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The effect of prolonged anoxia on enzyme activities in oysters (*Crassostrea virginica*) at different seasons

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Abstract

The effect of prolonged anoxia (96 h under a N_2 atmosphere) during either winter (November) or summer (July) was investigated by measuring the maximal activities of 20 metabolic enzymes in gill, mantle, hepatopancreas, and phasic and catch adductor muscles of the oyster, *Crassostrea virginica*. The enzymes analyzed are involved in carbohydrate and amino acid metabolism, the pentose phosphate shunt, anaplerotic reactions of the TCA cycle, and phosphagen/adenylate metabolism. The data demonstrate that oyster metabolism is influenced by both long-term seasonal change and by shorter-term environmental insult (anoxia). Seasonal changes were concentrated among enzymes involved in glycogen metabolism whereas the prominent response to anoxia was suppression of PK activity. Anoxia exposure induced tissue-specific changes in enzyme activities suggesting a substantial metabolic reorganization involving both coarse controls on enzyme amount and reversible covalent modification. In addition, the effects of anoxia on enzymes of intermediary metabolism were seasonally dependent and more widespread in the winter. These results demonstrate the interaction of two environmental variables (season, anoxia) and suggest the importance of season as a modifying factor in the anoxic response.

Keywords: Anaerobiosis; Marine mollusc; Seasonality

1. Introduction

Many marine bivalve and gastropod molluscs display excellent anoxia tolerance.

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Oxygen limitation can arise in several ways including aerial exposure of intertidal species at low tide, burrowing into O_2-depleted substrate or, especially for sessile species, exposure to noxious environmental conditions that reduce seawater O_2 levels (e.g. algal blooms) or stimulate prolonged periods of valve closure (e.g. high silt levels, toxins and pollutants, predator attack). Oysters of the *Crassostrea* genus show good anoxia tolerance. For example, *C. virginica* readily endured 4 days under a nitrogen gas atmosphere at 20°C (Eberlee et al., 1983). Like most other anoxia-tolerant marine bivalve molluscs (De Zwaan, 1983; De Zwaan et al., 1991; Brooks et al., 1991), anaerobic metabolism in oysters relies on the coupled fermentation of glycogen and aspartate with an accumulation of succinate and alanine as end products (Collicutt and Hochachka, 1977; Foreman and Ellington, 1983; Eberlee et al., 1983). Upon return to oxygenated water, however, *C. virginica* cleared end products and restored aspartate pools within 6–12 h (Eberlee et al., 1983). Another of the key factors in anaerobic survival for marine molluscs is anoxia-induced metabolic rate depression. Anoxic metabolic rate is frequently less than 10% of resting, aerobic rate at the same temperature and there is a good correlation between anoxia survival time and the extent of metabolic rate depression in various species (De Zwaan et al., 1991).

The mechanisms of metabolic rate depression and the switch from aerobic to fermentative pathways of energy generation in molluscs have been traced to controls over various enzymes and functional proteins including covalent modification by reversible protein phosphorylation, allosteric regulation of key enzymes, and the reversible association of enzymes with subcellular structures (Storey and Storey, 1990; Storey, 1993). In particular, anoxia-induced covalent modification is a major factor in glycolytic rate control in anoxia-tolerant molluscs and alters the function of glycogen phosphorylase, phosphofructokinase and pyruvate kinase (PK) in anoxia (Storey, 1993). Indeed, PK phosphorylation in anoxia has been widely documented in molluscs and appears to be the major mechanism that regulates the phosphoenolpyruvate (PEP) branchpoint to control the switch from aerobic, via PK, to anaerobic, via phosphoenolpyruvate carboxykinase (PEPCK), routes of PEP catabolism.

Another mechanism of enzyme control that can have a major influence is changes in the amounts of enzyme present in tissues via modification of the rates of enzyme synthesis and/or degradation. Such coarse control is often used to make long-term changes in metabolic make-up such as seasonal adjustments (e.g. with respect to nutrient availability, the reproductive cycle, and environmental parameters such as temperature) (Galtsoff, 1964; Kluytmans et al., 1980; Gabbott, 1983; Newsholme and Crabtree, 1986; Ballantyne and Berges, 1991) but could also have a role in animal adjustment to prolonged severe hypoxia or anoxia lasting several days or more such as may occur under selected circumstances in nature or for commercial species during harvesting and shipping to market. The present study analyzes the maximal activities of numerous metabolic enzymes (involved in carbohydrate and amino acid metabolism, the pentose phosphate shunt, anaplerotic reactions of the TCA cycle, phosphagen and adenylate metabolism) in five tissues of oysters *C. virginica* and considers the influences of two factors on the organization of metabolic enzymes, seasonal adjustments and the effects of 4 days of anoxia exposure.

