Estimation of CO_2 partial pressure and bicarbonate concentration in the hemolymph of the black-lip pearl oyster *Pinctada margaritifera*

Takeshi Handa† and Ken-ichi Yamamoto

Abstract : We examined hemolymph pH, total CO_2 content (Tco_2, mM/*l*), CO_2 partial pressure (Pco_2, mmHg) and bicarbonate concentration ($[HCO_3^-]$, mM/*l*) in order to evaluate the acid-base balance of the black-lip pearl oyster *Pinctada margaritifera* under normoxic conditions. Hemolymph was collected anaerobically through a cannula after catheterization of the adductor muscle. The mean values of the hemolymph pH and Tco_2 were 7.563 and 2.04 mM/*l*, respectively. Using the CO_2 solubility coefficient and apparent dissociation constant of carbonic acid determined in this study, Pco_2 and $[HCO_3^-]$ were calculated as 1.50 mmHg and 1.98 mM/*l*, respectively. These values were in same range as those of the hemolymph of the akoya pearl oyster *P. fucata*.

Key words : *Pinctada margaritifera*, cannulation, hemolymph, acid-base balance, Pco_2, dissociation constant of carbonic acid

Introduction

The black-lip pearl oyster *Pinctada margaritifera* is a filibranchial bivalve classified in the Pteriidae.[1] *P. margaritifera* 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] where the annual seawater temperatures in the coastal zone of Wakayama, Kochi and Okinawa prefectures are 15–31℃.[3]

The black-lip pearl oyster has nacreous aragonite in the inner layer of its shell valves, and it is used for black pearl production worldwide. The process of pearl production is similar to the growth of the shell valves and is directly related to metabolism. The metabolism of the black-lip pearl oyster has been studied in terms of regulation of gill ventilation volume and oxygen uptake under normoxic, hypoxic and anathermal conditions.[4–7] The anatomical structures of the ctenidium, circulatory system, digestive diverticula and labial palp have been studied.[8,9] However, there are few reports on the respiratory mechanism from the viewpoint of CO_2 dynamic phase and acid-base balance. Research into the acid-base status could contribute to efficient CO_2 utilization, which is related to calcification for pearl formation and growth of the shell valve. The estimated CO_2 partial pressure of the hemolymph was 0.9 mmHg in sea mussel *Mytilus edulis*,[10] 2.3 mmHg in Asian freshwater clam *Corbicula fluminea*,[11] and 1.7–2.3 mmHg in akoya pearl oyster *P. fucata*.[12,13] Because the CO_2 partial pressures in bivalves are very low, it was supposed that the CO_2 partial pressure in the black-lip pearl oyster would also be similarly very low; however, the direct measurement of CO_2 partial pressure is difficult. The estimation of CO_2 partial pressure by application of the Henderson–Hasselbalch equation is practiced in studies of the acid-base balance owing to the relative ease and accuracy of the estimates.[14] In the equation, the characteristic values of the CO_2 solubility coefficient (αCO_2) and apparent dissociation constant of carbonic acid (pKapp) in the hemolymph are required for the experimental animal. Therefore, we examined

Department of Applied Aquabiology, National Fisheries University, Shimonoseki, Yamaguchi, Japan
†Corresponding author: handat@fish-u.ac.jp (T. HANDA)

black-lip pearl oyster hemolymph pH, total CO_2 content, CO_2 partial pressure and bicarbonate concentration using the hemolymph αCO_2 and pKapp, which were determined in this study. By means of catheterization of the adductor muscle, hemolymph was collected anaerobically from black-lip pearl oysters underwater.

