

Review

Effects of CO₂ on Marine Fish: Larvae and Adults

ATSUSHI ISHIMATSU^{1*}, TAKASHI KIKKAWA^{1,2}, MASAHIRO HAYASHI¹, KYOUNG-SEON LEE¹
and JUN KITA³

¹Marine Research Institute, Nagasaki University, Taira-machi, Nagasaki 851-2213, Japan

²Central Laboratory, Marine Ecology Research Institute, Onjuku, Chiba 299-5105, Japan

³Research Institute of Innovative Technology for the Earth (RITE),
Kizugawadai, Kizu-cho, Kyoto 619-0292, Japan

(Received 13 October 2003; in revised form 30 March 2004; accepted 6 April 2004)

CO₂-enriched seawater was far more toxic to eggs and larvae of a marine fish, silver seabream, *Pagrus major*, than HCl-acidified seawater when tested at the same seawater pH. Data on the effects of acidified seawater can therefore not be used to estimate the toxicity of CO₂, as has been done in earlier studies. Ontogenetic changes in CO₂ tolerance of two marine bony fishes (*Pag. major* and Japanese sillago, *Sillago japonica*) showed a similar, characteristic pattern: the cleavage and juvenile stages were most susceptible, whereas the preflexion and flexion stages were much more tolerant to CO₂. Adult Japanese amberjack, *Seriola quinqueradiata*, and bastard halibut, *Paralichthys olivaceus*, died within 8 and 48 h, respectively, during exposure to seawater equilibrated with 5% CO₂. Only 20% of a cartilaginous fish, starspotted smoothhound, *Mustelus manazo*, died at 7% CO₂ within 72 h. Arterial pH initially decreased but completely recovered within 1–24 h for *Ser. quinqueradiata* and *Par. olivaceus* at 1 and 3% CO₂, but the recovery was slower and complete only at 1% for *M. manazo*. During exposure to 5% CO₂, *Par. olivaceus* died after arterial pH had been completely restored. Exposure to 5% CO₂ rapidly depressed the cardiac output of *Ser. quinqueradiata*, while 1% CO₂ had no effect. Both levels of ambient CO₂ had no effect on blood O₂ levels. We tentatively conclude that cardiac failure is important in the mechanisms by which CO₂ kills fish. High CO₂ levels near injection points during CO₂ ocean sequestration are likely to have acute deleterious effects on both larvae and adults of marine fishes.

Keywords:

- Physiological effects of CO₂,
- CO₂ mortality,
- marine fish,
- developmental stage,
- acid-base regulation,
- blood circulation.

1. Introduction

Although fish physiologists have investigated the effects of environmental hypercapnia (elevated ambient CO₂) on several physiological functions of this major group in the aquatic environment, studies have dealt mostly with freshwater species and not much with marine species (Ishimatsu and Kita, 1999). Apart from the relative inaccessibility of marine species in most laboratories, there has been little incentive for the researchers to study effects of CO₂ on marine fish, because under natural conditions, both aquatic hypercapnia and hypoxia (low ambient O₂) are more common in freshwater environ-

ments, most typically in stagnant water bodies in the tropics, but are relatively infrequent in marine environments (see Dejours, 1988).

However, the idea of CO₂ ocean sequestration as a method to mitigate adverse effects of global warming has created an urgent need for a thorough understanding of how elevated levels of CO₂ in the ocean affect marine life. CO₂ causes a wide range of mostly negative influences on many aspects of animal physiology (see Pörtner *et al.*, 2004). Short-term effects, for example, include disturbance of acid-base status, respiration, blood circulation and nervous activities of the exposed animals, while long-term effects include reduced growth rate, reproduction, and calcification, although there are few data available on the long-term effects of CO₂ (Ishimatsu and Kita, 1999).

* Corresponding author. E-mail: a-ishima@net.nagasaki-u.ac.jp

When a fish is exposed to environmental hypercapnia, body fluid pH rapidly decreases due to increased body fluid PCO_2 , but is subsequently compensated by accumulation of bicarbonate ions in body fluids as long as PCO_2 remains sublethal. The gills are the principal site of bicarbonate uptake from and/or hydrogen ion excretion into the ambient water (Heisler, 1986). Marine fish generally show faster pH compensation than freshwater species because of the higher bicarbonate (Heisler, 1993) and sodium chloride concentrations (Iwama and Heisler, 1991) of seawater. Hypercapnia generally enhances gill ventilation of fish. It has been postulated that the CO_2 -driven hyperventilation is due to lowered blood oxygen content caused by both Bohr and Root effects, but recent evidence has demonstrated that CO_2 itself stimulates gill ventilation (Burleson and Smatresk, 2000; McKendry *et al.*, 2001). Chemoreceptors responsible for the response appear to reside in the gills. Cardiovascular responses to hypercapnia have not been examined until relatively recently. Studies reported variable cardiac and blood pressure responses, probably due to interspecific variability of CO_2 sensitivities and the different experimental protocols used (Perry and Gilmour, 2002).

Much less is known on the effects of higher, possibly lethal levels of CO_2 on fish. Cruz-Neto and Steffensen (1997) reported a decrease of oxygen uptake in freshwater European eel, *Anguilla anguilla*, subjected to water PCO_2 of 4.0 kPa, while McKenzie *et al.* (2002) reported no effect of water PCO_2 of up to 10.66 kPa on oxygen uptake, arterial PO_2 and cardiac output for the same species. Under extremely high CO_2 concentrations, fish will be anesthetized (Yoshikawa *et al.*, 1994; Bernier and Randall, 1998), and eventually die (Takeda and Itazawa, 1983).

This paper summarizes our recent findings on the effects of both lethal and sublethal levels of CO_2 on ma-

rine larval and adult fish. Fish in the early developmental stages are generally more susceptible to environmental toxicants than adults (McKim, 1977). It is therefore conceivable that CO_2 exerts greater negative impacts on fish eggs, larvae and juveniles, but this was a totally unexplored area before we started CO_2 exposure studies on the early stages (Kikkawa *et al.*, 2003, 2004). Even if the severity of environmental hypercapnia due to CO_2 sequestration is made tolerable to adults, a gradual reduction of population size, and changes in marine ecosystem structures are unavoidable consequences when young individuals cannot survive. Therefore, evaluation of both acute and chronic influences of CO_2 on marine animals should take all life stages of susceptible marine species into consideration.

