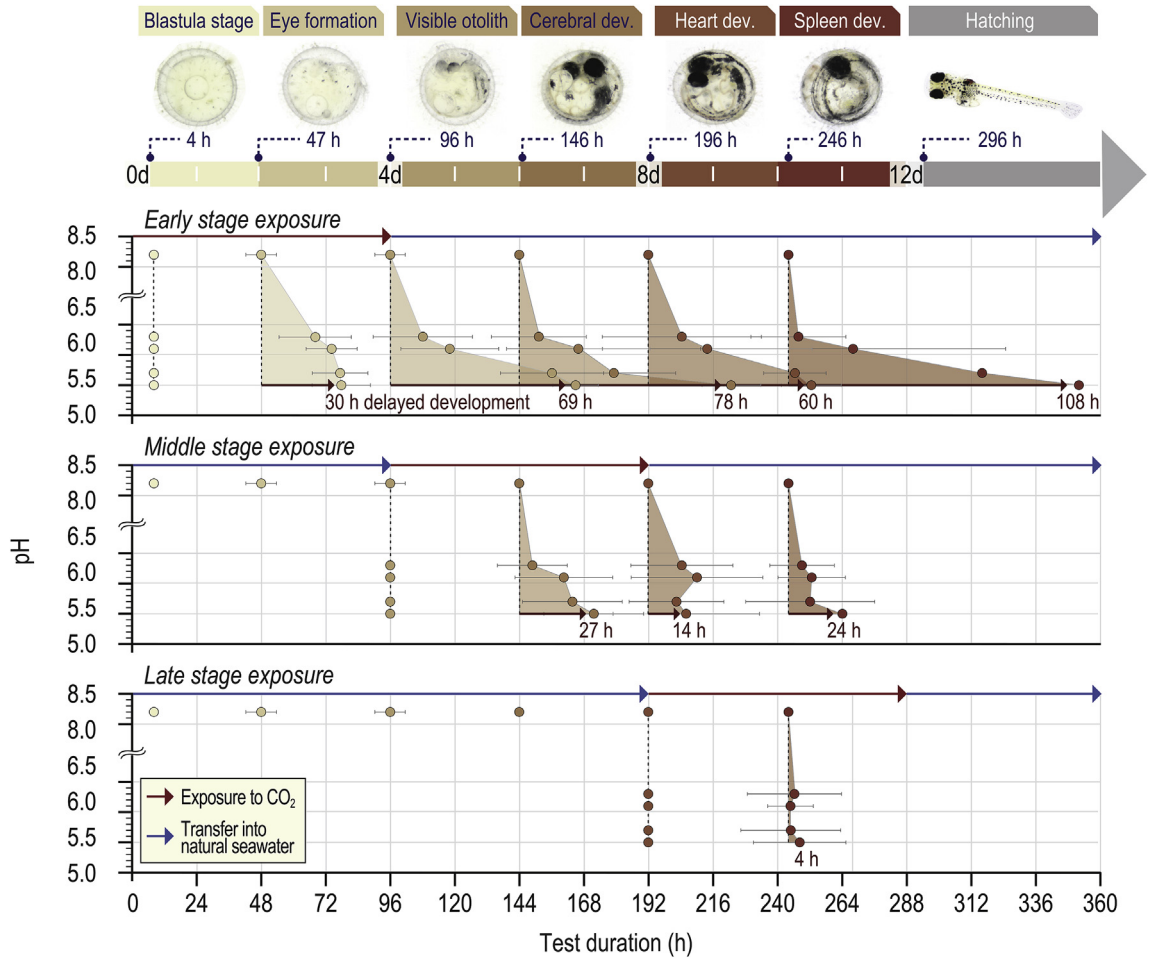

Fig. 2. Comparisons of developmental toxicities of marine medaka embryos in response to varing CO_2 exposure senarios; values/units given as pH. Developmental period of 14 d (360 h) was divided into 7-major stages from blastula formation to final hatching. Data were collected for the three following developmental stages for all the test individuals during the CO_2 exposure, viz., early 0–4 d; middle 4–8 d; and late 8–12 d. The time of developmental delay (h) is denoted (red) in each treatment. The detailed description of the daily developmental stages from spine formation (day 1) to 1st fry stage (day 12) is shown for 12 days in Fig. S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

analyzed the effective pH values. Metadata and data generated from the present study were compiled and generally covered well-established test species of marine fish with various sub-lethal effects at different developmental stages, including embryo, larvae, juvenile, and adult fish (Table S7). A previous study reported chronic deformity in juvenile marine medaka due to elevated CO_2 concentrations at pH = 7.2 (Mu et al., 2015). But hatching, heart rates, and embryonic development were not significantly different from those of the control group. In comparison with the present study, Mu et al. (2015) reported the effective pH values for acute sub-lethal and lethal effects on marine medaka embryos were pH < 6.3. We found embryonic development was delayed at pH < 6.3 and a time-cumulative effect was found from pH 5.5 to 6.3 (Table S7 and Fig. 2).

