

# Acid–Base Balance of Hemolymph in Pacific oyster *Crassostrea gigas* in Normoxic Conditions

Takeshi Handa<sup>1†</sup>, Akira Araki<sup>1</sup>, Koji Kawana<sup>2</sup> and Ken-ichi Yamamoto<sup>1</sup>

**Abstract** : We examined hemolymph O<sub>2</sub> partial pressure (P<sub>O<sub>2</sub></sub>), pH, total CO<sub>2</sub> content (Tco<sub>2</sub>), CO<sub>2</sub> partial pressure (Pco<sub>2</sub>), and bicarbonate concentration ([HCO<sub>3</sub><sup>-</sup>]) in order to evaluate the ability of the acid–base balance of the Pacific oyster *Crassostrea gigas* in normoxic conditions. Hemolymph was collected anaerobically through a cannula inserted into the adductor muscle of Pacific oyster submerged in experimental seawater. The mean values of hemolymph P<sub>O<sub>2</sub></sub>, pH, and Tco<sub>2</sub> were 62.0 torr, 7.414, and 1.87 mM/l, respectively. The apparent dissociation constant of carbonic acid (pK<sub>app</sub>) was estimated using the following equation: pK<sub>app</sub> = 33.462 – 13.032 • pH + 2.065 • pH<sup>2</sup> – 0.1088 • pH<sup>3</sup>. Using aco<sub>2</sub> (40.51 μM/l/torr) and pK<sub>app</sub> determined in this study, the hemolymph Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] were calculated as 2.18 torr and 1.78 mM/l, respectively. The non-bicarbonate buffer value (β<sub>NB</sub>) was 0.732 Slykes. These hemolymph properties were compared with those of other marine bivalves. The Pacific oyster should have an acid–base balance that is similar to Pectinidae and Pteriidae bivalves, but which is different from Mytilidae bivalves.

**Key words** : *Crassostrea gigas*, hemolymph, acid–base balance, normoxia, cannulation, adductor muscle

## Introduction

Pacific oyster *Crassostrea gigas* inhabits the intertidal and subtidal gravel to mud bottom of brackish-water embayments and often forming oyster reefs.<sup>1)</sup> Pacific oyster is an important cultured species, and worldwide production amounted to 662,513 tons in 2010.<sup>2)</sup> In Japan, the production volume in 2016 was 106,111 tons in Hiroshima, 19,366 tons in Okayama and 11,581 tons in Miyagi prefectures.<sup>3)</sup> Pacific oyster has been a subject of a previous study in terms of anatomy and respiratory physiology. The anatomical structures of the digestive diverticula, ctenidium and circulatory system were clarified recently.<sup>4,5)</sup> The regulation of the ventilation volume, oxygen uptake, and ciliary movement of the ctenidium in normoxic, hypoxic, hypotonic, anathermal, and feeding conditions have been studied.<sup>6–10)</sup> However, there are few reports on the respiratory mechanism from the viewpoint of CO<sub>2</sub> dynamic phase and acid–base

balance in Pacific oyster *C. gigas*. Handa et al. (2017A) developed surgical procedures, cannulation of the adductor muscle of Pacific oyster, and examined the hemolymph oxygen and acid–base status postoperation.<sup>11)</sup> The Pacific oyster temporarily showed slight hypoxemia without acidosis just after surgery, but the fluctuation disappeared 1 h after surgery. In this study, we elucidated the hemolymph acid–base balance of Pacific oyster in normoxic conditions. Research into the acid–base balance could contribute to efficient CO<sub>2</sub> utilization, which is related to respiration and calcification for the formation of the shell valves. The acid–base balance and CO<sub>2</sub> dynamic phase of Pacific oyster is useful for evaluation of cultivation environments, and of the effects of ocean acidification and increases in CO<sub>2</sub> level. In some marine bivalves in normoxic and normocapnic conditions, the CO<sub>2</sub> partial pressure (Pco<sub>2</sub>) of the hemolymph was 0.57–2.3 torr.<sup>12–18)</sup> The hemolymph Pco<sub>2</sub> of Pacific oyster was supposed to be low as in other bivalves; therefore,

