Acid–Base Balance of Hemolymph in Disk Abalone *Haliotis* (*Nordotis*) *discus discus* in Normoxic Conditions

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Abstract : We examined hemolymph pH, total CO_2 content (Tco_2), CO_2 partial pressure (Pco_2), and bicarbonate concentration ($[HCO_3^-]$) in order to evaluate the acid–base balance of disk abalone *Haliotis* (*Nordotis*) *discus discus* in normoxic conditions. Hemolymph from disk abalone submerged in experimental seawater was collected anaerobically from the vein located near the margin of the shell using a cannula. The mean values of hemolymph pH and Tco_2 were 7.320 and 1.78 mM/L, respectively. The apparent dissociation constant of carbonic acid (pKapp) was estimated using the following equation: $pKapp = -7.322 + 2.367 \bullet pH + 0.176 \bullet pH^2 - 0.0335 \bullet pH^3$. Using αco_2 (37.13 μM/L/torr) and pKapp determined in this study, the hemolymph Pco_2 and $[HCO_3^-]$ were calculated as 4.21 torr and 1.63 mM/L, respectively. The non-bicarbonate buffer value was 3.62 Slykes. These hemolymph properties were compared with those of other molluscan species, Pteriidae bivalves. Disk abalone could have a hemolymph acid–base balance that is similar to other Haliotidae, and have higher buffer capacity of non-bicarbonate buffer system than bivalves.

Key words : *Haliotis* (*Nordotis*) *discus discus*, hemolymph, acid–base balance, normoxia, carbonic acid, Pco_2

Introduction

Disk abalone *Haliotis* (*Nordotis*) *discus discus* is a marine mollusc classified in the Haliotidae, Vetigastropoda, GASTROPODA.[1)] Haliotidae abalones are economically important species worldwide, and the production volume amounted to about 162,770 tons from fisheries and aquaculture in Asia, Africa, Europe, America, and Oceania in 2016.[2,3)] In Japan, disk abalone inhabit the intertidal zone at a depth of about 20 m around the whole of the Japan Sea and the Pacific coast from Ibaraki Prefecture to Kyushu,[1)] and is produced as an expensive food. The disk abalone has been the subject of previous research in terms of the growth of juvenile abalone,[4)] ammonia excretion,[5)] oxygen consumption,[5)] amyotrophia,[6-9)] and immune responses to bacterial and viral stresses.[10,11)] The anatomical and histological structures of the digestive diverticula, ctenidium, and circulatory system were clarified recently in this species.[12,13)] The regulation of ventilation volume and O_2 uptake of the disk abalone ctenidium in normoxic and hypoxic conditions has been studied.[14-17)] However, there are few reports on the respiratory mechanism from the viewpoint of CO_2 dynamic phase and acid–base balance in disk abalone. Research into the acid–base balance could contribute to understanding efficient CO_2 utilization, which is related to respiration, and calcification for the formation of the shell in this species. The acid–base status and CO_2 dynamic phase of disk abalone was useful for the evaluation of cultivation environments, and of the effects of ocean acidification and increasing CO_2 levels. In some marine bivalves classified in mollusca, CO_2 partial pressure (Pco_2) of the hemolymph was 0.57–2.3 mmHg (torr) in normoxic and normocapnic conditions.[18-25)] The hemolymph Pco_2 of disk abalone was supposed to be low and similar to those molluscs; therefore, direct measurements of Pco_2 would be difficult. The estimation of Pco_2 by application of the Henderson–Hasselbalch equation is practiced in studies of acid–base balance owing to the relative ease and accuracy of the estimates.[26)] In the equation, the characteristic values of the CO_2 solubility coefficient (αco_2) and apparent

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dissociation constant of carbonic acid (pKapp) in the hemolymph were required for experimental animals. Therefore, we determined hemolymph αco_2 and pKapp, and estimated hemolymph Pco_2 and bicarbonate concentration ($[HCO_3^-]$), and evaluated acid–base balance of disk abalone hemolymph in normoxic conditions.

Materials and Methods

Experimental animals and conditions

The experiments used 19 disk abalone *Haliotis* (*Nordotis*) *discus discus* (total wet weight: 94.3 ± 20.2 g (mean ± SD)). The animals were obtained from a commercial marine farm in Yamaguchi prefecture, Japan. After cleaning the surface of the shell, the animals were reared by feeding the seaweed (*Sargassum macrocarpum, Ecklonia kurome* and *Ulva pertusa*) for 2 months in aerated seawater at 28℃. Twenty-four hours before collecting hemolymph, the disk abalone were transferred to particle-free (>0.45 μm) seawater without seaweed. All experiments were conducted in seawater with a salinity of 30 psu, water temperature 28℃, O_2 saturation 98%, pH 8.1, and total CO_2 concentration 1.5 mM/L.

