Estimation of hemolymph αco_2 and pKapp in disk abalone *Haliotis (Nordotis) discus discus* between 10℃ and 20℃

Takeshi Handa † and Akira Araki

Abstract: We investigated the effect of temperature on disk abalone *Haliotis (Nordotis) discus discus* hemolymph CO_2 solubility coefficient (αco_2) and the apparent dissociation constant of carbonic acid (pKapp). The disk abalone hemolymph was equilibrated with a standard CO_2 gas mixture between 10℃ and 20℃, to obtain expressions for αco_2 and pKapp as a function of temperature. The relationship between αco_2 and temperature (T) is expressed as follows: $\alpha co_2 = 74.005 - 1.2936 \bullet T - 0.00944 \bullet T^2$. And the relationship between pKapp and temperature expressed as follows: $pKapp = 6.4675 - 0.08682 \bullet T + 0.003996 \bullet T^2$. In these equations, the parameter units are ℃ for T and μM/L/torr for αco_2. The non-bicarbonate buffer values (β_{NB}), obtained as a regression coefficient relating pH and $[HCO_3^-]$, were 2.5 Slykes at 10℃, 2.2 Slykes at 15℃, and 3.4 Slykes at 20℃. These equations enable estimation of hemolymph αco_2 and pKapp between temperatures of 10℃ and 20℃.

Key words: *Haliotis (Nordotis) discus discus*, disk abalone, hemolymph acid–base balance, CO_2 solubility (αco_2), apparent dissociation constant (pKapp), temperature effects

Introduction

Disk abalone *Haliotis (Nordotis) discus discus* is a marine mollusc classified in the Haliotidae, Vetigastropoda, GASTROPODA, and inhabits intertidal zones at a depth of about 20 m throughout the whole of the Japan Sea and the Pacific coast from Ibaraki Prefecture to Kyushu[1]. In Japan, the disk abalone is traded as an economical importance species, and was previously evaluated in terms of juvenile abalone growth[2-4], ammonia excretion[5], oxygen consumption[5], amyotrophia[6-9], and immune responses to bacterial and viral stresses[10,11]. The anatomical structures of the digestive diverticula, ctenidium, and circulatory system are also well studied in this species[12,13]. Gill ventilation volume regulation and O_2 uptake in the disk abalone was evaluated for both normoxic and hypoxic conditions[14-17]. The disk abalone hemolymph acid–base balance under resting conditions was also studied[18]. In normoxic and normocapnic seawater at 28℃, the disk abalone has a hemolymph pH of 7.320 and total CO_2 concentration (Tco_2) of 1.78 mM/L[18]. The CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) for the hemolymph, calculated by the Henderson–Hasselbalch equation, was reported as 4.2 torr and 1.63 mM/L, respectively[18]. The Henderson–Hasselbalch equation is often used in studies involving acid–base balance to calculate Pco_2 owing to the relative ease and accuracy of this method[19]. In the equation, the CO_2 solubility coefficient (αco_2) and apparent dissociation constant of carbonic acid (pKapp) of the hemolymph are required for the experimental animals. The αco_2 and pKapp vary with temperature[19] but there are few reports on the effect of temperature on the hemolymph αco_2 and pKapp in Haliotidae. Therefore, we undertook a preliminary investigation to evaluate the effect of temperature on disk abalone hemolymph αco_2 and pKapp. If the relationships between temperature on the αco_2 and pKapp are simple for disk abalone hemolymph, the Pco_2 and $[HCO_3^-]$ may be easily calculated, which is useful in understanding the hemolymph acid–base balance,

Received date:29 November 2023, Accepted date 16 January 2024
Affiliation: Department of Applied Aquabiology, National Fisheries University, Nagata-honmachi, Shimonoseki, Yamaguchi Pref., JAPAN
† Corresponding author: handat@fish-u.ac.jp (T. HANDA)

respiratory physiology, and interpreting the effects of temperature on disk abalone aquaculture environments. In this study, we equilibrated disk abalone hemolymph with CO_2 standard gas mixes between 10℃ and 20℃ to obtain relational expressions between the coefficients (αco_2 and pKapp) and temperature.

