Acute CO_2 Tolerance During the Early Developmental Stages of Four Marine Teleosts

T. Kikkawa,[1,2] A. Ishimatsu,[2] J. Kita[1]

[1]Central Laboratory, Marine Ecology Research Institute, Onjuku, Chiba 299-5105, Japan

[2]Marine Research Institute, Nagasaki University, Taira, Nagasaki 851-2213, Japan

Received 7 April 2003; revised 18 July 2003; accepted 23 July 2003

ABSTRACT: Ocean sequestration of CO_2 is proposed as a possible measure to mitigate climate changes caused by increasing atmospheric concentrations of the gas, but its impact on the marine ecosystem is unknown. We investigated the acute lethal effect of CO_2 during the early developmental stages of four marine teleosts: red sea bream (*Pagrus major*), Japanese whiting (*Sillago japonica*), Japanese flounder (*Paralichthys olivaceus*), and eastern little tuna (*Euthynnus affinis*). The percentages of larvae that hatched and survived were not affected by exposure to water with a PCO_2 of 1.0 kPa (= 7.5 mmHg) within 24 h. Median lethal PCO_2 values for a 360-min exposure were 1.4 kPa (cleavage), 5.1 kPa (embryo), 7.3 kPa (preflexion), 4.2 kPa (flexion), 4.6 kPa (postflexion), and 2.5 kPa (juvenile) for red sea bream; 2.4 kPa (cleavage), 4.9 kPa (embryo), 5.9 kPa (preflexion), 6.1 kPa (flexion), 4.1 kPa (postflexion), and 2.7 kPa (juvenile) for Japanese whiting; 2.8 kPa (cleavage) and $>$ 7.0 kPa (young) for Japanese flounder; and 11.8 kPa (cleavage) for eastern little tuna. Red sea bream and Japanese whiting of all ontogenetic stages had similar susceptibilities to CO_2: the most susceptible stages were cleavage and juvenile, whereas the most tolerant stages were preflexion and flexion. Environ Toxicol 18: 375–382, 2003.
Keywords: CO_2 ocean sequestration; carbon dioxide; marine fish; CO_2 toxicity; early development

INTRODUCTION

As atmospheric CO_2 concentration increases, CO_2 will diffuse into the hydrosphere. The partial pressure of dissolved CO_2 will increase and thereby lower the pH of seawater. As a possible measure to reduce the rate of increase of atmospheric CO_2, ocean sequestration of CO_2 has been proposed, and its feasibility as well as its potential impacts on the marine ecosystem has been discussed (Handa and Ohsumi, 1995; Ormerod and Angel, 1996). Four proposed methods of CO_2 sequestration are: (1) releasing gaseous or liquid CO_2 at shallow fixed points (depth of 1000 m) from the coast by pipelines, (2) discharging liquid CO_2 into intermediate ocean depths (1000–2000 m) from a moving ship by a towed pipe, (3) dropping dry ice, and (4) storing liquid CO_2 in a restricted depression of the deep seafloor (>3000 m, Ohsumi, 1995). Nakashiki et al. (1995) concluded that the moving ship method would be the best because the dispersion area is unlimited, enabling CO_2 to be diluted for dissolution to very low concentrations to minimize adverse effects on marine organisms. In contrast, Omori et al. (1998) concluded that confined release of CO_2 in a restricted depression of the deep seafloor would be preferable because it would localize the impact on marine fauna. Whichever method may be applied, some harmful influences on marine

Correspondence to: T. Kikkawa; e-mail: kikkawa@kaiseiken.or.jp

Contract grant sponsor: New Energy and Industrial Technology Development Organization (NEDO).

Contract grant sponsor: Research Institute of Innovative Technology for the Earth (RITE).

Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/tox.10139

TABLE I. Developmental stages, experimental temperature, size, and time after fertilization or hatching of experimental materials

Species	Stage	Water Temperature (°C)	Diameter (mm)		Total Length (mm)	Hours after Fertilization	Days after Hatching
			Eggshell	Oil Globule			
Pagrus major	Egg (cleavage)	20	0.88 (0.043)	0.20 (0.007)	—	1	—
	Egg (embryo)		0.90 (0.021)	0.20 (0.005)	—	21	—
	Larva (preflexion)		—	—	5.32 (0.53)	—	10–12
	Larva (flexion)		—	—	6.75 (0.42)	—	14–15
	Larva (postflexion)		—	—	8.72 (1.17)	—	20–22
	Juvenile		—	—	14.29 (2.11)	—	28–33
Sillago japonica	Egg (cleavage)	26	0.67 (0.022)	0.15 (0.007)	—	1	—
	Egg (embryo)		0.68 (0.022)	0.15 (0.005)	—	12.5	—
	Larva (preflexion)		—	—	4.55 (0.64)	—	10–12
	Larva (flexion)		—	—	5.65 (0.82)	—	11–13
	Larva (postflexion)		—	—	9.09 (1.69)	—	17–19
	Juvenile		—	—	14.53 (2.00)	—	24–28
Paralichthys olivaceus	Egg (cleavage)	17	0.94 (0.008)	0.16 (0.003)	—	2.5	—
	Young	18	—	—	744 (62)	—	—
Euthynnus affinis	Egg (cleavage)	24	0.93 (0.027)	0.25 (0.005)	—	4	—