Affiliation: 1 Department of Applied Aquabiology, National Fisheries University, Nagata-honmachi, Shimonoseki City, Yamaguchi Pref., JAPAN

2 Hiroshima Yanmar Co., Ltd., Motoujina-machi, Minami Ward, Hiroshima City, Hiroshima Pref., JAPAN

† Corresponding author: handat@fish-u.ac.jp (T. HANDA)

direct measurement of  $P_{\text{CO}_2}$  would be difficult. The estimation  $\text{CO}_2$  partial pressure by application of the Henderson–Hasselbalch equation is practiced in studies of acid–base balance owing to the relative ease and accuracy of such estimates.<sup>19)</sup> In the equation, the characteristic values of the  $\text{CO}_2$  solubility coefficient ( $a_{\text{CO}_2}$ ) and apparent dissociation constant of carbonic acid ( $\text{pK}_{\text{app}}$ ) in the hemolymph are required for the experimental animal. Therefore, we determined hemolymph  $a_{\text{CO}_2}$  and  $\text{pK}_{\text{app}}$  of Pacific oyster, and evaluated acid–base balance of hemolymph in normoxic conditions.

## Materials and Methods

### *Experimental animals and conditions*

The experiments used 54 Pacific oysters *Crassostrea gigas* (shell length:  $60.3 \pm 2.2$  mm (mean  $\pm$  SE), shell height:  $120.2 \pm 6.1$  mm, total wet weight:  $112.8 \pm 7.6$  g). The animals were obtained from a marine farm in the western sea area of Hiroshima Prefecture, Japan. After cleaning the shell valves, they were reared for 1 month at  $23^\circ\text{C}$  in aerated seawater with added cultivated phytoplankton.<sup>6,9,20)</sup> Twenty-four hours before collecting hemolymph, the Pacific oysters were transferred to particle-free ( $>0.45 \mu\text{m}$ ) seawater. All experiments were conducted in seawater with a salinity of 32 psu, water temperature  $23^\circ\text{C}$ ,  $\text{O}_2$  saturation 98%, pH 8.19, and total  $\text{CO}_2$  content 1.4 mM/l.

### *Surgical procedures and hemolymph collection*

Hemolymph was collected from the adductor muscle using a cannula (polyethylene tubing, 0.96 mm outer diameter, 0.58 mm inner diameter, PE-50, Clay Adams).<sup>11)</sup> A small hole (2 mm diameter) was made on adjacent shell valves, which was at the center of the posterior margin. The cannula with a stylet was inserted through the hole into the adductor muscle and was advanced 5 mm toward the center of the adductor muscle. The stylet was removed, and the outside of the cannula was closed. The cannula was gently fixed to the left shell valve using denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent effects from movement of the shell valves. This surgical operation was completed

within 7 minutes. The cannulated oyster was transferred to a darkened acrylic respiratory chamber and was allowed to recover for 2 hr at  $23.0 \pm 0.2^\circ\text{C}$  in normoxic conditions. A hemolymph sample was then drawn through the cannula using a gas-tight micro syringe (Model 1750, Hamilton Co.). The volume of hemolymph collected was 0.5 ml.

### *Hemolymph analysis*

The hemolymph oxygen partial pressure ( $P_{\text{O}_2}$ , torr), pH, and total  $\text{CO}_2$  content ( $T_{\text{CO}_2}$ , mM/l) were measured immediately after each collection.  $P_{\text{O}_2}$  was measured using a blood gas meter (BGM200, Cameron Instruments) and  $P_{\text{O}_2}$  electrode (E101, Cameron Instruments). The pH was measured using the blood gas meter with pH glass and reference electrodes (E301, E351, Cameron Instruments). The  $P_{\text{O}_2}$  and pH electrodes were installed in a water jacket maintained at  $23.0^\circ\text{C}$ .  $T_{\text{CO}_2}$  was measured using a total  $\text{CO}_2$  analyzer (Capnicon 5, Cameron Instruments). The hemolymph  $\text{CO}_2$  partial pressure ( $P_{\text{CO}_2}$ , torr) and bicarbonate concentration ( $[\text{HCO}_3^-]$ , mM/l) were calculated by rearranging the Henderson–Hasselbalch equation.<sup>19,21)</sup> In the equation, the  $\text{CO}_2$  solubility coefficient ( $a_{\text{CO}_2}$ ,  $\mu\text{M}/\text{l}/\text{torr}$ ) and apparent dissociation constant of carbonic acid ( $\text{pK}_{\text{app}}$ ) of Pacific oysters were required. The determinations of  $a_{\text{CO}_2}$  and  $\text{pK}_{\text{app}}$  were performed by *in vitro* experiments.

