INTRODUCTION

When oxygen flux is reduced below some critical level in most animals, full or partial anaerobic metabolism begins to occur (Gnaiger, 1991). For more than two decades the topic of anaerobiosis has been intensively investigated in mussel physiology. The intertidal species *Mytilus edulis* has drawn the most attention, and the results show that different biochemical pathways are used for ATP generation under immersed, emersed, prolonged emersed and reimmersed conditions (de Zwaan, A. 1983). However, there are far fewer studies on the biochemistry of hypoxia in freshwater mussels.

During severe hypoxia, the degradation of glycogen via the Embden-Meyerhof pathway to lactate (i.e., anaerobic glycolysis) is recognized as an important metabolic route for providing energy in many species, especially vertebrates (Withers, 1992). In mussels, however, traditional anaerobic glycolysis may not be of major importance, particularly for those species capable of withstanding prolonged periods of environmental hypoxia or anoxia, such as intertidal bivalves. Lactate does not accumulate during anaerobic glycolysis in marine bivalves such as *Crassostrea*, *Mytilus* and *Rangia* (Hochachka and Mustafa, 1973; de Zwaan, 1977). Instead, other anaerobic pathways have been revealed which are alternatives to lactate formation. Phosphoenolpyruvate (PEP) is a branch point for the four main degradation pathways (Fig. 1) as follows: (1) glucose-lactate pathway (end-product: lactate); (2) glucose-opine pathway (end-product: opines); (3) glucose-succinate pathway (end products: succinate); and (4) aspartate-succinate pathway (end products: succinate

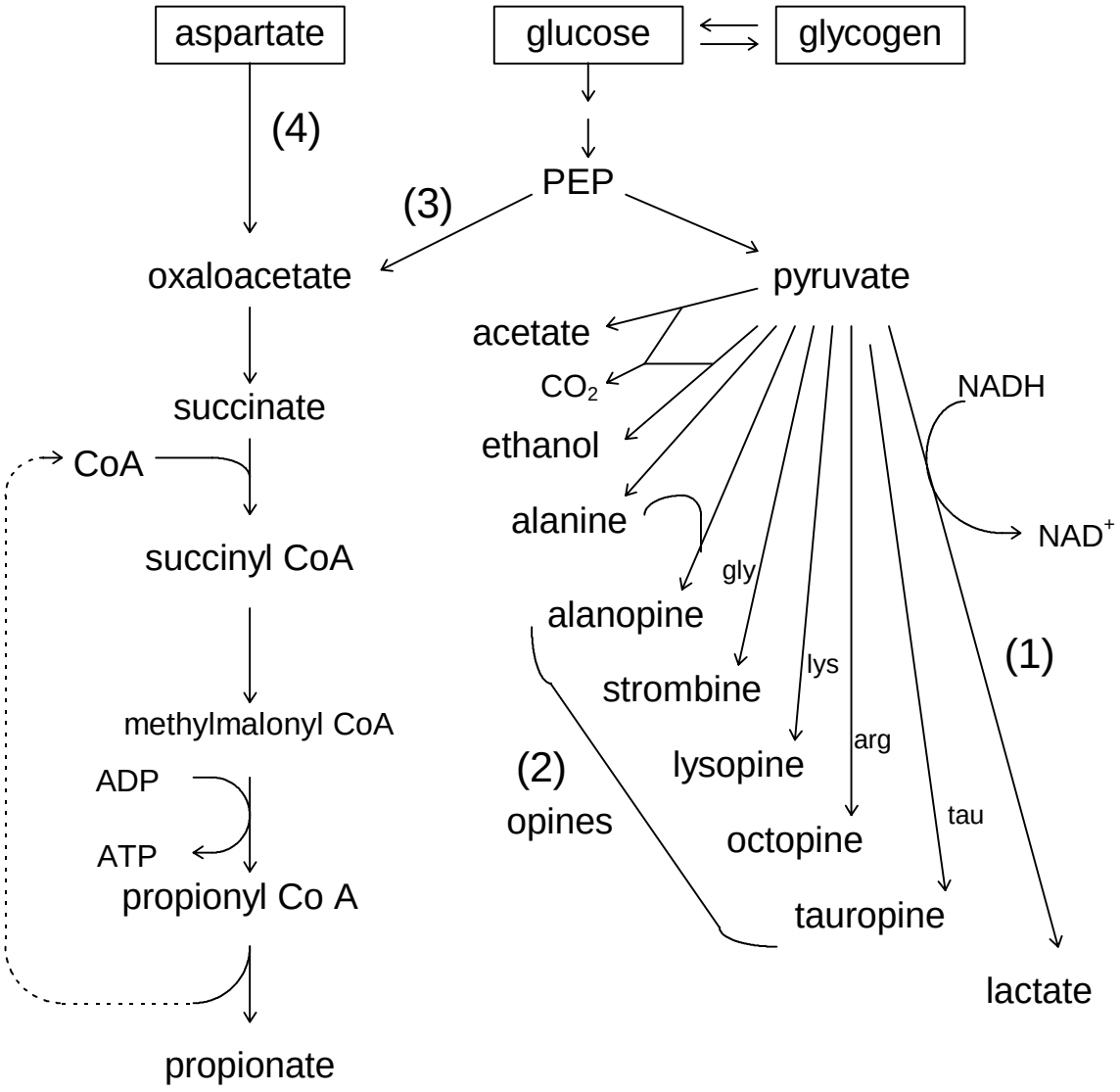

Fig. 1 The PEP branch point in bivalve molluscs. The four main anaerobic pathways were indicated by number in parentheses according to Hochachka and Somero (1984) and Livingstone (1983).

and alanine) (Hochachka and Somero, 1984; Livingstone, 1983). In the first and second pathway, lactate dehydrogenase (LDH) and other pyruvate reductases (i.e. opine dehydrogenases) ensures the continuous flux of glycolysis and a constant supply of ATP by maintaining the NADH/NAD ratio during exercise and hypoxia, as well as in the subsequent recovery period (Gäde and Grieshaber, 1986). The energy can be produced faster in the first and second pathways than in the third and fourth ones; however, energy production is more efficient in the third and fourth pathways. In these latter two, malate dehydrogenase, succinate dehydrogenase, ketoglutarate dehydrogenase and succinic thiokinase have functions similar to LDH and pyruvate reductases. Both pathways accumulate succinate and can yield almost twice as much energy (ATP) as the first two pathways. All of the four anaerobic pathways have been demonstrated in various marine bivalves under long-term and short-term hypoxia (de Zwaan, 1983), and the end products of lactate, succinate and alanine are the most important accumulated compounds under anaerobic stress (de Zwaan and Wijsman. 1976; Brodey, M. M. and Bishop, S.H., 1992).

Limited data suggest that the biochemical responses of marine mollusks to hypoxia are different from those of freshwater mussels. Marine mollusks have high intracellular free amino acid pools that contribute substantially to the osmotic pressure of the cell (Zurburg and de Zwaan, 1981). Some of these amino acids can be used along with carbohydrate during anaerobiosis (Meinardus and Gäde, 1981). In freshwater bivalves, it appears that different biochemical mechanisms are involved during hypoxia; one of these is anoxic endogenous oxidation (Holwerda et al., 1981). In anoxic endogenous oxidation, glycolysis,

the citric acid cycle, and the electron-transfer chain still operate, but oxygen is replaced by an unknown prestored electron acceptor. Holwerda et al. (1981) found that anaerobic oxidative capacity was increased greatly in freshwater clams kept under hypoxic conditions for 2 to 5 days. They suggested that anoxic endogenous oxidation is especially important to animals living on the bottom of small and shallow freshwater basins that show gradual seasonal changes in oxygen tension. Although the fermentative pathways utilizing pyruvate reductases such as alanopine dehydrogenase, and strombine dehydrogenase are widely distributed among marine invertebrates, they seem to be absent in freshwater molluscs (de Zwaan, 1983). Thus, hypoxic mechanisms of freshwater mussels cannot be directly extrapolated from those of marine species.