Hemolymph collection and analysis

The disk abalone was submerged in $MgCl_2$ solution (29–31 psu) in order to prevent the contraction of the muscle.[27] After the muscle relaxed, a polyethylene tube (0.96 mm outer diameter, 0.58 mm inner diameter, PE-50, Clay Adams) was inserted into the vein located near the margin of the shell. The cannulated animal was transferred to normoxic seawater in a respiratory chamber and allowed to recover for 1–3 hr at 28.0 ± 0.1℃. The hemolymph sample was then drawn anaerobically through the cannula using a gas-tight microsyringe (Model 1750, Hamilton Co.). The volume of collected hemolymph was 0.4–0.5 mL.

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) with pH glass and reference electrodes (E301, E351, Cameron Instruments). The pH electrodes were installed in a water jacket maintained at 28.0℃. Tco_2 was measured using a total CO_2 analyzer (Capnicon 5, Cameron Instruments). Hemolymph CO_2 partial pressure (Pco_2, torr) and bicarbonate concentration ($[HCO_3^-]$, mM/L) were calculated by rearranging the Henderson–Hasselbalch equation.[26,28] In the equation, CO_2 solubility coefficient (αco_2, μM/L/torr) and apparent dissociation constant of carbonic acid (pKapp) of disk abalone were required. The determinations of αco_2 and pKapp were performed by *in vitro* experiments.

αco_2 was determined using hemolymph that was adjusted 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 was used for αco_2 analysis. The supernatant 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 28.0℃, and subsequently the Tco_2 of each equilibrated sample was measured using a total CO_2 analyzer. The Pco_2 of the equilibrated sample was calculated from known CO_2 concentration standard gas (15.0%), prevailing barometric pressure, and water vapor pressure at 28.0℃. The αco_2 was calculated using the equation:

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

For the determination of pKapp, the hemolymph sample was transferred to a tonometer flask and equilibrated with humidified standard CO_2 gases (CO_2, 0.1%, 0.2%, 0.5%, 1.0%, 2.0%, and 5.0%; O_2, 20.9%; N_2 Balance) using an equilibrator at 28.0℃. After equilibration, the pH and Tco_2 of the sample were measured using a blood gas meter and total CO_2 analyzer. Using the sample pH, Tco_2, and αco_2 calculated from the above equation, pKapp was determined by rearrangement of the Henderson–Hasselbalch equation [26,28] 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 known CO_2 concentration

standard gases.

The αco_2 and pKapp obtained in this study were used for the 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. Kruskal–Wallis test was performed for changes in hemolymph properties using the standard gases. The comparison of two parameters used Mann–Whitney *U* test. Statistically significant differences were set at $P<0.05$.

Results

Hemolymph samples were collected anaerobically from disk abalones through a cannula. The mean values of hemolymph pH and Tco_2 in normoxic conditions were 7.320 and 1.78 mM/L, respectively (Table 1). The hemolymph αco_2 was 37.13 μM/L/torr. The hemolymph pKapp at known CO_2 partial pressures (standard gases) and the corresponding measured pH and Tco_2 values are shown in Table 2. The calculated pKapp from all hemolymph samples was 6.302168 ± 0.017538. Hemolymph Pco_2 and $[HCO_3^-]$ were calculated by substitution of the mean values of αco_2 and pKapp in the rearranged Henderson–Hasselbalch equation as follows:

$$Pco_2 = Tco_2 \bullet [0.03713 \bullet (1+10^{(pH-6.302168)})]^{-1}$$
$$[HCO_3^-] = Tco_2 - 0.03713 \bullet Pco_2$$

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

Hemolymph Pco_2 and $[HCO_3^-]$ at 28°C in normoxic conditions were 4.21 torr and 1.63 mM/L, respectively (Table 3). In *in vitro* experiments (Table 2), the changes in pH, Tco_2 and pKapp were statistically significant with the increase in Pco_2 (Kruskal–Wallis test, $P<0.05$). At the same time, the interaction between pKapp and pH was analyzed (Fig. 1), and the correction equation for pKapp was obtained as follows:

$$pKapp = 33.462 - 13.032 \bullet pH + 2.065 \bullet pH^2 - 0.1088 \bullet pH^3$$

For comparison, Pco_2 and $[HCO_3^-]$ were estimated using the mean value of pKapp and the correction equation. There was no significant difference in hemolymph Pco_2 and $[HCO_3^-]$ calculated by the two methods (Mann–Whitney *U* test, $P>0.05$, Table 4). The non-bicarbonate buffer value (β_{NB}), which was obtained as a regression coefficient relating pH and $[HCO_3^-]$, was 3.62 Slykes (Table 5).

