

## Effect of Air Exposure on Acid-Base Balance of Hemolymph in Hard-shelled Mussel *Mytilus coruscus*

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# Effect of Air Exposure on Acid–Base Balance of Hemolymph in Hard-shelled Mussel *Mytilus coruscus*

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**Abstract :** We investigated the hemolymph acid–base status of the hard-shelled mussel *Mytilus coruscus* exposed to air for 24–48 h at 24°C. *M. coruscus* exposed to air showed a decrease in hemolymph pH from 7.625 to 7.118 after 24 h and to 6.702 after 48 h. The hemolymph total CO<sub>2</sub> concentration increased from 1.43 mM/L to 3.12 mM/L during the first 24 h of air exposure, and increased to 6.32 mM/L after 48 h. The hemolymph CO<sub>2</sub> partial pressure increased from 1.50 torr to 9.48 torr during the first 24 h of air exposure, and increased to 41.8 torr for 48 h. The hemolymph [HCO<sub>3</sub><sup>-</sup>] increased to 2.74 mM/L at 24 h and to 4.62 mM/L at 48 h. These results indicated that during prolonged air exposure *M. coruscus* showed hemolymph acidosis with partial compensation. *M. coruscus* were immersed in seawater after air exposure for 24 h, and the acid–base status recovered to initial levels within 3–24 h.

**Key words :** *Mytilus coruscus*, hemolymph, acid–base balance, air exposure, normoxia, respiratory physiology

## Introduction

The hard-shelled mussel *Mytilus coruscus* is a Mytilidae bivalve.<sup>1)</sup> *M. coruscus* is distributed across East Asia and is cultivated commercially as a food in China and Korea. In Japan, *M. coruscus* inhabits the rocky bottom of intertidal zones from Hokkaido to Kyushu,<sup>1)</sup> and it is caught as a local specialty foodstuff. Because the animal is sold as a living bivalve, it is often exposed to air when handling in fishery and aquaculture production during transportation and sale. *M. coruscus* has been a subject of previous research in terms of the morphology of larvae,<sup>2)</sup> polymorphic microsatellite loci,<sup>3)</sup> microsatellite markers,<sup>4)</sup> biochemical response to heavy metal exposure,<sup>5)</sup> the effect of natural biofilm on the settlement mechanism,<sup>6)</sup> and the immune activities of hemocytes.<sup>7)</sup> In the field of respiratory physiology, *M. coruscus* in normoxic conditions has been studied to determine the hemolymph acid–base status.<sup>8)</sup> However, there are few reports on the respiratory physiology of air-exposed *M. coruscus* from the viewpoint of CO<sub>2</sub> dynamic phase and acid–base balance. Therefore, we conducted three experiments in

order to evaluate the effect of air exposure: experimental animals were exposed to air for 24–48 h; immersion after exposure to air for 24 h; and non-exposed to air as the control. These results should be useful for understanding the hemolymph acid–base balance, the effect of air exposure, and the recovery process, and for the development of the method which reduces the effect of air exposure on *M. coruscus*.

## Materials and Methods

### Experimental animals and conditions

The experiments used 36 hard-shelled mussels *M. coruscus* (mean total wet weight: 186 g). The animals were collected from the coastal sea area of Tana marine biological laboratory of the National Fisheries University in the Seto Inland Sea, Yamaguchi Prefecture, Japan. After cleaning the shell valves, they were reared for 1 month at 24°C in aerated seawater with added cultivated phytoplankton.<sup>9–11)</sup> Twenty-four hours before collecting hemolymph, the mussels were transferred to a respiratory chamber with flowing particle-free (>0.45 µm)

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seawater. The experimental seawater had a salinity of 33 psu, water temperature 24°C, O<sub>2</sub> saturation 99%, pH 8.15, and total CO<sub>2</sub> concentration 1.4 mM/L.

### Experimental procedure

The effect of air exposure on hemolymph acid–base status was investigated using the following procedures.

#### Series I. Air exposure

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. Other experimental animals were exposed to air for 24 h or 48 h, and hemolymph was similarly collected from the adductor muscle. The temperature and humidity of the air were maintained by passing air through the experimental seawater, and adjusted air flowed into the respiratory chamber. Five different individual experimental animals were used for each analysis (n=15).