The purpose of this study was to investigate the anaerobic pathways of freshwater mussel species occurring in habitats which typically experience different dissolved oxygen (DO) conditions. In previous study (Chapter 2), it was found that species from different habitats have different abilities to regulate the oxygen consumption rate under declining DO. In order to evaluate possible metabolic indicators of hypoxic stress using the metabolite changes, different tissues were analyzed. The anaerobic metabolites, lactic acid and succinic acid, were determined after exposing the mussels to different dissolved oxygen (DO) levels and to air exposure. Glucose and glycogen also were measured as an indicator of energy flow and energy store under hypoxia and air exposure.

MATERIALS AND METHODS

Three mussel species were collected by snorkeling in various water bodies in Virginia. Specimens of *Pyganodon grandis* were collected from the profundal zone of Claytor Lake, a reservoir on the New River, Montgomery County. *Elliptio complanata* were collected from pools in the Nottoway River, Nottoway County. *Villosa iris* were taken from riffles in the North Fork Holston River, Smyth County. All of the mussels were acclimated in 30L aquaria with sand substratum and a flow-through temperature controlled (24 ±1 °C) system for 1 wk before an experiment was started. The animals were fed a commercial algal diet (SUN Chlorella "A" granules by YSK international Corp., Japan) daily in the morning at a concentration of approximately 60,000 cells/30L tank.

A photoperiod of 12 light: 12 dark was used, and the light period started at 0730. Animals were exposed to different levels of dissolved oxygen (DO) for 8 hr (Figure 2). Different levels of DO exposure were obtained with a gas-mixing flow meter (Cameron Instrument Company. Model GF3mp) to control the flow and ratio of air and nitrogen aerated in a 20 L mixing chamber. The water then was pumped into the 0.5 L exposure chambers equipped with an oxygen probe (YSI 5750) in the lid, a screen above the floor, and a magnetic stirring bar on the floor. The animals were acclimated in the exposure chamber for 3 hr before any reduction in DO was implemented. The exposure began at 2000 hr (0.5 hr after the dark period started), which simulates the environmental hypoxia often encountered

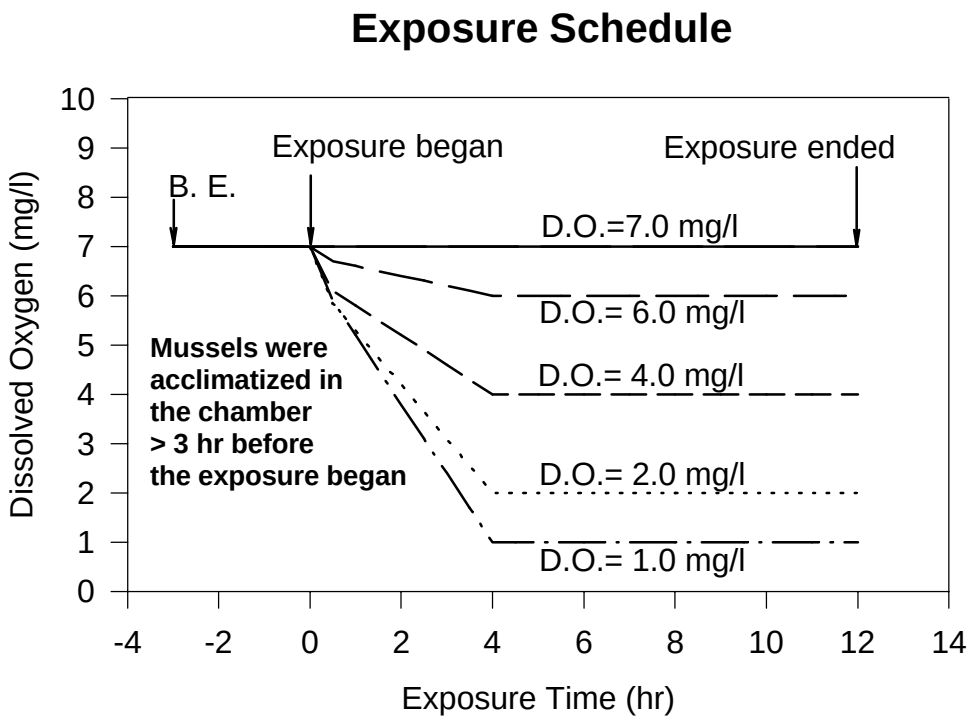

Fig. 2 Schematic design for mussels exposed to different levels of dissolved oxygen; **B. E.** is before exposure.

in nature at night. DO was reduced at a constant rate according to a pre-calculated DO time table by manually adjusting the gas mixture. The desired DO was reached at midnight 0000 and maintained until the morning of the second day 0800 (total of 12 hr). Animals from each species also were exposed to extremely low DO (anoxia) and air for 12 hr (and some for 36 hr) to examine the extreme responses under both conditions. Three (of *V. iris* and *E. complanata*) or four (of *P. grandis*) were used for each DO level and air exposure at the end of the exposure period. After exposure, the mussels were quickly cut open and placed in liquid nitrogen (-195.8 °C) until frozen thoroughly. The samples then were stored in a -80 °C freezer until analyzed. The experiment was repeated with fresh-collected samples of *V. iris* (recent sample), as those used in the first analysis (old sample) had been kept in the laboratory for several months before the exposures and showed low levels of glycogen in the tissues. Those data are included in this report because they illustrate the effect that poor holding conditions can have on subsequent responses to hypoxic stress.

Samples for the determination of metabolite concentrations were deproteinized immediately after homogenization according to Diangelo and Heath (1987) and Gäde and Grieshaber (1989). About 0.05 ~ 0.1 g of tissue from posterior adductor muscle (P), anterior adductor muscle (A), foot (F) and gill (G) were weighed and homogenized in pre-cooled perchloric acid to denature the proteins in the tissues and to stop all biochemical reactions. For *P. grandis* and the second batch of *V. iris*, the mantle tissues (M) also were homogenized for metabolite measurements. The homogenate was slowly neutralized with

potassium bicarbonate, then centrifuged at 1,500 g at 4 °C for 30 min. The precipitates were discarded, and the supernates were analyzed for metabolites.

Lactic acid and succinic acid levels were measured to delineate the changes in anaerobic metabolism of mussels, because lactic acid is the most common anaerobic end-product in animal tissues (Hochachka and Somero, 1984), and succinic acid is one of the most common end-products in marine mussels (Livingstone and Bayne, 1977; Demers and Guderley, 1994). All of the metabolites were determined by spectrophotometry. Lactate and succinic acid were measured according to Noll (1984) and Beutler (1985), respectively. The sensitivities of these methods were described in the references. Glucose and glycogen concentrations were measured using the enzymatic method of Keppler and Decker (1984); the method was slightly modified, and Sigma kits (Catalog No. 510-A) were used for measuring glucose levels. The sensitivity of the modified method can detect glycogen at 0.01 mg/ml in 5 ul of homogenized and neutralized extract. From 0.05 to 0.1g of wet tissues were used to make 200 - 300 ul of extract, which was diluted more than 5 times for glycogen measurements of mantle tissue. The statistical comparisons were done by an unpaired student's t-test, and the criterion for statistical significance was $p \leq 0.05$.

RESULTS

In the presentation of results, tables have been used to summarize data with little or no change in metabolites. Graphs have been utilized to illustrate trends and significant changes in metabolite values.

A. *Villosa iris* (old sample)

The lactic acid concentrations in this group of *V. iris* showed a declining trend in the lower DO levels for four different tissues; however, no significant differences between the control group and different DO treatment groups were observed (Table 1). For some groups, the lactic acid levels were too low to be detectable. The concentrations of succinic acid did not show significant changes under different DO levels, nor were there any changes in glucose levels in the four different tissues. There were no significant changes among glycogen contents at different DO levels, although they tended to decline in low DOs.