Table 1. Hemolymph pH and total CO_2 content (Tco_2) of disk abalone (*Haliotis* (*Nordotis*) *discus discus*) at 28℃ in normoxic conditions

		Mean	SE	N
pH		7.320	0.0217	7
Tco_2	mM/L	1.78	0.049	7

Water temperature, 27.8 ± 0.1 °C (Mean ± SD)

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

Standard gas		Hemolymph			
CO_2 (%)	Pco_2 (torr)	pH	Tco_2 (mM/L)	pKapp	N
0.102	0.740	7.608	0.840	6.1321892	9
0.203	1.48	7.500	1.043	6.2580713	12
0.515	3.76	7.361	1.808	6.2927812	12
1.01	7.36	7.232	2.623	6.3077984	10
2.00	14.6	7.144	3.472	6.4182550	10
5.01	36.6	6.803	4.877	6.3976120	10

Barometric pressure, 758.3 ± 0.9 torr; water temperature. 27.8 ± 0.2 °C (Mean ± SD)
Mean value of pKapp, 6.302168

Table 3. Hemolymph CO_2 partial pressure (Pco_2) and bicarbonate concentration ($[HCO_3^-]$) of disk abalone (*Haliotis* (*Nordotis*) *discus discus*) in normoxic conditions

		Mean	SE	N
Pco_2	torr	4.21	0.149	7
$[HCO_3^-]$	mM/L	1.63	0.049	7

Water temperature. 27.8 ± 0.1 °C (Mean ± SD)

Table 4. Compariosn of the values calculated using the correction equation and from the mean pKapp in the hemolymph Pco_2 and $[HCO_3^-]$

	Pco_2 (torr)	$[HCO_3^-]$ (mM/L)	N
the mean value of pKapp	4.21 ± 0.149	1.63 ± 0.049	7
pKapp calculated by the correction equation	4.29 ± 0.224	1.62 ± 0.051	7

Data show mean ± SE. No statistically significant difference (Mann–Whitney *U* test, P>0.05)

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

Standard gas		Hemolymph		
CO_2 (%)	Pco_2 (torr)	pH	$[HCO_3^-]$ (mM/L)	N
0.102	0.740	7.608	0.859	9
0.203	1.48	7.500	0.988	12
0.515	3.76	7.361	1.668	12
1.01	7.36	7.232	2.350	10
2.00	14.6	7.144	2.930	10
5.01	36.6	6.803	3.519	10

Barometric pressure, 758.3 ± 0.9 torr; water temperature. 27.8 ± 0.2 °C (Mean ± SD)
Non-bicarbonate buffer value (β_{NB}), 3.624

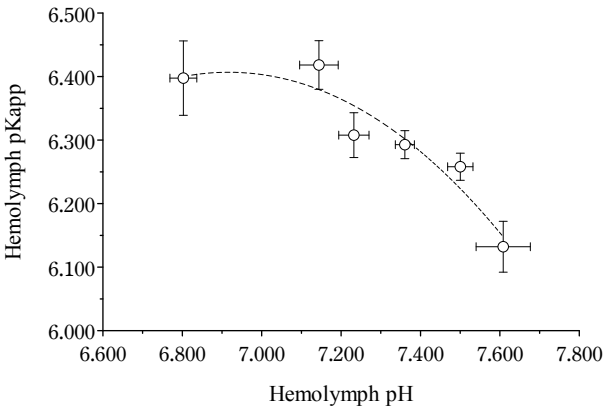

Fig.1. Relationship between pH and apparent dissociation constant of carbonic acid (pKapp) of hemolymph in disk abalone *Haliotis* (*Nordotis*) *discus discus* at 28°C. Values are means ± standard error. Dashed line fitted to the data and the equation: pKapp = 33.462 – 13.032• pH + 2.065 • pH^2 – 0.1088 • pH^3 (r^2 = 0.9036)

