Acid–base Balance of the Hemolymph in Hard-shelled Mussel *Mytilus coruscus* in Normoxic Conditions

Takeshi Handa†, Akira Araki and Ken-ichi Yamamoto

**Abstract**: We examined hemolymph pH, total CO₂ content (Tco₂), CO₂ partial pressure (Pco₂) and bicarbonate concentration ([HCO₃⁻]) in order to evaluate the acid–base balance of the hard-shelled mussel *Mytilus coruscus* in normoxic conditions. The hemolymph was collected anaerobically through a cannula by pretreatment of the adductor muscle by catheterization. The mean values of the hemolymph pH and Tco₂ were 7.617 and 1.44 mM/l, respectively. The CO₂ solubility coefficient (αco₂) was 40.6 μM/l/mmHg. The apparent dissociation constant of carbonic acid (pKapp) was able to be expressed using the estimated equation as follows: pKapp = - 6371.321 + 3923.163 • pH ‒ 856.100 • pH² + 82.978 • pH³ ‒ 3.014 • pH⁴. Using αco₂ and pKapp determined in this study, hemolymph Pco₂ and [HCO₃⁻] were calculated as 0.57 mmHg and 1.42 mM/l, respectively. The non-bicarbonate buffer value (β NB) was 0.44 Slykes.

**Key words**: *Mytilus coruscus*, acid-base balance, cannulation, dissociation constant of carbonic acid, CO₂ partial pressure, hemolymph

**Introduction**

The hard-shelled mussel *Mytilus coruscus* is a Mytilidae bivalve classified in the Mytiloida, PTERIOMORPHIA. *Mytilus coruscus* is distributed in East Asia and is cultivated commercially as food in China and Korea. In Japan, *M. coruscus* inhabits the rocky bottom of intertidal zones up to 20 m deep from Hokkaido to Kyushu, and it is caught as a local specialty of the littoral region. *Mytilus coruscus* has been a subject of previous research in terms of the morphology of larvae, polymorphic microsatellite loci, microsatellite markers, biochemical response to heavy metal exposure, the effect of natural biofilm on the settlement mechanism and immune activities of hemocytes. However, there are few reports on the respiratory mechanism from the viewpoint of CO₂ dynamic phase and acid–base balance in *M. coruscus*. Research into the acid–base status could contribute to efficient CO₂ utilization, which is related to respiration, and calcification for the formation of the shell valves. The acid–base balance and CO₂ dynamic phase of *M. coruscus* is useful for evaluation of fishery environments, and of the effects of ocean acidification and increase in CO₂ level. In some bivalves in normoxic and normocapnic conditions, the CO₂ partial pressure (Pco₂) of the hemolymph was 0.9 mmHg in blue mussel *Mytilus edulis*, 1.7‒2.3 mmHg in akoya pearl oyster *Pinctada fucata*, and 1.55 mmHg in noble scallop *Mimachlamys nobilis*. Because the Pco₂ values of bivalves are very low, it was supposed that the Pco₂ in *M. coruscus* would also be similarly low; however, the direct measurement of Pco₂ is difficult. The estimation CO₂ 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 estimates. In the equation, the characteristic values of the CO₂ solubility coefficient (αco₂) and apparent dissociation constant of carbonic acid (pKapp) in the hemolymph are required for the experimental animal. Therefore, we examined *M. coruscus* hemolymph pH, total CO₂ content, CO₂ partial pressure, and bicarbonate concentration using the hemolymph αco₂ and pKapp, which were determined in this study. By pretreatment...
with adductor muscle catheterization, the hemolymph was anaerobically from *M. coruscus* underwater.