2. Materials and methods

2.1. Chemicals and animals

All biochemicals and coupling enzymes were obtained from Sigma Chemical, St. Louis, MO or Boehringer–Mannheim, Montreal, PQ. Distilled, deionized water was used throughout for the preparation of solutions. All ATP stock solutions contained added $MgCl_2$ in 1:1 molar amounts. Malpeque oysters, *C. virginica*, of $\sim 10 \times 5$ cm shell dimension from Prince Edward Island were obtained from a local seafood supplier. Winter animals were purchased in early to mid-November and summer animals were obtained in July or early August. Animals were acclimated in aerated artificial seawater (1000 mOsmol/l) at 10°C for at least 1 week prior to experimentation.

2.2. Preparation of experimental animals

Control oysters were taken directly from the aerated seawater. The valves were opened, the adductor muscle severed, and then gills, mantle, hepatopancreas, and phasic and catch adductor muscles were quickly excised and immediately frozen in liquid nitrogen. All tissues were stored at -70°C until use. For experimental anoxia, oysters were placed in large jars that had a small amount of seawater (~ 0.5 cm depth) on the bottom to maintain a humid environment. The water had been previously bubbled with nitrogen gas (100%) for 20–30 min to displace oxygen and after oysters were the put in the jar, bubbling was continued for a further 30 min, after which the lid was sealed, and the jar was placed in a 10°C incubator. Oysters were maintained under anoxic conditions for 96 h after which the animals were rapidly dissected and tissues frozen, as described above.

2.3. Tissue extraction and enzyme assay

Tissue extracts for enzyme assay were prepared by homogenizing frozen tissue samples with a Pro200 homogenizer (Diamed Lab Supplies, Mississauga, Ontario) using a 1:5 w/v ratio for all tissues. The same homogenization buffer was used for all tissues and enzymes and contained 50 mM imidazole–HCl buffer (pH 7.2), 100 mM NaF, 5 mM EDTA, 5 mM EGTA and 15 mM 2-mercaptoethanol. A few crystals of phenylmethylsulfonyl (PMSF) were added immediately prior to homogenization. NaF and EDTA/EGTA inhibit protein phosphatases and kinases, respectively, and prevent a change in the phosphorylation state of enzymes during the extraction and assay processes.

2.4. Glycogen phosphorylase and synthetase

Homogenates were allowed to settle on ice for a minimum of 30 min and then the supernatant was removed for assay; for adductor muscle extracts it was necessary to centrifuge homogenates at low speed in a benchtop centrifuge for 30 s in order to

retrieve a supernatant. Assay conditions for the active, phosphorylated form of glycogen phosphorylase (GP*a*) were 50 mM potassium phosphate buffer (pH 7.0), 0.25 mM EDTA, 10 mM $MgCl_2$, 0.4 mM NADP, 10 μM glucose-1,6-bisphosphate, 2 mg/ml glycogen (previously dialyzed), 0.2 unit/ml (U/ml) glucose-6-phosphate dehydrogenase, and 0.7 U/ml phosphoglucomutase. For total phosphorylase ($a + b$), 1.6 mM AMP was also present in the assay. Assay conditions for the active, glucose-6-phosphate independent *I* form of glycogen synthetase (GS*I*) were 20 mM imidazole–HCl buffer (pH 7.0), 20 mM KCl, 5 mM $MgCl_2$, 0.15 mM NADH, 2 mg/ml oyster glycogen (previously dialyzed), 5 mM uridine 5′ diphosphoglucose, 1 mM phosphoenolpyruvate, 1 U/ml pyruvate kinase and 1 U/ml lactate dehydrogenase. For total synthetase activity ($I + D$), 5 mM glucose-6-phosphate (G6P) was included in the assay mixture. GP and GS assays were conducted by monitoring changes in NAD(P)H absorbance at 340 nm using a Gilford 240 recording spectrophotometer. Assays were initiated by addition of the enzyme preparation and were performed at 21±1°C, with a final cuvette volume of 1 ml plus added enzyme extract.

