**Morphological changes in branchial mitochondria-rich cells of the
teleost *Paralichthys olivaceus* as a potential indicator of CO₂ impacts**

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Abstract

We studied the morphological and biochemical changes of mitochondria-rich cells (MRCs) of a demersal teleost, *Paralichthys olivaceus*, during exposure to 0.98, 2.97 and 4.95 kPa pCO₂. The apical opening area of MRCs increased 2.2 and 4.1 times by 24 hr exposure to 2.97 and 4.95 kPa pCO₂, respectively, while the cross-sectional area or density of MRCs did not change. Gill Na⁺/K⁺-ATPase activity more than doubled at 72 hr and then returned to the pre-exposure level at 168 hr in 0.98 kPa pCO₂, while it increased 1.7 times at 24 hr at 4.95 kPa. These results indicate that the apical opening area of MRCs and the gill Na⁺/K⁺-ATPase activity may be used as an indicator of acute (up to 72 hr), but not chronic, impacts of high (> 1 kPa) seawater CO₂ conditions in *P. olivaceus*. Limitations of those parameters as indices of CO₂ impacts are discussed.

Keywords: carbon dioxide; CO₂ leakage; environmental hypercapnia; mitochondria-rich cell; Na⁺/K⁺-ATPase activity; *Paralichthys olivaceus*

1. Introduction

Geological storage of CO₂ has received greater attention as a potential mitigation measure against global warming, with which CO₂ is injected into different types of geological formations such as deep saline-water saturated formations, depleted oil and gas fields, and un-mineable coals (CSLF, 2011; IPCC, 2005; Pires et al., 2011). Several projects of CO₂ geological storage have already been operating and many more are being planned (CSLF, 2011). The CO₂ is being monitored after injection in the storage sites, but potential leakage of CO₂ and its ecological and environmental consequences have always been a concern against large-scale implementation (Upham and Roberts, 2011), even though the probability of the leakage from the CO₂ stored in a geological formation is considered to be extremely low (IPCC, 2005).

Monitoring CO₂ leakage is more problematic when CO₂ is stored under the seabed, and novel sensors and devices are being developed to improve the precision of detecting changes in seawater CO₂ and pH (Shitashima et al., 2012). Biological monitoring could be used to complement those engineering approaches. Among different marine

47 organisms, fishes have been most extensively investigated with respect to biological
48 responses to environmental perturbations including elevations in water CO₂
49 concentrations, making them candidates for biological monitoring of CO₂ leakage. On
50 the other hand, drawbacks of using fishes for this purpose include the relatively high
51 mobility (particularly pelagic species) and thus possible avoidance from a leakage site
52 and their higher tolerance to CO₂ than most marine invertebrates (Ishimatsu et al., 2005,
53 2008).

54 Fishes have well developed pH-regulating mechanisms, which largely rely upon ion
55 transfer processes across the gills by mitochondria-rich cells (MRCs, Evans et al., 2005;
56 Heisler, 1986; Perry and Gilmour, 2006). When fishes are exposed to environmental
57 hypercapnia (elevated CO₂ conditions), CO₂ will diffuse into the body fluids along
58 pCO₂ gradient mainly through the gills, arterial blood pH decreases rapidly (within
59 minutes) but it then recovers towards pre-exposure levels within several hours (in
60 marine species) or longer (in many freshwater fishes). During the pH recovery, plasma
61 Cl⁻ decreases with an almost equimolar increase of plasma HCO₃⁻ in both seawater and