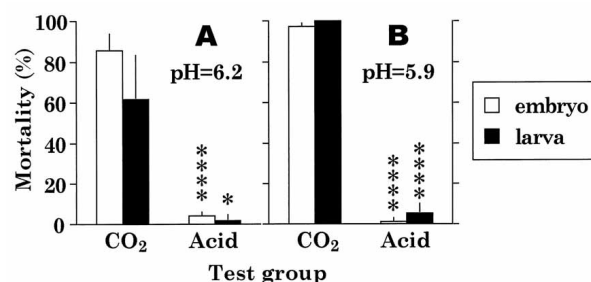


Fig. 1. Comparison of mean mortalities of embryo ($n = 5$) and larva ($n = 3$) of silver seabream, *Pagrus major*, exposed to two seawater pH levels lowered by either CO_2 or HCl (Condition A: pH 6.2, Condition B: pH 5.9 in Table 1). Exposure periods for embryo and larva were 6 h and 24 h, respectively. Asterisks show significant difference between test groups (A: Welch's t-test, B: Student's t-test, *: $p < 0.05$, ****: $p < 0.0001$, from Kikkawa *et al.*, 2004).

Table 1. Seawater carbonic systems of CO_2 and acid groups.

Parameters	Condition A		Condition B	
	CO ₂ (5%)	Acid	CO ₂ (10%)	Acid
pH	6.16	6.19	5.86	5.87
[H ⁺] (mmol L ⁻¹)	$6.92 \cdot 10^{-7}$	$6.46 \cdot 10^{-7}$	$1.38 \cdot 10^{-6}$	$1.35 \cdot 10^{-6}$
PCO_2 (kPa)	4.95	0.037	9.90	0.037
[CO ₂] (mmol kg ⁻¹)*	1.58	$1.17 \cdot 10^{-2}$	3.16	$1.17 \cdot 10^{-2}$
[HCO ₃ ⁻] (mmol kg ⁻¹)	2.16	$1.71 \cdot 10^{-2}$	2.16	$0.82 \cdot 10^{-2}$
[CO ₃ ²⁻] (mmol kg ⁻¹)	$2.05 \cdot 10^{-3}$	$1.74 \cdot 10^{-5}$	$1.03 \cdot 10^{-3}$	$4.00 \cdot 10^{-6}$

pK_1 (6.026) and pK_2 (9.181) from Mehrbach *et al.* (1973). CO_2 solubility ($0.03241 \text{ mol kg}^{-1} \text{ atm}^{-1}$) from Weiss (1974).

*Includes negligible concentration of H_2CO_3 (Heisler, 1986). Assuming atmospheric pressure of 101.3 kPa. From Kikkawa *et al.* (2004).

2. Larvae

2.1 Acid and CO₂

We exposed eggs and larvae of silver seabream, *Pagrus major*, to seawater pre-acidified either by equilibrating with a CO₂ gas mixture or addition of hydrochloric acid to give the same pH levels (pH 6.2 and 5.9). Eggs were judged dead if hatching did not occur, while the death of larvae was judged on cessation of heartbeat. When tested at the same pH levels, seawater acidified by elevated PCO₂ exerted a far more severe effect on survival of eggs (Fig. 1A) and larvae (Fig. 1B) than seawater acidified by addition of hydrochloric acid. Mortalities were significantly higher in the CO₂ groups than in the acid groups, irrespective of developmental stage. The use of acid toxicity data of nonvolatile acids, such as HCl or H₂SO₄, would therefore greatly underestimate the impacts of CO₂, as has been done in earlier studies (Auerbach *et al.*, 1997). CO₂ must be used in both laboratory and field experiments to properly assess the environmental impacts of CO₂ ocean sequestration on marine organisms, and we must urgently accumulate CO₂ toxicity data for various species with different physiological and ecological characteristics under different environmental conditions, e.g. temperature, pressure, and dissolved O₂ levels.

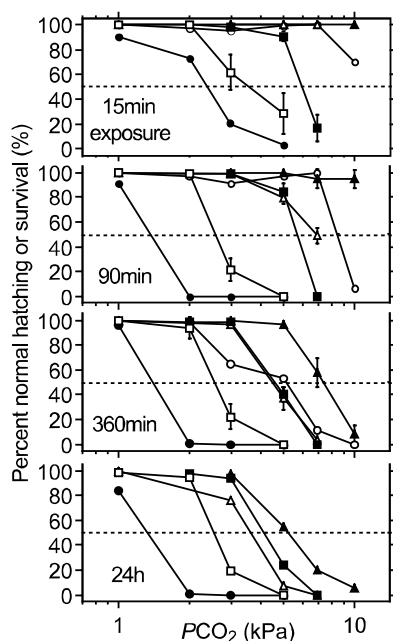


Fig. 2. Percent normal hatching or survival of eggs, larvae and juveniles of *Pagrus major* in hypercapnic seawater for 15 min to 24 h exposure. Vertical lines indicate standard deviation. Solid circles: cleavage stage, open circles: embryo stage, solid triangles: preflexion stage, open triangles: flexion stage, solid squares: postflexion stage, open squares: juvenile stage (from Kikkawa *et al.*, 2003).

Table 1 lists the calculated concentrations of CO₂-derived molecular species in the test seawater. All CO₂-derived molecular species were ca. 130-fold (5%) and 260-fold (10%) higher in the CO₂ group than in the acid group, while the H⁺ concentration was nearly identical for each pH level. Compared with HCO₃⁻ and CO₃²⁻ ions, uncharged CO₂ molecules readily diffuse through the epithelium into the body according to PCO₂ gradient (Vandenberg *et al.*, 1994), and then hydrate to form carbonic acid, which immediately dissociates into H⁺ and HCO₃⁻, the latter further dissociating into H⁺ and CO₃²⁻. Thus, elevation of ambient PCO₂ will rapidly increase H⁺ concentrations of body fluids and decrease their pH. The resulting intracellular acidosis will affect a number of physiological processes (Roos and Boron, 1981), to the extent that it may be lethal to marine organisms. In addition to these acidic toxicities, CO₂ itself may be toxic to animal cells (Max, 1991).

2.2 Ontogenetic changes in CO₂ susceptibility

Both hatching (for cleavage and embryo stages) and survival (preflexion stage and thereafter) declined with water PCO₂ and exposure period when *Pag. major* was exposed to different levels of ambient CO₂ levels for varying periods of time (Fig. 2). The effects of water PCO₂ on percentage hatching and survival of Japanese sillago, *Sillago japonica*, were generally similar to those for *Pag. major*, although the trend was less clear.