In addition, embryonic, larval, and juvenile stages of various fish taxa were more sensitive to pH changes caused by elevated CO_2 concentrations compared with adult fish, but significant sensitivity to pH changes were only detected in the juvenile stage group (Table S8). Acute adult fish mortality was observed in gilthead seabream (*Sparus aurata*), marine medaka (*O. melastigma*), and yellowtail (*Seriola quinqueradiata*) at pH values ranging from 5.7 to 6.3 (Jutfelt et al., 2013; Lee et al., 2003; this study). Furthermore, metadata showed fish eggs, larvae, and juveniles (corbia, *Rachycentron canadum*) exhibited higher sensitivity to elevated CO_2 concentrations compared with adult fish, with body length and shape, enzyme activity, blood acid-base status, and swimming ability effected by pH of 7.2–7.8 (Bignami et al., 2013). In addition, Tseng et al. (2013) reported acid-base regulatory gene expression varied between medaka embryos and larvae (Tseng et al., 2013). Increased CO_2 induced mRNA expression of the HCO_3^- regulation gene (i.e. AE1, which was associated with bicarbonate absorption) occurred during hatching rather than the early embryonic stage. Therefore, results indicated developmental delays during the embryonic stage were potentially associated with the absence of the AE1 regulation gene during the late embryonic stage, close to the hatching period.

Previous studies showed developmental stages of marine fish were generally more sensitive to the sub-lethal effects (mean pH 7.3–7.6) of elevated CO_2 concentrations than lethal effects (mean pH 6.1–7.4) (Table S9 and Fig. 4a) (Allan et al., 2013, 2014; Basallote et al., 2012; Castro et al., 2017; Chambers et al., 2014; Duteil et al., 2016; Franke and Clemmesen, 2011; Frommel et al., 2016; Hamilton et al., 2017; Heuer et al., 2016; Heuer and Grosell, 2016; Hurst et al., 2013, 2017; Jutfelt et al., 2013; Kim et al., 2015; Lee et al., 2003; Lopes et al., 2016; Maneja et al., 2014; Michaelidis et al., 2007; Milazzo et al., 2016; Neves and Brown, 2015; Ou et al.,

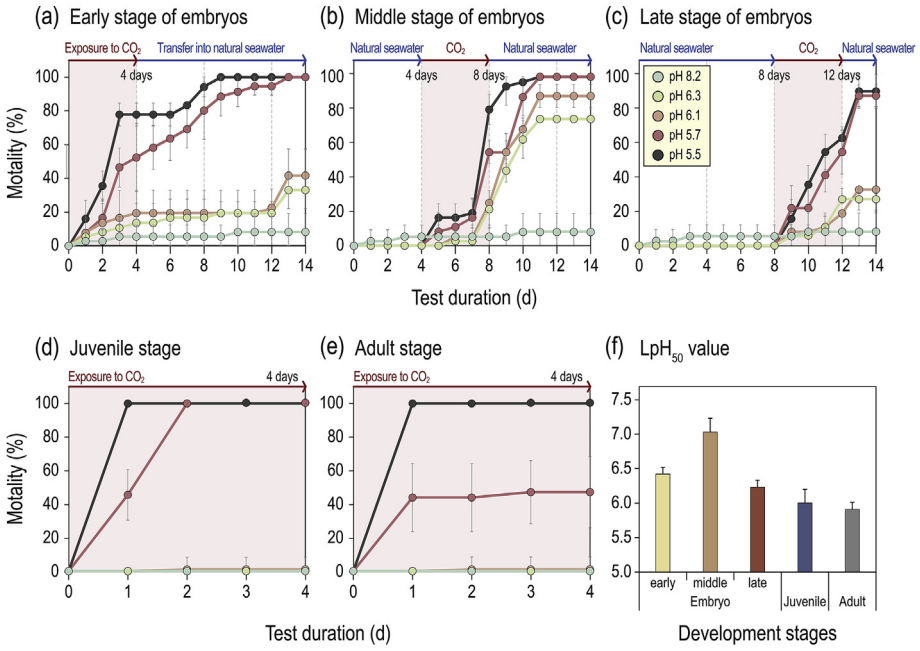

Fig. 3. Lethal toxicity (%-mortality) data from varying CO_2 exposure treatments (pH 5.5–8.2) on marine medaka was obtained as the following developmental stages; (a) early embryonic (n = 36 per CO_2 treatment), (b) middle embryonic (n = 36 per CO_2 treatment), (c) late embryonic (n = 36 per CO_2 treatment), (d) juvenile (n = 30 per CO_2 treatment), and (e) adult (n = 30 per CO_2 treatment). Mean values of (f) LpH_{50} for each stage of marine medaka was summarized in a separate panel (refer to Table 1 for the detailed experimental conditions).