Values in parentheses show *SD*.

organisms and the ecosystem are unavoidable; therefore, careful assessment of the biological impact of ocean CO_2 sequestration is indispensable before embarking on this debatable practice (Seibel and Walsh, 2003). Unfortunately, information on the physiological performance of deep-sea organisms is scarce and is largely limited to descriptions of fauna, biomass, and diel and ontogenetic vertical migration in deep-sea fishes (Haedrich, 1997; Weitzman, 1997) and plankton (Omori et al., 1998).

Fish are an essential component of the marine ecosystem and provide an important protein source in many countries, including Japan. Although some data are available on the effects of CO_2 on fish, most studies have focused on adults of freshwater species, and information is critically lacking on the effects of CO_2 on the eggs, larvae, and juveniles of both freshwater and seawater species (Ishimatsu and Kita, 1999). Fish survival during the early developmental stages contributes to the population size of a species; hence, it is important to assess susceptibility to elevated CO_2 concentrations during the early developmental stages of fish.

Auerbach et al. (1997) studied the biological impact of CO_2 ocean sequestration and derived mortality curves based on data from the literature about marine organisms of limited mobility (e.g., fish larvae, copepods, and mollusks). However, their analysis was based on data from experiments in which organisms were exposed to acidified seawater, not to CO_2 per se. Acidified seawater and CO_2 exert significantly different physiological influences on aquatic organisms because of the different permeabilities of hydrogen ions and gaseous CO_2 (Heisler, 1986; Morris et al., 1989). Therefore, it is probably inappropriate to use data for pH–mortality relationships to evaluate the effects of CO_2. To our knowledge, there is no information about the effects of CO_2 on marine fish during their early life stages.

The purpose of this study was to examine ontogenetic changes in the median lethal PCO_2 in acute exposure experiments for commercially important marine teleosts. The species we chose were red sea bream, *Pagrus major* (Perciformes: Sparidae); Japanese whiting, *Sillago japonica* (Perciformes: Sillaginidae); Japanese flounder, *Paralichthys olivaceus* (Pleuronectiformes: Paralichthyidae), and eastern little tuna, *Euthynnus affinis* (Perciformes: Scombridae).

MATERIALS AND METHODS

Experimental Fish

Table I summarizes the experimental materials used in this study. The eggs of *Pagrus major*, *Sillago japonica,* and *Paralichthys olivaceus* were obtained from fish that spawned naturally at the Marine Ecology Research Institute (MERI). Some eggs were used for tests at the cleavage stage, 1–2.5 h after fertilization. The remaining eggs of *Pag. major* and *S. japonica* were incubated in 100-L tanks supplied with aerated, sand-filtered seawater, and used for tests at the embryonic stage, when the auditory vesicles had formed. The eggs of *Euthynnus affinis* were tested at the

TABLE II. Median lethal PCO_2 of four teleosts

Species	Stage	Median Lethal PCO_2 (kPa)							
		15 min	90 min	360 min	1000 min	24 h	45 h	48 h	72 h
Pagrus major	Egg (cleavage)	2.20	1.35	1.38	—	1.31	—	—	—
	Egg (embryo)	>9.90	8.39	5.08	—	—	—	—	—
	Larva (preflexion)	>9.90	>9.90	7.33	—	5.22	—	—	—
	Larva (flexion)	>6.93	6.84	4.24	—	2.94	—	—	—
	Larva (postflexion)	5.96	5.68	4.56	—	4.09	—	—	—
	Juvenile	3.56	2.56	2.54	—	2.52	—	—	—
Sillago japonica	Egg (cleavage)	2.52	2.39	2.40	2.38	—	—	—	—
	Egg (embryo)	>9.80	>9.80	4.88	—	—	—	—	—
	Larva (preflexion)	>9.80	>9.80	5.87	—	2.98	—	—	—
	Larva (flexion)	>9.80	9.10	6.13	—	4.73	—	—	—
	Larva (postflexion)	>4.90	4.34	4.06	—	3.63	—	—	—
	Juvenile	3.72	2.81	2.66	—	2.57	—	—	—
Paralichthys olivaceus	Egg (cleavage)	>7.95	2.93	2.78	—	2.82	2.29	—	—
	Young	>6.95	>6.95	>6.95	—	4.96	—	4.61	4.61
Euthynnus affinis	Egg (cleavage)	>14.75	9.96	11.84	—	9.28	—	—	—

cleavage stage and were obtained from fish that spawned naturally at the Tokyo Sea Life Park, Japan. Within 4 h of fertilization, they were transferred to MERI and immediately used for tests.