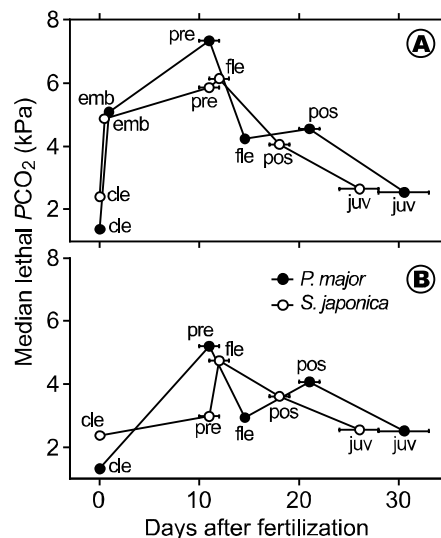


Fig. 3. Ontogenetic changes of the median lethal PCO₂ in *Pagrus major* (solid circles) and *Sillago japonica* (open circles). Horizontal lines show range. A: 360 min exposure, B: 24 h exposure. cle: cleavage stage, emb: embryo stage, pre: preflexion stage, fle: flexion stage, pos: postflexion stage, juv: juvenile stage (from Kikkawa *et al.*, 2003).

Table 2. 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
<i>Pagrus major</i>	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	—	—	—
<i>Sillago japonica</i>	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	—	—	—
<i>Paralichthys olivaceus</i>	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
<i>Euthynnus affinis</i>	Egg (cleavage)	>14.75	9.96	11.84	—	9.28	—	—	—

From Kikkawa *et al.* (2003).

The pattern of ontogenetic changes in CO_2 tolerance, expressed as median lethal PCO_2 (PCO_2 at which 50% of test animals die in a specified exposure period), was similar for *Pag. major* and *Sil. japonica*, in that median lethal PCO_2 peaked in the preflexion stage (*Pag. major*) or one day after, i.e. the flexion stage (*Sil. japonica*), with CO_2 sensitivity much higher in the preceding and following stages (Table 2 and Fig. 3). 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 while *Sil. japonica* is a coastal demersal fish.

It is currently unclear why CO_2 susceptibility changes during early development. Our hypothesis is that developmental changes in ion-transporting chloride cell population and respiratory surface area are responsible for the changes. We postulate that gill chloride cells are involved in pH regulation in marine species (see Subsection 3.1 Acid-base regulation). Chloride cells develop in the embryo stage in a number of teleosts (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. Our recent studies have demonstrated a significant increase in chloride cell size in the yolk sac membrane of *Sil. japonica* exposed to 1% CO_2 for 21 h and in the gills of young *Pag. major* subjected to both short-term (1% CO_2

for 24 h) and long-term (0.6 and 1.1% CO_2 for 30 days) hypercapnia (Kikkawa *et al.*, unpublished).

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; *Sil. japonica*: Oozeki *et al.*, 1992), which dramatically increases the surface area available for diffusion. In these early developmental stages, gas transfer across the body surface should be diffusion-limited (Perry and Gilmour, 2002).

3. Adults

3.1 Acid-base regulation

Teleosts died during 5% CO_2 exposure (water PCO_2 4.9 kPa): cumulative mortality was 17% at 8 h, 33% at 24 h and 100% at 48 h for the bastard halibut (*Paralichthys olivaceus*), whereas the Japanese amberjack (*Seriola quinqueradiata*) died considerably earlier (20% mortality recorded at 3 h and 100% at 8 h). In contrast, a cartilaginous fish, the star-spotted smooth-hound (*Mustelus manazo*), died only at 7% CO_2 (6.9 kPa) with 20% mortality recorded at 72 h. Thus, *M. manazo* was more tolerant to CO_2 than the two teleosts, although pH_a (arterial pH) regulation was less efficient in this fish than in the teleosts (see below). This may indicate that blood pH itself is not a direct cause of acute fish death during hypercapnia.

Figure 4 summarizes acid-base changes during CO_2

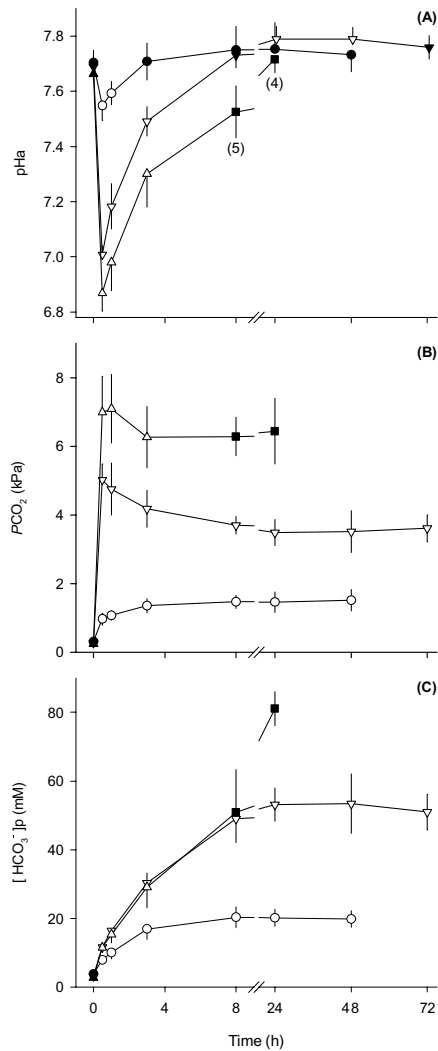


Fig. 4. Arterial pH (A), PCO_2 (B) and hematocrit (Hct, C) of *Paralichthys olivaceus* during exposure to seawater equilibrated with 1% (circles), 3% (upward triangles) and 5% CO_2 (downward triangles) in air ($N = 5$). Open symbols indicate significant differences from corresponding 0-h values ($p < 0.05$; Dunnett test). N decreased due to mortality at 5% (squares), to which no statistical comparison was applied (from Hayashi *et al.*, 2004).

exposure for *Par. olivaceus*. When exposed to elevated ambient CO_2 , PCO_2 gradient is transiently reversed until a new steady-state condition is established by continued production of metabolic CO_2 to resume outward diffusion of CO_2 through the gills. Elevations of blood PCO_2 resulted in a transient but significant drop of pHa, which was subsequently recovered within 3 to 24 h by CO_2 -dependent increases in plasma $[HCO_3^-]$ ($[HCO_3^-]_p$). Importantly, fish died at 5% CO_2 after pHa had already been completely restored to the pre-exposure level within 24 h. Qualitatively, two other fish (*Ser. quinqueradiata* and