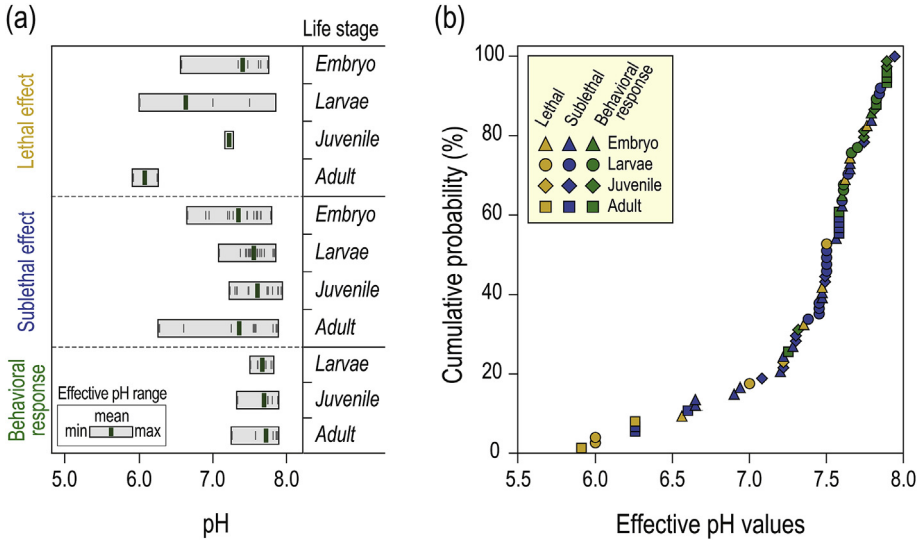

Fig. 4. Range of pH values resulting in lethal, sub-lethal, and behavioral effects in various marine fish species associated with elevated CO_2 concentration exposure; metadata collected from published and the present study. The effects given were from embryo, larvae, juvenile, and adult fish. The original compiled metadata, including scientific names of marine fish species, experimental conditions, and pH ranges showing lethal and sub-lethal effects are given in Table S7.

2015; Pimentel et al., 2014; Pistevos et al., 2017; Schade et al., 2014; Schunter et al., 2016; Silva et al., 2016; Stiasny et al., 2016; Tirsgaard et al., 2015; Tix et al., 2017). In addition, the pH values exhibiting the greatest deleterious effects on marine fish species varied depending on developmental stage, particularly for lethal toxicity. However, developmental stages were not associated with behavioral response sub-lethal effects, which instead was species and exposure duration specific (Allan et al., 2013, 2014; Bignami et al., 2013, 2014; Castro et al., 2017; Hamilton et al., 2017; Heuer et al., 2016; Jutfelt et al., 2013; Lopes et al., 2016; Pimentel et al., 2014; Pistevos et al., 2017; Tix et al., 2017). Marine fish species larval, juvenile, and adult stage behavioral responses were not notably different among stages, with a narrow range of pH values detected (7.6–7.9) (Table S9 and Fig. 4a). Species swimming behavior in predator–prey relationships (i.e., prey reaction distances and predator success rates) under elevated CO_2 exposure conditions (pH 7.8–7.9) of larvae (*Coryphaena hippurus*), juvenile (*Pomacentrus amboinensis*), and adult piscivorous fish (*Pseudochromis fuscus*) was reduced compared to controls (Allan et al., 2013; Bignami et al., 2014). However, a slight CO_2 concentration increase (0.2 pH unit) might have caused a behavioral response delay in all developmental stages. Overall, behavioral responses and sub-lethal toxicities were the least sensitive effects against CO_2 exposure compared to lethal toxicities (one-way ANOVA; $p < 0.01$; Fig. 4b and Table S9).

Marine habitats occupied by fish species, particularly in early life developmental stages, varied widely and might play a specific role in each species biological sensitivities to CO_2. It now requires further attention that species distributed in estuarine areas (marine medaka) and inner shelves (summer flounder), areas with relatively high ambient CO_2 concentrations exhibited different sensitivities to experimentally elevated CO_2 during early life stages. Furthermore, results indicated embryonic developmental stages of marine medaka were relatively tolerant to lethal elevated CO_2 concentration exposure effects, but sensitive to sub-lethal effects.

Ultimately, it was difficult to ascertain the total impacts of elevated CO_2 exposure on marine fish species due to various uncertainty factors (e.g. species, tested pH ranges, and exposure duration, among other variables). However, collection and evaluation of toxicological data was necessary as baseline data to predict biological impacts of marine species against increased CO_2 for the future risk assessment and management. We highly encourage further study addressing predator–prey interactions and/or trophic pathway relationships associated with the effects of CO_2 exposure to characterize the natural adverse effects of possible CO_2 leakage in marine ecosystems.

4. Conclusions

Ocean acidification associated with CO_2 leakage at CCS sites could potentially raise lethal and/or sub-lethal toxicities at acute or chronic levels on various marine fish species. Our results showed exposure of elevated CO_2 concentrations caused abnormal embryo development and therefore negative effects might occur on marine fish populations. Phenotypic effects on subsequent generations, i.e. altered structure and/or function should be of particular concern. Our results generally supported elevated CO_2 exposure caused development-specific toxicological effects on marine fish, with adverse effects strongly dependent on the following: 1) waterborne chemistry based on H^+ concentration, namely pH; 2) initial exposure time and exposure duration; 3) marine fish species developmental stage; and 4) CO_2 tolerance of fish species in varied marine habitats. The results of the present study provide valuable contributions to the existing database of the toxicological effects of elevated CO_2 concentrations on marine species, most notably fish taxa. Additional study is recommended to examine CO_2 toxicities in various fish species and marine invertebrates for use in ecological risk assessment and ecosystem management for existing and future implementation of CCS sites.