62 freshwater teleosts (Brauner and Baker, 2009; Fivelstad, 2012; Hayashi et al., 2004;
63 Larsen and Jansen, 1997; Toews et al., 1983; Elasmobranchs appear to employ different
64 acid-base regulatory mechanisms, because plasma Cl^- ion concentrations usually does
65 not or only slightly decrease during pH compensation from hypercapnic acidosis. See
66 Ishimatsu et al., 2005). The mechanisms for ion regulation in fishes have been
67 intensively investigated and are discussed in many extensive review papers (Dymowska
68 et al., 2012; Evans et al., 2005; Hwang et al., 2011; Kaneko et al., 2008). In comparison,
69 relatively few studies examined morphological changes in MRCs in response to CO_2
70 exposure; Cameron and Iwama (1987) found a 30% increase in number and a 75%
71 increase in the apical opening area of MRCs after a 4-day exposure up to 8% CO_2 (7.84
72 kPa pCO_2) in the freshwater channel catfish. In contrast, Goss et al. (1992)
73 demonstrated a 90% reduction in the apical opening area of MRCs and a 50% reduction
74 in cell density, which was in concert responsible for a 95% reduction in MRC fractional
75 surface area (% in a unit epithelial area), in the congeneric brown bullhead exposed to
76 2% CO_2 (1.96 kPa) for 2 days. Goss and Perry (1993) reported a similar, but much less

pronounced decrease in MRC fractional area in hypercapnia rainbow trout (1% CO₂, 4 days). More recently, Baker et al. (2009) reported a significant decrease in MRC apical opening area, density and fractional surface area in white sturgeon exposed to 1.53% CO₂ (1.50 kPa). Thus, there is a possibility that morphological, biochemical and/or molecular characteristics of MRCs are useful indicators of CO₂ impacts on fishes.

The present study was aimed to examine this possibility by investigating the effects of elevated seawater CO₂ levels on the morphology of branchial MRCs and the activity of gill Na⁺/K⁺-ATPase, in the Japanese flounder, *Paralichthys olivaceus*. The Na⁺/K⁺-ATPase is the enzyme that provides an electrochemical gradient that drives ion fluxes through other transport pathways in MRC (Evans, 2005; Hwang et al., 2011) and therefore commonly used as an index of MRC activity.

2. Materials and methods

2.1 Experimental fish

Japanese flounder with mean ($\pm$ S.D.) weight of 116 ± 16 g ($n = 84$) were purchased from two local aquaculture farms, and kept in an indoor fiberglass tank (1,000 l capacity) with filtered, recirculating well-aerated natural seawater at 20°C. Seawater was pumped at 20 m depth from the coast near the Institute for East China Sea Research, Nagasaki University, where all experiments were carried out. The fish would not accept food for extended periods under captivity and therefore were used within 2 weeks after purchase.

2.2 Experimental protocol

The experimental setup consisted of two fish chambers (internal dimension; $475 \times 240 \times 45$ (H) mm), a water reservoir (ca. 50 l capacity) and two gas equilibration columns (90 (internal diameter) $\times 795$ (H) mm). The total volume of the recirculating water was ca. 65 l. Six individuals were placed in each chamber supplied with continuous flow of well-aerated seawater (20°C, salinity 35 ppt) at a rate of 4 l min^{-1} . Fresh seawater was continuously supplied into the reservoir at a rate of $0.5\text{-}1 \text{ l min}^{-1}$ to

avoid buildup of waste substances in water. After 1 day acclimation, the fish were exposed to normocapnia (water equilibrated with air) or different levels of hypercapnia (water equilibrated with a gas mixture containing 1%, 3% or 5% CO₂ in air; 1% CO₂ = 0.98 kPa pCO₂ under the barometric pressure of 101.3 kPa, at 20°C). The gas mixtures (flow rates, 6 l min⁻¹) were prepared with a gas-mixing flowmeter (GF-3/MP, Cameron Instruments, USA). Hayashi et al. (2004) have shown that seawater pH would attain new stable levels within 1 hour of gas bubbling at the same flow rates of gas and at the same recirculation rate of seawater as used in this study. The measured seawater pH values (7.01 at 0.98 kPa pCO₂ (1% CO₂), 6.41 at 2.97 kPa (3% CO₂), and 6.18 at 4.95 kPa (5% CO₂), control seawater pH 8.18) were nearly identical with the value predicted from the pCO₂ of the gas mixture used, temperature, salinity, and alkalinity. Thus, we presumed that nearly complete equilibration was attained between partial pressures of the gas mixtures and seawater in this study. Tissue samples were taken at 0, 24, 72 and 168 hr (except in the 4.95 kPa pCO₂ treatment in which survival rates were 100% at 24 hr but 0% at 72 hr) at each exposure level. No mortality occurred in the other treatments.