#### Series II. Non-exposure

As a control for Series I, animals were not exposed to air without stopping flow in the respiratory chamber, and hemolymph was collected at the same time as in Series I. Five different individual experimental animals were used for each analysis (n=15).

#### Series III. Immersion after air exposure

After exposure of the experimental animals to air for 24 h, the inflow of experimental seawater was resumed into the respiratory chamber, and the animals were immersed in seawater. Hemolymph was collected at 3 h and 24 h after immersion in seawater (R3 h, R24 h). Three different individual experimental animals were used for each analysis (n=6).

### Hemolymph collection and analysis

Hemolymph was collected anaerobically once from each individual from the adductor muscle by direct puncture using a gas-tight microsyringe (Model 1750LTN, Hamilton Co.), and the volume of each hemolymph sample was 0.3 mL. Hemolymph pH and total CO<sub>2</sub> concentration (Tco<sub>2</sub>, mM/L) were measured

immediately after collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments) with glass and reference electrodes (E301, E351; Cameron Instruments) at 24°C. Tco<sub>2</sub> was measured using a total CO<sub>2</sub> analyzer (Capnicon 5; Cameron Instruments).

### Calculation

The hemolymph CO<sub>2</sub> partial pressure (Pco<sub>2</sub>, mmHg) and bicarbonate concentration ([HCO<sub>3</sub><sup>-</sup>], mM/L) were calculated by rearranging the Henderson–Hasselbalch equation.<sup>12)</sup> In the equation, the CO<sub>2</sub> solubility coefficient (aco<sub>2</sub>) and apparent dissociation constant of carbonic acid (pKapp) of *M. coruscus* hemolymph required. Handa et al. (2017) described hemolymph aco<sub>2</sub> and pKapp of *M. coruscus* were 40.6 μM/L/torr and 6.2609,<sup>8)</sup> respectively. The hemolymph Pco<sub>2</sub> and [HCO<sub>3</sub><sup>-</sup>] were calculated using the following equations:

$$\begin{aligned} P_{\text{CO}_2} &= T_{\text{CO}_2} \cdot [0.0406 \cdot (1 + 10^{(\text{pH} - 6.2609)})]^{-1} \\ [\text{HCO}_3^-] &= T_{\text{CO}_2} - 0.0406 \cdot P_{\text{CO}_2} \end{aligned}$$

The non-bicarbonate buffer value (β<sub>NB</sub>, slykes) and the related expression of the hemolymph non-bicarbonate buffer were calculated from the *in vitro* experiments<sup>8)</sup> on hard-shell mussel.

### Statistical analysis

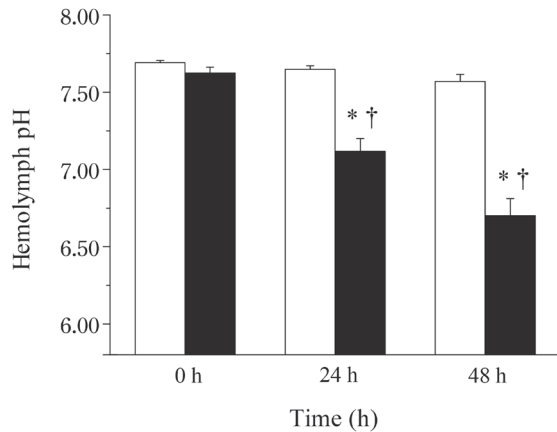
All data are expressed as means ± standard error of the means. Kruskal–Wallis test was performed for changes in hemolymph properties over the experimental time course. The comparison of two mean values used Mann–Whitney's *U* test. Statistically significant differences were set at *P* < 0.05 (KyPlot 5.0, KyensLab Inc.)