Acknowledgments

This work was supported by projects entitled “Marine ecosystem-based analysis and decision-making support system development for marine spatial planning (20170325)” and “Integrated management of marine environment and ecosystems around Saemangeum (20140257)” funded by the Ministry of Oceans and Fisheries of Korea (MOF) granted to JSK. This work was also supported by the project entitled “Quantification of self-purification capacity of coastal wetlands through understanding of chemodynamics of land-driven coastal pollutants (NRF-2017R1E1A1A01075067)” funded by the Ministry of Science and ICT.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.envpol.2018.05.091.

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<Supplementary Materials>

Sub-lethal and lethal toxicities of elevated CO_2 on embryonic, juvenile, and adult stages of the marine medaka *Oryzias melastigma*

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Table of Contents

Supplementary Tables

Supplementary Figures

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Table S1. Summary of carbonate system speciation in the test seawater.

Temp (˚C)	Sal (psu)	pH (NBS scale)	A_T	TC	HCO_3^-	CO_3^{2-}	CO_2	pCO_2 (µatm)	Ω_{Cal}	Ω_{Arag}
			(µmol kg^{-1} SW)							
Embryo										
25(±0.1)	35(±1)	8.17(±0.04)	2321.22	1933.7	1654.7	271.23	7.69	271.7	6.53	4.30
25(±0.1)	35(±1)	6.36(±0.03)		3005.6	2307.4	5.86	692.38	24464.6	0.14	0.09
25(±0.1)	35(±1)	6.07(±0.04)		3672.1	2314.7	3.01	1354.35	47854.6	0.07	0.05
25(±0.1)	35(±1)	5.70(±0.03)		5503.7	2320.1	1.29	3182.28	112443.0	0.03	0.02
25(±0.1)	35(±1)	5.53(±0.03)		7034.0	2322.1	0.87	4710.99	166458.2	0.02	0.01
Juvenile										
25(±0.1)	35(±1)	8.18(±0.04)	2233.48	1849.8	1578.0	264.67	7.17	253.2	6.37	4.20
25(±0.1)	35(±1)	6.29(±0.01)		3010.5	2222.2	4.80	783.45	27682.5	0.12	0.08
25(±0.1)	35(±1)	6.05(±0.04)		3595.1	2227.6	2.77	1364.78	48223.2	0.07	0.04
25(±0.1)	35(±1)	5.69(±0.04)		5367.4	2232.6	1.21	3133.56	110721.3	0.03	0.02
25(±0.1)	35(±1)	5.55(±0.01)		6563.7	2234.2	0.88	4328.64	152948.4	0.02	0.01
Adults										
25(±0.1)	35(±1)	8.25(±0.06)	2276.7	1839.1	1531.4	301.77	5.92	209.2	7.26	4.79
25(±0.1)	35(±1)	6.31(±0.09)		3032.3	2264.7	5.12	7.62	22908.3	0.12	0.08
25(±0.1)	35(±1)	6.11(±0.04)		3483.9	2269.6	3.24	1211.3	42792.3	0.08	0.05
25(±0.1)	35(±1)	5.71(±0.03)		5326.9	2275.5	1.29	3050.09	107772.1	0.03	0.02
25(±0.1)	35(±1)	5.56(±0.01)		6589.9	2277.3	0.92	4311.67	152349.0	0.02	0.01

A_T: Total alkalinity; TC: total inorganic carbon content; Ω_{Cal}: saturation state of calcite; Ω_{Arag}: saturation state of aragonite.

Table S2. Two-way ANOVA of pH values in the treatment versus experimental setup.

Variable	Degrees of freedom	Sum of squares (% of total)	*F* value	Pr(>*F*)
CO_2 (%)	1	63.54	189.773	<0.05
Tank *Tank 1; embryo* *Tank 2; juvenile* *Tank 3; adult*	2	0.00	0.003	0.997
Residuals	105	35.15		

Table S3. A result of Shapiro-Wilk normality test for lethal and sub-lethal end-points on each stage of marine medaka embryos.

End-point	Statistic	Degrees of freedom	*P* value
Mortality	.706	195	< 0.01
Cardiac edema	.597	195	< 0.01
Tail abnormality	.615	195	< 0.01
Abnormal pigmentation	.678	195	< 0.01

Table S4. Spearman rank correlation results between mortality rate and other end-points on each stage of marine medaka embryos. Values in bold (*r*) indicate correlation significance at the $p < 0.0l$ level (matched with ++).

End point		Early				Middle				Late			
		Mortality	Cardiac edema	Tail abn.	Abnormal pig.	Mortality	Cardiac edema	Tail abn.	Abnormal pig.	Mortality	Cardiac edema	Tail abn.	Abnormal pig.
Early	Mortality		**.734**	**.962**	**.786**								
	Cardiac edema	++		**.845**	**.743**								
	Tail abnormality	++	++		**.798**								
	Abnormal pigmentation	++	++	++									
Middle	Mortality						**.914**	**.843**	**.826**				
	Cardiac edema					++		**.946**	**.864**				
	Tail abnormality					++	++		**.861**				
	Abnormal pigmentation					++	++	++					
Late	Mortality										**.877**	**.765**	**.447**
	Cardiac edema									++		**.819**	.271
	Tail abnormality									++	++		.037
	Abnormal pigmentation									++	+		

++ Significantly correlated at the p < 0.01 level (2-tailed).
+ Significantly correlated at the p < 0.05 level (2-tailed).