2.5. Survey of enzyme maximal activities

Tissue homogenates were prepared as above and then centrifuged for 20 min at 18 000 *g* in a Biofuge 15 centrifuge (Canlab) at 5°C. Blanks were run and subtracted; the specific substrate omitted in the blank is indicated by an asterisk in the assays below. Changes in NAD(P)H absorbance at 340 nm were monitored using a MR 5000 Microplate Reader (Dynatech Laboratories, Chantilly, VA) connected to a 486 computer running Biolinx v2.0 software. Assays were conducted at 21±1°C and initiated by addition of the enzyme preparation with a final microplate well volume of 0.250 ml plus added homogenate. Assay conditions were based on those of Brooks and Lampi (1995, 1996), as designed for the microplate reader, with optimal substrate concentrations determined for the oyster enzymes in preliminary tests.

Hexokinase (HK; EC 2.7.1.1): 100 mM Tris buffer (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 5 mM glucose*, 1 mM Mg · ATP, 0.2 mM $NADP^+$, and 1 U/ml $NADP^+$-dependent G6PDH.

Phosphofructokinase (PFK; EC 2.7.1.11): 100 mM imidazole–HCl buffer (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM fructose-6-phosphate*, 0.1 mM Mg · ATP, 0.15 mM NADH, 0.1% (v/v) rotenone-saturated ethanol, 0.2 U/ml aldolase, 0.16 U/ml triosephosphate isomerase and 1.2 U/ml G3PDH. To desalt coupling enzymes, aliquots of concentrated enzymes were centrifuged for 2 min in a Brinkman 5412 microcentrifuge and the pellet was resuspended in assay buffer (100 mM imidazole, pH 7.2, 50 mM KCl, 5 mM $MgCl_2$); this was then centrifuged (benchtop centrifuge at top speed for 1 min) through a 5-ml column of fine Sephadex G-25 equilibrated in assay buffer.

Aldolase (EC 4.1.2.13): 50 mM imidazole–HCl buffer (pH 7.0), 2 mM $MgCl_2$, 0.2 mM fructose-1,6-P_2*, 0.15 mM NADH, 1 U/ml triosephosphate isomerase and 2 U/ml G3PDH.

Glycerol-3-phosphate dehydrogenase (G3PDH; EC 1.1.1.8): 500 mM triethanolamine buffer (pH 7.9), 5% (w/v) bovine serum albumin, 26 mM glyceraldehyde-3-phosphate*, 0.15 mM NADH, and 1 U/ml triosephosphate isomerase.

Pyruvate kinase (PK; EC 2.7.1.40): 100 mM imidazole–HCl buffer (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM phosphoenolpyruvate*, 2 mM ADP, 0.15 mM NADH, 0.2% (v/v) rotenone-saturated ethanol, and 1 U/ml LDH.

Phosphoenolpyruvate carboxykinase (PEPCK; EC 4.1.1.32): 100 mM imidazole–HCl buffer (pH 6.6), 30 mM 2-mercaptoethanol, 5 mM phosphoenolpyruvate*, 50 mM $NaHCO_3$, 1.25 mM IDP, 1 mM $MnCl_2$, 0.15 mM NADH, and 2.5 U/ml MDH. Solutions were degassed prior to assay.

Lactate dehydrogenase (LDH; EC 1.1.1.27): 50 mM imidazole–HCl buffer (pH 7.0), 2 mM $MgCl_2$, 4 mM pyruvate*, 0.15 mM NADH.

Fructose-1,6-bisphosphatase (FBPase; EC 3.1.3.11): 25 mM imidazole–HCl buffer (pH 7.0), 5 mM $MgSO_4$, 1.4 mM fructose-1,6-P_2^*, 0.2 mM $NADP^+$, 1 U/ml phosphoglucose isomerase and 1 U/ml G6PDH.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49): 100 mM Tris–HCl buffer (pH 7.5), 3.3 mM G6P*, 6 mM $MgCl_2$, and 0.4 mM $NADP^+$.

6-Phosphogluconate dehydrogenase (6PGDH; EC 1.1.1.44): 50 mM Tris–HCl (pH 7.0), 0.5 mM 6-phospho-D-gluconate*, and 0.4 mM $NADP^+$.