Larvae of *Pag. major* and *S. japonica* were reared in 500-L tanks under a natural photoperiod at MERI. Young *Par. olivaceus* were obtained from Nisshin Marine Tech Company, Ltd., Aichi, Japan (body weight: 3.5 ± 1.0 g). Fish were fed either rotifers *Brachionus plicatilis* (S and L types), *Artemia salina* nauplii, fish eggs, or an artificial diet, depending on their developmental stage. The developmental stages of eggs and larvae were classified according to Oozeki and Hirano (1985) and Kendall et al. (1984), respectively.

Egg Tests

For the tests of *Pag. major* and *S. japonica*, two PVC tanks (capacity 14 L), one for CO_2 exposure and the other as a control, were placed in a water bath (100 L). The PVC tanks were filled with 11 L of seawater bubbled with gas mixtures of O_2 (20.95%) and CO_2 (0.3–15.0%) balanced with N_2 (CO_2 exposure tank) or air (control tank). The gas mixtures were supplied by a gas mixing flowmeter (GF-3/MP, Cameron Instrument Company, TX, USA), at a flow rate of 300 mL/min. Cylindrical polycarbonate vessels (85 mL) with open ends covered with a fine mesh net were used as containers for the eggs. Eggs were placed in the vessels filled with hypercapnic seawater and were submerged in the PVC tank.

All tests were conducted in duplicate. At the start of an exposure test, the vessels, containing approximately 45 eggs each, were placed in the PVC tanks. At the end of the exposure periods (from 15 min to 24 h, Table II), the vessels in the CO_2 exposure tank were transferred to the control tank. The eggs were transferred from the vessels to 300-mL beakers filled with control seawater after the longest exposure period was completed and then were incubated overnight.

Numbers of normally hatched larvae, dead eggs, malformed eggs, dead larvae, and malformed larvae were counted 1 day after hatching. Percent hatching was calculated as the ratio of normally hatched larvae to the total number of eggs.

About 30–40 eggs of *Par. olivaceus* and *E. affinis* were placed in polycarbonate vessels similar to those used for the above two species and were subjected to the same hypercapnia test as described above, except that the test for the *Par. olivaceus* eggs lasted up to 45 h. Experimental temperatures are shown in Table I.

Larvae and Juvenile Tests

Polycarbonate bottles (1000 mL) with round net-covered windows on the sidewall were used to contain larvae and juveniles. Gas mixtures containing 1–10% CO_2 were used to test the CO_2 susceptibilities of larvae and juveniles.

About 1 h before starting an experiment, larvae or juveniles were collected from the rearing tanks in a plastic cup and were placed in the water bath in order to allow recovery

TABLE III. Relationships of PCO_2 (kPa) of gas mixture and seawater pH at equilibrium, pH = a log(PCO_2) + b

Water Temperature (°C)	a	b	r^2
17	−0.868	7.017	0.970
18	−1.055	6.856	0.995
20	−1.011	6.858	0.991
24	−1.481	7.765	0.970
26	−1.004	6.894	0.995

from handling stress. About 20 larvae and juveniles of *Pag. major* were placed in each bottle, with different numbers of larvae (ca. 15) and juveniles (ca. 10) used to test *S. japonica*. Four bottles, each containing the above numbers of fish, were simultaneously immersed in hypercapnic seawater, with one bottle as the control, because mortality in the control group was nearly always 0%. When handling the test fish, care was taken not to expose them to air. Survival was checked at the prescheduled intervals shown in Table II with a stereomicroscope. An individual was regarded as dead when the heart was not beating.

Young Test

To test the CO_2 susceptibility of young *Par. olivaceus*, we used a CO_2 exposure tank (7 L), a control tank (7 L), and a seawater reservoir (14 L).

One day before an experiment, 12 fish were placed in each of the tanks, which were filled with aerated seawater at 18°C. At the start of an experiment, the CO_2 exposure tank was flushed for about 5 min with hypercapnic seawater prepared in the reservoir. Thereafter, the CO_2 levels in the CO_2 exposure tank were maintained by bubbling the CO_2 gas mixture directly into the tank. The effects of CO_2 were assessed as above.

Water Quality

Seawater pH was measured to the third decimal place at the start and end of tests with a pH meter (HM-60G and combination electrode GST-5721C, TOA, Co., Ltd., Tokyo, Japan) and was monitored continuously during each test (MP125 and polymer combination electrode InLab413-IP67, Mettler Tolledo Japan, Co., Ltd., Osaka, Japan).

Control seawater had a mean (*SD*) salinity of 33.84 (0.62) psu, a mean (*SD*) alkalinity of 2.294 (0.025) meq/L, and a mean (*SD*) pH of 8.111 (0.062). Seawater pH equilibrated with different concentrations of CO_2 used in the study is shown in Table III. Water PCO_2 was calculated from fractional CO_2 concentrations of the gas mixtures, barometric pressure, and temperature.