Materials and Methods

Experimental animals and conditions

These experiments used 102 disk abalone *Haliotis (Nordotis) discus discus* (total wet weight: 97.8 ± 16.6 g (mean ± SD)). The animals were obtained from a commercial marine farm in Yamaguchi prefecture, Japan. After cleaning the shell surface, the animals were reared over 1 month in aerated seawater at 10℃, 15℃, or 20℃ and fed seaweed (*Sargassum macrocarpum, Ecklonia kurome* and *Ulva pertusa*). Twenty-four hours before hemolymph collection, the disk abalone were transferred to particle-free (>0.45μm) seawater without seaweed. All experiments were conducted in seawater with a salinity of 30 psu, O_2 saturation 98%, pH 8.0, and total CO_2 concentration 1.5 mM/L.

Animal surgery and hemolymph collection

Disk abalone, reared at each temperature (10℃, 15℃, or 20℃), were subjected to a surgical operation to collect the hemolymph[18]. The disk abalone was submerged in $MgCl_2$ solution (29-31 psu) to prevent muscle contraction[20]. After muscle relaxation, a polyethylene tube (0.96 mm outer diameter, 0.58 mm inner diameter, PE-50, Becton Dikinson CO.) was inserted into the vein located near the margin of the shell[18]. The cannulated animals were then transferred to a respiratory chamber supplied with flowing aerated water, and allowed to recover overnight at the assigned temperatures. Hemolymph was collected through the cannula for *in vitro* experiments.

Experimental protocols

Hemolymph αco_2 was determined by first adjusting to pH 2.5 by the addition of lactic acid (Wako Pure Chemical Industries, Ltd.). The hemolymph with lactic acid was centrifuged, and the supernatant used for αco_2 analysis. The supernatant sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gas (CO_2, 5.0% or 15.0%; O_2, 20.9%; N_2 Balance) using an equilibrator (DEQ-1, Cameron Instruments) at 10℃, 15℃, or 20℃, and measuring the Tco_2 for each equilibrated sample. The Pco_2 of the equilibrated sample was calculated using the known standard gas CO_2 concentration, barometric pressure, and water vapor pressure at each experimental temperature. The αco_2 was calculated using the equation:

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

For pKapp determination, the hemolymph was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gases (CO_2, 0.1-5.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 10℃, 15℃, or 20℃. After equilibration, the pH and Tco_2 of the sample were measured. Using the sample pH, Tco_2, and αco_2 which calculated from the above equation, pKapp was determined by rearrangement of the Henderson–Hasselbalch equation[19,21] as follows:

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

where Pco_2 was calculated from the standard gas' known CO_2 concentration.

Hemolymph analysis

Tco_2 was measured using a total CO_2 analyzer (Capnicon 5, Cameron Instruments). The pH was measured using a blood gas meter (BGM200, Cameron Instruments) with pH glass and reference electrodes (E301, E351, Cameron Instruments). The pH electrodes were installed in a water jacket and maintained at experiment temperatures. Hemolymph Pco_2 and [HCO_3^-] were calculated by rearranging the Henderson–Hasselbalch equation[19,21]. The αco_2 and pKapp obtained here were then used for hemolymph Pco_2 calculation

from 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, or from αco_2, Pco_2, pH, and pKapp using the equation:

$$[HCO_3^-] = Tco_2 - \alpha co_2 \bullet Pco_2$$
$$[HCO_3^-] = \alpha co_2 \bullet Pco_2 \bullet 10^{(pH - pKapp)}$$

For assessment of the relationship between hemolymph pH and $[HCO_3^-]$ in the experimental animals, the non-bicarbonate buffer values (β_{NB}) were calculated from the slope of the relational expression between pH and $[HCO_3^-]$.

Statistical analysis

All data are expressed as mean ± standard error. The Kruskal–Wallis test was performed for changes in hemolymph sample properties and the calculated αco_2 or pKapp. Multiple comparison for all pairs used the Steel–Dwass test. Statistically significant differences were set at $P < 0.05$. All analyses were carried out using the statistical software Kyplot v. 6.0 (KyensLab Inc., Japan).

Results

The mean hemolymph αco_2 values between 10℃ and 20℃ are 44.4 − 60.1 μM/L/torr (Table 1) and are significantly different ($P<0.05$). A polynomial equation was fitted to the αco_2 data obtained at the experimental temperatures (Fig. 1). The equation of αco_2 and temperature is expressed as follows:

$$\alpha co_2 = 74.005 - 1.2936 \bullet T - 0.00944 \bullet T^2$$

where T is the temperature, and the parameter units in the equations are μM/L/torr for αco_2 and ℃ for T.

