Effect of Air Exposure on the Acid–Base Balance of Hemolymph in Black-lip Pearl Oyster Pinctada margaritifera

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EFFECT OF AIR EXPOSURE ON THE ACID–BASE BALANCE OF HEMOLYMPH IN AKOYA PEARL OYSTER *PINCTADA FUCATA MARTENSII*

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ABSTRACT The hemolymph acid–base status of Akoya pearl oyster *Pinctada fucata martensii* exposed to air at 20°C was investigated. Air-exposed Akoya pearl oyster showed a decrease in hemolymph pH from 7.568 to 6.825 after 24 h. The hemolymph total CO_2 concentration increased from 2.25 mM/L to 4.50 mM/L during 24 h of air exposure. The hemolymph CO_2 partial pressure (Pco_2) was calculated by rearranging the Henderson–Hasselbalch equation. The hemolymph Pco_2 increased from 1.0 torr to 14.8 torr, and bicarbonate ion concentration increased from 2.21 mM/L to 3.91 mM/L during 24 h of air exposure. The hemolymph calcium ion concentration ($[Ca^{2+}]$) increased from 9.4 mM/L to 12.8 mM/L. These results indicated that Akoya pearl oysters showed hemolymph acidosis with partial metabolic compensation by mobilization of bicarbonate from the shell valve during prolonged air exposure. Immersion in seawater for between 4 h and 24 h decreased the effect of air exposure on hemolymph acid–base status, except for $[Ca^{2+}]$. The hemolymph $[Ca^{2+}]$ of immersed Akoya pearl oysters was slightly higher than the initial level upon air exposure although hemolymph $[Ca^{2+}]$ had already decreased after immersion in seawater for 4 h and 24 h. The hemolymph acid–base balance of immersed Akoya pearl oysters recovered to the initial level after 4–24 h, even if the animals were exposed to the air for a prolonged time (24 h).

KEY WORDS: hemolymph acid–base balance, respiratory physiology, air exposure, Akoya pearl oyster, *Pinctada fucata martensii*

INTRODUCTION

Akoya pearl oyster *Pinctada fucata martensii* is a filibranchial bivalve classified in the Pteriidae and is endemic to Japan (Hayami 2017). Akoya pearl oyster is distributed from the Boso Peninsula on the Pacific coast and the Oga Peninsula on the Japan Sea coast south to Okinawa (Hayami 2017). Akoya pearl oyster has nacreous aragonite in the inner layer of its shell valves, and it is used for the production for Akoya pearls. The process of pearl production is directly related to metabolism. The metabolism of Akoya pearl oyster has been studied in terms of regulation of oxygen uptake, gill ventilation volume, and filtration rate in hypoxic, anathermal, and feeding conditions (Numaguchi 1994, Yamamoto et al. 1999a, Yamamoto 2000, Yamamoto et al. 2002, 2010). The structures of the digestive diverticula and ctenidium were clarified by observation of tissue sections and corrosion resin-cast samples (Handa & Yamamoto 2003, Yamamoto et al. 2008, 2019). There are, however, few reports of the effect of air exposure on the respiratory physiology from the viewpoint of CO_2 dynamic phase and acid–base balance in Akoya pearl oyster. In the pearl production, Akoya pearl oysters are often exposed to air for the surgery, preparation, and maintenance (Taylor & Strack 2008). Therefore, research into the effect of air exposure may contribute to the elucidation of the acid–base balance and in the handling of the Akoya pearl oysters. In some marine bivalves, the CO_2 partial pressure (Pco_2) of the hemolymph is very low (0.57–2.3 torr) in normoxic and normocapnic conditions (Booth et al. 1984, Michaelidis et al. 2005, Handa & Yamamoto 2012, 2016, Handa et al. 2018). The estimation of Pco_2 by application of the Henderson–Hasselbalch equation is practiced in studies of the acid–base balance owing to its relative ease and accuracy (Boutilier et al. 1985). In the equation, the CO_2 solubility coefficient (αco_2) and apparent dissociation constant (pKapp) of carbonic acid in the hemolymph are required for the experimental animal. Thus, this study determined αco_2 and pKapp of the hemolymph in *in vitro* experiment, and examined the hemolymph Pco_2 and acid–base balance of the air-exposed Akoya pearl oyster in *in vivo* experiment.