Due to the limited number of fish chambers, exposure periods of each fish group were staggered.

2.3 Tissue sampling for morphological observations

The gills on the eyed (top) side were excised under benzocaine anesthesia (ethyl-m-aminobenzoate) at a final concentration of 0.1%. The excised gills were washed rapidly three times in cold saline (0.9% NaCl solution). For scanning-electron microscopy (SEM), the first gill arch was fixed in 4% paraformaldehyde (PFA)-2% glutaraldehyde in 0.1 M phosphate buffer (PB) solution (pH 7.4) for 1 day, then washed overnight in 0.1 M PB (containing 10% sucrose) solution, and preserved in 70% ethanol. Fixation, washing and preservation were all done at 4°C. Fish exposed to the 2.97 kPa pCO₂ were used only for SEM analysis. For confocal laser-scanning microscopy (LSM), the second gill arch was fixed in 4% PFA in 0.1 M PB (pH 7.4) for 1 day at 4°C and treated in the same way as for the SEM observations. For the measurement of gill Na⁺/K⁺-ATPase activity, the third gill arches were stored in 1 ml homogenizing solution

(250 mM sucrose, 6 mM EDTA-2Na and 20 mM imidazole) of pH 6.8 at –80°C until use.

2.4 SEM determination of the apical opening area of MRCs

A portion of the first arch (containing several gill filaments) was severed from the central section of the trisected first gill arches, dehydrated in ethanol, and immersed in 2-methyl-2-propanol. The gill filaments were carefully excised, freeze-dried (JFD-310, JEOL, Japan), mounted on specimen stubs, and coated with gold in an ion sputter (JFC-1200, JEOL, Japan). Then, the apical opening area of MRCs from three randomly selected filaments was determined with a scanning electron microscope (JSM-5310LV, JEOL, Japan) at a magnification of 3500×. Observation sites were two non-contiguous fields in a filament randomly selected from a flat region of the afferent-vascular edge, which lacked secondary gill lamellae. A total of six micrographs per individual was taken and saved as TIFF files. The apical opening area of MRCs was determined using a graphics tablet system (Intuos2 i-420, Wacom, Japan) with an image analyzing software

(Scion Image Beta 4.0.2, Scion Corp, USA). Mean apical opening area (μm^2) of

individual fish ($n = 6$) was obtained from all the apical openings in the six micrographs.

2.5 LSM determination of the cross-sectional area and density of MRCs

Tissues of the second gill arches were processed according to the method of Katoh et

al. (2000), and the cross-sectional area and density of MRCs were observed with a

confocal laser-scanning microscope. For the detection of MRCs in the whole-mount

preparations of the gill filaments, the tissues were immunocytochemically stained with

an antibody specific for Na^+/K^+ -ATPase (Ura et al., 1996). The specific antibody was

affinity-purified and labeled with Alexa fluor 488 (A-11034, Molecular Probes, USA)

as a fluorescent marker. Before the whole-mount immunocytochemistry, a portion of

the second gill arch was severed as described for SEM, and was treated as follows: (1)

washed in distilled water and then in 0.01 M phosphate-buffered saline (PBS, pH 7.2);

(2) incubated overnight at 4°C with anti- Na^+/K^+ -ATPase serum diluted 1:1000 v/v with