The hemolymph pKapp at known Pco_2 (standard gases), with corresponding measured pH and Tco_2 are shown for each temperature in Table 2-4. The changes in pH, Tco_2, and pKapp between 10℃ and 20℃ significantly associated with the increase in Pco_2 ($P<0.05$), except for pKapp at 10℃. The distribution of pKapp mean values and the corresponding pH are shown for each temperature in Fig. 2. The mean values of pKapp at 20℃ were higher than that at 10℃ and 15℃ ($P<0.05$). The mean hemolymph pKapp values were 5.99896 ± 0.01936 at 10℃, 6.06434 ± 0.02193 at 15℃, and 6.32955 ± 0.03880 at 20℃. The polynomial equation was fitted to the mean pKapp values at the experimental temperatures (Fig. 3). The relationship between pKapp and temperature is expressed as follows:

$$pKapp = 6.4675 - 0.08682 \bullet T + 0.003996 \bullet T^2$$

where T is temperature in ℃.

The hemolymph pH and calculated $[HCO_3^-]$ values are listed in Table 5. The non-bicarbonate buffer values (β_{NB}), obtained as a regression coefficient relating pH and $[HCO_3^-]$, were 2.5 Slykes at 10℃, 2.2 Slykes at 15℃, and 3.4 Slykes at 20℃.

Table 1. Disk abalone *Haliotis (Nordotis) discus discus* hemolymph CO_2 solubility (αco_2) at three water temperatures

WT	CO_2 solubility (μM/L/torr)		
(°C)	Mean	SE	N
10	60.1	3.08	6
15	52.5	1.59	6
20	44.4	0.79	5

Table 2. Mean values of measured pH, total CO_2 content (Tco_2) and calculated apparent dissociation constant of carbonic acid (pKapp) of disk abalone *Haliotis (Nordotis) discus discus* hemolymph with known Pco_2 standard gases at 10℃

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/L)	pKapp	N
0.102	0.770	7.396	1.20	6.0061130	5
0.203	1.54	7.237	1.40	6.0946147	5
1.01	7.65	6.751	3.26	5.9680550	5
2.00	15.1	6.526	4.00	5.9902950	5
5.01	37.8	6.182	6.41	5.9270075	5

Barometric pressure, 766.6 ± 6.6 torr; water temperature, 10.3 ± 0.3°C (Mean ± SD).
Mean value of pKapp, 5.99896 ± 0.019368.

Table 3. Mean values of measured pH, total CO_2 content (Tco_2) and calculated apparent dissociation constant of carbonic acid (pKapp) of disk abalone *Haliotis (Nordotis) discus discus* hemolymph with known Pco_2 standard gases at 15℃

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/L)	pKapp	N
0.203	1.53	7.320	1.388	6.125411	6
0.515	3.87	7.050	1.860	6.151023	6
1.01	7.60	6.834	2.736	6.067520	6
2.00	15.0	6.576	3.657	6.018804	6
5.01	37.6	6.220	5.586	5.958987	6

Barometric pressure, 765 ± 3.3 torr; water temperature, 15.0 ± 0.1°C (Mean ± SD).
Mean value of pKapp, 6.06434 ± 0.021930.

Table 4. Mean values of measured pH, total CO_2 content (Tco_2) and calculated apparent dissociation constant of carbonic acid (pKapp) of disk abalone *Haliotis (Nordotis) discus discus* hemolymph with known Pco_2 standard gases at 20℃

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/L)	pKapp	N
0.203	1.51	7.500	1.043	6.258071	6
0.515	3.83	7.361	1.808	6.292781	6
1.01	7.51	7.232	2.623	6.307798	6
2.00	14.9	7.144	3.472	6.418255	6
5.01	37.2	6.803	4.877	6.397612	6

Barometric pressure, 760 ± 1.6 torr; water temperature, 20.0 ± 0.2°C (Mean ± SD).
Mean value of pKapp, 6.32955 ± 0.03880.

