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

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Abstract : We investigated the hemolymph acid-base status of the black-lip pearl oyster *Pinctada margaritifera* exposed to air at 23℃. Air-exposed *P. margaritifera* showed a decrease in hemolymph pH from 7.563 to 6.965 after 36 h. The hemolymph total CO_2 concentration (Tco_2) increased from 2.01 mM/L to 5.63 mM/L during 36 h of air exposure. The hemolymph CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) were calculated, by rearranging the Henderson-Hasselbalch equation with substitution of CO_2 solubility coefficient (αco_2, 40.49 mM/L/torr) and apparent dissociation constant of carbonic acid (pKapp, 5.98351). The hemolymph Pco_2 increased from 1.28 torr to 13.68 torr, and $[HCO_3^-]$ increased from 1.96 mM/L to 5.08 mM/L during 36 h of air exposure. The hemolymph calcium concentration ($[Ca^{2+}]$) increased from 7.96 mM/L to 11.2 mM/L. These results indicated that during prolonged air exposure *P. margaritifera* showed hemolymph acidosis with partial metabolic compensation by the mobilized bicarbonate from the shell valve. When the experimental animals were immersed in seawater after air exposure, *P. margaritifera* needed 24 hours at least for recovery of the hemolymph acid-base status to the initial level.

Key words : *Pinctada margaritifera*, black-lip pearl oyster, hemolymph, acid-base balance, air exposure, respiratory physiology

Introduction

The black-lip pearl oyster *Pinctada margaritifera* is a filibranchial bivalve classified in the Pteriidae[1)] and is distributed in the Indo-West Pacific region near the equator, including in the Red Sea, Arabian Sea, Persian Gulf, and around India, Sri Lanka, New Guinea, Hawaii and Madagascar.[2)] In Japan, *P. margaritifera* is present southwards from the Kii Peninsula.[1)] The black-lip pearl oyster has nacreous aragonite in the inner layer of its shell valves, and it is used for black pearl production. The process of pearl production is similar to the growth of the shell and is related directly to metabolism. The metabolism of the black-lip pearl oyster has been studied in terms of regulation of gill ventilation volume and oxygen uptake in normoxic, hypoxic, and anathermal conditions.[3-6)] The anatomical structures of the ctenidium, circulatory system, digestive diverticula, and labial palp have been studied.[7,8)] Handa et al. (2015) described the estimation of hemolymph CO_2 partial pressure (Pco_2) of black-lip pearl oyster in normoxic conditions.[9)] However, there are few reports of the effect of air exposure on the respiratory physiology from the viewpoint of CO_2 dynamics phase and acid-base balance in black-lip pearl oyster. In the pearl production, pearl oysters are often exposed to the air for the surgical implantation of a nucleus. The host pearl oysters are removed from the seawater, and a wedge is placed between the shell valves to keep the pearl oyster open.[10)] The oyster is clamped open, placed in the shell stand, and undergoes a surgical operation.[10)] Therefore, research into the effect of air exposure on the acid-base balance may contribute to efficient CO_2 utilization and growth, which are related to pearl formation and the handling of black-lip pearl oysters. We examined the hemolymph acid-base status of the black-lip pearl oyster *P. margaritifera* pre- and post-air exposure, and evaluated the acid-base balance and CO_2 dynamics *in vivo*. The hemolymph Pco_2 values of marine bivalves, including *P. margaritifera*, are very low

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(0.57–2.3 torr) in normoxic and normocapnic conditions.[9,11-19] 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.[20] 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, we determined the coefficient and constant of the hemolymph *in vitro*, and calculated the hemolymph Pco_2 of *P. margaritifera*, and available for the evaluation of the hemolymph acid–base balance.