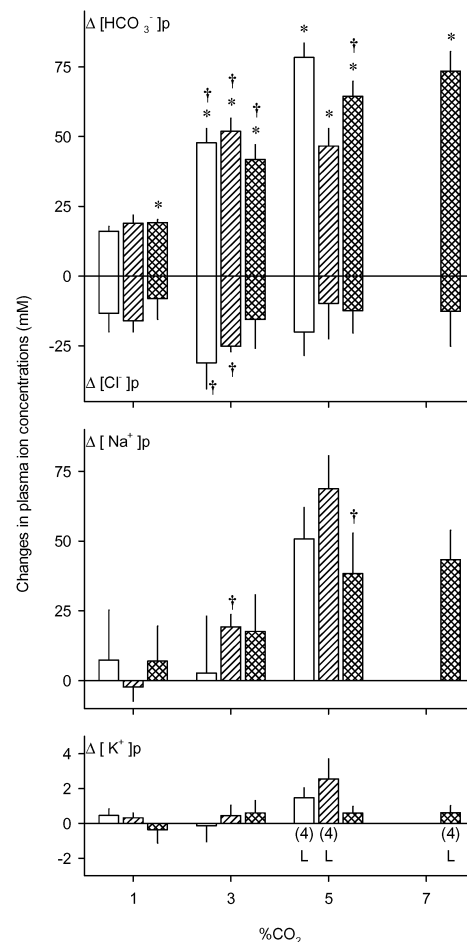


Fig. 5. Changes in plasma ion concentrations in response to environmental hypercapnia for *Paralichthys olivaceus* (open bars, $N = 6$), *Seriola quinqueradiata* (hatched bars, $N = 5$), and *Mustelus manazo* (cross hatched bars, $N = 5$). The data are shown as differences between normocapnic control values and data obtained at the end of each CO_2 exposure (72 h for sublethal exposures and the last sampling time for lethal exposures (L), i.e. 5% for *Par. olivaceus* and *Ser. quinqueradiata*, and 7% for *M. manazo*). Numbers in parentheses indicate the numbers of fish at the last sampling time. Vertical lines indicate SD. Asterisks indicate that absolute changes in $[HCO_3^-]_p$ were significantly different from those in $[Cl^-]_p$ under the same condition ($p < 0.05$; paired t-test). Daggers represent significant differences from the corresponding 1% values ($p < 0.05$; Dunnett test, no comparison from 1% values was applied where partial mortality occurred: from Hayashi *et al.*, 2004).

M. manazo) showed a similar pattern of pHa recovery during CO_2 exposure. However, there were noticeable quantitative differences in acid-base and plasma ion changes during CO_2 exposure among fish as well as among applied CO_2 levels (Table 3). The pHa recovery found in this study is relatively more rapid than those

Table 3. Depression (Δ pHa) and recovery time (R.T.) of pHa during CO₂ exposure.

Experimental fish	Water temp. (°C)	%CO ₂	Δ pHa	R.T. (h)	References
Halibut (SW)	20	1	-0.16 ± 0.06 a	3	Hayashi <i>et al.</i> , 2004
		3	-0.68 ± 0.05 b	8	
		5	-0.80 ± 0.08 c	24	
Amberjack (SW)	20	1	-0.10 ± 0.03 a	1	Hayashi <i>et al.</i> , 2004
		3	-0.27 ± 0.03 d	8	
		5	-0.45 ± 0.10 d	No recovery	
Smooth-hound (SW)	17	1	-0.33 ± 0.03 d	72	Hayashi <i>et al.</i> , 2004
		3	-0.68 ± 0.05 b	72 (-0.14 ± 0.04)	
		5	-0.82 ± 0.04 c	72 (-0.19 ± 0.05)	
		7	-0.96 ± 0.10 d	No recovery	
Conger (<i>Conger conger</i>) (SW)	17	1	-0.40	10	Toews <i>et al.</i> , 1983
Cod (<i>Gadus morhua</i>) (SW)	12	1	-0.20	24	Larsen <i>et al.</i> , 1997
Carp (<i>Cyprinus carpio</i>) (FW)	15	1	-0.22	>456	Claiborne and Heisler, 1986

Recovery time is defined as the time it needed before pHa became insignificantly different from the control, pre-exposure value. Δ pHa values at the same CO₂ levels with same letters are not significantly different from each other ($p < 0.05$; Tukey test). From Hayashi *et al.* (2004).

previously reported, especially compared with the freshwater carp (Table 3).

Figure 5 compares changes in plasma ion concentrations from respective control values during exposure (Δ [ions]p). The data are calculated as the difference between the final values (72 h for sublethal conditions or the last sampling time for lethal conditions) and the corresponding pre-exposure values. As far as water PCO₂ remains at sublethal levels, Δ [HCO₃⁻]p increased in a CO₂-dependent manner for all fish, values being more or less similar at a given CO₂ level. Δ [Cl⁻]p was significantly more negative at 3% than at 1% for the teleosts, whereas Δ [Cl⁻]p was nearly constant irrespective of water CO₂ levels for *M. manazo*. At 1% CO₂, Δ [HCO₃⁻]p was significantly larger than $-\Delta$ [Cl⁻]p only for *M. manazo*, while this was the case at higher CO₂ levels for all fish. Δ [Na⁺]p values were significantly larger at 3% (*Ser. quinqueradiata*) and 5% (*M. manazo*) than corresponding 1% values. There were no significant differences in Δ [K⁺]p. Exposure to lethal levels of hypercapnia resulted in further increases in Δ [HCO₃⁻]p for *Par. olivaceus* and *M. manazo*, but not for *Ser. quinqueradiata*. Δ [Cl⁻]p did not show any further decrease. Δ [Na⁺]p increased substantially for the teleosts but not for *M. manazo*. Δ [K⁺]p increased only in the teleosts during lethal CO₂ exposure.

A net absorption of HCO₃⁻ from and/or an excretion of H⁺ ions to surrounding water across the gills are re-

sponsible for pHa recovery during hypercapnia in both marine teleosts (Toews *et al.*, 1983) and elasmobranchs (sharks and rays, Claiborne and Evans, 1992). The transport of HCO₃⁻ and/or H⁺ across the body surface inevitably accompanies transport of counterions to maintain the electroneutrality of body fluids, but the precise mechanisms of, and the cell types responsible for the transport process have yet to be firmly established, particularly for marine fish. For freshwater fish, the role of H⁺-ATPase coupled with Na⁺ channels for acid-base regulation has gained growing support in recent years, but the role of H⁺-ATPase in marine fish is generally thought to be minor (Claiborne *et al.*, 2002). The current model of acid-base regulation in marine fish emphasizes Na⁺/H⁺ exchange brought about by Na⁺/H⁺ ion exchangers (Claiborne *et al.*, 2002). However, it should be noted that [Cl⁻]p invariably decreases with a nearly equimolar increase in [HCO₃⁻]p under low (1 to 2%) levels of hypercapnia in both freshwater and marine teleosts, with little or no change in [Na⁺]p (Toews *et al.*, 1983; Larsen *et al.*, 1997). This attests to the importance of Cl⁻ ions as the main counterion accompanying acid-base compensation at least at sublethal moderate levels of hypercapnia.