### Materials and Methods

**Experimental animals and conditions**

The experiments used 40 hard-shelled mussels *Mytilus coruscus* (shell length: 123.1 ± 22 mm (mean ± SE), shell height: 58.5 ± 0.9 mm, total wet weight: 186.1 ± 6.3 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 3 months at 24℃ in aerated seawater with added cultivated phytoplankton. Twenty-four hours before collecting hemolymph, the mussels were transferred to particle-free (>0.45 μm) seawater. All experiments were conducted in seawater with a salinity of 32 psu, water temperature 24℃, O₂ saturation 99%, pH 8.15, and Tco₂ 1.2 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). The small hole (2 mm diameter) was made adjacent to the shell valves near the adductor muscle at the posterior margin. A cannula with a stylet was inserted through the hole into the adductor muscle and was advanced 0.3-0.5 cm toward the center of the adductor muscle. The stylet was removed, and the end of the cannula was closed. The cannula was gently fixed to the left shell valve with denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent any effect of the movement of the shell valves. This surgical operation was completed within 8 minutes. The cannulated mussel was transferred to a darkened respiratory chamber and was allowed to recover for 3 h at 23.7 ± 0.3℃ 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 0.3-0.4 ml.

### Hemolymph properties analysis

The hemolymph pH and Tco₂ (mM/l) were measured immediately after each collection. The pH was measured using a blood gas meter (BGM200; Cameron Instruments) using glass and reference electrodes (E301, E351; Cameron Instruments) at 23.7±0.3℃. Tco₂ was measured using a total CO₂ analyzer (Capnicon 5; Cameron Instruments). The hemolymph CO₂ partial pressure (Pco₂, mmHg) and bicarbonate concentration ([HCO₃⁻], mM/l) were calculated by rearranging the Henderson–Hasselbalch equation.

In the equation, the αco₂,μM/l/mmHg) and pKapp of the *M. coruscus* hemolymph were required. The determinations of the αco₂ and pKapp were performed by in vitro experiments. The αco₂ was determined using *M. coruscus* hemolymph adjusted to pH 2.5 by the addition of the lactic acid (Wako Pure Chemical Industries, Ltd.). The acidified sample was transferred to a tonometer flask, and equilibrated with humidified standard CO₂ gases (CO₂, 0.2, 0.5, 1.0, 2.0 and 5.0%; O₂, 20.9%; N₂ Balance) using the equilibrator (DEQ-1; Cameron Instruments) at 23.7±0.3℃, and subsequently the total CO₂ content of each equilibrated sample was measured using the total CO₂ analyzer. The αco₂ was calculated using the equation:

\[
\alpha_{CO₂} = \text{Total CO₂ content} \cdot \text{CO₂ Partial pressure}^{-1}
\]

For determination of the pKapp, hemolymph was transferred to a tonometer flask and equilibrated with humidified standard CO₂ gases (CO₂, 0.2, 0.5, 1.0, 2.0 and 5.0%; O₂, 20.9%; N₂ Balance) using an equilibrator at 23.7±0.3℃. After equilibration, the pH and total CO₂ content of the sample were measured with the blood gas meter and the total CO₂ analyzer. Using the sample pH, total CO₂ content and αco₂, calculated using the above equation, the pKapp was determined by rearrangement of the Henderson–Hasselbalch equation as follows:
pKapp = pH - log [(total CO₂ content - αco₂) • CO₂ partial pressure] - (αco₂ • CO₂ partial pressure)⁻¹

where CO₂ partial pressure is calculated from the known CO₂ concentration of standard gases.

The αco₂ and pKapp obtained in this study were used for the calculation of hemolymph Pco₂ from measured pH and Tco₂:

\[ P_{\text{CO}_2} = T_{\text{CO}_2} \cdot \left( \frac{\alpha_{\text{CO}_2} \cdot (1+10^{pH-pK_{\text{CO}_2}})}{\alpha_{\text{CO}_2} \cdot \text{CO}_2 \text{ partial pressure}} \right)^{-1} \]

The hemolymph [HCO₃⁻] was calculated from Tco₂, αco₂, and Pco₂ using the following equation (20):

\[ [\text{HCO}_3^-] = T_{\text{CO}_2} - \alpha_{\text{CO}_2} \cdot \text{P}_{\text{CO}_2} \]

The non-bicarbonate buffer value \( (\beta_{\text{NB}, \text{Slykes}}) \), which is usually described at the absolute value, was calculated as the regression coefficient relating \([\text{HCO}_3^-]\) and pH in \textit{in vitro} experiments with the standard gases.