MATERIALS AND METHODS

Experimental Animals and Conditions

Akoya pearl oysters (n = 65; mean total wet weight, 53.8 g; shell height, 69.6 mm; shell length, 64.1 mm) were obtained from a marine farm in Tsushima, Nagasaki Prefecture, Japan. After cleaning the shell valves, they were reared for 2 mo at 20°C in aerated seawater with added cultivated phytoplankton (Yamamoto et al. 1999b). Twenty-four hours before collecting hemolymph, the Akoya pearl oysters were transferred to a respiratory chamber with a flow of particle-free (>0.45 μm) seawater. All experiments were conducted in seawater with a salinity of 34, water temperature 20°C, O_2 saturation 98%, pH 8.18, and total CO_2 content 2.2 mM/L.

Experimental Procedure

The effect of air exposure on hemolymph acid–base status was investigated *in vivo*, and hemolymph Pco_2 and bicarbonate ion concentration ($[HCO_3^-]$) were calculated using the results of *in vitro* experiments in this study.

Series I. Air exposure in *in vivo* experiment

Experimental animals in the respiratory chamber were exposed to air by stopping the flow into the chamber and siphoning out the water. When the air exposure started (0 h), hemolymph was collected from the adductor muscle as a control (AE0h). Other experimental animals were exposed to air for 24 h. The temperature and humidity of the air were maintained

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by passing air through the experimental seawater, and adjusted air flowed into the respiratory chamber. After exposure to air for 24 h, hemolymph was collected from the adductor muscle (AE24h). The inflow of experimental seawater was resumed into the respiratory chamber after exposing the experimental animals to air for 24 h, and the animals were immersed in seawater. Hemolymph was collected at 4 h or 24 h after immersion in seawater (Im4h, Im24h).

Series II. Determination of αco_2 and pKapp of the hemolymph in *in vitro* experiment

The hemolymph Pco_2 and $[HCO_3^-]$ were calculated by rearranging the Henderson–Hasselbalch equation (Davenport 1974, Boutilier et al. 1985). In the equation, the αco_2 and pKapp were required for Akoya pearl oyster. *In vitro* determination of αco_2 and pKapp was performed on hemolymph drawn from the adductor muscle of animals without air exposure. The αco_2 value was determined using hemolymph, which was adjusted to pH 2.5 by the addition of lactic acid (Wako Pure Chemical Co., Japan). The acidified sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gas (CO_2, 5.0%; O_2, 20.9%; N_2 Balance) using an equilibrator (DEQ-1, Cameron Instruments Co.) at 20°C, and subsequently the total CO_2 concentration (Tco_2) of each equilibrated sample was measured. The Pco_2 of the equilibrated sample was calculated from known CO_2 concentration standard gas (5.0%), prevailing barometric pressure, and water vapor pressure at 20°C. For the determination of pKapp, the hemolymph sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gases (CO_2, 0.1, 0.2, 0.5, 1.0, and 2.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 20°C. After equilibration, the pH and Tco_2 of the sample were measured. Using the sample pH and Tco_2, αco_2 and pKapp were determined by rearrangement of the Henderson–Hasselbalch equation.

Hemolymph Collection and Analysis

Hemolymph was collected anaerobically from the adductor muscle of each experimental animal by direct puncture with a gas-tight microsyringe (Model 1750LTN; Hamilton Co.). The needle of a gas-tight syringe was inserted into the adductor muscle from the posterior margin of the shell valve, and was advanced 5 mm toward the center part of the adductor muscle (Handa & Yamamoto 2012). The volume of each hemolymph sample was approximately 0.3 mL. Hemolymph pH and Tco_2 were measured immediately after collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments Co.) with glass and reference electrodes (E301 and E351; Cameron Instruments Co.) at 20°C. The Tco_2 was measured using a total CO_2 analyzer (Capnicon 5; Cameron Instruments Co.). Hemolymph calcium ion concentration ($[Ca^{2+}]$, mM/L) was determined with a test kit (Calcium E-test; Wako Pure Chemical Co., Japan) and a spectrophotometer (610 nm, Spectronic 20A; Shimadzu Co., Japan).