RESULTS

Percent hatching (for cleavage and embryo stages) and survival (preflexion stage and thereafter) declined with water PCO_2 and exposure period for *Pagrus major* (Fig. 1). The cleavage stage was consistently most susceptible to CO_2, followed by the juvenile stage. The embryo, preflexion, and flexion stages were far more tolerant, particularly for short-term exposures. For long-term exposures, susceptibility of the postflexion stage was similar to that of the embryo, preflexion and flexion stages. The effects of water PCO_2 on percent hatching and survival of *Sillago japonica* were generally similar to those for *Pag. major*, although the trend was less clear (Fig. 2).

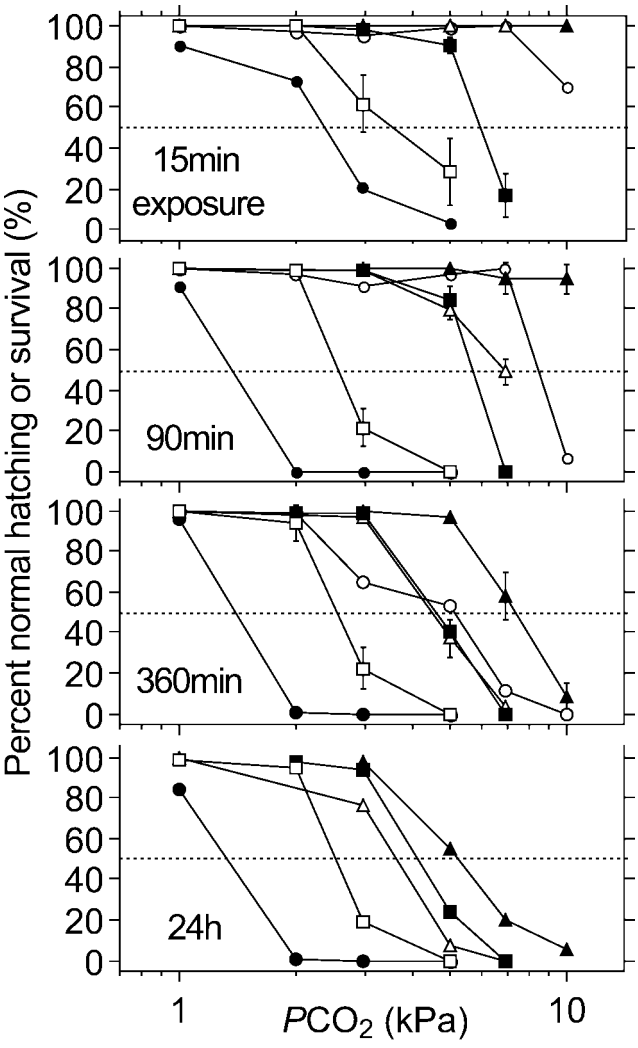

Fig. 1. Percent normal hatching or survival of eggs, larvae, and juveniles of *Pagrus major* in hypercapnic seawater for exposure of from 15 min to 24 h. Vertical lines indicate *SD*. Solid circles: cleavage stage, open circles: embryo stage, solid triangles: preflexion stage, open triangles: flexion stage, solid squares: postflexion stage, open squares: juvenile stage.