## Results

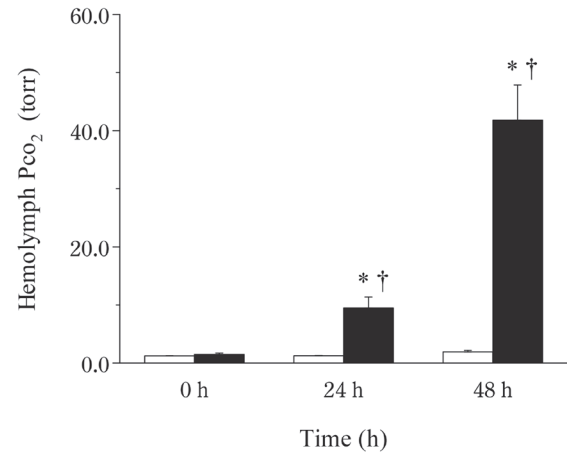
The hemolymph pH of air-exposed animals (Series I) was 7.118 at 24 h and 6.702 at 48 h (Fig. 1). These mean values were statistically significantly lower than the value at 0 h (*P* < 0.01). The hemolymph Tco<sub>2</sub> increased significantly during air exposure (*P* < 0.01), reaching 3.12 mM/L at 24 h and 6.32 mM/L at 48 h (Fig. 2). The

hemolymph  $P_{\text{CO}_2}$  increased from 1.50 torr to 9.48 torr during the first 24 h of air exposure, and increased to 41.8 torr after 48 h ( $P < 0.01$ , Fig. 3). The hemolymph  $[\text{HCO}_3^-]$  increased significantly during air exposure ( $P < 0.01$ ), reaching 2.74 mM/L at 24 h and 4.62 mM/L at 48 h (Fig. 4). In the control group (Series II), the mean values

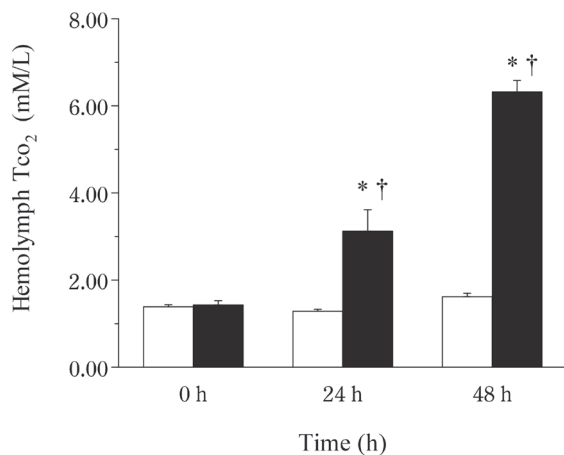
of hemolymph pH were 7.569–7.691 from 0 h to 48 h, and those for  $\text{Tco}_2$  were 1.29–1.62 mM/L. The mean  $P_{\text{CO}_2}$  and  $[\text{HCO}_3^-]$  from 0 h to 48 h were 1.22–1.93 torr and 1.24–1.54 mM/L (Series II). The hemolymph pH of immersed animals (Series III) was higher than the pH of air-exposed animals after 24 h and 48 h (Fig. 5). The



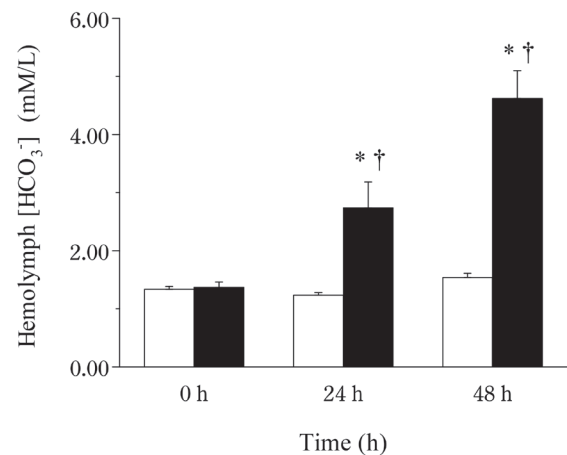
**Fig. 1** The hemolymph pH of hard-shelled mussel *Mytilus coruscus* after air exposure (closed column) for 0 h, 24 h, and 48 h, and in normoxic water as a control (open column). Hemolymph was collected from each experimental animal ( $n=5$  in each column). Values are means  $\pm$  SEM. Asterisks indicate values that are statistically significantly different from the value at 0 h, and daggers indicate the statistical significance compared with the control value at each time point.