The  $a_{\text{CO}_2}$  was determined using hemolymph, which was adjusted to pH 2.5 by the addition of lactic acid (Wako Pure Chemical Industries, Ltd.). The acidified sample was transferred to a tonometer flask, and equilibrated with humidified standard  $\text{CO}_2$  gas ( $\text{CO}_2$ , 15.0%;  $\text{O}_2$ , 20.9%;  $\text{N}_2$  Balance) using an equilibrator (DEQ-1, Cameron Instruments) at  $23.0^\circ\text{C}$ , and subsequently the  $T_{\text{CO}_2}$  of each equilibrated sample was measured using a total  $\text{CO}_2$  analyzer. The  $P_{\text{CO}_2}$  of the equilibrated sample was calculated from known  $\text{CO}_2$  concentration standard gas (15.0%), prevailing barometric pressure, and water vapor pressure at  $23.0^\circ\text{C}$ . The  $a_{\text{CO}_2}$  was calculated using the equation:

$$a_{\text{CO}_2} = T_{\text{CO}_2} \cdot P_{\text{CO}_2}^{-1}$$

For determination of the pKapp, the hemolymph sample was transferred to a tonometer flask and equilibrated with humidified standard CO<sub>2</sub> gases (CO<sub>2</sub>, 0.1, 0.2, 0.5, 1.0, 2.0 and 5.0%; O<sub>2</sub>, 20.9%; N<sub>2</sub> Balance) using an equilibrator at 23.0°C. After equilibration, the pH and Tco<sub>2</sub> of the sample were measured using the blood gas meter and total CO<sub>2</sub> analyzer. Using the sample pH, Tco<sub>2</sub>, and aco<sub>2</sub> calculated from the above equation, and pKapp was determined by rearrangement of Henderson–Hasselbalch equation<sup>19,21)</sup> as follows:

$$\text{pKapp} = \text{pH} - \log [(T\text{co}_2 - a\text{co}_2 \cdot \text{Pco}_2) \cdot (a\text{co}_2 \cdot \text{Pco}_2)^{-1}]$$

where Pco<sub>2</sub> was calculated from known CO<sub>2</sub> concentration standard gases. The aco<sub>2</sub> and pKapp obtained in this study were used for calculation of hemolymph Pco<sub>2</sub> from measured pH and Tco<sub>2</sub>:

$$\text{Pco}_2 = T\text{co}_2 \cdot [a\text{co}_2 \cdot (1 + 10^{(\text{pH}-\text{pKapp})})]^{-1}$$

[HCO<sub>3</sub><sup>-</sup>] was calculated from Tco<sub>2</sub>, aco<sub>2</sub> and Pco<sub>2</sub> using the equation:

$$[\text{HCO}_3^-] = T\text{co}_2 - a\text{co}_2 \cdot \text{Pco}_2$$

### Statistical analysis

All data are expressed as means ± standard error. Normality of distribution in hemolymph properties was assessed through use of the Shapiro–Wilk test. The homoscedasticity was assessed using Bartlett's test or F-test. Kruskal–Wallis test was performed for changes in hemolymph properties using the standard gases. The comparison of two parameters used unpaired *t*-test in normal distribution and homoscedasticity. Statistically significant differences were set at *P*<0.05.