Materials and Methods

Experimental animals and conditions

The experiments used 29 black-lip pearl oysters (mean total wet weight: 188 g). After cleaning the shell valves, they were reared for one month at 23°C in aerated seawater with added cultivated phytoplankton.[21-23] Twenty-four hours before collecting hemolymph, the black-lip 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 33 psu, water temperature 23°C, O_2 saturation 98%, pH 8.15, and total CO_2 content 1.5 mM/L.

Experimental procedure

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

In vivo experiments

Experimental animals in the respiratory chamber were exposed to air for 36 h by stopping the flow into the chamber and siphoning out the seawater. The temperature and humidity of the air were maintained by passing air through the experimental seawater, and adjusted air flowed into the respiratory chamber. Hemolymph was collected from the adductor muscle of the air-exposed animals at 36 h (AE36h). The inflow of experimental seawater was resumed into the respiratory chamber after exposure to air for 36 h, and the animals were immersed in seawater. The hemolymph of immersed animals was collected at 6 h (Im6h) and 24 h (Im24h). As a control for the experimental animals, hemolymph was collected before air exposure (AE0h). Six different individual animals were used for each analysis (n=24).

In vitro experiments

The hemolymph Pco_2 and $[HCO_3^-]$ were calculated by rearranging the Henderson–Hasselbalch equation.[20,24] In the equation, the αco_2 and pKapp were required in the black-lip pearl oyster. *In vitro* determination of αco_2 and pKapp were performed on hemolymph drawn from the adductor muscle of the experimental animals before exposure to air (n=5). αco_2 was determined using hemolymph, which was adjusted to pH 2.5 by the addition of lactic acid (Wako Pure Chemical Co., JP). The acidified sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gas (CO_2, 15.0%; O_2, 20.9%; N_2 Balance) using an equilibrator (DEQ-1, Cameron Instruments) at 23°C, and subsequently the total CO_2 concentration (Tco_2, mM/L) of each equilibrated sample was measured. The Pco_2 (torr) of the equilibrated sample was calculated from known CO_2 concentration standard gas (15.0%), prevailing barometric pressure and water vapor pressure at 23°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, 0.2, 0.5, 1.0, and 2.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 23°C. After equilibration, the pH and Tco_2 of the sample were measured. Using the sample pH, Tco_2, and αco_2, the pKapp was determined by rearrangement of the Henderson–Hasselbalch equation.

Hemolymph collection and analysis

Hemolymph was collected once from each individual from the adductor muscle by direct puncture with a gas-tight microsyringe (Model 1750LTN, Hamilton Co., US), and the volume of each hemolymph sample was 0.4–0.5

mL. Hemolymph pH and Tco_2 were measured immediately after collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments Co., US) with glass and reference electrodes (E301, E351; Cameron Instruments Co., US) at 23°C. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5; Cameron Instruments Co., US). Hemolymph calcium concentrations ($[Ca^{2+}]$, mM/L) were determined with a test kit (Calcium E-test, Wako Pure Chemical Co., JP) and a spectrophotometer (Spectronic 20A, Shimadzu Co., JP).

Calculation

αco_2 of the experimental animals was calculated using the equation:

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

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

Using the sample pH, Tco_2, and αco_2 calculated using the above equation, pKapp was determined by rearrangement of Henderson–Hasselbalch equation[20,24] as follows:

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

where Pco_2 is calculated from known CO_2 concentration standard gases.

The hemolymph Pco_2 and $[HCO_3^-]$ (mM/L) were calculated using the equations:

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

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

where Tco_2 and pH were measured values, and αco_2 and pKapp were obtained in *in vitro* experiments.

The non-bicarbonate buffer value (β_{NB}, slykes) and the relational expression of the hemolymph non-bicarbonate buffer were calculated from pH and $[HCO_3^-]$ of the *in vitro* experiment. Apparent buffer value of the *in vivo* experiment (β_{vivo}, slykes) was calculated from the hemolymph pH and $[HCO_3^-]$ of the animals exposed to air at AE0h and AE36h.