T-PBS (PBS containing 0.05% Triton X-100, 10% normal goat serum, 0.1% bovine

167 serum albumin, 0.02% keyhole limpet hemocyanin and 0.01% sodium azide); (3) rinsed
168 in 0.01 M PBS; (4) incubated overnight at 4°C with Alexa fluor diluted 1:1000 v/v with
169 T-PBS; (5) rinsed in 0.01 M PBS; (6) incubated with Propidium iodide (P-3566,
170 Molecular Probes, USA) diluted 1:20 v/v with T-PBS for 20 min at room temperature
171 (for staining nucleic acid); and (7) rinsed in 0.01 M PBS. Subsequently, the gill
172 filaments were incised from a segment, placed in a chamber slide with a cover slip over,
173 and observed with a confocal laser-scanning microscope (LSM 510 META-Ver. 3.0,
174 Carl Zeiss, Germany). The 488 nm line of an argon-ion laser and the 543 nm line of a
175 helium/neon-ion laser were used as the excitation wavelength. Observations were made
176 from three non-contiguous fields randomly selected from the surface of the trailing edge
177 of each of the three excised filaments (nine digital images per individual). The obtained
178 images were saved as above. The cross-sectional area and density of immunopositive
179 MRCs were measured using an image-processing software (HGK-ST, HOGA, Japan).
180 Mean cross-sectional area (μm^2) of individual fish (n = 6) was obtained from all the
181 cells in the nine digital images. For determination of MRC density, an area was

randomly selected from a digital image and all the MRCs present were counted except for the uncompleted cell outlines found at the edge of the predefined boundary. Mean cell density of individual fish ($n = 6$) was obtained from nine digital images and was expressed as cells per mm^2 .

2.6 Measurement of gill Na^+/K^+ -ATPase activity

Na^+/K^+ -ATPase activity was measured as described by Soyano et al. (1988). The stored gill arches were rapidly thawed and several gill filaments were removed. The filaments were placed in a microtube containing fresh homogenizing solution, homogenized on ice, and centrifuged at $12,000 \times g$ for 20 min at 4°C . The supernatant was assayed for Na^+/K^+ -ATPase activity and protein content. The gill homogenate (40 μl) was added into the tubes containing 160 μl of the assay buffer (160 μl ; 250 mM imidazole, 12.5 mM Na_2ATP , 337.5 mM NaCl, 162.5 mM KCl and 50 mM MgCl_2) with or without 2.5 mM ouabain, and incubated at 37°C for 20 min. The reaction was terminated by addition of 4 ml of ice-cold Iron-TCA (100 g l^{-1} TCA, 10 g l^{-1} Thiourea and 30 g l^{-1}

197 Mohr's salt). Finally, 500 µl of a coloring reagent (90 ml l⁻¹ H₂SO₄ and 44 g l⁻¹
198 (NH₄)₆Mo₇O₂₄·4H₂O) was added to the tubes to determine the concentration of
199 inorganic phosphate of the supernatant using the method of Goldenberg and Fernandez
200 (1966). The protein content of the sample was determined with a commercial kit
201 (Bio-Rad Protein Assay, Bio-Rad, USA).

202

203 2.7 Statistics

204 All values are expressed as means ± standard deviation (S.D.). Statistical
205 comparisons among sampling time were made using one-way analysis of variance
206 (ANOVA) followed by Tukey's test (Sigmastat 2.0, Jandel, USA).

207

208

209 3. Results

210 The scanning electron micrographs of gill filaments in the Japanese flounder exposed
211 to hypercapnic seawater are shown in Fig. 1 (A-D, 2.97 kPa pCO₂; E and F, 4.95 kPa).

The apical opening area of MRCs did not change significantly in control (normocapnia) and at 0.98 kPa pCO₂, but increased 2.2 ($p < 0.05$; Tukey's test) and 4.1 ($p < 0.05$; Tukey's test) times by 24 hr exposure to 2.97 and 4.95 kPa pCO₂, respectively (Fig. 2). The area subsequently decreased but still remained significantly high than the 0 hr value by 72 hr at 2.97 kPa (Fig. 2). Neither the cross-sectional area nor density of MRCs changed significantly at any CO₂ treatment until 168 hr of exposure (Fig. 3). Gill Na⁺/K⁺-ATPase activity more than doubled ($p < 0.05$; Tukey's test) at 72 hr and then decreased at 0.98 kPa pCO₂, while it increased 1.7 times ($p < 0.05$; Tukey's test) at 24 hr during exposure to 4.95 kPa pCO₂ (Fig. 4).