## Results

Hemolymphs were collected anaerobically from the adductor muscles of Pacific oysters through cannulae. The mean values of hemolymph Po<sub>2</sub>, pH, and Tco<sub>2</sub> in

normoxic conditions were 62.0 torr, 7.414, and 1.87 mM/l, respectively (Table 1). The hemolymph aco<sub>2</sub> was 40.51 ± 0.24 μM/l/torr. The hemolymph pKapp at known CO<sub>2</sub> partial pressures (standard gases) and the corresponding measured pH and Tco<sub>2</sub> values are shown in Table 2. The calculated pKapp from all hemolymph samples was 6.07343 ± 0.01756. Hemolymph Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] were calculated by substitution of the mean value of aco<sub>2</sub> and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

$$\text{Pco}_2 = T\text{co}_2 \cdot [0.04051 \cdot (1 + 10^{(\text{pH}-6.07343)})]^{-1}$$

$$[\text{HCO}_3^-] = T\text{co}_2 - 0.04051 \cdot \text{Pco}_2$$

where the units of the parameters in the equations are torr for Pco<sub>2</sub> and mM/l for Tco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>]. Hemolymph Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] at 23°C in normoxic conditions were 2.18 torr and 1.78 mM/l, respectively (Table 3). In *in vitro* experiments (Table 2), the changes in pH, Tco<sub>2</sub>, and pKapp were statistically significant with the increase in Pco<sub>2</sub> (*P*<0.05). At the same time, the interaction between pKapp and pH was analyzed (Fig. 1), and the correction equation for pKapp was obtained as follows:

$$\text{pKapp} = 33.462 - 13.032 \cdot \text{pH} + 2.065 \cdot \text{pH}^2 - 0.1088 \cdot \text{pH}^3$$

For comparison, Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] were estimated using the mean value of pKapp and the correction equation. There was no significant difference in hemolymph Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] calculated by the two methods (Table 4). The mean values of pH and [HCO<sub>3</sub><sup>-</sup>] in *in vitro* experiments and the non-bicarbonate buffer value (β<sub>NB</sub>), which was obtained as the regression coefficient relating pH and [HCO<sub>3</sub><sup>-</sup>], was 0.732 Slykes (Table 5).

## Discussion

We collected Pacific oyster hemolymph from the adductor muscle, and examined hemolymph Po<sub>2</sub>, pH, Tco<sub>2</sub>, Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] in order to evaluate the acid–base

balance of Pacific oyster in normoxic conditions. The hemolymph was collected anaerobically through a cannula from submerged experimental animals after pretreatment by adductor muscle catheterization. The

hemolymph  $P_{O_2}$  in this study was  $62.0 \pm 6.86$  torr at  $23^\circ\text{C}$  (Table 1). Allen and Burnett (2008) and Tran et al. (2008) reported Pacific oyster hemolymph  $P_{O_2}$  in the adductor muscle by direct puncture as  $7.09 \pm 0.53$  kPa ( $53.17 \pm 3.98$

**Table 1.** Hemolymph oxygen partial pressure ( $P_{O_2}$ ), pH and total  $\text{CO}_2$  content ( $T_{\text{CO}_2}$ ) of the Pacific oyster *Crassostrea gigas* in normoxic condition

		Mean	SE	N
$P_{O_2}$	torr	62.0	6.86	10
pH		7.414	0.0592	10
$T_{\text{CO}_2}$	mM/l	1.87	0.104	10

Water temperature.  $23.0 \pm 0.2^\circ\text{C}$  (Mean  $\pm$  SE)

**Table 2.** Mean values of measured pH, total  $\text{CO}_2$  content ( $T_{\text{CO}_2}$ ) and calculated apparent dissociation constant of carbonic acid ( $\text{pK}_{\text{app}}$ ) of the hemolymph in adductor muscle of the Pacific oyster *Crassostrea gigas* with known  $P_{\text{CO}_2}$  standard gases

Standard gas		Hemolymph			
$\text{CO}_2$ (%)	$P_{\text{CO}_2}$ (torr)	pH	$T_{\text{CO}_2}$ (mM/l)	$\text{pK}_{\text{app}}$	N
0.102	0.758	7.476	1.05	5.98178197	8
0.203	1.509	7.278	1.13	6.04599521	8
0.515	3.831	7.031	1.48	6.10528476	8
1.01	7.519	6.804	1.84	6.10272056	8
2.00	14.860	6.579	2.31	6.12683108	6
5.00	37.190	6.202	3.38	6.10923144	6