Calculation

The αco_2 of the experimental animals was calculated using the equation:

$$\alpha co_2 = Tco_2 \cdot Pco_2^{-1}$$

where the units of the parameters are mM/L/torr for αco_2, mM/L for Tco_2, and torr for Pco_2.

Using the sample pH, Tco_2, and αco_2 calculated with the above equation, pKapp was determined by the rearrangement of the Henderson–Hasselbalch equation (Davenport 1974, Boutilier et al. 1985) as follows:

$$pKapp = pH - \log [(Tco_2 - \alpha co_2 \cdot Pco_2) \cdot (\alpha co_2 \cdot Pco_2)^{-1}]$$

where Pco_2 is calculated from known CO_2 concentration standard gases.

The hemolymph Pco_2 and $[HCO_3^-]$ were calculated using the equations:

$$Pco_2 = Tco_2 \cdot [\alpha co_2 \cdot (1 + 10^{(pH - pKapp)})]^{-1}$$

$$[HCO_3^-] = Tco_2 - \alpha co_2 \cdot Pco_2$$

where Tco_2 and pH were measured values, and αco_2 and pKapp were obtained in *in vitro* experiments. The units of the parameters are torr for Pco_2, mM/L for Tco_2 and $[HCO_3^-]$, and mM/L/torr for αco_2.

The nonbicarbonate buffer value (β_{NB}, slykes) and the relational expression of the hemolymph nonbicarbonate buffer were calculated using pH and $[HCO_3^-]$ of the *in vitro* experiment. The buffering capacity of the hemolymph *in vivo* was calculated by pH and $[HCO_3^-]$ at AE0h and AE24h of air-exposed animals.

Statistical Analysis

Data are expressed as means ± SD. Kruskal–Wallis test was performed for changes in hemolymph properties over the experimental time course. Multiple comparison of all pairs used the Steel–Dwass test. Statistically significant differences were set at $P < 0.05$. All analyses were carried out with the statistical software Kyplot v. 5.0 and 6.0 (KyensLab Inc., Japan).

RESULTS

Akoya pearl oysters exposed to air showed significant changes in hemolymph properties (Series I). In air-exposed animals, the mean values of hemolymph pH showed a statistically significant decrease from 7.568 to 6.825 during air exposure for 24 h ($P < 0.05$, Fig. 1). The hemolymph Tco_2 increased from 2.25 mM/L to 4.50 mM/L during air exposure for 24 h ($P < 0.05$, Fig. 1). When the experimental animals were immersed in seawater after air exposure, the hemolymph pH increased and Tco_2 decreased at Im4h and Im24h ($P < 0.05$). There were no significant differences between control and immersed animals for hemolymph pH and Tco_2. In *in vitro* experiments (Series II), the mean value of hemolymph αco_2 was 40 μM/L/torr as a result of analysis with a tonometer. The hemolymph pKapp at known Pco_2 (standard gases) and the corresponding measured pH and Tco_2 are shown in Table 1. The changes in pH, Tco_2, and pKapp were statistically significant with the increase in Pco_2 ($P < 0.05$, Table 1). The interaction between pKapp and pH was analyzed (Fig. 2), and the relational expression for pKapp was obtained as follows:

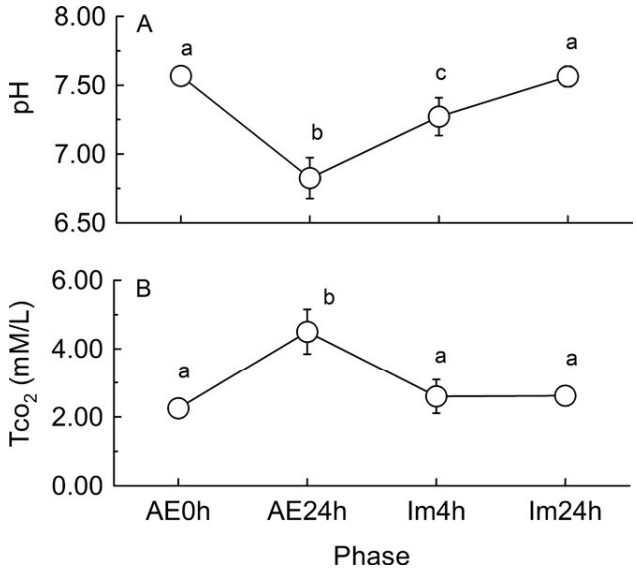

Figure 1. Effect of air exposure on hemolymph pH (A) and total CO_2 concentration (Tco_2, B) in Akoya pearl oyster *Pinctada fucata martensii* after air exposure. AE0h: air exposure for 0 h (control); AE24h: air exposure for 24 h; Im4h and Im24h: immersion for 4 h or 24 h after air exposure, respectively. Hemolymph from the adductor muscle was collected from each experimental animal (n = 6 in each symbol). Values are means ± SD. Different lowercase letters indicate statistically significant differences (P < 0.05, Steel–Dwass multiple comparison test).

$$pKapp = 183.939 - 77.811 \cdot pH + 11.340 \cdot pH^2 - 0.5508 \cdot pH^3$$

The Pco_2 and $[HCO_3^-]$ were calculated by substituting the values of αco_2 and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \cdot [0.040 \cdot (1 + 10^{(pH - pKapp)})]^{-1}$$
$$[HCO_3^-] = Tco_2 - 0.040 \cdot Pco_2$$

where the pKapp applies the value obtained by the relational expression as earlier, and the units of the parameters are torr for Pco_2, and mM/L for Tco_2 and $[HCO_3^-]$. Hemolymph Pco_2 and $[HCO_3^-]$ at AE0h were 1.0 torr and 2.21 mM/L, respectively. The hemolymph Pco_2 and $[HCO_3^-]$ increased significantly during air exposure (P < 0.05), reaching 14.8 torr and 3.91 mM/L at AE24h, respectively (Fig. 3). When the experimental animals were immersed in seawater after air exposure, the hemolymph Pco_2 and $[HCO_3^-]$ decreased at Im4h and Im24h (P < 0.05). There were no significant differences between control and immersed animals for hemolymph Pco_2 and $[HCO_3^-]$. The hemolymph $[Ca^{2+}]$ of air-exposed animals increased from 9.4 mM/L to 12.8 mM/L for 24 h (P < 0.05, Fig. 3). When the experimental animals were immersed in seawater after air exposure, the hemolymph $[Ca^{2+}]$ decreased at Im4h and Im24h. The hemolymph $[Ca^{2+}]$ at Im4h and Im24h were higher than $[Ca^{2+}]$ at AE0h (P < 0.05). The progress of change in acid–base balance in experimental animals is summarized in a pH-$[HCO_3^-]$ diagram (Fig. 4). The hemolymph $[HCO_3^-]$ of air-exposed animals rose with decreasing pH, and the point at

TABLE 1.

Mean values of measured pH, Tco_2, and calculated pKapp of carbonic acid of the hemolymph of the Akoya pearl oyster *Pinctada fucata martensii* with known Pco_2 standard gases.

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/L)	pKapp	n
0.102	0.76	7.631	2.21	5.77881	7
0.203	1.51	7.477	2.43	5.88952	7
0.515	3.82	7.201	2.60	6.00382	7
1.01	7.50	6.938	2.88	6.00971	7
2.00	14.9	6.662	3.26	6.01492	7

CO_2 solubility coefficient (αco_2): 40 µM/L/torr; Temperature: 20°C. pKapp, apparent dissociation constant; Pco_2, CO_2 partial pressure; Tco_2, total CO_2 concentration.