**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 of variance was assessed using Bartlett’s test for comparison the properties of hemolymph, which was equilibrated with standard CO₂ gases. One-way analysis of variance (ANOVA) was performed for changes in hemolymph properties using the standard CO₂ gases. Statistically significant differences were set at \( P<0.01 \).

**Results**

Hemolymph samples were collected from the adductor muscles of \textit{M. coruscus} through cannulae. The collection volume was 0.3–0.4 ml from each individual. The hemolymph pH and Tco₂ in normoxic conditions were 7.617±0.0225 and 1.44±0.047 mM/l, respectively (Table 1). In \textit{in vitro} experiments, the hemolymph αco₂ was 40.6 ±0.37 μM/l/mmHg. The hemolymph pKapp at known CO₂ partial pressures (standard gases) and the corresponding measured pH and Tco₂ values are shown in Table 2. The mean value of all pKapp was 6.2609. However, the pH was statistically significantly lowered with the rise in Pco₂, and the values of pKapp with each CO₂ standard gas were statistically significantly different (Table 2). Therefore, the interaction between pKapp and pH was analyzed, and the estimated equation of pKapp was obtained as follows:

| Table 1. Hemolymph pH, total CO₂ content (Tco₂), CO₂ partial pressure (Pco₂) and bicarbonate concentration ([HCO₃⁻]) of Mytilus coruscus at 24℃ in normoxic conditions |
|---------------|--------|-----|---|
|               | Mean   | SE  | N  |
| pH            | 7.617  | 0.0225 | 16 |
| Tco₂          | 1.44   | 0.047 | 16 |
| Pco₂          | 0.57   | 0.158 | 16 |
| [HCO₃⁻]       | 1.42   | 0.043 | 16 |

Mean temperature 23.7 ℃; αco₂ 40.6 μM/l/mmHg; see the details of the pKapp equation in the Result section.
Table 2. Mean values of measured pH, total CO₂ content (Tco₂) and calculated apparent dissociation constant of carbonic acid (pKapp) of hemolymph in the adductor muscle of *Mytilus coruscus* with known Pco₂ standard gases

<table>
<thead>
<tr>
<th>Standard gas CO₂ (%)</th>
<th>Pco₂ (mmHg)</th>
<th>pH</th>
<th>Tco₂ (mM/l)</th>
<th>pKapp</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.203</td>
<td>1.51</td>
<td>7.483</td>
<td>1.549</td>
<td>6.10449</td>
<td>24</td>
</tr>
<tr>
<td>0.509</td>
<td>3.79</td>
<td>7.290</td>
<td>1.660</td>
<td>6.31157</td>
<td>24</td>
</tr>
<tr>
<td>0.993</td>
<td>7.39</td>
<td>7.074</td>
<td>1.984</td>
<td>6.33207</td>
<td>24</td>
</tr>
<tr>
<td>1.99</td>
<td>14.8</td>
<td>6.732</td>
<td>2.156</td>
<td>6.33513</td>
<td>24</td>
</tr>
<tr>
<td>4.97</td>
<td>37.0</td>
<td>6.336</td>
<td>3.569</td>
<td>6.22109</td>
<td>24</td>
</tr>
</tbody>
</table>

*Water temperature 23.7 °C; barometric pressure 765.7 mmHg; water vapor pressure 21.98 mmHg*

\[
pKapp = -6371.321 + 3923.163 \cdot \text{pH} - 856.100 \\
\quad \cdot \text{pH}^2 + 82.978 \cdot \text{pH}^3 - 3.014 \cdot \text{pH}^4
\]

Pco₂ and [HCO₃⁻] were calculated by substitution of the hemolymph αco₂ and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

\[
Pco₂ = Tco₂ \cdot \left\{ 0.0406 \cdot \left( \frac{1}{1 + 10^{(\text{pH} - pKapp)}} \right) \right\}^{-1}
\]

\[
[HCO₃⁻] = Tco₂ - 0.0406 \cdot Pco₂
\]

where the units of the parameters in the equations were mmHg for Pco₂ and mM/l for Tco₂ and [HCO₃⁻].