Marine fish actively secrete Na⁺ and Cl⁻ through chloride cells to counterbalance diffusional entry of the ions (Zadunaisky, 1984). We have recently found that Na⁺/K⁺ ATPase activity in the gills increased significantly

upon exposure to 1% and 5% CO₂ and that apical opening area of branchial chloride cells increased significantly upon exposure to 5% CO₂ in *Par. olivaceus* (Hayashi *et al.*, unpublished). Hypercapnia also increased chloride cell size in larval *Sil. japonica* and young *Par. major* (Kikkawa *et al.*, unpublished). In addition, there is some morphological evidence for the involvement of chloride cells in acid-base regulation in freshwater fish. Goss *et al.* (1995) demonstrated that decreases in [Cl⁻]_p during hypercapnic exposure in brown bullhead, *Ictalurus nebulosus*, coincided with a reduction in chloride cell surface area caused by the extension of adjacent pavement cells, thereby limiting active uptake of the ion. Kaneko *et al.* (1999) found a remarkable proliferation of branchial chloride cells in Japanese dace, *Tribolodon hakonensis*, in acid water. We speculate that the lowering of [Cl⁻]_p observed in marine fish during hypercapnia was brought about by stimulated chloride cell activity.

Most previous studies employed only moderate levels of hypercapnia (mostly 1 to 2% CO₂), and few data are available on acid-base responses and plasma ion status under different levels of hypercapnia. No direct comparison has been made on acid-base adjustments among different groups of fish under various CO₂ conditions. At 3% or higher CO₂ conditions, a Na⁺-involving mechanism appears to participate in pH_a recovery as attested by the significant rise in [Na⁺]_p (Fig. 5). Grøttum and Sigholt (1996) also reported a 20 mM rise in [Na⁺]_p during 7% CO₂ exposure for the seabass, *Dicentrarchus labrax*, when [Cl⁻]_p decreased by about 50 mM. Elasmobranch fish appear to employ different acid-base regulatory mechanisms, because responses of plasma sodium and chloride ion concentrations to different levels of hypercapnia differ from those in teleosts (Fig. 5). Previous studies on elasmobranchs reported either no change in [Cl⁻]_p during 1% hypercapnia for a skate, *Raja ocellata* (Graham *et al.*, 1990), and 0.7% for a dogfish, *Scyliorhinus stellaris* (Randall *et al.*, 1976), or a small but significant decrease for a dogfish, *Squalus acanthias* (Cross *et al.*, 1969).

3.2 Cardiovascular function

No Japanese amberjack, *Seriola quinqueradiata*, died at 1% CO₂ until the end of the exposure (72 h), while all fish died within 8 h at 5%. The exposure to 5% CO₂ had deleterious effects on cardiac function (Fig. 6). Cardiac output (Q) and stroke volume (SV) rapidly decreased, while heart rate (HR) was maintained throughout the experiment.

Neither arterial PO₂ (PaO₂) (Fig. 7A) nor oxygen content (CaO₂) (Fig. 7B) changed significantly during exposure to either level of hypercapnia. Hematocrit (Hct) increased significantly only at 5% CO₂ (Fig. 7C). Acid-base responses were essentially the same as reported

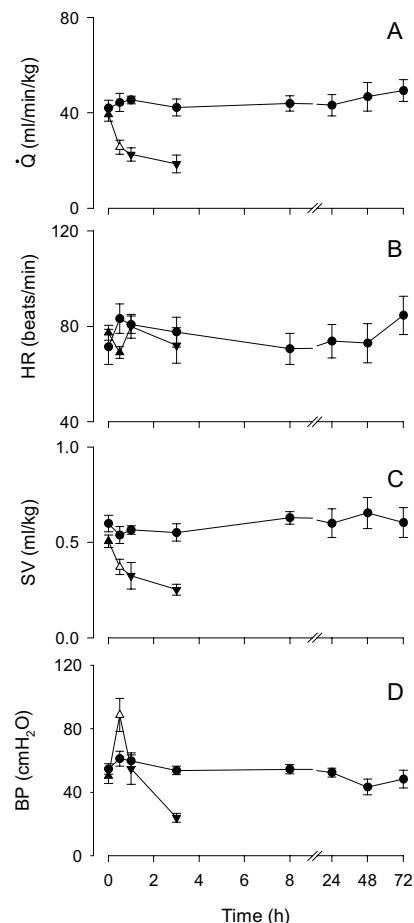


Fig. 6. Time-dependent changes in cardiac output (Q: A), heart rate (HR: B), stroke volume (SV: C) and arterial blood pressure (BP: D) in *Seriola quinqueradiata* exposed to two levels of hypercapnia. Circles represent data at 1% CO₂ (water PCO₂ = 0.9 kPa, N = 5), and triangles data at 5% CO₂ (water PCO₂ = 5.1 kPa, N = 6). Open symbols indicate a significant difference from control values ($P < 0.05$). Downward triangles indicate that N was decreased due to fish death. No statistical analysis was applied to these points. Means \pm SEM. From Lee *et al.* (2003).

above. Although the oxygen affinity of amberjack blood is sensitive to CO₂/pH (Bohr factor -0.74 , Lee *et al.*, 2003), CaO₂ was probably maintained by the release of stored red blood cells into circulation, as suggested by the large increase in hematocrit. The pH_a was only about 0.3 pH unit lower than the pre-exposure level before the fish died at 5% CO₂, making it unlikely to be the cause of CO₂ mortality. In addition, *Par. olivaceus* died during exposure to 5% CO₂, when pH_a had already been restored to the normocapnic level (Fig. 4). From these findings, we tentatively conclude that cardiac failure, and not blood acidosis, is the major physiological perturbation leading