Materials and Methods

Experimental animals and conditions

The experiments used 78 black-lip pearl oysters (shell length: 90.4 ± 1.8 mm (Mean ± SE), shell height: 108.1 ± 1.9 mm, and total wet weight: 188.2 ± 10.5 g). The animals were obtained from the coast of Kochi prefecture, Japan. After cleaning the shell valves, they were reared for one month at 26℃ in aerated seawater with added cultivated phytoplankton.[13,15] Twenty-four hours before collecting hemolymph, the black-lip pearl oysters were transferred to seawater that was particle-free (>0.45 μm). All experiments were conducted in seawater with a salinity of 33 psu, water temperature 26℃, O_2 saturation 98%, pH 8.10, and total CO_2 content 1.8 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). 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, which is the part of the large anterior muscle, and was advanced 0.7-1.0 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 using denture adhesive (Kobayashi Pharmaceutical Co., Ltd.) in order to prevent effects from movement of the shell valves. This surgical operation was completed within 5 minutes. The cannulated oyster was transferred to a darkened acrylic respiratory chamber, and was allowed to recover for 2 hr at 26℃ under 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 pH and total CO_2 content (Tco_2, mM/*l*) were measured immediately after each collection. The pH was measured using a blood gas meter (BGM200, Cameron Instruments) using pH glass and reference electrodes (E301, E351, Cameron Instruments) at 26.0℃. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5, Cameron Instruments). The hemolymph CO_2 partial pressure (Pco_2, mmHg) and bicarbonate concentration ($[HCO_3^-]$, mM/*l*) were calculated by rearranging the Henderson-Hasselbalch equation.[14,16] In the equation, the CO_2 solubility coefficient (αCO_2, μM/*l*/torr) and apparent dissociation constant of carbonic acid (pKapp) of black-lip pearl oysters were required. The determinations of αCO_2 and pKapp were performed *in vitro*.

αCO_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 CO_2 gas (CO_2, 15.0%; O_2, 20.9%; N_2 Balance) using an equilibrator (DEQ-1, Cameron Instruments) at 26.0℃, and subsequently the total CO_2 content of each equilibrated sample was measured with a total CO_2 analyzer. The CO_2 partial pressure of the equilibrated sample was calculated from known CO_2 concentration standard gas (15.0%), prevailing barometric pressure and water vapor pressure at 26.0℃. The CO_2 solubility coefficient was calculated using the equation:

$$\alpha CO_2 = \text{Total } CO_2 \text{ content} \bullet CO_2 \text{ Partial pressure}^{-1}$$

For determination of the apparent dissociation constant of carbonic acid, 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, 2.0 and 5.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 26.0℃. After equilibration, the pH and total CO_2 content of the sample were measured with a blood gas meter and a total CO_2 analyzer. Using the sample pH, total CO_2 content and αCO_2 calculated by the above equation, the pKapp was

determined by rearrangement of Henderson-Hasselbalch equation[14,16] as follows:

$$pKapp = pH - \log [(\text{Total } CO_2 \text{ concentration} - \alpha CO_2 \bullet CO_2 \text{ Partial pressure}) \bullet (\alpha CO_2 \bullet CO_2 \text{ Partial pressure})^{-1}]$$

where CO_2 partial pressure is calculated from known CO_2 concentration standard gases.

The αCO_2 and pKapp obtained in this study were used for calculation of hemolymph Pco_2 from measured pH and Tco_2:

$$Pco_2 = Tco_2 \bullet [\alpha CO_2 \bullet (1+10^{(pH-pKapp)})]^{-1}$$

$[HCO_3^-]$ was calculated from Tco_2, αCO_2 and Pco_2 using the equation:

$$[HCO_3^-] = Tco_2 - \alpha CO_2 \bullet Pco_2$$

Statistical analysis

All data are expressed as means ± standard error. Analysis of variance was used to test for changes in hemolymph properties using the standard CO_2 gases. Unpaired t-test was used for the comparison of mean values of hemolymph parameters. Statistically significant differences were set at $P<0.01$.

Results

Hemolymph samples were collected from the adductor muscles of black-lip pearl oysters through cannulae. The collection volume was 0.4-0.5 *ml*. The hemolymph pH and Tco_2 under normoxic conditions were 7.563 ± 0.0142 and 2.04 ± 0.058 mM/*l*, respectively. The hemolymph αCO_2 was 36.27 ± 0.740 μM/*l*/mmHg. The hemolymph pKapp at known CO_2 partial pressures (standard gases) and the corresponding measured pH and Tco_2 values are shown in Table 1. The calculated pKapp from all hemolymph samples was 5.99878 ± 0.01756. Pco_2 and $[HCO_3^-]$ were calculated by substitution of the mean value of hemolymph αCO_2 and pKapp in the rearranged Henderson-Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \bullet [0.03627 \bullet (1+10^{(pH-5.99878)})]^{-1}$$

$$[HCO_3^-] = Tco_2 - 0.03627 \bullet Pco_2$$

where the units of the parameters at the equations are mmHg in Pco_2, mM/*l* in Tco_2 and mM/*l* in $[HCO_3^-]$.