Barometric pressure, 764.1 torr; water temperature,  $23.0^\circ\text{C}$ ;  $a_{\text{CO}_2}$ ,  $40.51 \mu\text{M/l/torr}$

**Table 3.** Hemolymph  $\text{CO}_2$  partial pressure ( $P_{\text{CO}_2}$ ) and bicarbonate concentration ( $[\text{HCO}_3^-]$ ) of the Pacific oyster *Crassostrea gigas* in normoxic condition

		Mean	SE	N
$P_{\text{CO}_2}$	torr	2.18	0.314	10
$[\text{HCO}_3^-]$	mM/l	1.78	0.097	10

Water temperature.  $23.0 \pm 0.2^\circ\text{C}$  (Mean  $\pm$  SE)

**Table 4.** The comparison of the values calculated by the correction equation and by the mean pKapp in hemolymph Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>]

	Pco <sub>2</sub> (torr)	[HCO <sub>3</sub> <sup>-</sup> ] (mM/l)	N
the mean value of pKapp	2.18 ± 0.314	1.78 ± 0.097	10
pKapp calculated by the correction equation	1.95 ± 0.346	1.79 ± 0.097	10

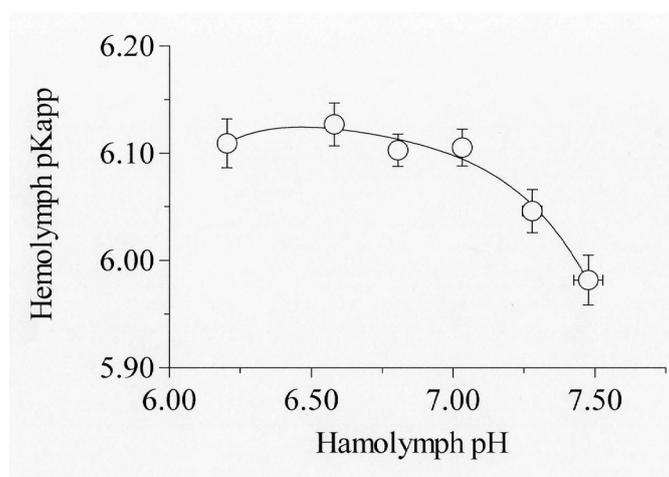
Data are shown mean ± SE. No statistically significant difference (unpaired *t*-test, *P*>0.05)

**Table 5.** Mean values of measured pH and calculated bicarbonate concentration ([HCO<sub>3</sub><sup>-</sup>]) of the hemolymph in adductor muscle of with known Pco<sub>2</sub> standard gases

Standard gas		Hemolymph		
CO <sub>2</sub> (%)	Pco <sub>2</sub> (torr)	pH	[HCO <sub>3</sub> <sup>-</sup> ] (mM/l)	N
0.102	0.758	7.476	1.023	8
0.203	1.509	7.278	1.071	8
0.515	3.831	7.031	1.323	8
1.01	7.519	6.804	1.538	8
2.00	14.860	6.579	1.711	6
5.00	37.190	6.202	1.880	6

Barometric pressure, 764.1 torr ; water temperature, 23.0 °C.

Non-bicarbonate buffer value ( $\beta_{NB}$ : the regression coefficient relating pH and bicarbonate), 0.732



**Fig 1.** Relationship between pH and apparent dissociation constant of carbonic acid (pKapp) of hemolymph collected from the adductor muscle of Pacific oyster *Crassostrea gigas* at 23°C. Values are means ± standard error (N=44). Solid line fitted to the data and the equation:  $pKapp = 33.462 - 13.032 \cdot pH + 2.065 \cdot pH^2 - 0.1088 \cdot pH^3$  ( $r^2 = 0.9790$ )

torr) at 18°C and 3.8–5.4 kPa (28.5–40.5 torr) at 26°C, respectively.<sup>2,23)</sup> In marine blue mussel *Mytilus edulis*, the adductor muscle comprises a large fraction of the total hemolymph volume,<sup>24)</sup> and the hemolymph samples collected from the adductor muscle probably contain a mixture of pre- and post-branchial hemolymph from various regions of the circulatory system.<sup>12)</sup> Pacific oyster hemolymph, which was collected from the adductor muscle, would circulate around various regions and perfuse to the adductor muscle.