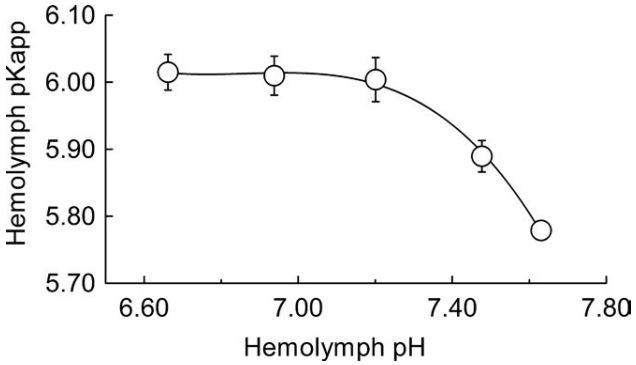

Figure 2. Relationship between pH and apparent dissociation constant of carbonic acid (pKapp) of hemolymph collected from the adductor muscle of Akoya pearl oyster *Pinctada fucata martensii* at 20°C. Values are means ± SE (n = 7 in each symbol). Solid curve fitted to the data and the equation: $183.939 - 77.811 \cdot pH + 11.340 \cdot pH^2 - 0.5508 \cdot pH^3$ (R^2 = 0.998)

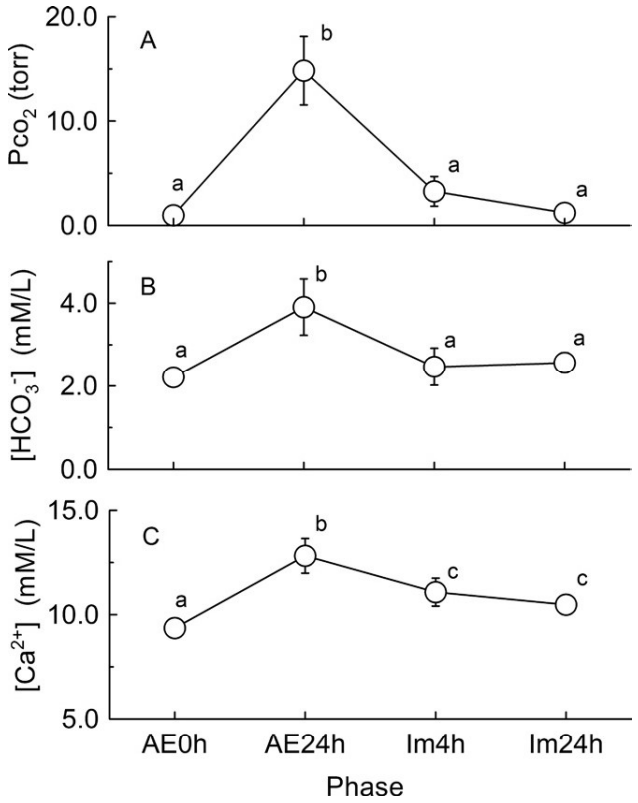

Figure 3. Effect of air exposure on hemolymph CO_2 partial pressure (Pco_2, A), bicarbonate concentration ($[HCO_3^-]$, B), and calcium ion concentration ($[Ca^{2+}]$, C) in Akoya pearl oyster *Pinctada fucata martensii* after air exposure. AE0h: air exposure for 0 h (control); AE24h: air exposure for 24 h; Im4h and Im24h: immersion for 4 h or 24 h after air exposure, respectively. Further details of the figure are given in Fig. 1.