Hemolymph Pco_2 and $[HCO_3^-]$ at 26℃ under normoxic condition were 1.50 ± 0.065 mmHg and 1.98 ± 0.056 mM/*l*, respectively (Table 2). In *in vitro* experiments (Table 1), the pH decreased significantly with the increase in Pco_2 ($P<0.01$). At the same time, the interaction between pKapp and pH was analyzed (Fig. 1), and the correction equation of pKapp was obtained as follows:

$$pKapp = 174.2725 - 73.0837 \bullet pH + 10.5906 \bullet pH^2 - 0.5118 \bullet pH^3$$

For the comparison, using the hemolymph pH and Tco_2 measured immediately after collection, Pco_2 and $[HCO_3^-]$ were estimated by the correction equation and are shown in Table 3. The hemolymph Pco_2 calculated from the mean value of pKapp was higher than that by the correction equation ($P<0.01$, Table 3). There was no difference in hemolymph $[HCO_3^-]$ calculated by the two methods.

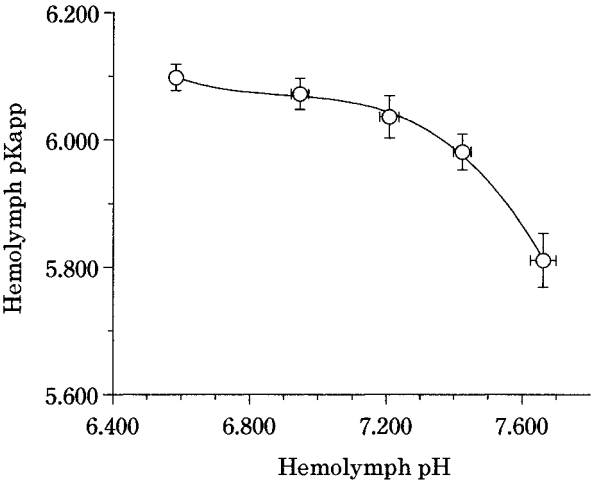

Fig. 1. Relationship between pH and apparent dissociation constant of carbonic acid (pKapp) in the hemolymph of the black-lip pearl oyster *Pinctada margaritifera* at 26℃. Values are means ± SE (N= 15). Solid line fitted to the data and the equation: $pKapp = 174.2725 - 73.0837 \bullet pH + 10.5906 \bullet pH^2 - 0.5118 \bullet pH^3$ (r=0.99)

Table 1. Mean values of measured pH, total CO_2 content (Tco_2) and calculated apparent dissociation constant of carbonic acid (pKapp) of the hemolymph in adductor muscle of the black-lip pearl oyster (*Pinctada margaritifera*) with known Pco_2 standard gases

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (mmHg)	pH	Tco_2 (mM/*l*)	pKapp	N
0.2	1.49	7.661	3.884	5.8107	15
0.5	3.72	7.422	3.874	5.9810	15
1.0	7.44	7.207	4.297	6.0358	15
2.0	14.88	6.946	4.602	6.0718	15
5.0	37.20	6.582	5.477	6.0982	15

Barometric pressure 765 mmHg, Water temperature 26.0℃

Table 2. Hemolymph pH, total CO_2 content (Tco_2), CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) of the black-lip pearl oyster (*Pinctada margaritifera*) at 26℃ under normoxic condition

		Mean	SE	N
pH		7.563	0.0142	18
Tco_2	mM/*l*	2.04	0.058	18
Pco_2	mmHg	1.50	0.065	18
$[HCO_3^-]$	mM/*l*	1.98	0.056	18

αco_2, 36.27 μM/*l*/mmHg; pKapp, 5.99878

Table 3. The compariosn of the hemolymph Pco_2 and $[HCO_3^-]$ calculated by the mean value of pKapp and by the correction equation

	Pco_2 (mmHg)	$[HCO_3^-]$ (mM/*l*)	N
the mean value of pKapp	1.50	1.98	18
the correction equation of the pKapp	1.20 *	1.99	18

* : statistically significant difference (unpaired *t*-test, $P<0.01$)