Pacific oyster hemolymph pH and  $T_{CO_2}$  measured immediately after hemolymph collection were 7.414 and 1.87 mM/l at 23.0°C, respectively. Previously reported hemolymph pH values include pH 7.65 in *M. edulis* at 12°C,<sup>12)</sup> pH 7.55 in Mediterranean mussel *Mytilus galloprovincialis* at 18°C,<sup>13)</sup> pH 7.617 in hard-shelled mussel *Mytilus coruscus* at 24°C,<sup>18)</sup> pH 7.284–7.375 in akoya pearl oyster *Pinctada fucata martensii* at 28°C,<sup>14,15)</sup> pH 7.563 in black-lip pearl oyster *Pinctada margaritifera* at 26°C<sup>16)</sup> and pH 7.442 in noble scallop *Mimachlamys nobilis* at 24°C.<sup>17)</sup> Handa and Yamamoto (2012, 2015, 2016) and Handa et al. (2017B) reported hemolymph  $T_{CO_2}$  in *P. fucata martensii*, *P. margaritifera*, *M. nobilis*, and *M. coruscus* as 1.90–2.10 mM/l,<sup>15)</sup> 2.04 mM/l,<sup>16)</sup> 1.50 mM/l<sup>17)</sup> and 1.44 mM/l,<sup>18)</sup> respectively. The hemolymph pH in Pacific oyster was almost the same as that in *M. nobilis* and lower than that in *M. edulis*, *M. galloprovincialis* and *M. coruscus*. The contents of carbonic acid and  $CO_2$  was approximately the same as *P. fucata martensii* and *P. margaritifera*, and higher than in *M. nobilis*, *M. galloprovincialis* and *M. coruscus*. The Pacific oyster should have the acid–base status that is similar to Pectinidae and Pteriidae bivalves, and which is different from Mytilidae bivalves.

Cameron (1986) reported  $CO_2$  solubility as a function of temperature and salinity, and the solubility coefficients were 39.2–42.3  $\mu M/l/torr$  at 22–24°C and 30–35 salinity (psu).<sup>25)</sup> The hemolymph  $a_{CO_2}$  in Pacific oyster (40.51  $\mu M/l/torr$ ) was in the range of the coefficient reported in Cameron (1986). The mean value of hemolymph pKapp in this study was 6.07343, whereas the hemolymph pKapp values of other marine bivalves were 5.8191 in the *P. fucata martensii* at 28°C,<sup>15)</sup> 5.9987 in *P. margaritifera* at 26°C,<sup>16)</sup>

6.0641 in *M. nobilis* at 23°C,<sup>17)</sup> 6.2609 in *M. coruscus* at 24°C<sup>18)</sup> and 6.114 in *M. edulis* at 12°C.<sup>12,26)</sup> The pKapp value is equal to the pH value at which it is most effective as a buffer.<sup>27)</sup> The most effective buffer pH in Pacific oyster hemolymph seemed to be similar to the value in *P. margaritifera* and *M. nobilis*.

Using the hemolymph  $a_{CO_2}$  and pKapp in this study,  $P_{CO_2}$  and  $[HCO_3^-]$  of the hemolymph of Pacific oyster were calculated. The pKapp was estimated by the relational expression corresponding to the change in pH because the pH changed significantly with an increase in  $P_{CO_2}$  in standard gases. The mean values of hemolymph  $P_{CO_2}$  and  $[HCO_3^-]$  in Pacific oyster were 2.18 torr and 1.78 mM/l, respectively. In other marine bivalves, the mean values of hemolymph  $P_{CO_2}$  and  $[HCO_3^-]$  were 0.9 torr and 1.8 mM/l in *M. edulis* at 12°C,<sup>12)</sup> 1.15 torr and 1.62 mM/l in *M. galloprovincialis* at 18°C,<sup>13)</sup> and 0.57 torr and 1.42 mM/l in *M. coruscus* at 24°C,<sup>18)</sup> 1.50 torr and 1.98 mM/l in *P. margaritifera* at 26°C,<sup>16)</sup> and 2.08–2.33 torr and 1.83–2.04 mM/l in *P. fucata martensii* at 28°C.<sup>15)</sup> The hemolymph acid–base status of Pacific oyster approximated that of *P. fucata martensii*.