Discussion

We collected the hemolymph and examined hemolymph pH, Tco_2, Pco_2, and $[HCO_3^-]$ in order to evaluate the acid–base balance of disk abalone in normoxic conditions. The hemolymph was collected anaerobically through a cannula, and the hemolymph pH and Tco_2 measured immediately were 7.320 and 1.78 mM/L at 28.0°C, respectively. Although there are few descriptions of hemolymph pH and Tco_2 in disk abalone, hemolymph pH of *Haliotis iris* was 7.16–7.17 (15°C), and *Haliotis diversicolor supertexta* had hemolymph pH 7.23–7.28 and Tco_2 1.82–2.18 mM/L (25°C) in normoxic conditions.[29,30] The hemolymph pH in disk abalone was almost the same as that in *H. diversicolor supertexta* and higher than in *H. iris*. The content of carbonic acid and CO_2 was approximately the same as *H. diversicolor supertexta*. Disk abalone could have a similar acid–base status to the hemolymph of *H. diversicolor supertexta*.

Cameron (1986) reported CO_2 solubility as a function of temperature and salinity, and the solubility coefficients were 35.49–38.12 μM/L/torr at 26–28°C and 30–35 salinity (psu).[31] The hemolymph αco_2 in disk abalone (37.13 μM/L/torr) was in the range of the coefficient reported in previous study.[31] The mean value of hemolymph pKapp in this study was 6.302168. There are few reports of hemolymph pKapp of disk abalone, but other molluscs, including marine bivalves, have reported hemolymph pKapp values of 5.8191–6.2609 at 12–28°C.[18-25] The pKapp value is equal to the pH value at which it is most effective as a buffer.[32] The effective buffer pH of disk abalone seemed to be higher than that of bivalves.

Using the hemolymph αco_2 and pKapp determined in this study, Pco_2 and $[HCO_3^-]$ of the hemolymph of disk abalone were calculated. The mean values of Pco_2 and

[HCO_3^-] in disk abalone were 4.21 torr and 1.63 mM/L, respectively. In *H. diversicolor supertexta*, hemolymph Pco_2 and [HCO_3^-] were 4.0–4.5 mmHg (torr) and 1.71–2.05 mM/L, respectively.[30] The hemolymph acid–base balance of disk abalone approximated to that of *H. diversicolor supertexta*.

The β_{NB} of disk abalone hemolymph (3.62 Slykes) was higher than that of bivalves, (akoya pearl oyster *Pinctada fucata martensii*, 1.35-1.45 Slykes;[20] blue mussel *Mytilus edulis*, 0.4-0.622 Slykes;[18,33] marine mussel *M. galloprovincialis*, 0.65 Slykes;[19] hard-shelled mussel *M. coruscus*, 0.44 Slykes;[23] Pacific oyster *Crassostrea gigas*, 0.73 Slykes[24]). Disk abalone hemolymph exhibited a higher non-bicarbonate buffer value than those of bivalves. 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. In disk abalone, changes of hemolymph pH would need greater quantities of acid or base in comparison with bivalves, and disk abalone may have a better ability to maintain hemolymph pH. Disk abalone seemed to be tolerant to some changes of water quality, such as the rise in CO_2 level.

References

1) Hayami I: Ostreidae. *In*: Okutani T (ed) Marine Mollusks in Japan. The second edition, Tokai University Press, Tokyo, 1182-1185 (2017)

2) Food and Agriculture Organization of the United Nations (FAO), Fishery and Aquaculture Department: Abalones, winkles, conchs. *In*: Fishery and Aquaculture Statistics, Aquaculture production by species group, B-52, 116 (2016)

3) Food and Agriculture Organization of the United Nations (FAO), Fishery and Aquaculture Department: Abalones, winkles, conchs. *In*: Fishery and Aquaculture Statistics, Capture production by species group, B-52, 386-387 (2016)

4) Ishida O: Effect of population density on the growth of juveniles of the abalone, *Haliotis discus*. *Suisanzoshoku* (*Aquaculture Sci*), **41**, 431-433 (1993)

5) Segawa S: Preliminary experiment on the effect of temperature on rates of oxygen consumption and ammonia excretion of young disk abalone, *Nordotis discus discus*. *Suisanzoshoku* (*Aquaculture Sci*), **43**, 219-224 (1995)

6) Nakatsugawa T: Infectious nature of a disease in cultured juvenile abalone with muscular atrophy. *Fish Pathol*, **25**, 207-211 (1990)

7) Nakatsugawa T, Okabe M, Muroga K: Horizontal transmission of amyotrophia in Japanese black abalone. *Fish Pathol*, **35**, 11-14 (2000)

8) Okada K, Nishimura M, and Kawamura T: Prevention of amyotrophia in mass production of 0-year-old abalone, *Haliotis discus*, by Quarantine. *Suisanzoshoku* (*Aquaculture Sci*), **48**, 657-663 (2000)