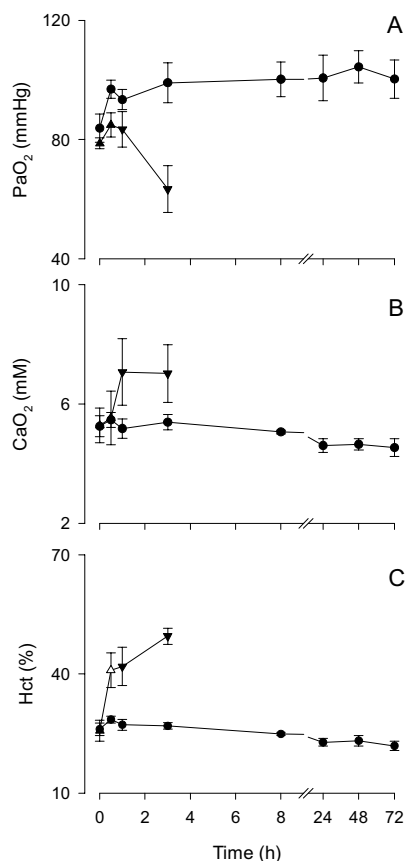


Fig. 7. Time-dependent changes in arterial PO_2 (PaO_2 : A), arterial oxygen content (CaO_2 : B) and hematocrit value (Hct: C) in *Seriola quinqueradiata*. Symbols are the same as in Fig. 6. From Lee *et al.* (2003).

to death of fish subjected to lethal levels of environmental CO_2 pressures.

Hypercapnic acidosis is known to negatively affect contraction of fish cardiac muscle cells (myocardium) *in vitro* (see Farrell and Jones, 1992 for review). The high solubility of CO_2 will rapidly lower intracellular pH of the myocardium, reducing contractility through an antagonism between hydrogen ions and intracellular calcium ion (Gesser and Poupa, 1983). It is conceivable, therefore, that the reduced myocardial contractility is responsible for the observed lowering of stroke volume. As shown above, CO_2 tolerance varied among fishes, i.e. the star-spotted smooth-hound (*M. manazo*) being the most tolerant, followed in turn by *Par. olivaceus* and *Ser. quinqueradiata* (Hayashi *et al.*, 2004). In this context, it may be worth pointing out that a flounder (*Platichthys* (= *Pleuronectes*) *flesus*) is exceptional among fish in that myocardial contractility restores under sustained *in vitro* hypercapnia, as in mammalian myocardium (Gesser and Poupa, 1983). Therefore, the higher tolerance of *Par.*

olivaceus, compared with that of *Ser. quinqueradiata*, might be due to this exceptional capacity of myocardial cells. However, one should be somewhat cautious about extrapolating these *in vitro* findings to *in vivo* conditions because these *in vitro* experiments compared myocardial forces at 2–3% CO_2 and above 10% CO_2 , the former “low” CO_2 level already being far higher than *in vivo* CO_2 levels under normocapnic conditions.

In spite of the established negative inotropic effect (i.e. reduction of contractility) of hypercapnia on fish myocardium *in vitro*, *in vivo* cardiac responses to hypercapnia varied among fishes (see Perry and Gilmour, 2002 for review). Perry *et al.* (1999) reported that rainbow trout (*Oncorhynchus mykiss*) exposed to water PCO_2 of 0.8 and 1.2 kPa for 30 min experienced no change in cardiac output, a 15–26% increase in stroke volume, but a significant drop in heart rate. In contrast, white sturgeon (*Acipenser transmontanus*) exposed to water PCO_2 of 2.6 kPa for 2 h showed a 31% increase in cardiac output, a 41% increase in stroke volume, and a smaller but significant (8%) increase in heart rate (Crocker *et al.*, 2000). McKendry *et al.* (2001) demonstrated that hypercapnia (water PCO_2 0.9 kPa for 20 min) elicited a 30% decrease in cardiac output, and a 64% reduction in heart rate in the Pacific spiny dogfish (*Squalus acanthias*), indicating that stroke volume was increased. McKenzie *et al.* (2002) reported acute cardiorespiratory responses of freshwater eel, *Anguilla anguilla*, to graded levels of CO_2 , and found no significant effect on cardiac output up to water PCO_2 as high as 10.7 kPa; a significant rise in stroke volume at PCO_2 higher than 5.33 kPa accompanied by a corresponding fall in heart rate. Obviously, *in vivo* cardiovascular responses to hypercapnia varies with the severity as well as the duration of hypercapnia imposed on fish, let alone interspecific variability, and probably experimental temperature. Furthermore, the above studies all examined an acute response (commonly shorter than 30 min), and no information is available on the effects of long-term exposure, during which respiratory acidosis is compensated, as described earlier. This may be particularly relevant in considering cardiac function under sustained hypercapnia because the *in vitro* depression of myocardial contractility by hypercapnic acidosis depends on bicarbonate concentration in the bathing medium. Therefore, cardiac output depressed by hypercapnia may be restored as bicarbonate concentration is increased by the acid-base compensation unless hypercapnic stress is so severe that death would ensue in a short time.

In vivo blood pressure responses to hypercapnia are similarly variable. Trout showed a significant increase in the dorsal aortic pressure at water PCO_2 of above 0.47 kPa accompanied by a water PCO_2 -dependent increases in systemic vascular resistance (Perry *et al.*, 1999). Changes in dorsal aortic pressure in white sturgeon were

significant, but only marginal, i.e. from 2.92 ± 0.093 kPa during normocapnia to 3.00 ± 0.11 in 2 h of hypercapnia. Systemic resistance decreased significantly (20%, Crocker *et al.*, 2000). The dogfish showed a small but significant decrease (11%) in dorsal aortic pressure, with no change in systemic resistance (McKendry *et al.*, 2001). The dramatic increase in dorsal aortic pressure during exposure to 5% CO₂ indicate a considerable hypertention of the ventral aorta, although no data are available for the latter. The very high pressure level is likely beyond the range of homeometric regulation, with which stroke volume is maintained over a range of output pressure (Fig. 6, see Farrell and Jones, 1992). In fact, cardiac output began to fall before blood pressure started to rise (Lee *et al.*, 2003). Certainly, more study is needed to understand neural and hormonal cardiovascular regulation during hypercapnia.