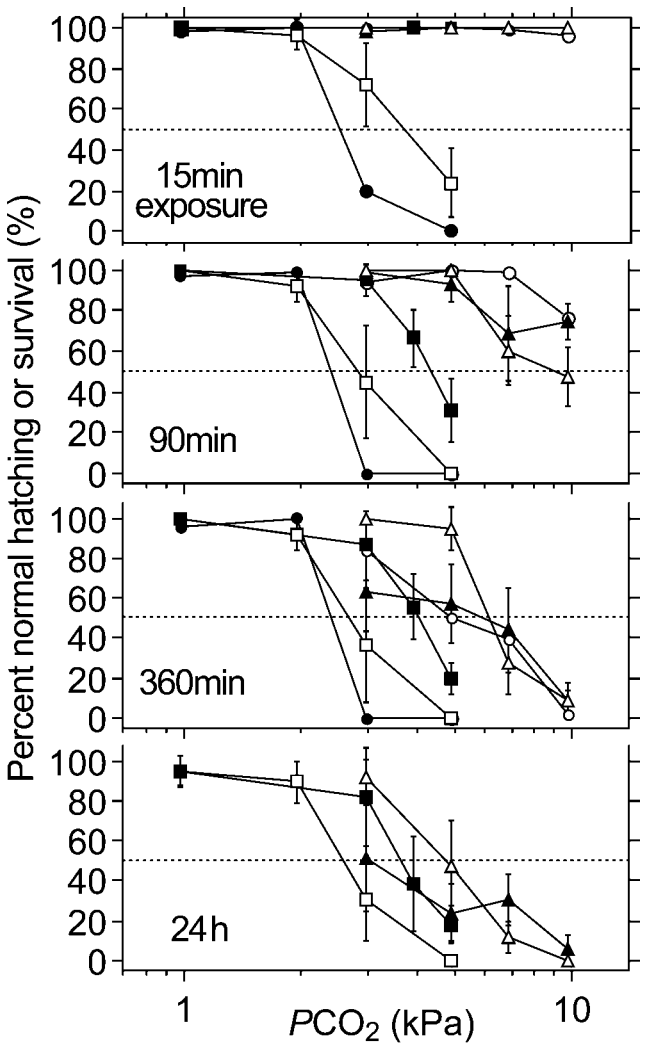

Fig. 2. Percent normal hatching or survival of eggs, larvae, and juveniles of *Sillago japonica* in hypercapnic seawater for exposure of from 15 min to 24 h. Vertical lines indicate *SD*. Solid circles: cleavage stage, open circles: embryo stage, solid triangles: preflexion stage, open triangles: flexion stage, solid squares: postflexion stage, open squares: juvenile stage.

Percent hatching of *Paralichthys olivaceus* was above 82% at a PCO_2 of 1.0 kPa (7.5 mmHg), irrespective of exposure duration. Above a PCO_2 of 3.0 kPa, the value dropped sharply with prolonged exposure, and no hatching occurred at a PCO_2 of 8.0 kPa for exposures longer than 24 h. Young fish were more tolerant to CO_2 than eggs, showing no mortality at a PCO_2 of 3.0 kPa even for the 72-h exposure. Survival rates of young were less than 50% and 0% at a PCO_2 of 5.0 kPa and 6.9 kPa, respectively, for exposures longer than 24 h.

Euthynnus affinis was considerably more tolerant to CO_2 than were the other species tested. Hatching rates remained above 90%, even when eggs were exposed to a PCO_2 of 7.9 kPa for 24 h. There was 100% mortality of eggs only with a PCO_2 of 14.8 kPa after a 24-h exposure.

Median lethal PCO_2 values were calculated from the above data according to the Japanese Industrial Standard (1998) (Table III), and changes with ontogenetic stage are given in Figure 3 for *Pag. major* and *S. japonica* [Fig. 3(A), 360-min data; Fig. 3(B), 24-h data].

DISCUSSION

The pattern of ontogenetic changes in CO_2 tolerance was similar for *Pagrus major* and *Sillago japonica*, in that median lethal PCO_2 peaked in the preflexion stage (*Pag. major*) or 1 day after, that is, the flexion stage (*S. japonica*), with CO_2 sensitivity much higher in the preceding and following stages. This pattern was especially clear when median lethal PCO_2 was calculated for 360 min and might be common among temperate shallow-water teleosts, considering the different taxonomy and life histories of the two species—*Pag. major* migrates between the coast and adjacent shelf waters, whereas *S. japonica* is a coastal demersal fish.

Freshwater fish are generally more susceptible to various toxicants during their early developmental stages (McKim, 1977). Although little is known about marine species in this

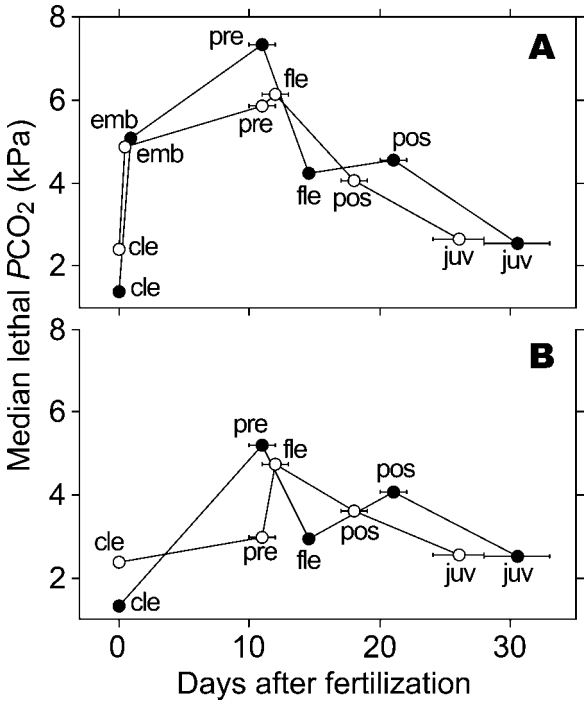

Fig. 3. Ontogenetic changes of the median lethal PCO_2 in *Pagrus major* (solid circles) and *Sillago japonica* (open circles): (A) 360-min exposure, (B) 24-h exposure (horizontal lines show range; cle: cleavage stage, emb: embryo stage, pre: preflexion stage, fle: flexion stage, pos: postflexion stage, juv: juvenile stage.

respect, McKim (1985) reviewed the results of chronic toxicity tests on a seawater-adapted sheepshead minnow, *Cyprinodon variegatus*, and found a similar relationship between development and toxicant susceptibility. However, his definitions of the developmental stages differed from those most commonly used (Kendall et al., 1984), including in the present study. Moreover, the larval substages were grouped together in each report reviewed by McKim (1985). Koyama et al. (1992) also found that median lethal concentration (LC_{50}) values for cadmium and fenitrothion generally increased with development for seven marine fish larvae and juveniles, although exact values varied among species and toxicants. Thus, the ontogenetic pattern of median lethal PCO_2 appears to be uniquely different from those known for other toxicants.