Discussion

We collected hemolymph from the adductor muscle and examined hemolymph pH, total CO_2 content, CO_2 partial pressure and bicarbonate concentration in order to evaluate the acid-base balance of black-lip pearl oysters. The hemolymph collected anaerobically through a cannula from black-lip pearl oysters underwater by pretreatment of the adductor muscle catheterization. The mean values of pH and Tco_2 measured immediately after hemolymph collection were 7.563 and 2.04 mM/*l*, respectively. The mean values of hemolymph pH were 7.65 in sea mussel *M. edulis* at 12℃,[10] 7.36 in Pacific oyster *Crassostrea gigas* at 15℃,[17] 7.55 in *M. galloprovincialis* at 18℃,[18] and 7.284–7.375 in the akoya pearl oyster *P. fucata* at 28℃.[13] Although there are few descriptions of the hemolymph total CO_2 content in marine bivalves, Handa and Yamamoto (2011, 2012) reported that the mean value in the akoya pearl oyster *P. fucata* was 1.90–2.10 mM/*l*.[12,13] The hemolymph pH and Tco_2 in the black-lip pearl oyster were almost the same as those in other marine bivalves.

Cameron (1986) reported the CO_2 solubility of seawater as a function of temperature, and the solubility coefficient was 35.49–38.12 μM/*l*/mmHg at 26–28℃.[19] The hemolymph αCO_2 in the black-lip pearl oyster (36.27 μM/*l*/mmHg) was in the range of the coefficient of seawater. The mean value of the hemolymph pKapp in this study was 5.99878, whereas that in the adductor muscle of akoya pearl oyster hemolymph at 28℃ was 5.8191,[13] and that in hemolymph of sea mussel *M. edulis* at 12℃ was 6.114.[10,20] The apparent dissociation constant of carbonic acid is equal to the pH at which it shows the most effective as a buffer.[21] The most effective buffer of the black-lip pearl oyster hemolymph seemed to be lower than that in sea mussel *M. edulis*, and was consistent with that in the akoya pearl oyster *P. fucata* which is classified into the same genus as the black-lip pearl oyster.

Using the αCO_2 and pKapp in this study, Pco_2 and [HCO_3^-] were calculated. The mean values of hemolymph Pco_2 and [HCO_3^-] were 1.50 mmHg and 1.98 mM/*l*, respectively (Table 2). In some marine bivalves, mean values of hemolymph Pco_2 and [HCO_3^-] were 0.9 mmHg and 1.8 mM/*l* in sea mussel *M. edulis* at 12 ℃[10], 0.15 kPa (2.0 mmHg) and 1.37 mM/*l* in Pacific oyster *C. gigas* at 15℃[17], 1.15 mmHg and 1.62 mM/*l* in *M. galloprovincialis* at 18℃[18], and 2.08–2.33 mmHg and 1.83–2.04 mM/*l* in the akoya pearl oyster *P. fucata* at 28℃.[13] The hemolymph acid-base status in the black-lip pearl oyster was similar to those in other marine bivalves. In teleosts, the blood Pco_2 in the carp *Cyprinus carpio* was 3.8–4.2 mmHg,[22,23] which is 10% that of humans (Pco_2 = 40 mmHg). The carp blood Pco_2 suggested that CO_2 is excreted rapidly from the blood, which enables common carp to live in ambient water where pH and dissolved gases often vary.[22,23] Therefore, the black-lip pearl oyster should excrete or utilize CO_2 rapidly from the hemolymph, which then regulates the acid-base balance and calcification for shell valves and pearl formation.

On the other hand, the pH decreased significantly with the increase in Pco_2 (Table 1). The relationship of hemolymph pH and pKapp was shown, and Pco_2 and [HCO_3^-] were estimated on the basis of the correction equation. The hemolymph Pco_2 estimated by the correction equation was lower than that from the mean value of pKapp, nevertheless there was no difference in [HCO_3^-] (Table 3). αCO_2 and pKapp vary with ionic strength and temperature,[14] and estimation of the Pco_2 could be affected by temperature and salinity. Therefore, it is necessary to examine these parameters at various temperatures and salinities in order to increase the accuracy of the calculation of Pco_2 and to formulate the correction equation for pH, salinity and water temperature.

References

1) Okutani T: Pterioida. *In*: Okutani T (ed) Marine Mollusks in Japan. Tokai University Press, Tokyo, 881 (2000)

2) Wada KT, Temkin Ilya: Taxonomy and Phylogeny.