**Fig. 3** The hemolymph  $\text{CO}_2$  partial pressure ( $P_{\text{CO}_2}$ ) of hard-shelled mussel *Mytilus coruscus* after air exposure (closed column) for 0 h, 24 h, and 48 h, and in normoxic water as a control (open column). Hemolymph was collected from each experimental animal ( $n=5$  in each column). Values are means  $\pm$  SEM. Asterisks indicate values that are statistically significantly different from the value at 0 h, and daggers indicate the statistical significance compared with the control value at each time point.



**Fig. 2** The hemolymph total  $\text{CO}_2$  concentration ( $\text{Tco}_2$ ) of hard-shelled mussel *Mytilus coruscus* in after exposure (closed column) for 0 h, 24 h, and 48 h, and in normoxic water as a control (open column). Hemolymph was collected from each experimental animal ( $n=5$  in each column). Values are means  $\pm$  SEM. Asterisks indicate values that are statistically significantly different from the value at 0 h, and daggers indicate the statistical significance compared with the control value at each time point.



**Fig. 4** The hemolymph bicarbonate concentration ( $[\text{HCO}_3^-]$ ) of hard-shelled mussel *Mytilus coruscus* after air exposure (closed column) for 0 h, 24 h, and 48 h, and in normoxic water as a control (open column). Hemolymph was collected from each experimental animal ( $n=5$  in each column). Values are means  $\pm$  SEM. Asterisks indicate values that are statistically significantly different from the value at 0 h, and daggers indicate the statistical significance compared with control value at each time point.

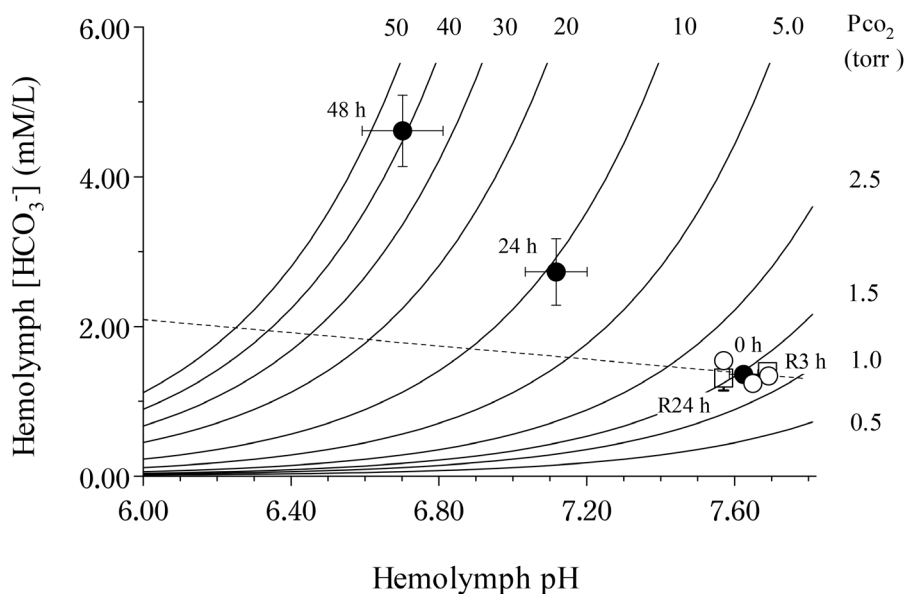
hemolymph  $[\text{HCO}_3^-]$  of immersed animals was lower than the  $[\text{HCO}_3^-]$  of air-exposed animals. There were statistically significant differences in pH,  $[\text{HCO}_3^-]$ , and  $\text{Pco}_2$  between immersed animals (R3 h, R24 h) and air-exposed animals (24 h, 48 h). There were no significant differences of hemolymph properties between the values for R3 h, R24 h, 0 h, and the control (Fig. 5). The  $\beta_{\text{NB}}$ , which is the slope of the non-bicarbonate buffer line, was 0.44 slykes, and the relationship with the expression of the hemolymph non-bicarbonate buffer line is indicated in Fig. 5.