Table S5. Multiple comparisons (Scheffe's test for one-way ANOVA) results for effective pH range under early, middle, and late stage of marine medaka embryo's mortality.

	Scheffe		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
	Group I	Group J				Lower Bounce	Upper Bounce
Mortality (pH 8.2)	Early	Middle	0.00000	0.52535	1.000	-1.3030	1.3030
		Late	0.00000	0.52535	1.000	-1.3030	1.3030
	Middle	Late	0.00000	0.52535	1.000	-1.3030	1.3030
Mortality (pH 6.3)	Early	Middle	-8.12308	4.14452	0.151	-18.4026	2.1564
		Late	10.03077	4.14452	0.057	-0.2487	20.3103
	Middle	Late	18.15385	4.14452	< 0.01	7.8743	28.4333
Mortality (pH 6.1)	Early	Middle	-9.4384	4.71514	0.140	-21.1332	2.2563
		Late	13.0154	4.71514	< 0.05	1.3206	24.7102
	Middle	Late	22.4538	4.71514	< 0.01	10.7591	34.1486
Mortality (pH 5.7)	Early	Middle	25.4154	7.09293	< 0.01	7.8231	43.0077
		Late	48.0692	7.09293	< 0.01	30.4769	65.6616
	Middle	Late	22.6538	7.09293	< 0.01	5.0615	40.2462
Mortality (pH 5.5)	Early	Middle	32.0538	7.74851	< 0.01	12.8355	51.2722
		Late	59.1846	7.74851	< 0.01	39.9663	78.4030
	Middle	Late	27.1308	7.74851	< 0.01	7.9124	46.3491

Table S6. Scheffe's test for one-way ANOVA results for mortality rate among the embryonic stage, juvenile, and adult stage on marine medaka.

Variable	Degrees of freedom	Sum of squares	*F* value	*P* value
Embryonic stage *1. embryo (n=39)* *2. juvenile (n=15)* *3. adult (n=15)*	2	9590.280	6.329	< 0.01
Residuals	66	1515.297		
Total	68			

Table S7. Acute and chronic toxicities on fish at various life stages of development, effective pH given at embryo, larvae, juvenile, and adult stages of fish.

Target organism	**Experimental condition**			**Lethal & sub-lethal effect**	**Effective pH**	**Reference**
Life stage/species	**Duration (d)**		**pH range**	**Endpoints**	**range**	
Embryo						
Coryphaena hippurus	7	Acute	7.38–8.0	Hatching rate	7.62	Bignami et al., 2014
Oryzias melastigma	20	Chronic	7.2–8.2	Deformity	7.2	Mu et al., 2015
Oryzias melastigma	4	Acute	5.6–8.3	Mortality (LpH_{50})	6.56	**This study**
Embryo to larvae						
Rachycentron canadum	21	Chronic	7.04–8.13	Swimming ability	7.79	Bignami et al., 2013
				Otolith deformation	7.79	
				Body length & shape	7.22	
Gadus morhua	22–25	Chronic	7.61–8.17	Mortality	7.76	Stiasny et al., 2016
Gobiusculus flavescens	7	Acute	7.6–8.1	Abnormal embryos	7.6	Forsgren et al., 2013
	9	Acute	7.6–8.1	Larval phototaxis	7.6	
Lepidopsetta polyxystra	87	Chronic	7.6–8.0	Body length & weight	7.6	Hurst et al., 2017
Thunnus albacares	7–9	Acute	6.9–8.21	Tissue damage	7.56	Frommel et al., 2016
				Survival	7.35	
				Length	6.90	
Paralichthys dentatus	28	Chronic	7.06–7.81	Survival	7.47	Chambers et al., 2014
				Size and shape	7.47	
	7	Acute	7.06–7.81	Tissue damage	7.47	
Clupea harengus	8	Acute	7.05–8.08	RNA/DNA ratio	7.28	Franke and Clemmesen., 2011
Oncorhynchus gorbuscha	140	Chronic	6.65–8.1	Body length	6.94	Ou et al., 2015
				Production efficiency	6.65	
				Wet/dry mass	6.65	
Theragra chalcogramma	33–38	Chronic	7.4–8.16	Hatch time	n.s	Hurst et al., 2013
				Survival	n.s	
				Growth rate	n.s	
Embryo to juvenile						
Gasterosteus aculeatus	90	Chronic	7.65–8.03	Survival	7.65	Schade et al., 2014
				Growth	7.65	
				Otolith size	7.65	
Dicentrarchus labrax	59–68	Chronic	7.82–8.03	Fish speed	n.s	Duteil et al., 2016
				Individual movements	n.s	