In *in vivo* and *in vitro* experiments, Hemolymph Pco₂ and [HCO₃⁻] at 23.7°C in normoxic conditions were 0.57 mmHg and 1.42 mM/l, respectively (Table 1). The mean values of Tco₂ and [HCO₃⁻] of hemolymph with known Pco₂ standard gases are shown in Table 3, and the non-bicarbonate buffer value (β₂bic) which was obtained as the regression coefficient relating [HCO₃⁻] and pH was 0.44 Slykes.

**Discussion**

We collected *M. coruscus* hemolymph from the adductor muscle, and examined hemolymph pH, Tco₂, Pco₂, and [HCO₃⁻] in order to evaluate the acid–base balance of *M. coruscus* in normoxic conditions. The hemolymph was collected anaerobically through a cannula from animals kept underwater after pretreatment by adductor muscle catheterization. The mean values of pH and Tco₂ measured immediately after hemolymph collection were 7.617 and 1.44 mM/l, respectively. Previously reported mean values of hemolymph pH include 7.65 in blue mussel *M. edulis* at 12°C, 7.36 in Pacific oyster *Crassostrea gigas* at 15°C, 7.55 in *M. galloprovincialis* at 18°C, 7.284–7.375 in *P. fucata* at 28°C, 7.563 in *P. margaritifera* at 26°C, and 7.442 in noble scallop *Mimachlamys nobilis* at 24°C.

Although there are few descriptions of hemolymph Tco₂ in marine bivalves, Handa and Yamamoto (2012, 2015, 2016) reported the mean values of Tco₂ in *P. fucata*, *P. margaritifera*, and *M. nobilis* as 1.90‒2.10 mM/l, 2.04 mM/l, and 1.50 mM/l, respectively. The hemolymph pH in *M. coruscus* was almost the same as that in *M. edulis* and higher than that in other marine bivalves, and the contents of carbonic acid and CO₂ in *M. coruscus* hemolymph appeared to be less than in pearl oysters.

Cameron (1986) reported the CO₂ solubility as a function of temperature and salinity, and the solubility coefficients were 39.2–42.3 μM/l/mmHg at 22–24°C and 30–35 salinity (psu). The hemolymph αco₂ in *M. coruscus* (40.6 μM/l/mmHg) was in the range of the coefficient reported in Cameron (1986). The mean value
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is 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 CO₂ and bicarbonate buffer systems with non-bicarbonate buffers is particularly advantageous when nonvolatile H⁺ ions are to be buffered in a buffer system. Therefore, the M. coruscus would experience a large change in hemolymph pH with a slight fluctuation of Pco₂. Mytilus coruscus seems to be sensitive to environmental changes in comparison with P. fucata and C. gigas from the viewpoint of acid–base balance of the hemolymph.

Acknowledgments

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References


2) Semenikhina OY, Kolotukhina NK and Evseev GA: Morphology of larvae of the family Mytilidae (Bivalvia) from the north-western part of the Sea
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正常酸素分圧条件におけるイガイヘモリンパ液の酸塩基平衡

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要  旨

イガイ（Mytilus coruscus）の酸塩基平衡を解明するため、供試貝の閉殻筋にヘモリンパ液を採取する為のカニュレーション手術を行った。手術から回復した供試貝から、カニューラを通じてヘモリンパ液を嫌気的に採取し、正常酸素分圧条件におけるイガイヘモリンパ液の酸塩基平衡を分析した。その結果、ヘモリンパ液のpH 7.617、全炭酸含量1.44 mM/l、二酸化炭素分圧0.57 mmHg、炭酸水素イオン濃度1.42 mM/lを示した（環境水の酸素飽和度99 %、pH 8.15、全炭酸含量 12 mM/l、水温24.0 ℃）。