B. *Villosa iris* (recent sample)

The lactic acid concentrations in fresh *V. iris* significantly increased as the DO was reduced in both anterior and posterior adductor muscles (Fig. 3). There appears to be a trend of increasing lactate in mantle, foot and gill tissues (Table 2), although it is not statistically significant. There were no evident changes in the succinic acid levels under declining DO stress among different tissues. The glucose content in the posterior adductor muscle under

Table 1. The metabolite changes under different concentrations of dissolved oxygen (DO) in poorly fed *V. iris*. Each mean value represents 3 mussel samples ± SEM. **P. add. muscle** Posterior adductor muscle. **A. add. muscle** Anterior adductor muscle. **B. E.** before exposure.

V. iris

Lactic Acid (nM/g wet wt.)

mean ± SEM	DO (mg/l) B.E.	7	6	4	2	1
P. add. muscle	63.28±10.58	106.05±26.66	65.72±19.68	30.93±11.52	43.61±8.72	-
A. add. muscle	94.93±9.05	82.62±22.07	41.60±8.94	-	42.36±10.93	66.91±3.18
Foot	56.06±19.72	67.15±15.30	47.32±16.11	34.29±4.23	25.43±4.80	29.60±7.50
Gill	89.35±36.01	40.33±7.76	48.39±22.07	-	37.79±6.33	22.31±9.51

Succinic Acid (uM/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1
P. add. muscle	5.48±0.83	4.14±0.90	5.50±0.34	-	4.04±0.23	4.68±0.14
A. add. muscle	5.50±1.25	-	5.91±0.49	5.36±0.51	5.80±0.18	5.94±0.55
Foot	2.49±0.63	3.12±0.22	5.27±0.07***	4.77±1.36	2.85±0.26	3.39±0.37
Gill	2.05±0.61	2.08±0.37	3.17±0.46	5.13±0.61*	3.06±0.51	3.49±0.67

Glucose (mg/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1
P. add. muscle	0.187±0.020	0.221±0.031	0.177±0.022	0.187±0.018	0.246±0.097	0.150±0.055
A. add. muscle	0.388±0.072	0.668±0.201	0.327±0.054	0.411±0.021	0.292±0.115	0.187±0.076
Foot	0.275±0.033	0.448±0.084	0.250±0.029	0.351±0.076	0.240±0.081	0.184±0.068
Gill	0.189±0.065	0.293±0.050	0.130±0.020	0.197±0.056	0.331±0.112	0.107±0.035

Glycogen (mg/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1
P. add. muscle	0.314±0.077	0.584±0.195	0.298±0.058	0.301±0.229	0.098±0.030	0.046±0.015
A. add. muscle	0.291±0.095	1.611±0.846	0.441±0.171	0.610±0.527	0.095±0.036	0.029±0.021
Foot	0.228±0.054	1.131±0.568	0.322±0.104	0.433±0.284	0.067±0.023	0.050±0.040
Gill	0.124±0.039	0.424±0.224	0.347±0.205	0.279±0.165	0.169±0.045	0.050±0.030

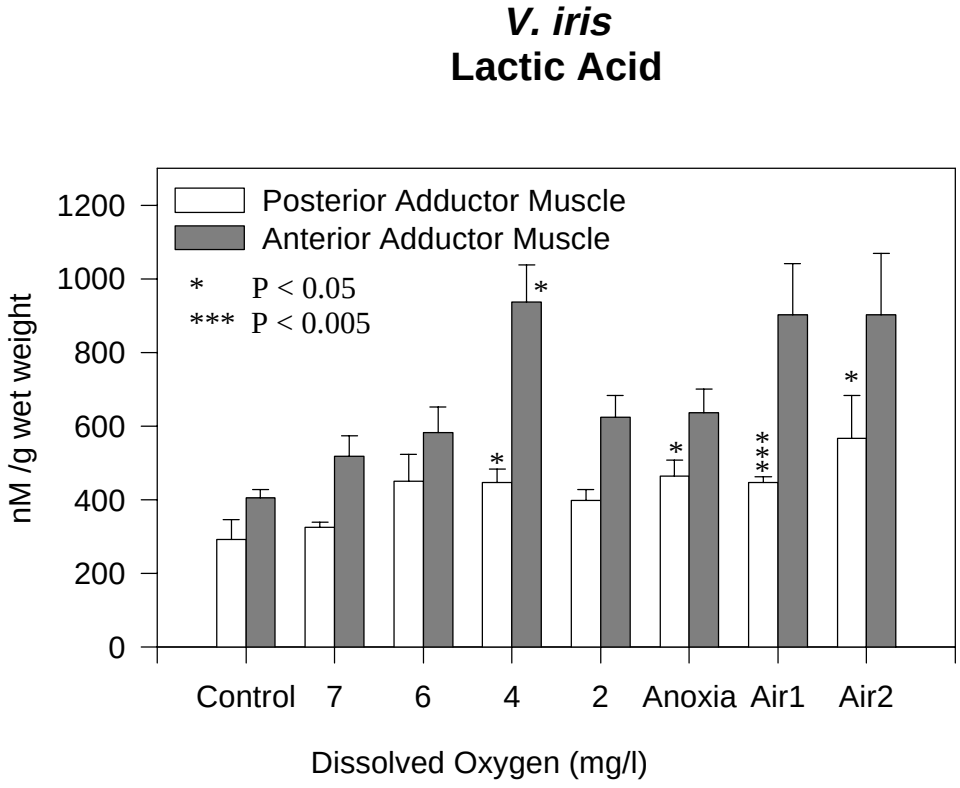

Fig. 3 Comparison of lactic acid levels in *V. iris* for posterior adductor muscle and anterior adductor muscle under different DO treatments. Error bars =± SEM. **B. E.**: before exposure. For the unpaired t-test, * P<0.05 and ***P<0.005 were used for the comparison between control (DO = 7 mg/l) and different exposure levels such as anoxia, 12 hr air exposure (Air1) and 36 hr air exposure (Air2).

Table 2. The metabolite change under different concentrations of dissolved oxygen (DO) in freshly sampled *V. iris*. Each mean value represents 3 mussel samples ± SEM. **P. add. muscle** Posterior adductor muscle. **A. add. muscle** Anterior adductor muscle. **B. E.** before exposure. **A. E.** Air exposure. For the unpaired t-test, $^{+}$ P<0.05, $^{++}$P<0.01, and $^{+++}$P<0.005 were used for the comparison between DO=1 mg/l and anoxia and air exposures , * P<0.05, and ***P<0.005 were used for the comparison between control (DO =7 mg/l) and different exposure levels.

V. iris

Lactic Acid (nM/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	12 hr A. E.	36 hr A. E.
Foot	246.39±22.20	253.64±32.82	220.44±24.99	320.35±84.03	327.88±51.23	-	347.39±45.46	347.95±44.04	247.18±39.64
Gill	85.32±0.80**	190.91±8.62	223.22±12.04	169.13±68.41	325.51±73.01	-	348.05±136.03	216.24±102.83	154.03±74.83
Mantle	272.54±12.32	385.64±29.54	354.66±56.14	301.09±28.01	454.39±95.95	-	596.66±90.45	674.98±138.60	444.40±87.23

Succinic Acid (uM/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	12 hr A. E.	36 hr A. E.
P. add. muscle	1.69±0.49	2.15±0.31	2.30±0.64	1.52±0.33	2.01±0.60	2.17±0.43	1.44±0.35	1.57±0.08	2.46±0.30
A. add. muscle	1.18±0.83	1.48±0.95	1.78±0.43	1.36±0.31	0.67±0.29	1.65±0.44	0.99±0.25	1.71±0.37	2.25±0.53
Foot	1.29±0.19	1.36±0.33	1.35±0.53	0.51±0.18	0.85±0.36	1.23±0.36	0.65±0.24	0.95±0.25	1.39±0.48
Gill	0.50±0.07	1.01±0.37	1.21±0.15	0.85±0.31	0.91±0.19	1.32±0.10	0.84±0.08	0.57±0.02	0.50±0.17
Mantle	1.14±0.22	1.99±0.58	1.62±0.61	1.19±0.02	2.05±0.33	1.88±0.42	1.52±0.37	2.78±0.56	2.03±0.35