				Social dynamics	n.s	
Larvae						
Atherina presbyter	7–15	Acute	7.64–8.03	Oxidative stress	7.85	Silva et al., 2016
				Energy metabolism	7.64	
				Critical swimming speed	n.s	
Paralichthys olivaceus	> 28	Chronic	7.73–8.05	Body length & weight	7.84	Kim et al., 2015
Coryphaena hippurus	20	Chronic	7.56–8.0	Swimming activity	7.82	Bignami et al., 2014
Lates calcarifer	7	Acute	7.70–8.13	Behavioral choice	7.70	Pistevos et al., 2017
Pomatoschistus pictus	3–4 min	Acute	7.66–8.06	Auditory response	7.66	Castro et al., 2017
Atherina presbyter	7	Acute	7.61–8.10	Behavioral lateralization	7.61	Lopes et al., 2016
	21	Chronic	7.61–8.10	Behavioral lateralization	7.61	
Solea senegalensis	30	Chronic	7.50–8.03	Hatching success	7.5	Pimentel et al., 2014
				Survival	7.5	
				Growth rate	7.5	
				Skeletal deformities	7.5	
				Otolith morphometrics	7.5	
				Oxygen consumption	7.5	
Clupea harengus	39	Chronic	7.07–8.08	Growth/development	7.45	Maneja et al., 2014
				Tissue damage	7.45	
Gadus morhua	46	Chronic	7.08–8.08	Growth	7.45	Frommel et al., 2013
				Tissue damage	7.08	
Coryphaena hippurus	21	Chronic	7.38–8.0	Otolith size	7.38	Bignami et al., 2014
Sparus aurata	3	Acute	5.5–8.0	Sediment elutriate mortality	7.0	Basallote et al., 2012
				Seawater mortality	6.0	
Juvenile						
Acanthochromis polyacanthus	150	Chronic	7.94–8.15	Transcriptomics (parents-offspring)	7.94	Schunter et al., 2016
Pomacentrus amboinensis	4	Acute	7.89–8.15	Prey reaction distance	7.89	Allan et al., 2013
				Apparent looming threshold	7.89	
Amphirion melanopus	10–11	Acute	7.81–8.15	Escape performance	7.81	Allan et al., 2014
Sebastes caurinus	70	Chronic	7.32–7.87	Behavioral lateralization	7.74	Hamilton., 2017
	147			Transcriptomics	7.49	
	98–119			Aerobic scope	7.49	
	35–56			Critical swimming speed	7.32	
Sebastes mystinus	70	Chronic	7.32–7.87	Behavioral lateralization	7.74	
	147			Transcriptomics	7.74	

	112–133			Aerobic scope	n.s	
	49–63			Critical swimming speed	n.s	
Sparus aurata	10	Acute	7.3–8.05	Blood acid–base status	7.3	Michaelidis et al., 2007
				Enzyme activity	7.3	
Gadus morhua	30	Chronic	7.03–7.44	Mortality	7.22	Neves et al., 2015
				Body length & weight	7.22	
Oryzias melastigma	4	Acute	5.6–8.3	Mortality (LpH_{50})	6.0	**This study**
Adult						
Pseudochromis fuscus	4	Acute	7.89–8.15	Predator success	7.89	Allan et al., 2013
				Predation rate	7.89	
				Predator attack	7.89	
Symphodus ocellatus	> 4	Chronic	7.82–8.18	Mating behavior	7.82	Milazzo et al., 2016
Gasterosteus aculeatus	>40	Chronic	7.65-8.08	Behavioral response	7.65	Jutfelt et al., 2013
				Mortality	n.s	
Acanthochromis polyacanthus	4	Acute	7.58–8.15	Behavioral response	7.58	Heuer et al., 2016
				Brain HCO_3^-/pH	7.58	
				Plasma HCO_3^-	7.58	
Opsanus beta	14–28	Chronic	7.58–8.15	Oxygen consumption	7.58	Heuer and Grosell., 2016
				HCO_3^- secretion	7.58	
Pimephales promelas	4–12	Acute	7.25–8.20	Behavioral impairments	7.25	Tix et al., 2017
Gadus morhua	3	Acute	6.6–7.7	Oxygen consumption	6.6	Tirsgaard et al., 2015
Seriola quinqueradiata	3	Acute	6.26–8.25	Mortality	6.26	Lee et al., 2003
				Heart capacity	6.26	
				Blood pH	6.26	
Oryzias melastigma	4	Acute	5.6–8.3	Mortality (LpH_{50})	5.91	**This study**

n.s; not significant.

Table S8. Multiple comparisons (Scheffe's test for one-way ANOVA; n=69, $p < 0.05$, f=3.89,) among results for effective pH range under adult stage and other stage (embryo, larvae, and juvenile) on various fishes.

Scheffe		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
Group I	Group J				Lower Bounce	Upper Bounce
Adult	Embryo	-0.42552	0.17760	0.136	-0.9353	0.0842
	Larvae	-0.45204	0.17760	0.101	-0.9618	0.0577
	Juvenile	-0.66438	0.19722	<0.05	-1.2304	-0.0983
Embryo	Larvae	-0.02652	0.13826	0.998	-0.4234	0.3703

Table S9. Multiple comparisons (Scheffe's test for one-way ANOVA; n=92, $p < 0.01$, f=13.13) among results for effective pH range under behavioral response group and other response groups (sub-lethal and lethal toxicity) on various fishes.