The  $\beta_{NB}$  of Pacific oyster hemolymph (0.732 Slykes) was lower than that of *P. fucata martensii* (1.35–1.45 Slykes)<sup>15)</sup> but higher than those of *M. edulis* (0.4–0.622 Slykes),<sup>12,26)</sup> *M. galloprovincialis* (0.65 Slykes),<sup>28)</sup> and *M. coruscus* (0.44 Slykes).<sup>18)</sup> The non-bicarbonate buffer value was decided by the buffer capacity of the non-bicarbonate buffer system (for example, protein buffer system), and used to quantify the amount of buffering of the solution component. The interaction of the  $CO_2$  and bicarbonate buffer systems with non-bicarbonate buffers is particularly advantageous when nonvolatile  $H^+$  ions are to be buffered in a buffer system.<sup>29)</sup> Therefore, Pacific oyster *C. gigas* could experience a change in hemolymph pH with an anaerobic metabolism or a slight fluctuation of seawater pH. Pacific oyster *C. gigas* seems to be sensitive to environmental changes in comparison with *P. fucata martensii*, but not more sensitive than Mytilidae bivalves from the viewpoint of acid–base balance of the hemolymph.

## References

- 1) Hayami I: Ostreidae. *In*: Okutani T (ed) Marine Mollusks in Japan. The second edition, Tokai University Press, Tokyo, 1182-1185 (2017)
- 2) Fishery and Aquaculture Department (FAO): Oyster. *In*: Fishery and Aquaculture Statistics, Aquaculture production by species group, B-53 (2010).
- 3) Statistics Department, Ministry of Agriculture, Forestry and Fisheries Japan (MAFF): Marine Aquaculture. *In*: Fishery and Aquaculture Production, The 90th statistical yearbook, Ministry of Agriculture, Forestry and Fisheries Japan, 4 (2016).
- 4) Yamamoto K, Handa T, Kondo M: Trial of corrosion casting to the digestive diverticula of the Pacific oyster *Crassostrea gigas*. *J Nat Fish Univ*, **51**, 95-104 (2003)
- 5) Yamamoto K, Handa T: Anatomical structure of ctenidia of the Pacific oyster *Crassostrea gigas*. *J Nat Fish Univ*, **61**, 190-210 (2013)
- 6) Yamamoto K, Adachi S, Tamura I, Aramizu T and Koube H: Effects of hypoxia and water temperature on ciliary movement of gills 5 bivalvia, *Mytilus edulis*, *Atrina pectinate*, *Pinctada fucata martensii*, *Chlamys nobilis* and *Crassostrea gigas*. *J Nat Fish Univ*, **44**, 137-142 (1996)
- 7) Yamamoto K, Handa T: Effect of hypoxia on ventilation in the Pacific oyster *Crassostrea gigas*. *Aquaculture Sci*, **59**, 1-4 (2011)
- 8) Yamamoto K, Handa T: Effect of low salinity on ventilation in the oyster *Crassostrea gigas*. *Aquaculture Sci*, **59**, 5-8 (2011)
- 9) Yamamoto K, Handa T: Effect of hypoxia on oxygen uptake in the Pacific oyster *Crassostrea gigas*. *Aquaculture Sci*, **59**, 199-202 (2011)
- 10) Yamamoto K, Handa T: Ventilation in the Pacific oyster *Crassostrea gigas* with feeding. *Aquaculture Sci*, **59**, 203-206 (2011)
- 11) Handa T, Araki A and Yamamoto K: Oxygen and acid–base status of hemolymph in the Pacific oyster *Crassostrea gigas* after cannulation of the adductor muscle. *J Nat Fish Univ*, **66**, 35-40 (2017A)
- 12) Booth CE, McDonald DG, Walsh PJ: Acid–base balance in the sea mussel, *Mytilus edulis*. I. Effects of hypoxia and air-exposure on hemolymph acid–base status. *Mar Bio Lett*, **5**, 347-358 (1984)
- 13) Michelidis B, Ozounis C, Paleras A, Portner HO: Effects of long-term moderate hypercapnia on acid–base balance and growth rate in marine mussels *Mytilus galloprovincialis*. *Mar Ecol Prog Ser*, **293**, 109-118 (2005)
- 14) Handa T, Yamamoto K: The blood acid–base balance in the pearl oyster, *Pinctada fucata martensii*, after the surgery. *J Nat Fish Univ*, **60**, 57-61 (2011)
- 15) Handa T, Yamamoto K: The acid–base balance of the hemolymph in the pearl oyster *Pinctada fucata martensii* under normoxic conditions. *Aquaculture Sci*, **60**, 113-117 (2012)
- 16) Handa T, Yamamoto K: Estimation of CO<sub>2</sub> partial pressure and bicarbonate concentration in the hemolymph of the black-lip pearl oyster *Pinctada margaritifera*. *J Nat Fish Univ*, **63**, 181-188 (2015)
- 17) Handa T and Yamamoto K: Estimation of CO<sub>2</sub> partial pressure and bicarbonate concentration in the hemolymph of the noble scallop *Mimachlamys nobilis*. *J Nat Fish Univ*, **64**, 188-194 (2016)
- 18) Handa T, Araki A, Yamamoto K: Acid–base balance of the hemolymph in hard-shelled mussel *Mytilus coruscus* in normoxic conditions. *J Nat Fish Univ*, **65**, 39-46 (2017B)
- 19) Boutilier RG, Iwama GK, Heming TA, Randall DJ: The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15°C. *Resp Physiol*, **61**, 237-254 (1985)
- 20) Yamamoto K, Handa T, Nakamura M, Kitukawa K, Kita Y, Takimoto S, Nishikawa S: Effects of ozone-produced oxidants on respiration of the pearl oyster, *Pinctada fucata martensii*. *Aquaculture Sci*, **47**, 241-248 (1999)
- 21) Davenport HW: Fundamental equation. *In*: The ABC of acid–base chemistry 6th edition. University of Chicago Press, Chicago, 39-41 (1974)
- 22) Allen SM, Burnett LE: The effects of intertidal air exposure on the respiratory physiology and the killing activity of hemocytes in the Pacific oyster,