Statistical analysis

Data are expressed as means ± standard error of the means. Kruskal–Wallis test was performed for changes in hemolymph properties over the experimental time course. The multiple comparison of all pairs used the Steel–Dwass test. Statistically significant differences were set at $P < 0.05$ (KyPlot 5.0, KyensLab Inc., JP).

Results

The mean values of hemolymph pH were statistically significantly decreased from 7.563 to 6.965 during air exposure for 36 h ($P<0.05$, Fig. 1). The hemolymph Tco_2 increased from 2.01 mM/L to 5.63 mM/L during air exposure for 36 h ($P<0.05$, Fig. 2). When the experimental animals were immersed in the seawater after air exposure, the hemolymph pH increased and Tco_2 decreased at Im6h and Im24h ($P<0.05$). There were no significant differences in hemolymph pH and Tco_2 between control and immersed animals (Figs. 1-2). In *in vitro* experiments, hemolymph αco_2 was 40.49 μM/L/torr. The hemolymph pKapp at known Pco_2 (standard gases)

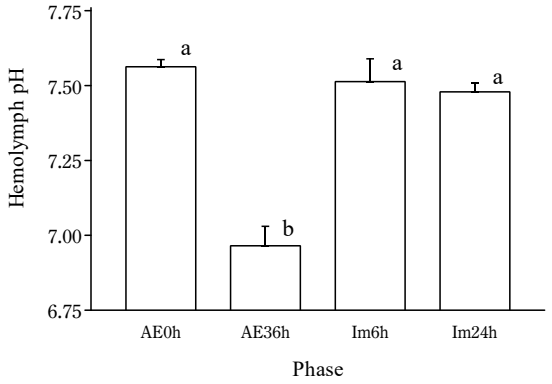

Fig. 1 Effect of air exposure on hemolymph pH in black-lip pearl oyster *Pinctada margaritifera*. AE0h: control; AE36h: air exposure for 36 h; Im6h: immersion for 6 h after air exposure; Im24h: immersion for 24 h after air exposure. Hemolymph was collected from each experimental animal (n=6 in each column). Values are means ± SEM. Different lowercase letters indicate statistically significant differences ($P<0.05$, Steel–Dwass multiple comparison test).

and the corresponding measured pH and Tco_2 are shown in Table 1. The calculated pKapp from all hemolymph samples was 5.98351 ± 0.014536. Pco_2 and $[HCO_3^-]$ were calculated by substitution of the values of αco_2 and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \bullet [0.04049 \bullet (1+10^{(pH-5.98351)})]^{-1}$$

$$[HCO_3^-] = Tco_2 - 0.04049 \bullet Pco_2$$

where the units of the parameters are torr for Pco_2 and mM/L for Tco_2 and $[HCO_3^-]$.

The mean values of the hemolymph Pco_2 and $[HCO_3^-]$ of the control values (AE0h) were calculated as 1.28 torr and 1.96 mM/L, respectively (Figs. 3, 4). The hemolymph Pco_2 and $[HCO_3^-]$ increased significantly during air exposure (P<0.05), reaching 13.6 torr and 5.08 mM/L at AE36h. When air-exposed animals were immersed in seawater, hemolymph Pco_2 and $[HCO_3^-]$ decreased at Im6h and Im24h. There were no significant differences in hemolymph Pco_2 and $[HCO_3^-]$ between control and immersed animals. Hemolymph $[Ca^{2+}]$ increased statistically significantly from 7.96 mM/L to 11.2 mM/L during air exposure for 36 h (P<0.05, Fig. 5). In immersed