Scheffe		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
Group I	Group J				Lower Bounce	Upper Bounce
Behavioral	Sub-lethal	0.22230	0.10800	0.126	-0.0465	0.4911
	Lethal	0.74648	0.19935	<0.01	0.3782	1.1148
Sub-lethal	Lethal	0.52417	0.12558	<0.01	-0.4911	0.0465

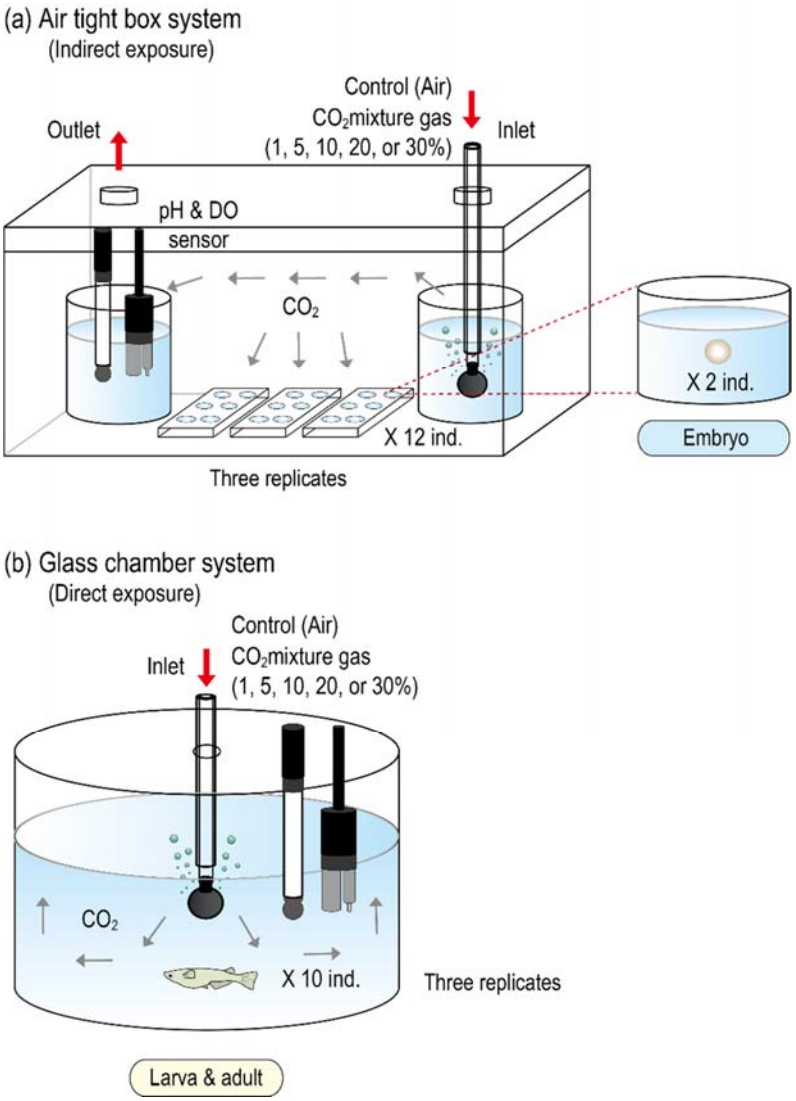

Fig. S1. Schematics design of the CO_2 exposure systems. (A) Air tight box system (indirect exposure) for embryo experiment, (B) Glass chamber system (direct exposure) for juvenile and adult experiment. Detailed experimental condition also given in Table 1.