4. Discussion

The results demonstrated that the surface morphology of branchial MRCs and the gill Na⁺/K⁺-ATPase responded significantly to the elevations of seawater CO₂ in *Paralichthys olivaceus*. To our knowledge, this is the first demonstration of the MRC

227 morphology in response to environmental hypercapnia in a marine teleost. In
228 comparison, two previous studies reported conflicting results on the effect of
229 environmental hypercapnia on the apical opening area of MRCs in two closely related
230 freshwater teleosts. Namely, Cameron and Iwama (1987) found a 30% increase in
231 number and a 75% increase in the apical opening area of MRCs after a 4-day stepwise
232 exposure up to 8% CO₂ (7.84 kPa pCO₂) in the channel catfish *Ictalurus punctatus*,
233 whereas Goss et al. (1992) demonstrated a 90% reduction in the apical opening area of
234 MRCs in the congeneric brown bullhead *I. nebulosus* exposed to 2% CO₂ (1.96 kPa) for
235 2 days. In agreement with the findings by Goss et al. (1992), Baker et al. (2009) recently
236 reported significant reductions in the apical opening area, density and fractional area (%
237 of a unit epithelial area) of MRCs in the freshwater white sturgeon *Acipenser*
238 *transmontanus* exposed to 1.53% CO₂ (1.50 kPa). Goss and Perry (1993) also reported a
239 similar, but much less pronounced decrease in MRC fractional area in rainbow trout
240 *Oncorhynchus mykiss* exposed to 1% CO₂ for 4 days. Morphological changes in the
241 apical opening area of MRCs can occur rapidly (in response to salinity change) as

242 shown for the mudskipper (*Periophthalmus modestus*) (within 30 min, Sakamoto and
243 Ando, 2002) and the silver sea bream (*Sparus sarba*) (in 6 hr, Kelly and Woo, 1999).
244 Thus, there is a possibility that our 24-hr sampling interval failed to detect rapid MRC
245 changes possibly occurred within 24 hr of hypercapnia.

246 In contrast to the possibly variable morphological responses of the MRCs to elevated
247 ambient CO₂, changes in plasma ion concentration during hypercapnia appear to be
248 highly consistent in teleosts irrespective of environmental salinities (freshwater or
249 seawater): the plasma Cl⁻ concentration decreases in a CO₂-concentration-dependent
250 manner with often equimolar increases in HCO₃⁻, whereas plasma Na⁺ concentration is
251 only marginally affected (Baker et al., 2009; Larsen and Jansen, 1997; Toews et al.,
252 1983; Truchot, 1987) unless ambient pCO₂ increases to lethal levels (Hayashi et al.,
253 2004). These results on plasma ionic changes lend support to the supposition that a
254 Cl⁻-modulating mechanism plays a predominant role in acid-base regulation during
255 hypercapnic pH compensation in both marine and freshwater teleosts, which also agrees
256 with the data from conventional flux studies (freshwater rainbow trout, Perry et al.,

1987; carp, Claiborne and Heisler, 1984). The observed reduction of MRC apical opening area in the freshwater brown bullhead was interpreted to be a mechanism to suppress Cl^- uptake/ HCO_3^- extrusion by restricting the functional surface area with overlying adjacent pavement cells, which would lead to a buildup of HCO_3^- and a decrease in Cl^- in the body fluids (Goss et al., 1992, 1995). If ion fluxes through MRCs are also modulated through an extension/retreat of adjacent pavement cells in a marine teleost, then one would predict that the apical opening areas of MRCs are enlarged when a marine teleost is subjected to an elevation of seawater pCO_2 , because marine teleosts must actively extrude Cl^- (and Na^+) against electrochemical gradients (Evans et al., 2005). The present results agree with this prediction. The HCO_3^- substitution study by Esbaugh et al. (2012) indicated that the seawater concentration of HCO_3^- ions significantly influenced pH recovery of fish under hypercapnia, again suggesting a predominant role of the $\text{Cl}^-/\text{HCO}_3^-$ exchange mechanism in acid-base regulation in marine teleosts. On the other hand, Claiborne et al. (2002) proposed that enhanced acid secretion during acidosis in marine teleosts was mostly due to Na^+/H^+ exchangers in the