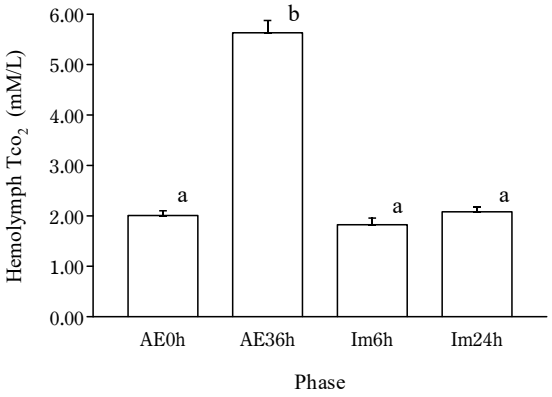

Fig. 2 Effect of air exposure on hemolymph total CO_2 concentration (Tco_2) in black-lip pearl oyster *Pinctada margaritifera*. AE0h: control; AE36h: air exposure for 36 h; Im6h: immersion for 6 h after air exposure; Im24h: immersion for 24 h after air exposure. Hemolymph was collected from each experimental animal (n=6 in each column). Values are means ± SEM. Different lowercase letters indicate statistically significant differences (P<0.05, Steel–Dwass multiple comparison test).

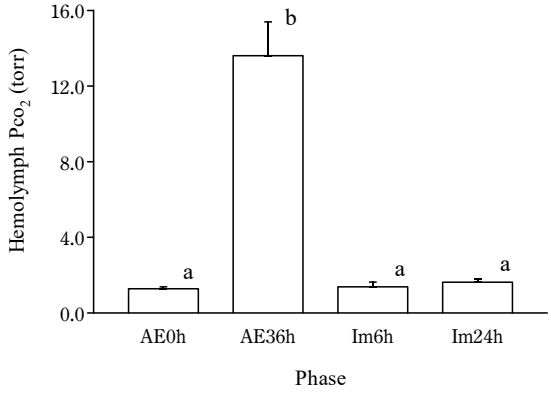

Fig. 3 Effect of air exposure on hemolymph CO_2 partial pressure (Pco_2) in black-lip pearl oyster *Pinctada margaritifera*. AE0h: control; AE36h: air exposure for 36 h; Im6h: immersion for 6 h after air exposure; Im24h: immersion for 24 h after air exposure. Hemolymph was collected from each experimental animal (n=6 in each column). Values are means ± SEM. Different lowercase letters indicate statistically significant differences (P<0.05, Steel–Dwass multiple comparison test).

Table. 1. Mean values of hemolymph pH, bicarbonate ion concentration ($[HCO_3^-]$) and apparent dissociation constant of carbonic acid (pKapp) of the black-lip pearl oyster (*Pinctada margaritifera*) with known CO_2 standard gases at 23°C

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	$[HCO_3^-]$ (mM/L)	pKapp	N
0.203	1.50	7.360	1.86	5.87958	5
0.515	3.81	7.119	1.94	6.02382	5
1.01	7.46	6.839	1.97	6.02678	5
2.00	14.8	6.589	2.32	6.00384	5

Barometric pressure , 767±4.1 torr; CO_2 solubility coefficient (αco_2), 40.49 μM/L/torr

animals, hemolymph [Ca^{2+}] at Im6h (10.4 mM/L) was statistically significantly higher than the control value (AE0h, 7.96 mM/L), although hemolymph [Ca^{2+}] at Im24h (8.94 mM/L) was approximately the same as the control value. The progress of change in acid–base balance in experimental animals is summarized in a pH-[HCO_3^-] diagram (Fig. 6). The hemolymph [HCO_3^-] of air-exposed animals rose with decreasing pH, and the point at AE36h was located above the non-bicarbonate buffer line, for which the relationship with the expression is indicated in Fig. 6. The hemolymph pH and [HCO_3^-] of immersed animals were similar to the control values, with the points located near the non-bicarbonate buffer line. The β_{NB}, which is the slope of the non-bicarbonate buffer line, was 0.53 slykes. The β_{vivo}, which was calculated from the hemolymph pH and [HCO_3^-] of air-exposed animals at AE0h and AE36h, was 5.22 slykes.