Although it may be premature to discuss the cause of the characteristic changes in CO_2 tolerance with development because the lethal mechanisms of CO_2 are unknown for fish, some speculation may be useful for future investigations. CO_2 molecules diffuse through the epithelium into the body according to the PCO_2 gradient and hydrate to form carbonic acid, which immediately dissociates into H^+ and HCO_3^-. The latter further dissociates into H^+ and CO_3^{2-}. The extent of the reactions depends on temperature, ionic strength, and pH. Thus, elevation of ambient PCO_2 will quickly increase H^+ concentrations of both extracellular and intracellular fluids and decrease their pH. The resultant respiratory acidosis would interfere with cellular metabolic pathways (Heisler, 1989). The current hypothesis on acid–base regulation in fish predicts that ion-transporting chloride cells in the gills are responsible for pH regulation in marine species (Claiborne et al., 2002). This contention is based on the immunohistochemical demonstration of Na^+/H^+ exchangers, which are thought to be responsible for proton extrusion in marine fishes, in cells that also are immunoreactive to Na^+/K^+ ATPase, the enzyme widely used as a reliable marker for fish chloride cells. Interestingly, the expression of Na^+/H^+ exchangers (NHE) was found to increase during hypercapnia in seawater-adapted mummichog (*Fundulus heteroclitus*, NHE1 and NHE3), suggesting a role of NHE in acid–base regulation, at least in this species.

Two studies have investigated the effects of hypercapnia on chloride cell morphology, with conflicting results. Goss et al. (1992) found a sharp decrease in the chloride cell fractional area and a concomitant fall in chloride influx in the gills of adult freshwater bullhead, *Ictalurus nebulosus* (PCO_2 of 2.0 kPa for 48 h). In contrast, Cameron and Iwama (1987) reported an increase in chloride cell area during exposure to hypercapnia (PCO_2 progressively increased to 7.5 kPa in 4 days) in a congeneric species, *I. punctatus*. However, the current model for acid–base regulation in freshwater fish differs from that proposed for marine species in that pavement cells, rather than chloride cells, are thought to be the site of H^+ extrusion. This model is supported by a number of immunohistochemical investigations that have demonstrated localization of H^+-ATPase in pavement cells in freshwater fishes (see Claiborne et al., 2002 for review).

Hypercapnia consistently induces a lowering of plasma Cl^- concentrations in both freshwater and marine fish (Heisler, 1989). We have also found a PCO_2-dependent fall in plasma Cl^- in three marine fishes (*Mustelus manazo*, *Par. olivaceus*, *Seriola quinqueradiata*; Hayashi et al., in press). This implies enhanced activity of chloride cells because they are the definite site of Cl^- extrusion in marine fishes (Zadunaisky, 1984). Chloride cells develop in the embryo stage in a number of teleosts (Hwang and Hirano, 1985; Alderdice, 1988; Ayson et al., 1994; Kaneko et al., 1995; Shiraishi et al., 1997; Sasai et al., 1998; Katoh et al., 2000), and this may explain the observed enhanced tolerance to CO_2 from the cleavage to the embryo stages. We recently found that exposure to a PCO_2 of 1.0 kPa for 24 h significantly increased the chloride cell area in young *Pag. major* (Kikkawa et al., 2002).

The gradual fall in CO_2 tolerance from the larval to juvenile stage may result from the development of gill lamellae in the preflexion stage (*Pag. major,* Oikawa et al., 1999; *S. japonica*, Oozeki et al., 1992), which dramatically increases the surface area for diffusion. In these early developmental stages, gas transfer across the body surface should be diffusion-limited (Perry and Gilmour, 2002). Although information is very limited on CO_2 tolerance in the various developmental stages of fish, the cleavage and juvenile stages appear to be most susceptible to CO_2. Takeda and Itazawa (1983) reported no mortality of young *Pag. major* exposed to a PCO_2 of 4.1–4.5 kPa for 22 h. Moreover, Hayashi et al. (in press) found that the tolerance of adult *Paralichthys olivaceus* was lower than that of young fish but higher than eggs in the cleavage stage. These results indicate that the egg cleavage and juvenile stages should be included in the assessment of acute lethal effects of CO_2 on marine fishes.