- Crassostrea gigas*. *J Exp Mar Boil Ecol*, **357**, 165-171 (2008)
- 23) Tran D, Massabuau JC, Vercelli C: Influence of sex and spawning status on oxygen consumption and blood oxygenation status in oysters *Crassostrea gigas* cultured in a Mediterranean lagoon (Thau, France). *Aquaculture*, **277**, 58-65 (2008)
- 24) Walsh JP, McDonald DG, Booth CE: Acid-base balance in the sea mussel, *Mytilus edulis*. II. Effects of hypoxia and air-exposure on intracellular acid-base status. *Mar Biol Lett*, **5**, 359-369 (1984)
- 25) Cameron JN: The solubility of carbon dioxide as a function of temperature and salinity (Appendix table): *In*: Cameron JN (ed) Principles of physiological measurement. Academic Press, United Kingdom, 258-259 (1986)
- 26) Lindinger MI, Lauren DJ, McDonald DG: Acid-base balance in the sea mussel, *Mytilus edulis*. III. Effects of environmental hypercapnia on intra- and extracellular acid-base balance. *Mar Bio Lett*, **5**, 371-381 (1984)
- 27) Thomas RC: Intracellular pH. *In*: Hainsworth R (ed) Acid-base balance. Manchester University Press, United Kingdom, 50-74 (1986)
- 28) Michaelidis B, Haas D, Grieshaber MK: Extracellular and intracellular acid-base status with regard to the energy metabolism in the oyster *Crassostrea gigas* during exposure to air. *Physiol Biochem Zool*, **78**, 373-383 (2005)
- 29) Heisler N: Acid-base regulation, Interrelationships between gaseous and ionic exchange. *In*: Boutilier RG (ed) Vertebrate gas exchange, Comparative & environmental physiology 6, Springer-Verlag Berlin Heidelberg, 211-251 (1990)