272 MRCs and that apically located $\text{Cl}^-/\text{HCO}_3^-$ exchangers may be responsible for base
273 excretion, on the basis of cellular and molecular data. The proposed predominance of
274 Na^+/H^+ exchangers in acid-base regulation in marine teleosts is however in conflict with
275 the plasma ion data described above, and the apparent discrepancy between the
276 mechanisms inferred from plasma ion and flux data and from molecular localization of
277 transporters proteins must be resolved by further investigations.

278 The current model for the extrusion of NaCl by MRCs in marine teleosts include
279 secondary active transport of Cl^- through Cl^- channels residing in the apical membrane
280 driven by basolateral Na^+/K^+ ATPase, and passive Na^+ transport through the leaky tight
281 junctions between the MRCs and adjacent accessory cells (see Evans et al., 2005,
282 Hwang et al., 2011, and Marshall and Grosell, 2006, for review). Although Na^+ and Cl^-
283 ions are generally considered to be transported in 1:1 ratio to maintain electroneutrality
284 of the body fluids, the strong ion difference theory predicts that an excess extrusion of
285 anions over cations should cause alkalization of the fluid, without any “actual”
286 movement of acid-base relevant ions (such as H^+ and HCO_3^-) across the body surface

287 (Stewart, 1981). Therefore, it is theoretically possible for the MRCs to restore acid-base
288 disturbance of marine teleosts by extruding Na^+ and Cl^- at different rates.

289 Effects of environmental conditions on the gill MRC morphology in freshwater
290 teleosts have been extensively investigated (see Goss et al., 1995). For example, both
291 proliferation of MRCs and increased apical opening area have been reported in rainbow
292 trout exposed to ion-poor water (Greco et al., 1996) or hyperoxia (Goss et al., 1994), in
293 rainbow trout injected with NaHCO_3 or HCl to induce metabolic alkalosis or acidosis
294 (Goss et al., 1994), in parr and smolt Atlantic salmon *Salmo salar* exposed to cadmium
295 ions (Devos et al., 1998), and in snakehead *Channa punctata* exposed to a mixture of
296 four trace metals (Cu, Cd, Fe and Ni, Pandey et al., 2008). Also, a fourfold increase in
297 the fractional surface area of branchial MRC was observed when rainbow trout was
298 exposed to alkaline water (pH 9.5) for 3 days (Wilkie and Wood, 1994). Goss et al.
299 (1995) summarized effects of acid and alkaline water pH on MRC morphology of
300 freshwater fishes. In comparison, effects of environmental conditions on MRC
301 morphology are only poorly known for marine fishes.

302 Table 1 summarizes the data on morphological, biochemical and molecular responses
303 in the gills of marine teleosts in response to environmental hypercapnia. Apparently, no
304 clear pattern emerges from this data set. For example, the activity of Na^+/K^+ ATPase
305 remained elevated for 42 days during hypercapnia exposure to 1.0 kPa pCO_2 in *Zoarces*
306 *viviparus* (Deigweiher et al., 2008). Similarly, the activity and protein abundance of
307 Na^+/K^+ ATPase were found significantly higher than control levels when *Gadus morhua*
308 was exposed to 0.6 kPa for 12 months (but no change under 3 kPa after 4 months,
309 Melzner et al., 2009). The Na^+/K^+ ATPase activity transiently increased or decreased in
310 *Paralichthys olivaceus* (this study) and *Opsanus beta* (Esbaugh et al., 2012),
311 respectively, and the activity was found to be depressed after 2 days of CO_2 exposure in
312 *Porichthys notatus* (Perry et al., 2010). Carbonic anhydrase showed opposite changes in
313 mRNA expression between *Opsanus beta* (a decrease, Esbaugh et al., 2012) and
314 *Porichthys notatus* (an increase, Perry et al., 2010). With respect to those proteins
315 related to Cl^- fluxes, SLC4A2 decreased in mRNA expression, while SLC26A3 or
316 SLC26A6 did not show any change in *Opsanus beta* (Esbaugh et al., 2012). Similarly,