Table 5. Mean values of measured pH and calculated bicarbonate concentration ($[HCO_3^-]$) of disk abalone *Haliotis (Nordotis) discus discus* hemolymph with known Pco_2 standard gases at 10-20℃

Standard gas	10°C		15°C		20°C	
CO_2 (%)	pH	$[HCO_3^-]$ (mM/L)	pH	$[HCO_3^-]$ (mM/L)	pH	$[HCO_3^-]$ (mM/L)
0.102	7.396	1.15	—	—	—	—
0.203	7.237	1.30	7.320	1.31	7.471	1.56
0.515	—	—	7.050	1.66	7.232	2.02
1.01	6.751	2.78	6.834	2.34	7.295	2.89
2.00	6.526	3.13	6.576	2.87	7.092	3.15
5.01	6.182	4.078	6.220	3.61	6.913	3.51

The non-bicarbonate buffer value (β_{NB}), 2.5 Slykes at 10°C; 2.2 Slykes at 15°C; 3.4 Slykes at 20°C.

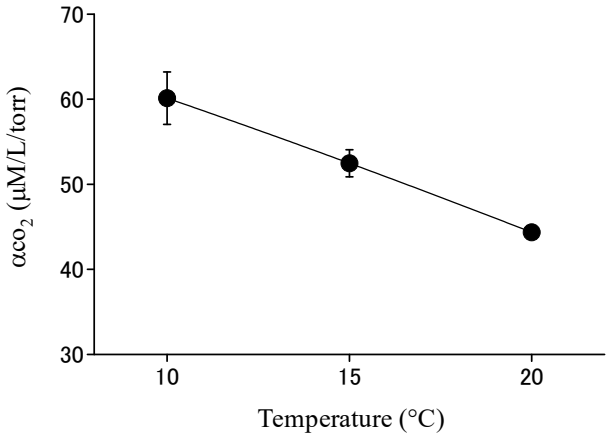

Fig. 1 Influence of temperature on CO_2 solubility coefficient (αco_2) for disk abalone *Haliotis (Nordotis) discus discus* hemolymph between 10℃ and 20℃. Data are Mean ± SE. Solid lines are fitted to the data and the equation: $\alpha co_2 = 74.005 - 1.2936 \bullet T - 0.00944 \bullet T^2$ ($R^2 = 0.9997$).

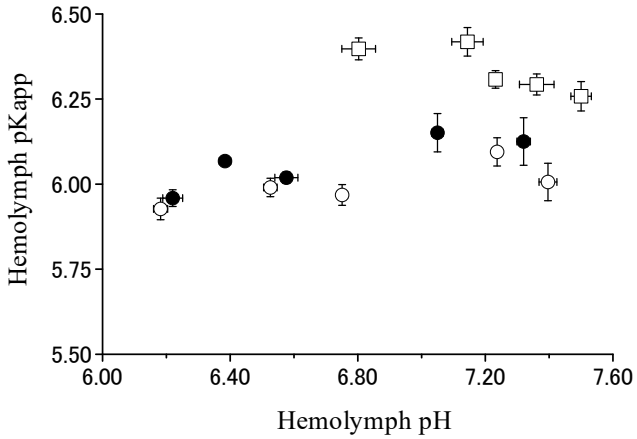

Fig. 2 Mean value distributions for pKapp and corresponding pH of the hemolymph in disk abalone *Haliotis (Nordotis) discus discus* at each temperature. Data are Mean ± SE. Open circle: 10℃; solid circle: 15℃; open square: 20℃. The mean values of pKapp at 20℃ were higher than that at 10℃ and 15℃ ($P<0.05$, Steel–Dwass multiple comparison test).

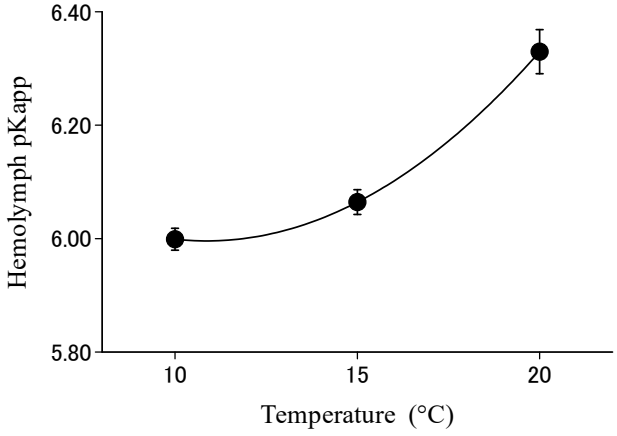

Fig. 3 Influence of temperature on the apparent carbonic acid dissociation constant (pKapp) of disk abalone *Haliotis (Nordotis) discus discus* hemolymph between 10℃ and 20℃. Data are Mean ± SE. The curved line is fitted to the data and the equation: $pKapp = 6.4675 - 0.08686 \bullet T + 0.003996 \bullet T^2$ ($R^2 = 0.8915$).