Environmental factors that should be considered in estimating the lethal effect of CO_2 at a depth are low temperature and high pressure. Water temperature in the ocean rapidly declines with depth and is below 4°C at depths for which CO_2 sequestration is being proposed (Lerman, 1986). Therefore, CO_2 exposure tests should be conducted at low temperatures, or results obtained at higher temperatures somehow should be extrapolated to low temperatures. In this study we used aquacultured species because they are readily available and culturing techniques have been established for all except *Euthynnus affinis*. To clarify the effects of temperature on CO_2 toxicity, experiments on a eurythermal species would be appropriate, as the eggs and larvae could be incubated over a wide range of temperatures. However, Sprague (1990) found that the effects of temperature on toxicity were difficult to generalize and were either small or had no overall pattern. The effects of high pressure

on CO_2 toxicity are more difficult to determine. However, we can estimate the effects by studying the effects of low temperature because high pressure exerts metabolic effects similar to those of low temperature (Sébert, 1997).

Another complicating factor in assessing the lethal effects of CO_2 under realistic conditions is the great differences in the taxonomies of bathypelagic and epipelagic fish. Bathypelagic fish are dominated by "ancient groups," whereas epipelagic species are mostly perciform (Weitzman, 1997). Although the susceptibility of bathypelagic fishes to CO_2 conceivably is different from that of epipelagic species, it is logistically very difficult to use these fishes for experiments, particularly individuals in early developmental stages. CO_2 susceptibility of deep-sea fishes may be indirectly estimated by using characteristics that correlate with CO_2 susceptibility in epipelagic fishes and can be obtained from both epipelagic and bathypelagic fishes, such as morphological characteristics. Thus, if we can identify some morphological variables that correlate with CO_2 susceptibility, it may be possible to estimate CO_2 susceptibility of deep-sea fishes using such information. Morphometric data of chloride cells may deserve scrutiny in this respect.

Finally, although CO_2 concentrations were fixed during experiments in this study, animals would be subjected to a fluctuating PCO_2 value when CO_2 droplets are released from the lower end of a pipe towed from a moving ship. The spatial and temporal patterns of PCO_2 fluxes in the surrounding waters would require them being analyzed with highly sophisticated numerical models (Sato and Sato, 2002). This should also be included in experimental protocols to assess the effects of CO_2 under realistic conditions.

We are grateful to the Tokyo Sea Life Park for providing materials and to Dr. M. Okiyama for critical reading of the manuscript.

REFERENCES

Alderdice DF. 1988. Osmotic and ionic regulation in teleosts eggs and larvae. In: Hoar WS, Randall DJ, editors. Fish physiology, 11A. San Diego, CA: Academic Press. p 163–251.

Auerbach DI, Caulfield JA, Adams EE, Herzog HJ. 1997. Impacts of ocean CO_2 disposal on marine life: I. A toxicological assessment integrating constant-concentration laboratory assay data with variable-concentration field exposure. Environ Model Assess 2:333–343.

Ayson FG, Kaneko T, Hasegawa S, Hirano T. 1994. Development of mitochondrion-rich cells in the yolk-sac membrane of embryos and larvae of tilapia, *Oreochromis mossambicus*, in fresh water and seawater. J Exp Zool 270:129–135.

Cameron JN, Iwama GK. 1987. Compensation of progressive hypercapnia in channel catfish and blue crabs. J Exp Biol 133:183–197.

Claiborne JB, Edwards SL, Morrison-Shetlar AI. 2002. Acid-base regulation in fishes: cellular and molecular mechanisms. J Exp Zool 293:302–319.

Goss GG, Laurent GP, Perry SF. 1992. Evidence for a morphological component in acid–base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). Cell Tiss Res 268:539–552.

Haedrich RL. 1997. Distribution and population ecology. In: Randall DJ, Angel M, editors. Deep-sea fishes. San Diego, CA: Academic Press. p 79–114.

Handa N, Ohsumi T. 1995. Direct ocean disposal of carbon dioxide. Tokyo: Terra Scientific Publishing Company. p 274.

Hayashi M, Kita J, Ishimatsu A. 2003. Acid–-base responses to lethal aquatic hypercapnia in three marine fish. Mar Biol, in press.

Heisler N. 1986. Acid–base regulation in animals. Amsterdam, The Netherlands: Elsevier. p 492.

Heisler N. 1989. Acid–base regulation in fishes: 1. Mechanisms. In: Morris R, Taylor EW, Brown DJA, Brown JA, editors. Acid toxicity and aquatic animals. Cambridge, UK: Cambridge University Press. p 85–97.

Hwang PP, Hirano R. 1985. Effects of environmental salinity on intercellular organization and junctional structure of chloride cells in early stages of teleosts development. J Exp Zool 236: 115–126.

Ishimatsu A, Kita J. 1999. Effects of environmental hypercapnia on fish. Japan J Ichthyol 46:1–13.

Japanese Standards Association. 1998. Testing methods for industrial wastewater. JIS K 0102. p 324.

Kaneko T, Hasegawa S, Takagi Y, Tagawa M, Hirano T. 1995. Hypoosmoregulatory ability of eyed-stage embryos of chum salmon. Mar Biol 122:165–170.