Glucose (mg/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	12 hr A. E.	36 hr A. E.
P. add. muscle	0.157±0.008	0.150±0.012	0.151±0.018	0.123±0.025	0.137±0.014	0.101±0.003*	0.090±0.002**	0.119±0.016	0.153±0.012++
A. add. muscle	0.371±0.083	0.185±0.010	0.236±0.061	0.272±0.094	0.243±0.048	0.133±0.022	0.309±0.088	0.198±0.030	0.238±0.049
Foot	0.154±0.019	0.160±0.015	0.153±0.028	0.159±0.030	0.126±0.023	0.095±0.009*	0.122±0.007	0.132±0.025	0.157±0.016+
Gill	0.120±0.020	0.139±0.039	0.099±0.018	0.107±0.066	0.085±0.015	0.084±0.024	0.089±0.047	0.083±0.027	0.070±0.014
Mantle	0.287±0.034	0.206±0.030	0.215±0.023	0.129±0.036	0.212±0.023	0.140±0.003	0.231±0.014+++	0.262±0.014+	0.125±0.023

Glycogen (mg/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	12 hr A. E.	36 hr A. E.
Foot	4.559±1.166	3.179±0.494	2.673±0.690	4.536±1.325	2.962±0.371	2.583±0.213	2.752±0.636	3.142±0.774	2.162±0.499
Gill	3.173±0.453	4.62±1.229	4.160±1.156	3.473±0.179	1.980±0.800	2.120±0.969	2.403±1.360	0.851±0.376*	1.290±0.080
Mantle	19.451±0.523	17.436±6.451	19.310±1.480	9.592±4.897	15.469±1.195	14.709±2.348	19.708±3.057	14.345±2.899	9.608±3.527

1 mg/l DO and anoxia was significantly lower than that of the control groups. This value might be an artifact of the declining health condition of the mussels acclimated in the laboratory, as the experiment started with high DO and progressed down to low DO and air exposure. The decrease in glucose contents also may have been caused by the activation of anaerobic metabolism. The glucose concentration following air exposure was not significantly different from that of the control value. However, they were significantly higher than that of DO= 1 mg/l and anoxia groups. An elevated glucose content has been recorded in mussels sampled from the field when they were exposed to air (unpublished data). The glycogen content in the anterior adductor muscle was significantly lower than in the control after anoxia, and after 12 hr and 36 hr air exposures (Fig. 4). Lactic acid levels in the same tissue and posterior adductor muscle tended to be higher than in the control for those groups (Fig. 3). The glycogen concentrations exhibited a declining trend under low DO exposure, although most of them were not statistically significant changes. Gill tissue had the least glycogen content, and mantle tissue had the highest glycogen content.

C. *Elliptio complanata*

The levels of lactic acid of *E. complanata* increased at the lower DOs for all of the tissues sampled except the foot. The gill showed the most consistent change as DO declined (Fig. 5). There also were significant increases in succinic acid in gill when DO $\leq$ 4 mg/l (Fig. 6). The level of succinic acid was significantly increased in all of the tissues for the anoxia and air exposure groups, with the exception of the anterior adductor muscle. The posterior

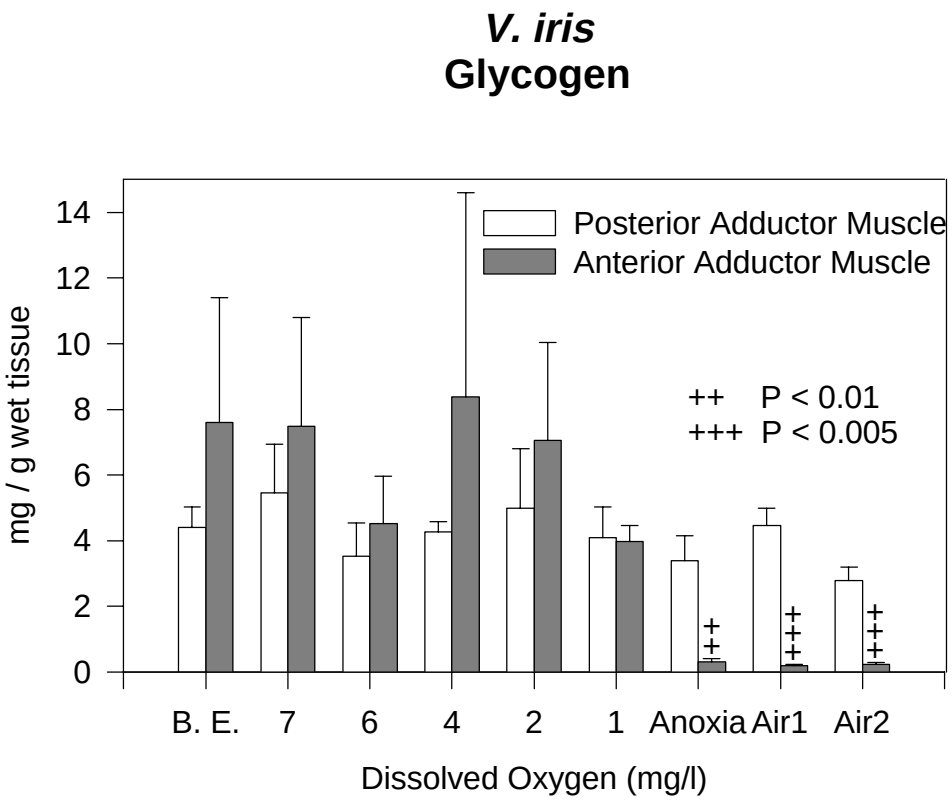

Fig. 4 Comparison of glycogen levels in *V. iris* for posterior adductor muscle and anterior adductor muscle under different DO treatments. Error bars =± SEM. **B. E.** before exposure. For the unpaired t-test, ++ P<0.01 and +++ P< 0.005 were used for the comparison between DO=1 and anoxia, 12 hr air exposure and 36 hr air exposure.

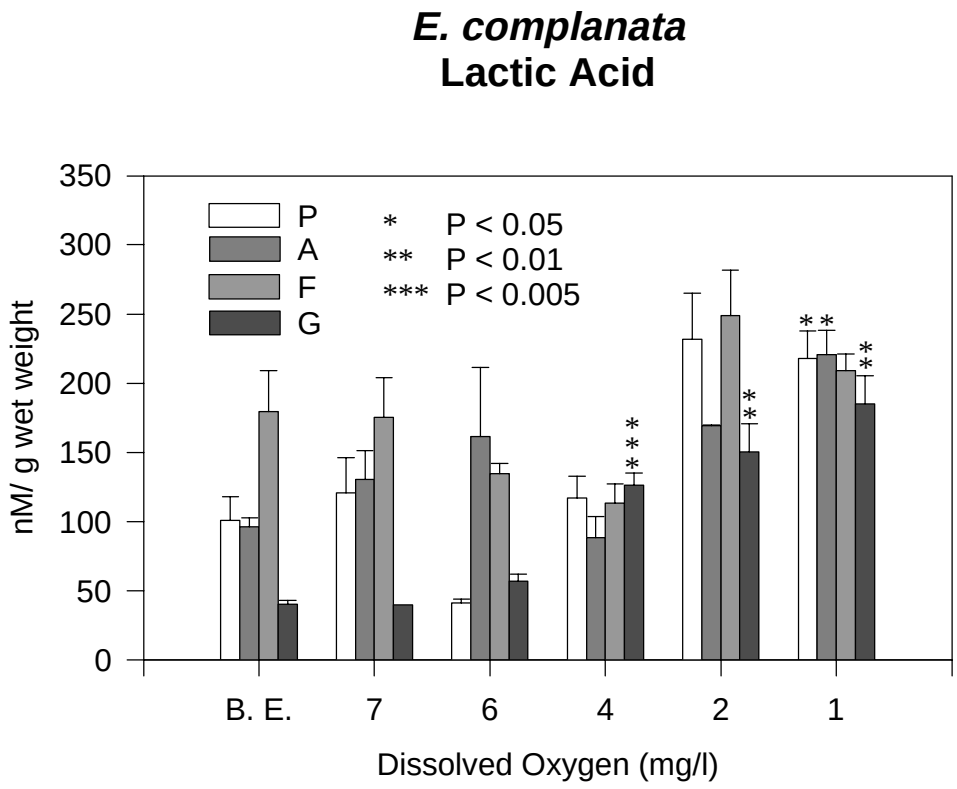

Fig. 5 Comparison of lactic acid levels in *E. complanata* for four tissues under different DO treatments. Error bars =± SEM. **P**: posterior adductor muscle. **A**: anterior adductor muscle. **F**: foot. **G**: gill. **B. E.**: before exposure. For the unpaired t-test, *P<0.05, **P<0.01, and ***P<0.005 were used for the comparison between control (DO = 7 mg/l) and different DO levels.