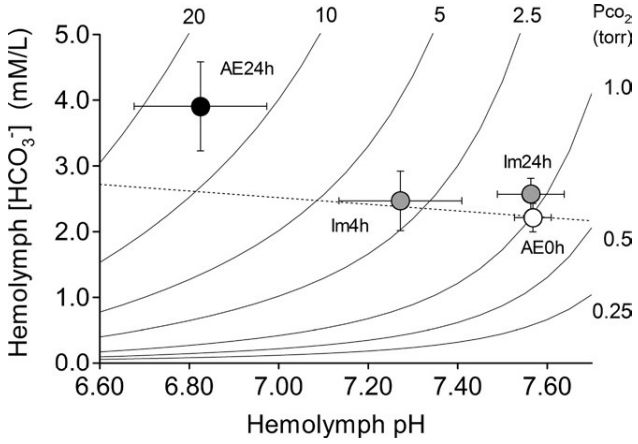

Figure 4. Hemolymph pH-$[HCO_3^-]$ diagram of the air exposure for 24 h (black circle, AE24h, n = 6), immersion (gray circles, Im4h, Im24h, n = 6 in each), and control (white circle, AE0h, n = 6) in Akoya pearl oyster *Pinctada fucata martensii*. Values are means ± SD. The CO_2 partial pressure (Pco_2) isopleths are derived from rearranging the Henderson–Hasselbalch equation. The dashed line is the nonbicarbonate buffer line: $[HCO_3^-] = 5.77 - 0.463 \cdot pH$ ($R^2 = 0.943$).

AE24h was located above the nonbicarbonate buffer line, which indicated the relationship between pH and $[HCO_3^-]$ in Figure 4. The point at Im4h located on the nonbicarbonate buffer line, and pH and $[HCO_3^-]$ at Im24h were similar to the values at AE0h. β_{NB} was 0.46 slykes, and the buffering capacity *in vivo* was 2.27 slykes which was calculated from the hemolymph pH and $[HCO_3^-]$ at AE0h and AE24h.

DISCUSSION

The hemolymph acid–base status of Akoya pearl oyster *Pinctada fucata martensii* was examined to evaluate the effect of air exposure on acid–base balance. Akoya pearl oysters showed a reduction in hemolymph pH and increase in Tco_2 and Pco_2 during air exposure for 24 h. The air-exposed animals were unable to ventilate the gill, which inhibited the release of CO_2. As a result, CO_2 gradually accumulated in the hemolymph, causing progressive acidosis. Therefore, Akoya pearl oysters during air exposure should experience respiratory acidosis as a result of the inhibition of CO_2 release. Funakoshi (1966) observed the decreasing pH of the pericardial fluid in Akoya pearl oyster during air exposure. The hemolymph pH at AE24h was similar to that of the pericardial fluid in Akoya pearl oyster. In some marine and freshwater bivalves, the hemolymph and pericardial fluid showed a drop in oxygen partial pressure (Jokumsen & Fyhn 1982, Booth et al. 1984, Byrne et al. 1991) and acidosis during air exposure (Jokumsen & Fyhn 1982, Booth et al. 1984, Byrne et al. 1991, Byrne & McMahon 1991, Michaelidis et al. 2005). Although the anaerobic end products were not measured, the results of biochemical studies on anaerobic metabolism (Zwaan & Wijsman 1976, Kluytmans et al. 1977, Livingstone & Bayne 1977, Livingstone 1978, Zwaan et al. 1983) suggested that air exposure in this study was sufficient to force anaerobic metabolism. Akoya pearl oysters exposed to air for a long time should undergo metabolic acidosis because of anaerobic metabolism. The Akoya pearl oysters increased hemolymph $[HCO_3^-]$ and $[Ca^{2+}]$ during air exposure for 24 h. The increased $[HCO_3^-]$ and $[Ca^{2+}]$ during air exposure seemed to be mobilized from $CaCO_3$ crystals in the shell valves of Akoya pearl oysters. In marine and freshwater bivalves, acidosis during air exposure induces increases in $[HCO_3^-]$ and $[Ca^{2+}]$ in the hemolymph or pericardial fluid (Jokumsen & Fyhn 1982, Booth et al. 1984, Byrne et al. 1991, Byrne & McMahon 1991). Research using radiolabeled markers indicated that the source of increased calcium is the shell valve (Crenshaw & Neff 1969). The increase in acidic end products of anaerobic metabolism could dissolve the shell valve of Akoya pearl oysters, and bicarbonate and calcium ions were mobilized from the shell valves to the hemolymph during air exposure in this study. The mobilized bicarbonate seemed to be effective for buffering acidosis in Akoya pearl oyster hemolymph.