4. Future Studies

Environmental factors that should be considered in estimating the lethal effect of CO₂ at depth are low temperature and high pressure. Water temperature in the ocean declines rapidly with depth, and is below 4°C at depths at which CO₂ sequestration is being proposed (Lerman, 1986). Therefore, CO₂ exposure tests should be conducted at low temperatures, or results obtained at higher temperatures should be somehow extrapolated to low temperatures. Another complicating factor is that the taxonomy of bathypelagic and epipelagic fish differs greatly (Weitzman, 1997). Although the susceptibility of bathypelagic fishes to CO₂ is conceivably different from that of epipelagic species, it may be logistically very difficult to use these fishes for experiments, particularly individuals in early developmental stages. However, we have recently found that adults of some deep-sea fish can be captured and kept alive under atmospheric pressure, such as rough snailfish, *Careproctus trachysoma* (Liparididae, distribution: 300–800 m), and Tanaka's eelpout *Lycodes tanakai* (Zoarcidae, distribution: >300 m) in Japan Sea (water temperature 1–2°C below depths of larger than 200 m). We have recently conducted a preliminary CO₂ exposure experiment on *C. trachysoma* using a high pressure chamber (max. pressure 50 MPa) at 5°C. Special care was necessary when handling the fish, but we successfully recorded electrocardiograms from fish exposed to seawater equilibrated with 1% CO₂ at 10 MPa (Ishimatsu *et al.*, unpublished).

Alternatively, the CO₂ susceptibility of deep-sea fishes may be estimated indirectly by using some morphological characteristics that correlate with CO₂ susceptibility. Our recent results suggest that chloride cell activity is stimulated by hypercapnia (Hayashi *et al.*, unpublished). Further, exceptionally CO₂-tolerant larval fish had a high density of chloride cells (Kikkawa *et al.*, unpublished). Thus, if interspecific correlation between

chloride cell morphometry and CO₂-susceptibility is established, then it may be possible to estimate the CO₂ susceptibility of deep-sea fishes without having live materials for experimental evaluation.

Finally, it should be noted that animals would be subjected to fluctuating PCO₂ when CO₂ droplets are released from the lower end of a pipe used for CO₂ ocean sequestration (Sato and Sato, 2002). Our preliminary studies indicated that mortality differed considerably when fish is exposed to unsteady levels of environmental CO₂ conditions, as compared with data obtained using steady CO₂ protocols (Kikkawa *et al.*, unpublished). This should also be included in experimental protocols to assess the effects of CO₂ under realistic conditions of CO₂ ocean sequestration.

Acknowledgements

This study was supported by the New Energy and Industrial Technology Development Organization (NEDO) and the Research Institute of Innovative Technology for the Earth (RITE).

References

- Auerbach, D. I., J. A. Caufield, E. E. Adams and H. J. Herzog (1997): Impacts of ocean CO₂ 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.
- Bernier, N. J. and D. J. Randall (1998): Carbon dioxide anaesthesia in rainbow trout: effects of hypercapnic level and stress on induction and recovery from anaesthetic treatment. *J. Fish Biol.*, **52**, 621–637.
- Burleson, M. L. and N. J. Smatresk (2000): Branchial chemoreceptors mediate ventilatory responses to hypercapnic acidosis in channel catfish. *Comp. Biochem. Physiol.*, **125A**, 403–414.
- Claiborne, J. B. and D. H. Evans (1992): Acid-base balance and ion transfers in the spiny dogfish (*Squalus acanthias*) during hypercapnia: A role for ammonia excretion. *J. Exp. Zool.*, **261**, 9–17.
- Claiborne, J. B. and N. Heisler (1986): Acid-base regulation and ion transfers in the carp (*Cyprinus carpio*): pH compensation during graded long- and short-term environmental hypercapnia, and the effect of bicarbonate infusion. *J. Exp. Biol.*, **126**, 41–61.
- Claiborne, J. B., S. L. Edwards and A. I. Morrison-Shetlar (2002): Acid-base regulation in fishes: cellular and molecular mechanisms. *J. Exp. Zool.*, **293**, 302–319.
- Crocker, C. E., A. P. Farrell, A. K. Gamperl and J. J. Cech, Jr. (2000): Cardiorespiratory responses of white sturgeon to environmental hypercapnia. *Am. J. Physiol.*, **279**, R617–R628.
- Cross, C. E., B. S. Packer, J. M. Linta, H. V. Murdaugh, Jr. and E. D. Robin (1969): H⁺ buffering and excretion in response to acute hypercapnia in the dogfish *Squalus acanthias*. *Am. J. Physiol.*, **216**, 440–452.