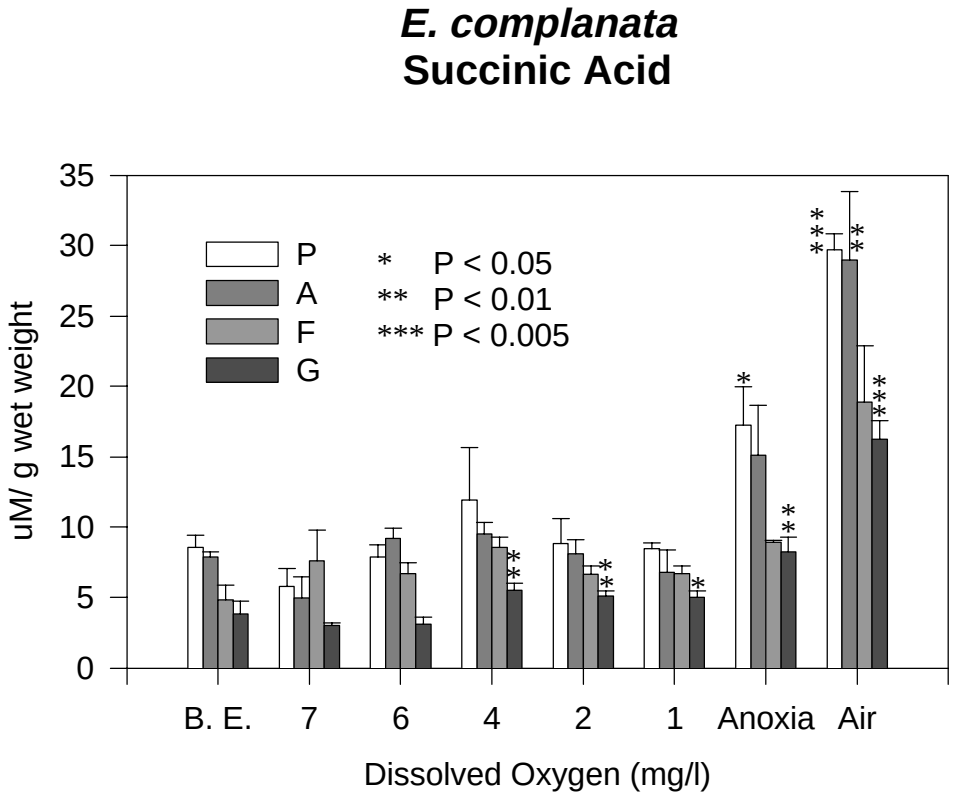

Fig. 6 Comparison of succinic acid levels in *E. complanata* for four tissues under different DO treatments. Error bars =± SEM. **P**: posterior adductor muscle. **A**: anterior adductor muscle. **F**: foot. **G**: gill. **B. E.**: before exposure. For the unpaired t-test, *P<0.05, **P<0.01, and ***P<0.005 were used for the comparison between control (DO = 7 mg/l) and different exposure levels.

adductor muscle exhibited the greatest increase following the air exposure test. There were no significant changes in the glucose concentrations under different levels of DO exposure (Fig. 7). However under both anoxia and air exposure, the glucose content in the four tissues declined greatly. The average glycogen content in the four different tissues of *E. complanata* (Fig. 8) was much higher than that of *V. iris*. There were no significant changes in glycogen content within the different treatment groups of *E. complanata*.

D. *Pyganodon grandis*

The lactic acid levels in *P. grandis* decreased significantly in posterior and anterior adductor muscles under anoxia and air exposure (Fig. 9). For the other tissues, no significant differences between the lactic acid levels of control group and different DO treatment groups were observed (Table 3). The lactic acid concentration in several animals, especially in the gill tissue, was too low to be detected. Succinic acid in the anterior adductor muscle decreased significantly under anoxia and air exposure (Fig. 10). There was no evidence of changes of succinic acid in the other tissues under different treatments. There also were no changes in glucose levels of the four tissue types, except for foot tissue under air exposure and gill tissue under anoxia, which had significantly higher glucose levels following exposure. The mantle tissue had the highest glycogen content (Fig. 11). The glycogen contents in different tissues of this species (Table 3) were higher than those of *V. iris* (Table 2), and there were no significant changes in the glycogen content of the tissues under different treatments in *P. grandis*.

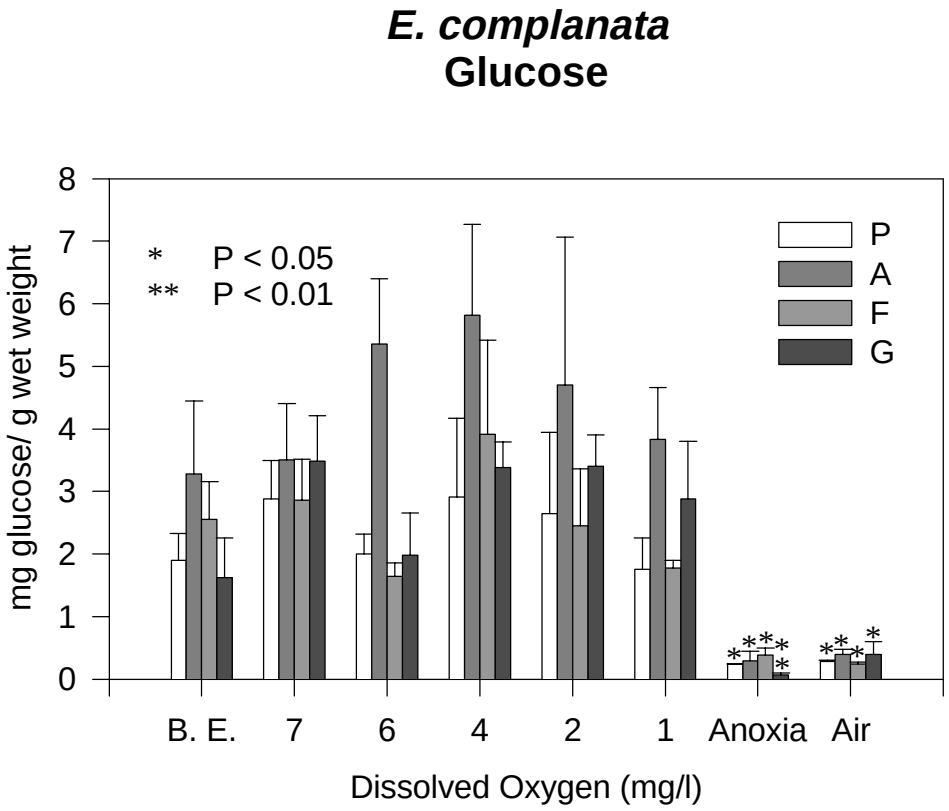

Fig.7 Comparison of glucose levels in *E. complanata* for four tissues under different DO treatments. Error bars =± SEM. **P**: posterior adductor muscle. **A**: anterior adductor muscle. **F**: foot. **G**: gill. **B. E.**: before exposure. For the unpaired t-test, * P<0.05 and **P<0.01were used for the comparison between control (DO = 7 mg/l) and different treatments.