In: Southgate PC, Lucas JS (eds) The Pearl Oyster. Elsevier, United Kingdom, 37–75 (2008)

3) Japan Oceanographic data center: Statistical analyzed result Water Temperature. Japan Coast Guard, http://www.jodc.go.jp/service.htm

4) Yamamoto K, Handa T, Minato Y, Odawara K, Sone K: Effect of low salinity on ventilation volumes in the black-lip pearl oyster *Pinctada margaritifera* and the pearl oyster *Pinctada fucata martensii*. *Aquaculture Sci*, **60**, 277–280 (2012)

5) Yamamoto K, Handa T, Araki A, Minato Y, Odawara K and Sone K: Effect of trichlorfon on ventilation in the Black-lip pearl oyster *Pinctada margaritifera*. *J Nat Fish Univ*, **61**, 11–14 (2012)

6) Yamamoto K, Handa T, Minato Y, Odawara K, Sone K: Effects of water temperature rise on ventilation in the black-lip pearl oyster *Pinctada margaritifera*. *J Nat Fish Univ*, **61**, 15–18 (2012)

7) Yamamoto K, Handa T, Minato Y, Odawara K, Sone K: Effect of hypoxia on oxygen uptake in the black-lip pearl oyster *Pinctada margaritifera*. *J Nat Fish Univ*, **61**, 19–22 (2012)

8) Yamamoto K, Handa T: Structure of the digestive diverticula of the black-lip pearl oyster *Pinctada margaritifera* (Mollusca: Bivalvia Pterioida = Pseudolamellibranchia). *J Nat Fish Univ*, **59**, 39–52 (2010)

9) Yamamoto K, Handa T: Structure of the ctenidium and labial palp of the black-lip pearl oyster *Pinctada margaritifera* (Pteriidae: Pterioida = Pseudolamellibranchia: Bivalvia). *J Nat Fish Univ*, **59**, 53–73 (2010)

10) 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)

11) Byrne RA, Shipman BN, Smatresk NJ, Dietz TH, McMahon RF : Acid-base balance during emergence in the freshwater bivalve *Corbicula fluminea*. *Physiol Zool*, **64**, 748–766 (1991)

12) 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)

13) 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)

14) Boutilier RG, Iwama GK, Heming TA, Randall DJ: The apparent pK of carbonic acid in rainbow trout blood plasma between 5 and 15℃. *Resp. Physiol*, **61**, 237–254 (1985)

15) 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)

16) Davenport HW: Fundamental equation. *In*: The ABC of acid-base chemistry 6th edition. University of Chicago Press, Chicago, 39–41 (1974)

17) 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 (2005a)

18) Michaelidis B, Ouzounis 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 (2005b)

19) 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)

20) 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)

21) Thomas RC: Intracellular pH. In: Hainsworth R (ed) Acid-base balance. Manchester University Press, United Kingdom, 50–74 (1986)

22) Itazawa Y, Takeda T: Gas exchange in the carp gills in normoxic and hypoxic conditions. *Resp Physiol*, **35**,

263–269 (1978)

23) Handa T, Yamamoto K: Blood and cardiovascular-respiratory properties of the common carp *Cyprinus carpio* under resting conditions. *J Nat Fish Univ*, **62**, 19–29 (2013)

クロチョウガイのヘモリンパ液における二酸化炭素分圧と炭酸水素イオン濃度の算出

半田岳志・山元憲一

要　旨

クロチョウガイの酸塩基平衡を明らかにするため、クロチョウガイ閉殻筋からポリエチレン細管を通してヘモリンパ液を嫌気的に採取し、pHと全炭酸含量を測定した。さらに二酸化炭素分圧と炭酸水素イオン濃度を算出するため、クロチョウガイヘモリンパ液の二酸化炭素溶解度と炭酸の解離定数を分析した。その結果、水温26度におけるクロチョウガイのヘモリンパ液pH は7.563（平均値）、全炭酸含量は2.04 mM/*l*と測定された。本研究で分析した二酸化炭素溶解度と炭酸解離定数を用いて二酸化炭素分圧と炭酸水素イオン濃度を算出すると、それぞれ1.50 mmHg、1.98 mM/*l*を示した。これらの値は、アコヤガイのヘモリンパ液での値に近似した。