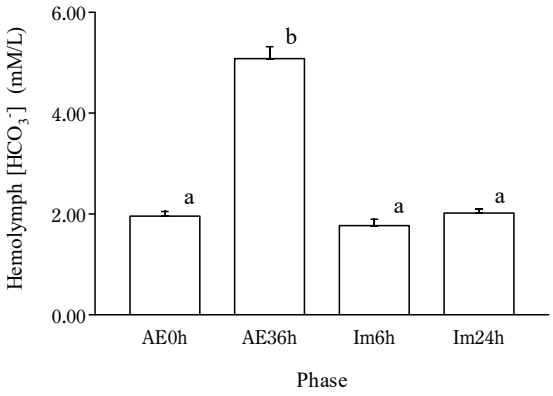

Fig. 4 Effect of air exposure on hemolymph bicarbonate concentration ([HCO_3^-]) in black-lip pearl oyster *Pinctada margaritifera*. AE0h: control; AE36h: air exposure for 36 h; Im6h: immersion for 6 h after air exposure; Im24h: immersion for 24 h after air exposure. Hemolymph was collected from each experimental animal (n=6 in each column). Values are means ± SEM. Different lowercase letters indicate statistically significant differences (P<0.05, Steel–Dwass multiple comparison test).

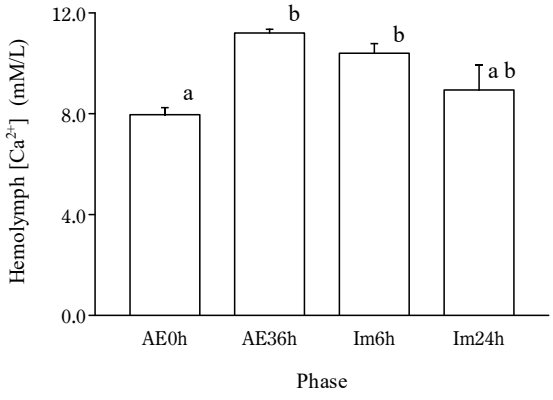

Fig. 5 Effect of air exposure on hemolymph calcium ion concentration ([Ca^{2+}]) in black-lip pearl oyster *Pinctada margaritifera*. AE0h: control; AE36h: air exposure for 36 h; Im6h: immersion for 6 h after air exposure; Im24h: immersion for 24 h after air exposure. Hemolymph was collected from each experimental animal (n=6 in each column). Values are means ± SEM. Different lowercase letters indicate statistically significant differences (P<0.05, Steel–Dwass multiple comparison test).

Discussion

We examined the hemolymph acid–base status of the black-lip pearl oyster *P. margaritifera* to evaluate the effect of air exposure on acid–base balance and CO_2 dynamics phase. *P. margaritifera* showed a reduction in hemolymph pH and increases in Tco_2 and Pco_2 during air exposure for 36 h. The air-exposed animals were unable to ventilate the gill, which inhibited the release of CO_2. CO_2 gradually accumulated in the hemolymph, causing progressive acidosis. Therefore, *P. margaritifera* during

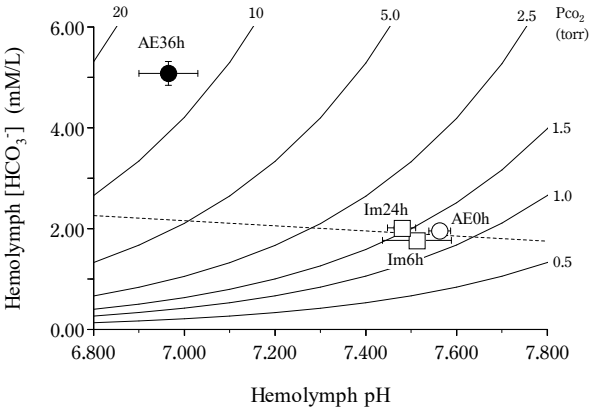

Fig. 6 Hemolymph pH-[HCO_3^-] diagram of air-exposed (closed circles), immersed (open squares), and control (open circles) black-lip pearl oysters *Pinctada margaritifera*. The Pco_2 isopleths are derived from rearranging the Henderson–Hasselbalch equation,[24] and αco_2 and pKapp of the experimental animals were 40.49 μM/L/torr and 5.98351, respectively. The dashed line is the non-bicarbonate buffer line: [HCO_3^-] = 5.7337 – 0.5319 • pH (R^2=0.7858). The line was obtained from the results of *in vitro* experiments in this study.