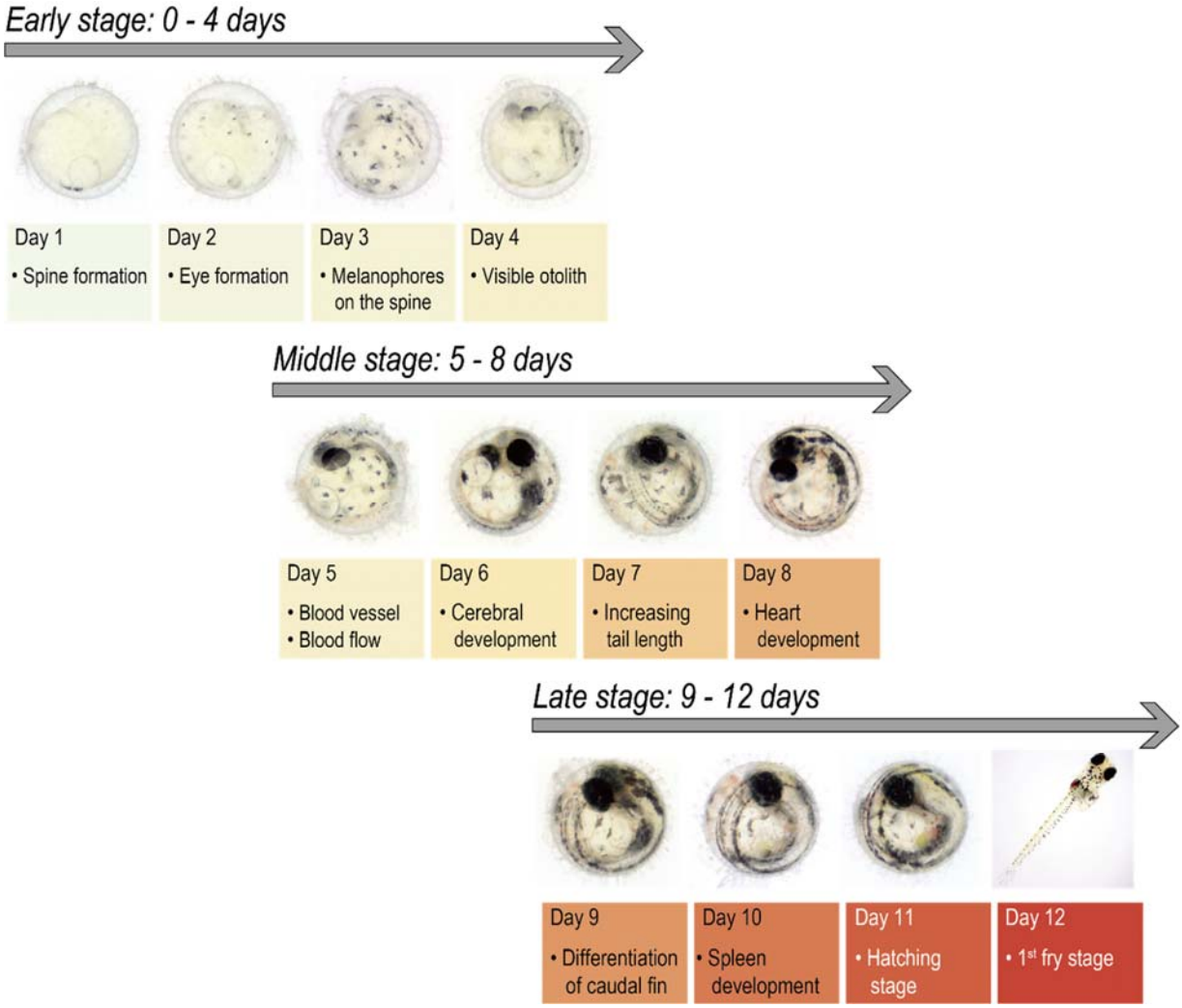

Fig. S2. Embryonic development stage of marine medaka.

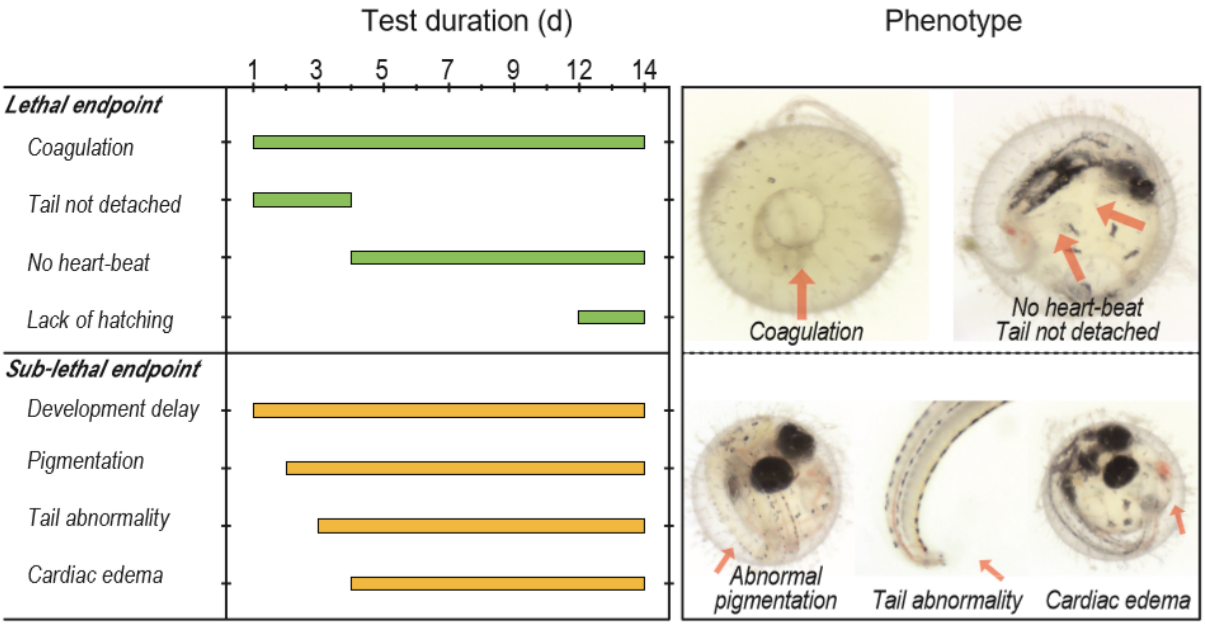

Fig. S3. Typical lethal and sub-lethal endpoints detected in marine medaka embryos exposed at varying concentration of CO_2. Arrows indicate lethal and sub-lethal endpoints.