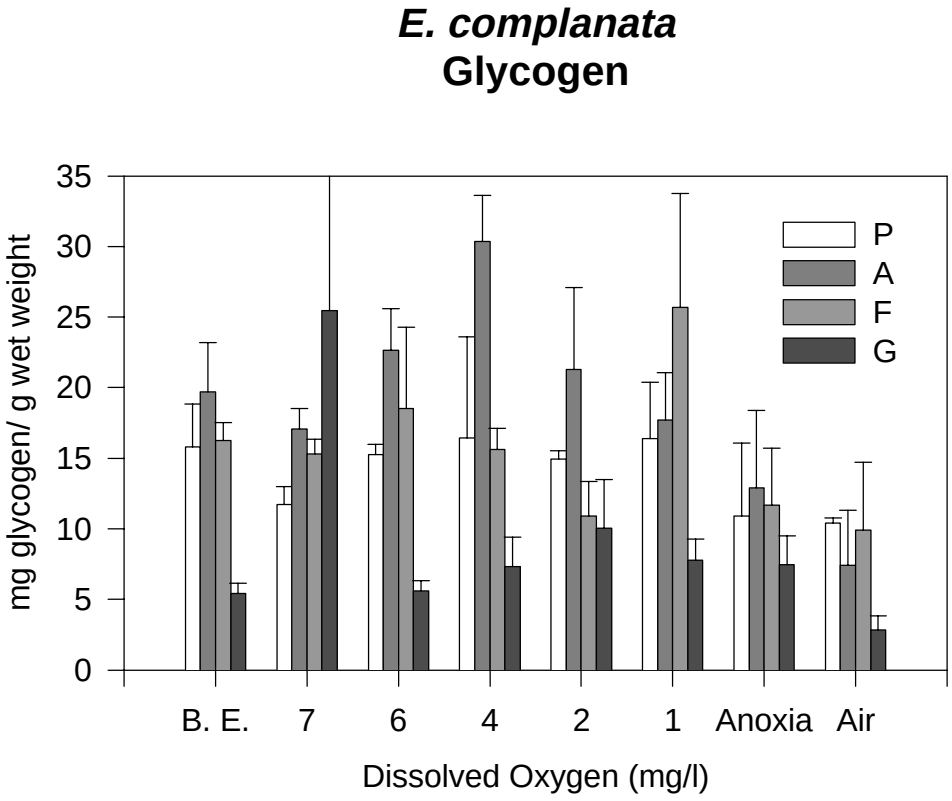

Fig.8 Comparison of glycogen levels in *E. complanata* for four tissues under different DO treatments. Error bars =± SEM. **P**: posterior adductor muscle. **A**: anterior adductor muscle. **F**: foot. **G**: gill. **B. E.**: before exposure. There is no significant difference between control (DO = 7 mg/l) and different treatments.

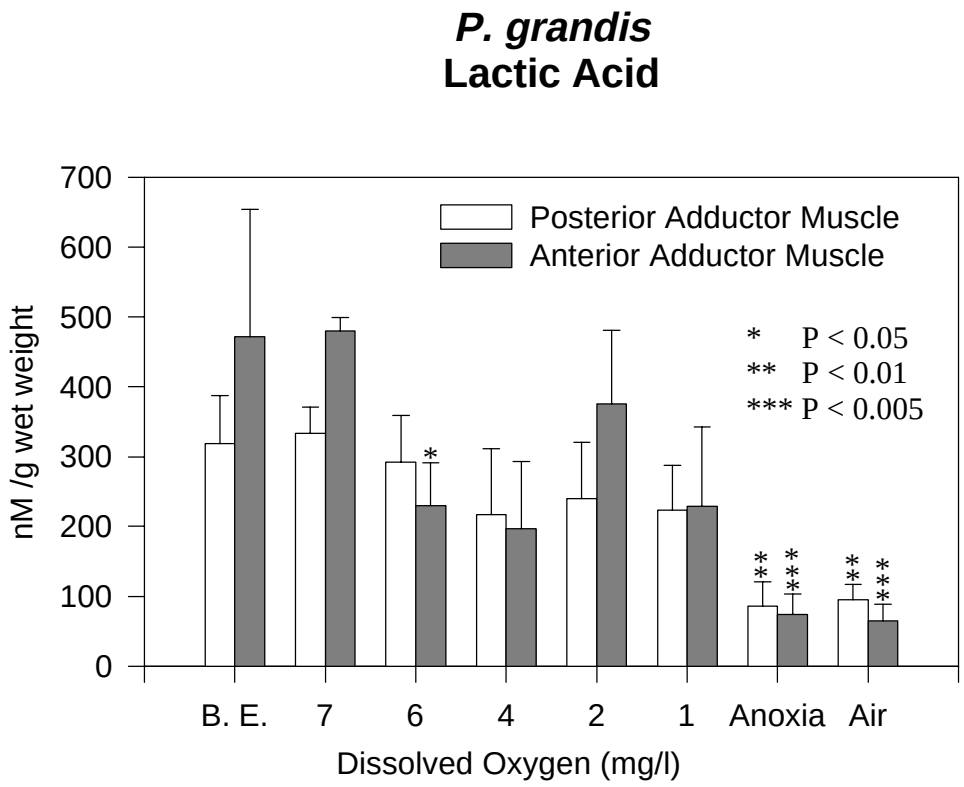

Fig. 9 Comparison of lactic acid levels in *P. grandis* for posterior adductor muscle and anterior adductor muscle under different DO treatments. Error bars =± SEM. **B. E.** before exposure. For the unpaired t-test, * P<0.05, **P<0.01, and ***P<0.005 were used for the comparison between control (DO = 7 mg/l) and different exposure levels.

Table 3. The metabolite change under different concentrations of dissolved oxygen (DO) in lab-acclimatized *P. grandis*. Each mean value represents 4 mussel samples ± SEM. **P. add. muscle** Posterior adductor muscle. **A. add. muscle** Anterior adductor muscle. **B. E.** before exposure. **A. E.** Air exposure. For the unpaired t-test, * $P<0.05$ and *** $P<0.005$ were used for the comparison between control (DO = 7 mg/l) and different exposure levels.

P. grandis

Lactic Acid (nM/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	A. E.
Foot	178.88±24.41	182.48±25.03	259.49±25.40	265.50±41.50	69.53±23.87*	234.30±15.01	180.53±45.04	122.75±78.99
Gill	22.22±12.94	47.47±12.68	65.14±25.46	-	-	-	-	24.00±14.69
Mantle	-	23.84±13.76	-	36.47±27.13	9.05±6.46	-	25.86±9.60	44.73±22.10

Succinic Acid (uM/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	A. E.
Foot	3.31±0.30	3.82±0.20	4.20±0.19	4.37±0.40	3.80±1.35	3.62±0.53	3.01±0.39	3.14±0.64
Gill	1.45±0.24	2.96±0.85	2.67±0.29	3.53±0.54	2.45±0.69	2.46±0.94	3.08±0.68	2.25±0.53
Mantle	2.60±0.32	3.79±0.77	3.87±0.37	3.83±0.34	3.18±1.39	2.29±0.65	1.89±0.46	2.12±0.72

Glucose (mg/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	A. E.
P. add. muscle	0.181±0.006	0.147±0.009	0.194±0.007	0.137±0.004	0.163±0.005	0.148±0.018	0.171±0.046	0.213±0.033
A. add. muscle	0.327±0.038	0.240±0.032	0.384±0.057	0.274±0.091	0.358±0.091	0.231±0.022	0.282±0.088	0.334±0.112
Foot	0.233±0.032	0.193±0.028	0.210±0.019	0.163±0.018	0.228±0.037	0.189±0.021	0.211±0.017	0.349±0.019***
Gill	0.088±0.017	0.063±0.012	0.122±0.023	0.077±0.017	0.075±0.004	0.089±0.009	0.146±0.027*	0.138±0.030
Mantle	0.204±0.029	0.197±0.047	0.330±0.055	0.142±0.020	0.218±0.038	0.227±0.043	0.207±0.068	0.214±0.055

Glycogen (mg/g wet wt.)