317 mRNA expression of zoarcid $\text{Cl}^-/\text{HCO}_3^-$ exchanger did not change in CO_2 -exposed *Z.*
318 *viviparus* (Deigweiher et al., 2008). These studies employed different exposure
319 protocols with the seawater pCO_2 ranging from 0.2 to 5.2 kPa, the exposure period from
320 only 1 hr to 12 months, and various sampling schedules. Therefore comparison of these
321 data cannot be made with certainty.

322 As shown in Table 1, some parameters changed only transiently in response to high
323 CO_2 exposure, and subsequently returned to pre-exposure, normocapnic levels. These
324 time-dependent variables may not be very useful as biological indices of CO_2 impacts
325 because the power of detection depends on the time between the onset of a hypercapnic
326 period and sampling, and because temporal patterns of responses are likely to vary with
327 test species, CO_2 levels, and other biotic and abiotic factors. Rather, it would be ideal if
328 a parameter remains deviated from its baseline level as long as environmental CO_2
329 levels are elevated or even after the ambient CO_2 concentration has returned to normal
330 levels of a locality. The opening area of the gill MRCs and the activity of branchial
331 Na^+/K^+ ATPase in *P. olivaceus* were both affected significantly within 72 hr but then

332 returned to pre-exposure levels (Figs. 2 and 4), which limits the usefulness of those
333 parameters as indices of CO₂ impact. Further, consideration must also be given to the
334 detection limits of environmental CO₂ by those biological indices. The threshold levels
335 at which these parameters showed significant changes were 3 and 1 kPa for apical
336 opening area (Fig. 2) and Na⁺/K⁺ ATPase activity (Fig. 4), respectively. At 1 kPa,
337 seawater pH dropped to 7.0, a drop of 1.2 pH units, which is easily detectable by pH
338 sensors (Shitashima et al., 2012). Nevertheless, considering the ecological and
339 economical importance of fishes, careful investigations must be continued to examine
340 effects of CO₂ on marine fishes, particularly using long-term (months or longer)
341 exposure protocols with pCO₂ levels lower than those used in the present study.
342 Tentatively, the present data would rank both of the parameters we studied as relatively
343 poor indicators of CO₂ impacts, but given the highly variable nature of biological
344 responses to CO₂ (Kroeker et al., 2010), more fish species must be tested for their
345 responses of MRC morphology and enzymatic activity before we can arrive at a solid
346 conclusion about the usefulness of MRC morphology and Na⁺/K⁺ ATPase activity as

347 indicators of CO₂ impact.

348 In the present study, we chose *P. olivaceu* because the fish was easily available from
349 aquaculture farms but also inhabits the depth range down to 200 m, the depth at which
350 subsea CCS may be implemented in the continental shelves. The fish is demersal and
351 less mobile than pelagic fish species (Kawabe et al., 2004), and may therefore be more
352 prone to be impacted by CO₂ leakage from a CCS site. Although demersal, inactive
353 fishes such as *P. olivaceus* could be useful model animals for monitoring CO₂
354 conditions around an injection site, it would be desirable also to explore suitable indices
355 of CO₂ impacts in sessile or poorly mobile invertebrates. Although biological
356 information is much less available than for fishes, marine invertebrates, particularly
357 those forming calcareous shells and skeletons (many of them have much less mobility
358 than fishes), are considered to be more vulnerable to CO₂ than fishes (Hofmann et al.,
359 2010). Some marine invertebrates such as sea urchins have been preferably used as
360 models in some fields of marine biology, which might facilitate search for suitable
361 biological indicators of CO₂ impacts (Ernst 1997; Lawrence, 2007). In addition, a rapid

expansion of ocean acidification research in recent years is predictive of the
identification of reliable biomarkers for CO₂ impacts in marine fishes and invertebrates
in the near future (Hardege et al., 2011).