When the experimental animals were immersed in seawater, they showed a gradual increase in hemolymph pH at Im4h and Im24h. The hemolymph Pco_2 was reduced with the increase in pH and the decrease in $[HCO_3^-]$. The immersed animals should resume gill ventilation and rapidly release CO_2 from the gill into the seawater for 24 h. The immersed animals might release CO_2 by diffusion from the surface of the soft body. Aerobic metabolism resumed in the immersed animals, and the production of

anaerobic acidic end products stopped. The increased [HCO_3^-] during air exposure was consumed to compensate for acidosis within 24 h in the immersed animals, and [HCO_3^-] decreased to the initial level (AE0h). The hemolymph [Ca^{2+}] levels at Im4h and Im24h were higher than [Ca^{2+}] at AE0h although the hemolymph [Ca^{2+}] decreased at Im4h and Im24h. The pericardial fluid Ca^{2+} concentration in Akoya pearl oyster increased during air exposure for 24 h, and returned to the initial level when the animals were immersed in the seawater for 48 h (Funakoshi 1966). Akoya pearl oyster may need 24–48 h for a decrease in hemolymph [Ca^{2+}] to the pre-exposure level by calcification. Silverman et al. (1983) reported that the freshwater mussel *Ligumia subrostrata* releases shell calcium in hypoxic conditions, and it reclaims Ca^{2+} as calcium phosphate concretions in the gill tissue. Akoya pearl oysters seemed to need over 24 h to reclaim surplus Ca^{2+} as a calcium phosphate concretion, although there were no results of histological analysis in this study.

According to the pH-[HCO_3^-] diagram of the hemolymph (Fig. 4), [HCO_3^-] and Pco_2 increased considerably with the reduction in pH, and the points at AE24h were located above the nonbicarbonate buffer line. Wood et al. (1977) expounded on the pH-[HCO_3^-] diagram of the blood. If a decrease in pH is due solely to a change in Pco_2, the blood will be simply titrated along the nonbicarbonate buffer line, and the point of the pH value moves on this line. If a decrease in pH is due solely to an increase in nonvolatile acid, then the blood will be titrated along a constant Pco_2 isopleth. Akoya pearl oysters showed hemolymph acidosis and high [HCO_3^-] during air exposure, and the point of AE24h located above the nonbicarbonate buffer line. The buffer value as a measure of the buffering capability is defined as the change in base or acid form of the buffer system per change in pH (Heisler 1993, Claiborne 1998). The β_{NB} is the buffer value of the nonbicarbonate buffer system (mainly protein residues). The buffering capacity *in vivo*, which was calculated by the hemolymph pH and [HCO_3^-] at AE0h and AE24h, was 2.27 slykes, and the capacity was 4.9-fold higher than the nonbicarbonate buffer capacity (β_{NB}, 0.46 slykes) determined by *in vitro* experiments. Therefore, air-exposed Akoya pearl oysters mobilized [HCO_3^-] from shell carbonate to the hemolymph and enhanced the buffering capacity of the nonbicarbonate buffer system. Byrne et al. (1991) reported that the resulting base mobilized (primarily bicarbonate) functions to increase the apparent "nonbicarbonate" buffering capacity almost 17-fold over that of isolated hemolymph of the freshwater clam *Corbicula fluminea* during air exposure for 72 h. This source of readily available buffering power compensates for the low inherent buffering capacity of native hemolymph (Byrne et al. 1991). Therefore, Akoya pearl oysters enhanced the low buffering capacity of the hemolymph using mobilized base (bicarbonate) from the shell, and provided a partial metabolic compensation for the acidosis during air exposure for 24 h in this study.

The results of this study clearly demonstrate the effect of air exposure on the hemolymph acid–base balance in Akoya pearl oyster. Akoya pearl oysters are often exposed to air for preparation, surgery, and maintenance during pearl production. After prolonged air exposure, Akoya pearl oysters showed disturbance of the hemolymph acid–base balance. When air-exposed Akoya pearl oysters are returned to seawater, the severe metabolic acidosis disappeared in between 4 h and 24 h, and [Ca^{2+}] showed a decline. The hemolymph acid–base status of immersed Akoya pearl oysters recovered to the initial level over 4–24 h, even if the oysters were exposed to the air for a prolonged time (24 h).

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