- Cruz-Neto, A. P. and J. F. Steffensen (1997): The effects of acute hypoxia and hypercapnia on oxygen consumption of the freshwater European eel. *J. Fish Biol.*, **50**, 759–769.
- Dejours, P. (1988): *Respiration in Water and Air: Adaptations-Regulation-Evolution*. Elsevier, New York, 179 pp.
- Farrell, A. P. and D. R. Jones (1992): The heart. p. 1–73. In *Fish Physiology Vol. XIIA*, ed. by W. S. Hoar, D. J. Randall and A. P. Farrell, Academic Press, San Diego.
- Gesser, H. and O. Poupa (1983): Acidosis and cardiac muscle contractility: comparative aspects. *Comp. Biochem. Physiol.*, **76A**, 559–566.
- Goss, G. G., S. F. Perry and P. Laurent (1995): Ultrastructural and morphometric studies on ion and acid-base transport processes in freshwater fish. p. 257–284. In *Fish Physiology Vol. 14: Cellular and Molecular Approaches to Fish Ionic Regulation*, ed. by C. M. Wood and T. J. Shuttleworth, Academic Press, San Diego.
- Graham, M. S., J. D. Turner and C. M. Wood (1990): Control of ventilation in the hypercapnic skate *Raja ocellata*: I. Blood and extradural fluid. *Respir. Physiol.*, **80**, 259–277.
- Grøttum, J. A. and T. Sigholt (1996): Acute toxicity of carbon dioxide on European seabass (*Dicentrarchus labrax*): Mortality and effects on plasma ions. *Comp. Biochem. Physiol.*, **115A**, 323–327.
- Hayashi, M., J. Kita and A. Ishimatsu (2004): Acid-base responses to lethal aquatic hypercapnia in three marine fish. *Mar. Biol.*, **144**, 153–160.
- Heisler, N. (1986): Acid-base regulation in fishes. p. 309–356. In *Acid-Base Regulation in Animals*, ed. by N. Heisler, Elsevier, Amsterdam.
- Heisler, N. (1993): Acid-base regulation. p. 343–378. In *The Physiology of Fishes*, ed. by D. H. Evans, CRC Press, Boca Raton.
- Ishimatsu, A. and J. Kita (1999): Effects of environmental hypercapnia on fish. *Japan. J. Ichthyol.*, **46**, 1–13.
- Iwama, G. K. and N. Heisler (1991): Effect of environmental water salinity on acid-base regulation during environmental hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.*, **158**, 1–18.
- Kaneko, T., S. Hasegawa, Y. Takagi, M. Tagawa and T. Hirano (1995): Hypoosmoregulatory ability of eyed-stage embryos of chum salmon. *Mar. Biol.*, **122**, 165–170.
- Kaneko, T., S. Hasegawa, K. Uchida, T. Ogasawara, A. Oyagi and T. Hirano (1999): Acid tolerance of Japanese dace (a cyprinid teleost) in lake Osorezan, a remarkable acid lake. *Zool. Sci.*, **16**, 871–877.
- Katoh, F., A. Shimizu, K. Uchida and T. Kaneko (2000): Shift of chloride cell distribution during early life stages in seawater-adapted killifish, *Fundulus heteroclitus*. *Zool. Sci.*, **17**, 11–18.
- Kikkawa, T., A. Ishimatsu and J. Kita (2003): Acute CO₂ tolerance during the early developmental stages of four marine teleosts. *Env. Toxicol.*, **18**, 375–382.
- Kikkawa, T., J. Kita and A. Ishimatsu (2004): Comparison of the lethal effect of CO₂ and acidification on red sea bream (*Pagrus major*) during the early developmental stages. *Mar. Pol. Bull.*, **48**, 108–110.
- Larsen, B. K., H.-O. Pörtner and J. B. Jensen (1997): Extra- and intracellular acid-base balance and ionic regulation in cod (*Gadus morhua*) during combined and isolated exposures to hypercapnia and copper. *Mar. Biol.*, **128**, 337–346.
- Lee, K. S., J. Kita and A. Ishimatsu (2003): Effects of lethal levels of environmental hypercapnia on cardiovascular and blood-gas status in yellowtail, *Seriola quinqueradiata*. *Zool. Sci.*, **20**, 417–422.
- Lerman, M. (1986): *Marine Biology*. The Benjamin/Cummings Publishing Company, California, 534 pp.
- Max, B. (1991): This and that: the neurotoxicity of carbon dioxide. *Trends Pharmacol. Sci.*, **12**, 408–411.
- McKendry, J. E., W. K. Milsom and S. F. Perry (2001): Branchial CO₂ receptors and cardiorespiratory adjustments during hypercarbia in Pacific spiny dogfish (*Squalus acanthias*). *J. Exp. Biol.*, **204**, 1519–1527.
- McKenzie, D. J., E. W. Taylor, A. Z. Dalla Valle and J. F. Steffensen (2002): Tolerance of acute hypercapnic acidosis by the European eel (*Anguilla anguilla*). *J. Comp. Physiol.*, **172B**, 339–346.
- McKim, J. M. (1977): Evaluation of tests with early life stages of fish for predicting long-term toxicity. *J. Fish. Res. Board Can.*, **34**, 1148–1154.
- Mehrbach, C., C. H. Culbertson, J. H. Hawley and J. E. Pytkowicz (1973): Measurement of the apparent dissociation constants of carbonic acid in seawater at atmospheric pressure. *Limnol. Oceanogr.*, **18**, 897–907.
- Oikawa, S., M. Hirata, J. Kita and Y. Itazawa (1999): Ontogeny of respiratory area of a marine teleost, porgy, *Pagrus major*. *Ichthyol. Res.*, **46**, 233–244.
- Oozeki, Y., P.-P. Hwang and R. Hirano (1992): Larval development of the Japanese whiting, *Sillago japonica*. *Japan. J. Ichthyol.*, **39**, 59–669.
- Perry, S. F. and K. M. Gilmour (2002): Sensing and transfer of respiratory gases at the fish gill. *J. Exp. Zool.*, **293**, 249–263.
- Perry, S. F., R. Fritsche, T. M. Hoagland, D. W. Duff and K. R. Olson (1999): The control of blood pressure during external hypercapnia in the rainbow trout (*Oncorhynchus mykiss*). *J. Exp. Biol.*, **202**, 2177–2190.
- Pörtner, H.-O., M. Langenbuch and A. Reipschläger (2004): Biological impact of elevated ocean CO₂ concentrations: Lessons from animal physiology and earth history. *J. Oceanogr.*, **60**, this issue, 705–718.
- Randall, D. J., N. Heisler and F. Drees (1976): Ventilatory responses to hypercapnia in the larger spotted dogfish *Scyliorhinus stellaris*. *Am. J. Physiol.*, **230**, 590–594.
- Roos, A. and W. F. Boron (1981): Intracellular pH. *Physiol. Rev.*, **61**, 296–434.
- Sasai, S., T. Kaneko and K. Tsukamoto (1998): Extrabranchial chloride cells in early life stages of Japanese eel, *Anguilla japonica*. *Ichthyol. Res.*, **45**, 95–98.
- Sato, T. and K. Sato (2002): Numerical prediction of the dilution process and its biological impacts in CO₂ ocean sequestration. *Mar. Sci. Technol.*, **6**, 169–180.
- Shiraishi, K., T. Kaneko, S. Hasegawa and T. Hirano (1997): Development of multicellular complexes of chloride cells in the yolk-sac membrane of tilapia (*Oreochromis mossambicus*) embryos and larvae in seawater. *Cell Tiss. Res.*, **288**, 583–590.
- Takeda, T. and Y. Itazawa (1983): Possibility of applying

- anesthesia by carbon dioxide in the transportation of live fish. *Nippon Suisan Gakkaishi*, **49**, 725–731.
- Toews, D. P., G. F. Holeton and N. Heisler (1983): Regulation of the acid-base status during environmental hypercapnia in the marine teleost fish *Conger conger*. *J. Exp. Biol.*, **107**, 9–20.
- Vandenberg, J. I., J. C. Metcalfe and A. A. Grace (1994): Intracellular pH recovery during respiratory acidosis in perfused hearts. *Am. J. Physiol.*, **266**, C489–C497.
- Weiss, R. F. (1974): Carbon dioxide in water and seawater: the solubility of a non-ideal gas. *Mar. Chem.*, **2**, 203–215.
- Weitzman, S. H. (1997): Systematics of deep-sea fishes. p. 43–78. In *Fish Physiology, Vol. XVI. Deep-Sea Fishes*, ed. by D. J. Randall and A. P. Farrell, Academic Press, San Diego.
- Yoshikawa, H., F. Kawai and M. Kanamori (1994): The relationship between the EEG and brain pH in carp, *Cyprinus carpio*, subjected to environmental hypercapnia at an anesthetic level. *Comp. Biochem. Physiol.*, **107A**, 307–312.
- Zadunaisky, J. A. (1984): The chloride cell: The active transport of chloride and the paracellular pathways. p. 130–176. In *Fish Physiology, Vol. 10B*, ed. by W. S. Hoar and D. J. Randall, Academic Press, Orlando.