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518

Figure Legends

Fig. 1 Scanning electron micrographs of gill filaments in the Japanese flounder exposed to hypercapnic seawater (2.97 kPa (A-D) and 4.95 kPa (E, F) pCO₂) at 0 (A, E), 24 (B, F), 72 (C) and 168 hr (D). Arrowheads, apical opening of MRCs. pc, pavement cell. Scale bars, 5 µm.

Fig. 2 The apical opening area of mitochondria-rich cells (MRCs) in the Japanese flounder exposed to normocapnic (white bars) and hypercapnic (0.98 kPa (hatched bars), 2.97 kPa (cross-hatched bars) and 4.95 kPa (black bars) pCO₂) seawater. Data are expressed as means ± S.D. (n = 6). Data at the same CO₂ levels with same letters are not significantly different from each other (p < 0.05; Tukey's test).

Fig. 3 The cross-sectional area (A) and cell density (B) of MRCs in the Japanese flounder exposed to normocapnic (white bars) and hypercapnic (0.98 kPa (hatched bars) and 4.95 kPa (black bars) pCO₂) seawater. Data are expressed as means ± S.D. (n = 6).

534

535 **Fig. 4** Na^+/K^+ -ATPase activity in the gills of the Japanese flounder exposed to
536 normocapnic (white bars) and hypercapnic (0.98 kPa (hatched bars) and 4.95 kPa (black
537 bars) pCO_2) seawater. Data are expressed as means $\pm$ S.D. (n = 6). Symbols are the
538 same as in Fig. 2.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Table 1

Morphological, biochemical, and molecular changes in the gills reported in response to hypercapnic exposure in marine teleosts

Species	Responses	pCO ₂ (kPa)	Time	Ref
<i>Dicentrarchus labrax</i>	Na ⁺ /H ⁺ exchanger (NHE1): mRNA expression decreased at 5.2 kPa (1 hr) but no change at 3.5 kPa (96 hr)	3.5/5.2*	96/1 hr	(1)
<i>Gadus morhua</i>	Na ⁺ /K ⁺ ATPase: activity and protein abundance both increased at 0.6 kPa after 12 months but not at 0.3 kPa after 4 months	0.3/0.6	4/12 months	(2)
<i>Opsanus beta</i>	Na ⁺ /K ⁺ ATPase: a transient drop in activity at 24 hr Carbonic anhydrase: mRNA expression depressed V-type H ⁺ -ATPase: a non-significant drop in activity at 24 hr SLC4A2: mRNA expression decreased at 8 and 72 hr SLC26A3, SLC26A6: mRNA expression did not change Na ⁺ /HCO ₃ ⁻ cotransporter: mRNA expression did not change	0.2	3 days	(3)
<i>Paralichthys olivaceus</i>	Na ⁺ /K ⁺ ATPase: a transient increase in activity above 1.0 kPa MRC apical opening area: a transient increase above 2.9 kPa MRC cross sectional area: no change MRC density: no change	< 4.9	7 days	(4)
<i>Porichthys notatus</i>	Na ⁺ /K ⁺ ATPase: no change in activity Carbonic anhydrase: activity and protein expression both increased	5.0	2 days	(5)
<i>Zoarcetes viviparus</i>	Na ⁺ /K ⁺ ATPase: mRNA expression, protein abundance, and activity all increased NHE1: a transient drop in mRNA expression with nearly no change in protein abundance Cl ⁻ /HCO ₃ ⁻ exchanger: no change in mRNA expression	1.0	42 days	(6)

$\text{Na}^+/\text{HCO}_3^-$ cotransporter: mRNA expression increased only after 42 days

(1) Rimoldi et al., 2009; (2) Melzner et al., 2009; (3) Esbaugh et al., 2012; (4) this study; (5) Perry et al., 2010; (6) Deigweier et al., 2008. *estimated from the given concentration of dissolved CO_2 and temperature. SLC4 and SLC26 families are various types of anion exchangers (see Romero et al., 2004 and Mount and Romero, 2004 for details)