9) Shibata T, Chikushi Y, Nakamoto T, Watanabe K, Nagashima T: Prevention of amyotrophia in large scale production of juvenile abalone, *Haliotis discus discus*, by ultraviolet irradiation of water supply. *Suisanzoshoku* (*Aquaculture Sci*), **50**, 227-232 (2002)

10) Zoysa DM, Whang I, Lee Y, Lee S, Lee JS, Lee J: Defensin from disk abalone Haliotis discus discus: Molecular cloning, sequence characterization and immune response against bacterial infection. *Fish Shellfish Immunol*, 28, 261-266 (2010)

11) Bathige SDNK, Umasuthan N, Godahewa GI, Thulasitha WS, Whang I, Won SH, Kim C, Lee J: Two variants of selenium-dependent glutathione peroxidase from the disk abalone *Haliotis discus discus*: Molecular characterization and immune responses to bacterial and viral stresses. *Fish Shellfish Immunol*, **45**, 648-655 (2015)

12) Yamamoto K, Handa T, Kondo M: Structure of the digestive diverticula of abalone *Haliotis* (*Nordotis*) *discus discus*. *J Nat Fish Univ*, **53**, 105-116 (2005)

13) Yamamoto K, Handa T, Kondo M: Ctenidum structure of the abalone *Haliotis* (*Nordotis*) *discus discus* (Gastropoda: Aspidobranchia). *J Nat Fish Univ*, **56**, 287-298 (2008)

14) Yamamoto K, Handa T: New method of direct measurement of ventilation volume of abalones (Mollusca, Haliotidae). *J Nat Fish Univ*, **49**, 59-65 (2001)

15) Yamamoto K, Handa T, Tsunoji T: Effect of seasonal

rise in water temperature on the respiration in the abalone *Haliotis* (*Nordotis*) *discus discus*. *Aquaculture Sci*, **59**, 529-534 (2011)

16) Yamaoto K, Handa T: Flow through respiratory pores of the abalone *Haliotis* (*Nordotis*) *discus discus*. *Aquaculture Sci*, **60**, 393-396 (2012)

17) Yamamoto K, Handa T: Effect of hypoxia on oxygen uptake in the abalone *Haliotis* (*Nordotis*) *discus discus*. *J Nat Fish Univ*, **61**, 81-85 (2013)

18) 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)

19) Michelidis B, Ozounis 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 (2005)

20) 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)

21) Handa T, Yamamoto K: Estimation of CO_2 partial pressure and bicarbonate concentration in the hemolymph of the black-lip pearl oyster *Pinctada margaritifera*. *J Nat Fish Univ*, **63**, 181-188 (2015)

22) Handa T and Yamamoto K: Estimation of CO_2 partial pressure and bicarbonate concentration in the hemolymph of the noble scallop *Mimachlamys nobilis*. *J Nat Fish Univ*, **64**, 188-194 (2016)

23) Handa T, Araki A, Yamamoto K: Acid–base balance of the hemolymph in hard-shelled mussel *Mytilus coruscus* in normoxic conditions. *J Nat Fish Univ*, **65**, 39-46 (2017)

24) Handa T, Araki A, Yamamoto K: Acid–base balance of the hemolymph in Pacific oyster *Crassostrea gigas* in normoxic conditions. *J Nat Fish Univ*, **66**, 103-110 (2018)

25) Handa T, Araki A, Yamamoto K: Oxygen and acid–base status of hemolymph in the densely lamellated oyster *Ostrea denselamellosa* in normoxic conditions. *J Nat Fish Univ*, **66**, 203-208 (2018)

26) 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)

27) Namba K, Kobayashi M, Aida S, Uematsu K, Yoshida M, Kondo Y, Miyata U: Persistent relaxation of the adductor muscle of oyster *Crassostrea gigas* induced by magnesium ion. *Fish Sci*, **61**, 241-244 (1995)

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

29) Ragg N, Taylor H: Oxygen uptake, diffusion limination, and diffusing capacity of the bipectinate gills of the abalone, *Haliotis iris* (Mollusca: Prosobranchia). *Comp Biochem Physiol*, **143A**, 299-306 (2006)

30) Cheng W, Liu CH, Cheng SY, Chen JC: Effect of dissolved oxygen on the acid–base balance and ion concentration of Taiwan abalone *Haliotis diversicolor supertexta*. *Aquaculture*, **231**, 573-586 (2004)

31) 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)

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

33) 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)