Katoh F, Shimizu A, Uchida K, Kaneko T. 2000. Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*. Zool Sci 17:11–18.

Kendall AW Jr., Ahlstrom EH, Moser HG. 1984. Early life history stages of fishes and their characters. In: Moser HG, Richards WJ, Cohen DM, Fahay MP, Kendall AW Jr., Richardson SL, editors. Ontogeny and systematics of fishes. American Society of Ichthyologists and Herpetologists, Special Publ 1. Lawrence, KS: Allen Press. p 11–22.

Kikkawa T, Kita J, Ishimatsu A. 2002. Effects of CO_2 on early development and growth of red sea bream (*Pagrus major*). Fish Sci 68 Suppl I:637–638.

Koyama J, Kuroshima R, Ishimatsu A. 1992. The seawater fish for evaluation of the toxicity of pollutants. J Jpn Soc Water Environ 15:804–813.

Lerman M. 1986. Marine biology. Menlo Park, CA: Benjamin/Cummings Publishing Company. p 534.

McKim JM. 1977. Evaluation of tests with early life stages of fish for predicting long-term toxicity. J Fish Res Board Can 34: 1148–1154.

McKim JM. 1985. Early life stage toxicity tests. In: Rand GM, editor. Fundamentals of aquatic toxicology: effects, environmental fate, and risk assessment, 2nd ed. Appendix B. Philadelphia, PA: Taylor & Francis. p 974–1011.

Morris R, Taylor EW, Brown DJA, Brown JA. 1989. Acid toxicity and aquatic animals, Society for Experimental Biology Seminar Series 34. Cambridge, UK: Cambridge University Press. p 282.

Nakashiki N, Ohsumi T, Katano N. 1995. Technical view on CO_2 transportation onto the deep ocean floor and dispersion at intermediate depth. In: Handa N, Ohsumi T, editors. Direct ocean disposal of carbon dioxide. Tokyo: Terra Scientific Publishing Company. p 183–193.

Ohsumi T. 1995. CO_2 storage options in the deep sea. MTS J 29:58–66.

Oikawa S, Hirata M, Kita J, Itazawa Y. 1999. Ontogeny of respiratory area of a marine teleost, porgy, *Pagrus major*. Ichthyol Res 46:233–244.

Omori M, Norman CP, Ikeda T. 1998. Oceanic disposal of CO_2: Potential effects on deep-sea plankton and micronekton—A review. Plankton Biol Ecol 45:87–99.

Oozeki Y, Hirano R. 1985. Effects of temperature changes on the development of eggs of the Japanese whiting *Sillago japonica* Temminck et Schlegel. Bull Jpn Soc Sci Fish 51:557–572.

Oozeki Y, Hwang P-P, Hirano R. 1992. Larval development of the Japanese whiting, *Sillago japonica*. Jpn J Ichthyol 39:59–669.

Ormerod B, Angel M. 1996. Ocean storage of carbon dioxide. Workshop 2 —Environmental Impact. Cheltenham, UK: IEA Greenhouse Gas R&D Programme. p 131.

Perry SF, Gilmour KM. 2002. Sensing and transfer of respiratory gases at the fish gill. J Exp Zool 293:249–263.

Sasai S, Kaneko T, Tsukamoto K. 1998. Extrabranchial chloride cells in early life stages of Japanese eel, *Anguilla japonica*. Ichthyol Res 45:95–98.

Sato T, Sato K. 2002. Numerical prediction of the dilution process and its biological impacts in CO_2 ocean sequestration. J Marine Sci Tech 6:169–180.

Seibel BA, Walsh PJ. 2003. Biological impacts of deep-sea carbon dioxide injection inferred from indices of physiological performance. J Exp Biol 206:641–650.

Sébert P. 1997. Pressure effects on shallow-water fishes. In: Randall DJ, Farrell AP, editors. Deep-sea fishes. San Diego, CA: Academic Press. p 279–323.

Shiraishi K, Kaneko T, Hasegawa S, Hirano T. 1997. Development of multicellular complexes of chloride cells in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) embryos and larvae in seawater. Cell Tissue Res 288:583–590.

Sprague JB. 1990. Aquatic toxicology. In: Schreck CB, Moyle PB, editors. Methods for fish biology. Bethesda, MD: American Fisheries Society. p 491–528.

Takeda T, Itazawa Y. 1983. Possibility of applying anesthesia by carbon dioxide in the transportation of live fish. Bull Jpn Soc Sci Fish 49:725–731.

Weitzman SH. 1997. Systematics of deep-sea fishes. In: Randall DJ, Farrell AP, editors. Deep-sea fishes. San Diego, CA: Academic Press. p 43–78.

Zadunaisky JA. 1984. The chloride cell: The active transport of chloride and the paracellular pathways. In: Hoar WS, Randall DJ, editors. Fish physiology, 10B. Orlando, FL: Academic Press. p 130–176.