mean ± SEM	DO (mg/l) B. E.	7	6	4	2	1	Anoxia	A. E.
P. add. muscle	11.083±1.292	11.874±2.717	13.450±6.243	7.065±1.330	8.075±1.178	7.904±2.420	7.583±2.995	9.468±2.530
A. add. muscle	9.244±0.436	10.097±1.970	9.116±0.198	4.596±0.492	6.191±1.044	6.209±0.980	8.096±3.788	9.070±1.423
Foot	6.153±0.561	6.569±0.292	4.542±0.768	3.456±0.390**	5.037±0.847	5.240±0.724	3.979±0.622***	5.804±2.096
Gill	2.707±0.583	2.230±0.546	1.977±0.357	1.610±0.177	1.721±0.416	1.697±0.220	2.086±0.640	2.108±0.849
Mantle	27.799±4.718	17.498±5.777	33.101±0.480	10.239±3.146	21.831±4.539	25.575±8.581	16.790±6.175	17.660±6.013

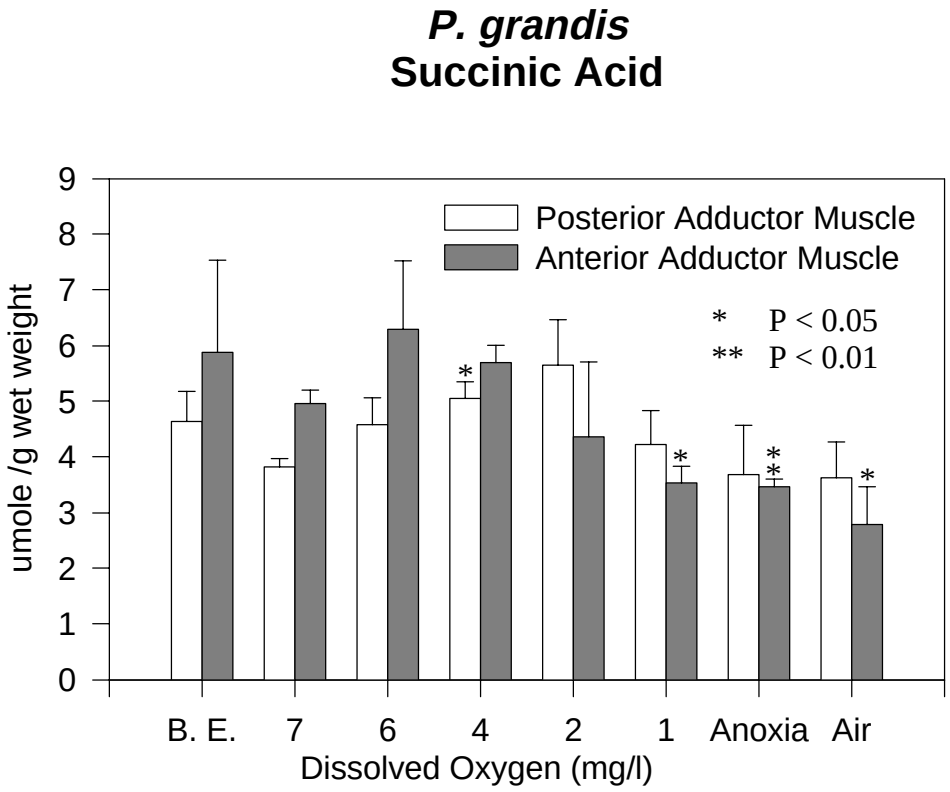

Fig. 10 Comparison of succinic acid levels in *P. grandis* for posterior adductor muscle and anterior adductor muscle under different DO treatments. Error bars =± SEM. **B. E.** before exposure. For the unpaired t-test, * P<0.05 and **P<0.01 were used for the comparison between control (DO = 7 mg/l) and anoxia, 12 hr air exposure.

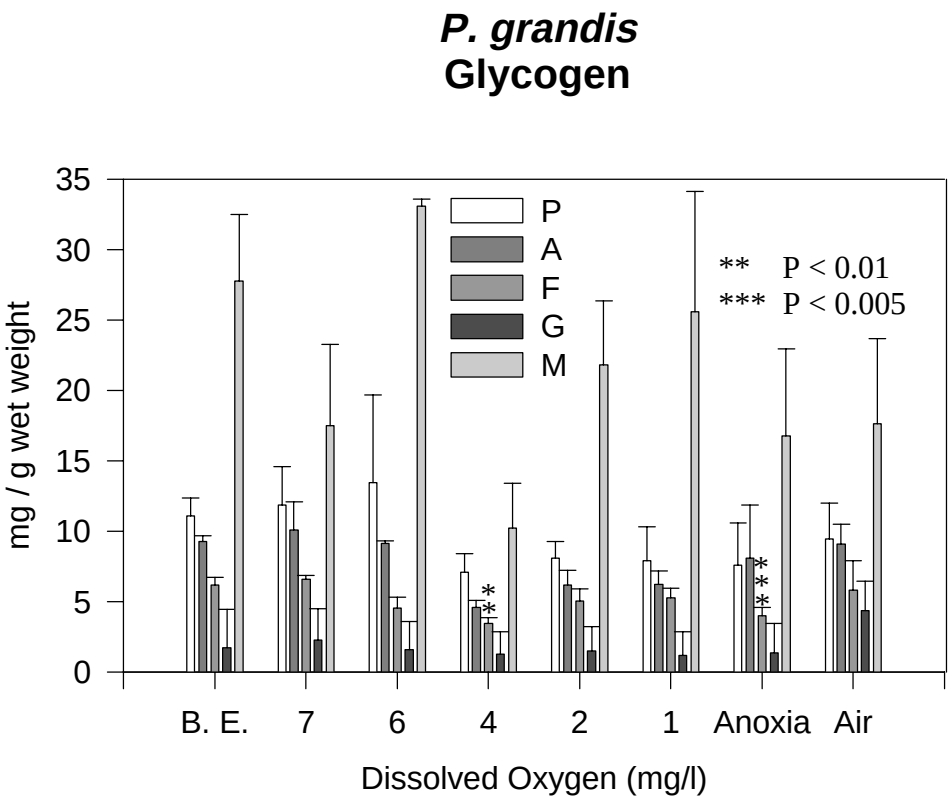

Fig.11 Comparison of glycogen levels in *P. grandis* for five tissues under different DO treatments. Error bars =± SEM. **P**: posterior adductor muscle. **A**: anterior adductor muscle. **F**: foot. **G**: gill. **M**: mantle. **B. E.**: before exposure. For the unpaired t-test, ** P<0.01 and ***P<0.005 were used for the comparison between control (DO = 7 mg/l) and different treatments.

DISCUSSION

The results of this study show that the anaerobic energy metabolism of freshwater mussels is different from that of many marine species. Unlike marine bivalves such as *Crassostrea*, *Mytilus* and *Rangia* (Stokes and Awapara, 1968; Chen and Awapara, 1969; Hammen, 1969; de Zwaan, 1972), lactic acid was accumulated in *E. complanata* and *V. iris* under some hypoxic and anoxic conditions. However, as in the hypoxia-tolerant marine species, the accumulation of succinic acid also was found in *E. complanata* following air exposure. From the depletion of the glycogen stores and accumulation of lactic acid in *V. iris* and *E. complanata* under anoxia and air exposure in this study, the energy source during the hypoxic condition must have come from degradation of glucose and glycogen. The possible participation of amino acids in the anaerobioses needs further research.

The three mussel species *V. iris, P. grandis* and *E. complanata* exhibited different biochemical responses under low DO conditions. In work presented in Chapter 2, *V. iris* proved to be sensitive to low DO whereas *P. grandis* and *E. complanata* were relatively tolerant of hypoxia. The results presented here indicate that *V. iris* also apparently has less anaerobic capacity, compared to the other two species. This can be judged from their relative ability to accumulate anaerobic metabolites and the level of glycogen content in their tissues. Freshly sampled specimens of *V. iris* used the lactate pathway under hypoxia, but this pathway can produce only 2 ATP per unit of glucose from glycolysis of glycogen. Glycogen is the major source of glucose units for glycolysis in animals (Gnaiger, 1983);

hence, glycogen concentration directly influences the capacity for anaerobic metabolism. In my study, the accumulation of lactic acid in the anterior adductor muscle (Fig. 3) occurred simultaneously with the depletion of glycogen (Fig 4). Since *V. iris* has a lower glycogen store in the various tissues than *E. complanata* and *P. grandis*, it has the lowest anaerobic capacity of these three species. The *V. iris* that had been poorly fed had glycogen concentrations in the posterior and anterior adductor muscles that were an order of magnitude less than that of the fresh samples. It is interesting that these animals seemed to be unable to metabolize anaerobically, as no obvious accumulation of the anaerobic end products (i.e., lactic acid and succinic acid) occurred in tissues under low DO. This would probably limit their ability to survive short periods of hypoxia, as might occur at night in eutrophic waters.