Discussion

We undertook a preliminary investigation of the effect of temperature on disk abalone hemolymph αco_2 and pKapp, and clarified these relationships between 10℃ and 20℃. Disk abalone hemolymph αco_2 was 44–60 μM/L/torr at the experimental temperature. Cameron (1986) reported CO_2 solubility as a function of temperature and salinity, with solubility coefficients of 44.74–60.80 μM/L/torr at a salinity of 30 (psu) between 10℃ and 20℃[22]. The obtained hemolymph αco_2 of the disk abalone between 10℃ and 20℃ reflects the coefficients reported by Cameron (1986). With increasing temperature, the hemolymph αco_2 decreased, with significantly different values at each temperature evaluated. Fitted to the disk abalone hemolymph αco_2 data at each experimental temperature we obtained the polynomial equation (Fig. 1). Thus, the hemolymph αco_2 equation enables αco_2 estimation in the temperature range between 10℃ and 20℃.

The distributions of the hemolymph pKapp differed with the corresponding pH at each temperature (Fig. 2). The pKapp at 20℃ was higher than that at other temperatures, with no significant differences in pKapp distribution between 10℃ and 15℃. The mean pKapp value was 5.999 at 10℃, 6.064 at 15℃, and 6.330 at 20℃. The pKapp value is equal to the pH at which it is most effective as a buffer[23]. Thus, the disk abalone hemolymph increases the buffer effectiveness at higher temperatures. This phenomenon may relate to seasonal variations in chemical properties of the hemolymph, such as ion or proteins. Although there are few reports of disk abalone hemolymph pKapp values, other reported marine bivalve hemolymph pKapp values include 6.114 for *Mytilus edulis* at 12℃[24,25], 6.073 for *Crassostrea gigas* at 23℃[26], 6.064 for Mimaclamys nobilis at 24℃[27], 5.998 for *Pinctada margaritifera* at 26℃[28], and 5.819 for *P. fucata martensii* at 28℃[29]. As the hemolymph pKapp value observed in this study was higher than those reported for bivalves, the disk abalone hemolymph appears a more effective buffer compared with the bivalves. The polynomial equation (Fig. 3) was obtained by fitting the disk abalone hemolymph pKapp data obtained at various experimental temperatures. Thus, hemolymph pKapp may be estimated for temperatures between 10℃ and 20℃.

The hemolymph $[HCO_3^-]$ in disk abalone was calculated using the hemolymph αco_2 and pKapp. The regression coefficient relating $[HCO_3^-]$ and the pH was the non-bicarbonate buffer value. The β_{NB} at 20℃ was 3.4 Slykes, and higher than that at 10-15℃ (2.2-2.5 Slykes). The non-bicarbonate buffer value was determined 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[23,30]. In disk abalone, hemolymph pH changes at 20℃ are predicted to require greater quantities of acid or base compared to the lower temperatures. Thus, at 20℃, the disk abalone may be better able to maintain hemolymph pH.

According to the public data of Japan Meteorological Agency, the coastal sea surface temperatures from Ibaragi prefecture to Kyusyu, Nagasaki prefecture are 13-28℃ [31], which is the habitat of the disk abalone. Komazawa et al. (2004a, 2004b) compared disk abalone juvenile growth rates in different water temperatures (13-28℃), reporting the highest juvenile growth rate was at 13℃ and 16-19℃[3,4]. Yamamoto et al. (2011) reported the effect of temperature on adult disk abalone respiration, suggesting an upper limit of suitable water temperature between 22℃ and 25 ℃ from gill ventilation volume and oxygen uptake measurements[15]. In this study, the hemolymph acid–base balance was examined between 10℃ and 20℃. This temperature range was narrower than the conditions of the previous reports and will be expanded to evaluate the acid–base equilibrium at other temperatures. Boutilier et al. (1985) described αco_2 and pKapp variations with temperature and ionic strength[19]. As hemolymph pKapp changes were influenced by the pH, it is necessary to study the relationships among temperature, pH, and pKapp of the disk abalone hemolymph.

Acknowledgments

We would like to express our sincere gratitude to Dr. Ken-ichi Yamamoto, Professor Emeritus, for securing the experimental animals for this study.

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