the air exposure should include respiratory acidosis due to the inhibition of CO_2 release. In some marine and freshwater bivalves, the hemolymph and pericardiac fluid showed a drop of oxygen partial pressure[11,25,26] and acidosis during air exposure.[11,25-29] Although we did not measure the anaerobic end-products, the results of biochemical studies on anaerobic metabolism[30-34] suggested that air exposure in this study was sufficient to force anaerobic metabolism. *P. margaritifera* exposed to air for a long time might undergo metabolic acidosis due to anaerobic metabolism. *P. margaritifera* exposed to the air increased hemolymph [HCO_3^-] and [Ca^{2+}] at AE36h. The increased [HCO_3^-] and [Ca^{2+}] during air exposure seemed to be mobilized from $CaCO_3$ crystals in the shell valves in *P. margaritifera*. In marine and freshwater bivalves, acidosis during air exposure induces increases in [HCO_3^-] and [Ca^{2+}] in the hemolymph or pericardiac fluid.[11,25-27] Research using radiolabeled markers indicated that the source of increased calcium was the shell valve.[35] The increase in acidic end-products of anaerobic metabolism could dissolve the shell valve of *P. margaritifera*, and the 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 *P. margaritifera* hemolymph.

When the experimental animals were immersed in seawater, *P. margaritifera* increased hemolymph pH at Im6h. 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 6 h. The immersed animals might release CO_2 by the 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 the acidosis within 6 h in the immersed animals, and [HCO_3^-] decreased to the control level. On the other hand, the increased [Ca^{2+}] during air exposure did not return to the control level within 6 h in immersed animals, although a declining trend in hemolymph [Ca^{2+}] was shown. 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.[36] *P. margaritifera* seemed to need a prolonged time to reclaim Ca^{2+} as concretions of calcium phosphate, and [Ca^{2+}] slowly decreased as a result, though there was no result of histology in this study. *P. margaritifera* required at least 24 h for the recovery of hemolymph [Ca^{2+}].

According to the pH-[HCO_3^-] diagram of the hemolymph (Fig. 6), [HCO_3^-] and Pco_2 increased considerably with the reduction in pH, and the points at AE36h were located above the non-bicarbonate buffer line. The buffer value as a measure for the buffering capability is defined as the change in base or acid form of the buffer system per change in pH.[37,38] The β_{NB} is the buffer value of the non-bicarbonate buffer system (mainly protein residues). If a decrease in hemolymph pH is due solely to a change in Pco_2, the hemolymph will be simply titrated along the non-bicarbonate buffer line,[39] and the point of pH value moves on this line. If a decrease in hemolymph pH is due solely to an increase in non-volatile acid, then the hemolymph will be titrated along a constant Pco_2 isopleth.[39] However, *P. margaritifera* showed hemolymph acidosis and high [HCO_3^-] during air exposure, and the point of AE36h located above the non-bicarbonate buffer line. β_{vivo}, which was calculated from hemolymph pH and [HCO_3^-] at AE0h and AE36h, was 5.22 slykes, and β_{vivo} was 10-fold higher than β_{NB} (0.53 slykes). Therefore, air-exposed *P. margaritifera* mobilized [HCO_3^-] from shell carbonate to the hemolymph, and enhanced the buffering capacity of the non-bicarbonate 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.[27] This source of readily available buffering power compensates for the low inherent buffering capacity of native hemolymph.[27] Therefore, *P. margaritifera* 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 in this study.

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