The lactic acid level of the hypoxia-tolerant species, *E. complanata*, showed a dose response as the hypoxic stress increased; the response was most evident in the gill tissue. The lactic acid levels in the foot of mussels from the control group were relatively high and exhibited high variation. This variability may be the result of the greater amount of movement at high DO levels. As the glycolytic production of lactic acid is the quickest way to generate energy (ATP) anaerobically (Hochachka and Guppy, 1987), it is reasonable that *E. complanata* uses this pathway first, when it is exposed to low DO.

During both anoxia and air exposure of *E. complanata*, succinic acid was accumulated in the tissues. However, there were no significant changes in succinic acid

levels for the low DO exposure groups. Hochachka and Somero (1984) reported that the production of succinic acid can yield twice the energy of the lactic acid pathway (4 moles ATP/mole glucose by producing succinic acid compared to 2 moles ATP/mole glucose by producing lactic acid). The utilization of the succinic acid pathway may therefore allow *E. complanata* to survive anoxic conditions in a manner similar to that found in some of the more hypoxia tolerant marine species. The accumulation of succinic acid was probably achieved by producing succinic acid from glucose instead of from the amino acid aspartate, because a marked decrease in glucose levels of the mussels exposed to anoxia and air was found (Fig. 7). Furthermore, there is some evidence that freshwater mussels use carbohydrate instead of protein as an energy source under air exposure (see below).

The responses of *E. complanata* to anoxia are similar to those recorded in air exposure, when the pattern of changes in succinic acid and glucose levels in different tissues are compared (Figs 6 & 7). However, it cannot be concluded that these two stresses always have the same effects on mussels. Under air exposure, mussels must face the stress of losing water and possibly obtaining some oxygen from the air. Some of the mussels when exposed to air were found to be slightly gaped, perhaps getting oxygen at the expense of losing water. The intertidal mussel *Mytilus edulis* maintains an aerobic metabolism responsible for up to 40% of the total (anaerobic and aerobic) metabolism while in air (Shick et al., 1986). In addition, several freshwater species exhibit "mantle edge exposure behavior" (MEB) (Heming et al., 1988; Bryne and McMahon, 1991; McMahon, 1991) for oxygen uptake during air exposure. Conversely, under anoxia (i.e., no oxygen in the water), mussels can use

only anaerobic metabolism for obtaining energy. Vitale and Friedl (1984) studied an *Elliptio* species and concluded that freshwater bivalves did not have the capacity, as do marine bivalves, to produce urea or uric acid under air exposure. Hence, utilizing amino acids for anaerobic metabolism under air exposure will result in the production of ammonia, which is extremely toxic to freshwater mussels. Bishop et al. (1983) also found that *C. fluminea* does not catabolize amino acids during air exposure, and protein catabolism was suppressed in the freshwater mussels *Lamellidens corrianus* and *L. marginalis*.

The glycogen content of *E. complanata* did not decline as the stress of hypoxia increased, and this may be because the amount of glycogen consumed through anaerobic metabolism was small when compared to the high store of glycogen present. Hochachka (1982) compared the glycogen content of *Mytilus* to that of other animals (i.e., turtles and goldfish) and concluded that animals with high anaerobic capacity normally have high glycogen levels in their tissues. This would appear to be the case for *E. complanata* and *P. grandis*. The glycogen content of these freshwater mussels is highest in the mantle tissue. In another study (Chen et al., 1997), the glycogen content in the mantle tissue of *E. complanata* collected in September was 24.2 ± 1.85 mg/g (± SEM mg/g, n=50), which was much higher than that of the gill and posterior adductor muscle in that study, and is also higher than most of the tissues in the current study.

Pyganodon grandis apparently utilizes a different metabolic strategy under hypoxic conditions. In Chapter 2, I reported that *P. grandis* can slow its heart rate from

approximately 18 beats/min down to 3 beats/min when the valves are closed. Simultaneous measurement of the heart rate and oxygen consumption rate revealed that *P. grandis* slowed heart rate when the DO was below the critical oxygen tension (DO below which the oxygen consumption declines as the DO is reduced). In this study, the reduced lactic acid and succinic acid levels in the anterior adductor muscle under anoxia and air exposure (Fig. 9 & 10) may have resulted from the suppression of overall metabolism, which has been found in anoxia-tolerant species (Hochachka and Guppy, 1987; Storey and Storey, 1990; Guppy et al. 1994) and also sublittoral marine bivalves under longer-term anoxia (Oeschger, 1990).

In a recent review, Hochachka (1997) indicated that there are two main defense strategies against hypoxia that tolerant animal species use: one is reduction in energy turnover (the same as metabolic depression), and the other is improvement in energetic efficiency. Both strategies were found in the freshwater mussels tested. *P. grandis* appears to use the former strategy, whereas *E. complanata* uses the latter one, in which anaerobic pathways which maximize the yield of ATP are favored (Hochachka, 1993).

In addition to the use of different metabolic strategies under hypoxic conditions, *E. complanata* and *P. grandis* may have different strategies for dealing with air exposure based on the difference in thickness of their shells. Byrne and McMahon (1994) found that unionid species with thin shells, such as *P. grandis*, are less tolerant of air exposure because of water loss. White (1979) indicated that *P. grandis* has the ability of rapid vertical migration through the substratum, which is an adaptation to avoid air exposure. In the current study, *P.*

grandis had an elevated glucose content in the foot during the air exposure (Table 3), which may be related to this adaptation. Byrne et. al. (1988) suggested that the unionid species with thicker shells such as *C. parva* and *U. tetralasmus*, which can be tightly sealed during air exposure, are much more emersion-tolerant than vertical migrating species. As *E. complanata* has a thick shell, it should be more tolerant of desiccation caused by air exposure than *P. grandis*.

The different metabolic strategies under air exposure used by *E. complanata* and *P. grandis* may be reflected in their behavioral responses. Byrne and McMahon (1994) found that *P. grandis* exhibited frequent mantle edge exposure behavior with increasing emersion duration, which results in high water loss but aerial respiration. On the other hand, thick-shelled mussels such as *E. complanata* close their shells and remain anaerobic for extended periods of air exposure. Such thick-shelled freshwater mussels probably rely on a more efficient anaerobic metabolism for energy supply.

For environmental monitoring and conservation of freshwater mussels, there is an increasing need for the use of biochemical indicators of stress or general health. Glycogen concentration has been used extensively as an indicator of the energetic status of freshwater mussels (Holopainen, 1987, Hemelraad et. al. 1990) and physiological condition (Haag et. al. 1993; Naimo et. al., 1998; Patterson, et. al. 1997). Glycogen is quite variable, however; it varies seasonally (Holopainen, 1987) and is usually high during the reproductive period (Jadhav and Lomte, 1982). In my study, I found that short-term stress is not reflected in

changes in glycogen content. However, for the more chronic stress, glycogen is a good indicator of physiological condition. Naimo et. al. (1998) used samples from the foot for glycogen analysis in a nonlethal evaluation of the health of mussels. My work suggests that mantle tissue is a better tissue for nonlethal measurements, as it has much higher concentrations than the other four tissues. Less tissue would be needed from specimens, and less stress would result from the sampling. In addition, Berg et al. (1995) found that mantle biopsy resulted in no significant difference in the survival rate of the samples in field experiments, while some mortality was found in foot tissue sampling by Burress (1995). When the accumulation of anaerobic metabolites are used as an indicator of stress, tissue samples from gill and anterior and posterior adductor muscles are better than foot tissue because the change in metabolite levels tends to be greater.

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