## Discussion

We examined the hemolymph acid-base status of the hard-shelled mussel *M. coruscus* to evaluate the effect of air exposure. *M. coruscus* demonstrated reduced hemolymph pH and increased  $\text{Tco}_2$  and  $\text{Pco}_2$  over the time course of air exposure (Figs. 1-3). Experimental animals exposed to the air were unable to ventilate the ctenidium, which inhibited the release of  $\text{CO}_2$ .  $\text{CO}_2$  gradually accumulated in the hemolymph, causing progressive acidosis. Therefore, acidosis in the animals during the air exposure could include respiratory

acidosis by the inhibition of  $\text{CO}_2$  release. In some marine and freshwater bivalves, the hemolymph and pericardiac fluid showed a drop of oxygen partial pressure<sup>13-15</sup> and acidosis during air exposure.<sup>13-17</sup> Although we did not measure the anaerobic end-products, the results of the biochemical studies on anaerobic metabolism<sup>18-22</sup> suggested that air exposure conducted in this study was sufficient to force anaerobic metabolism in the experimental animals. Therefore, *M. coruscus* exposed to air for a prolonged time might have undergone metabolic acidosis in this study. *M. coruscus* seemed to rapidly reduce  $\text{Po}_2$  and deplete stored oxygen when air exposure was started, and causing anaerobic metabolism and metabolic acidosis.

*M. coruscus* exposed to the air increased hemolymph  $[\text{HCO}_3^-]$  (Fig. 4). The increased in  $[\text{HCO}_3^-]$  during exposure to air should derive from mobilization of the shell valves ( $\text{CaCO}_3$  crystal). In marine and freshwater bivalves, acidosis during air exposure involved increased bicarbonate and calcium ion concentrations in the hemolymph or pericardiac fluid.<sup>13-16</sup> Research using radiolabeled markers indicated that the source of increased calcium was a shell valve.<sup>23</sup> Bicarbonate that dissolved from the shell valve could buffer the



**Fig. 5** A hemolymph pH- $[\text{HCO}_3^-]$  diagram of air-exposed (closed circles, Series I), control (open circles, Series II), and immersed *Mytilus coruscus* (open squares, Series III). The  $\text{Pco}_2$  isopleths are derived from rearranging the Hendersson-Hasselbalch equation,<sup>12)</sup> and  $a_{\text{CO}_2}$  and  $\text{pKapp}$  of the experimental animals were  $40.6 \mu\text{M/L/torr}$  and  $6.2609$ ,<sup>8)</sup> respectively. The dashed line is the *in vitro* non-bicarbonate buffer line:  $[\text{HCO}_3^-] = 4.7413 - 0.4412 \cdot \text{pH}$  ( $R^2=0.7045$ ). The line was obtained from the results of *in vitro* experiments.<sup>8)</sup>

hemolymph of *M. coruscus*. According to the pH-[HCO<sub>3</sub><sup>-</sup>] diagram of *M. coruscus* hemolymph (Fig. 5), [HCO<sub>3</sub><sup>-</sup>] and Pco<sub>2</sub> increased considerably with the reduction in pH, and the points at 24 h and 48 h were located above the non-bicarbonate buffer line. The increase in acidic end-products during air exposure should dissolve the shell valve, and the bicarbonate that was mobilized from the shell compensated partially for the acidosis. Therefore, [HCO<sub>3</sub><sup>-</sup>] derived from the shell valve was applied as metabolic compensation for acidosis. The increased Pco<sub>2</sub> during air exposure derived from the liberated CO<sub>2</sub> when an acidic end-product was buffered by shell carbonates.

*M. coruscus* showed increased hemolymph pH when the experimental animals were immersed in seawater after air exposure (Fig. 5). The aerobic metabolism of the experimental animals resumed, and anaerobic metabolite were not produced. The increased [HCO<sub>3</sub><sup>-</sup>] during air exposure was consumed to compensate for acidosis, and [HCO<sub>3</sub><sup>-</sup>] decreased within 24 h in the immersed animals. The hemolymph Pco<sub>2</sub> reduced with the increase in pH. The experimental animals should resume ventilation and rapidly release CO<sub>2</sub> from the ctenidium after immersion. There were no significant differences in pH, [HCO<sub>3</sub><sup>-</sup>] and Pco<sub>2</sub> between the values at R3 h, R24 h, 0 h, and the control ( $P > 0.05$ ). *M. coruscus* during air exposure for 24–48hr showed respiratory and metabolic acidosis with partial compensation by bicarbonate mobilized from the shell, and the acid-base status recovered to the initial levels within 3–24 h after immersed in the seawater again.

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