Glutamate pyruvate transaminase (GPT; EC 2.6.1.2): 500 mM imidazole–HCl buffer (pH 7.3), 30 mM 2-mercaptoethanol, 500 mM L-alanine*, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 15 mM 2-oxoglutarate, and 1 U/ml LDH.

Glutamate oxaloacetate transaminase (GOT; EC 2.6.1.1): 500 mM imidazole–HCl buffer (pH 7.8), 30 mM 2-mercaptoethanol, 250 mM L-aspartate*, 0.11 mM pyridoxal phosphate, 15 mM 2-oxoglutarate, 0.15 mM NADH, and 1 U/ml MDH.

Serine dehydratase (SDH; EC 4.2.1.13): 200 mM K_2HPO_4 buffer (pH 8.0), 2 mM EDTA, 100 mM L-serine*, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 0.2% (v/v) rotenone-saturated ethanol, and 1 U/ml LDH.

Malic enzyme (ME; EC 1.1.1.40): 150 mM triethanolamine buffer (pH 7.4), 5 mM L-malate*, 4 mM $MnCl_2$, and 0.2 mM $NADP^+$.

Malate dehydrogenase (MDH; EC 1.1.1.37): 50 mM imidazole–HCl buffer (pH 7.2), 10 mM $MgCl_2$, 20 mM oxaloacetate*, and 0.15 mM NADH. This assay determines the maximal activity of malate dehydrogenase using its preferred cofactor NADH. MDH activity with NADPH as the coenzyme was also determined under identical conditions but substituting 0.15 mM NADPH.

Arginine kinase (ArgK; EC 2.7.3.3): 50 mM imidazole–HCl (pH 7.2), 10 mM $MgCl_2$, 15 mM arginine phosphate*, 15 mM glucose, 2 mM ADP, 0.45 mM $NADP^+$, 1 U/ml hexokinase and 1 U/ml G6PDH.

Adenylate kinase (AK; EC 2.7.4.3): 50 mM potassium phosphate buffer (pH 7.4), 20 mM KCl, 5 mM $MgSO_4$, 2 mM AMP, 2 mM ATP*, 2 mM phosphoenolpyruvate, 0.15 mM NADH, 2 U/ml PK and 2 U/ml LDH.

2.6. Data and statistics

All data are presented as means±S.E.M. with statistical significance ($P < 0.05$) determined using the Student's *t*-test (two-tailed). For determination of enzyme activity as units per gram wet weight (U/gww), kinetic data from Biolinx 2.0 were imported into the Microplate Analysis Program (Brooks, 1994) which performed a linear regression on

the ΔOD/time readings. One unit of enzyme activity is defined as the amount that uses 1 μmole substrate per min at 21°C.

3. Results

3.1. Glycogen phosphorylase and glycogen synthetase

In winter oysters total GP activity (active *a* + inactive *b* forms) was not affected by anoxia exposure in any of the five tissues tested (Tables 1–5). However, anoxia stimulated significant changes in the amount of active phosphorylase *a* (GP*a*) in two tissues, a 43% increase in gill (Table 1) and a 71% decrease in hepatopancreas (Table 3). This raised the percentage of GP present as the active *a* form from 63% to 74% in anoxic gill and lowered %GP*a* from 68% to 25% in anoxic hepatopancreas. Comparable analysis of GP in summer animals revealed no significant effect of anoxia on either total GP or GP*a* in any tissue.

Anoxia exposure during the winter led to a 50% decrease in total GS activity (active

Table 1
Maximal activities of enzymes from gill of aerobic, control and 96 h anoxic winter (November) and summer (July) *C. virginica*[a]

Enzyme	Winter		Summer	
	Control	Anoxic	Control	Anoxic
GP (*a* + *b*)	1.03±0.13	1.25±0.10	0.92±0.08	0.93±0.05
GP*a*	0.65±0.03	0.93±0.09[b]	0.50±0.04	0.48±0.05
GS (*I* + *D*)	0.60±0.06	0.86±0.16	1.01±0.07[c]	0.91±0.13
GS*I*	0.45±0.04	0.67±0.17	0.61±0.06	0.54±0.05
HK	0.68±0.04	0.50±0.02[b]	0.59±0.02	0.60±0.04
PFK	1.57±0.08	1.09±0.13[b]	1.39±0.06	1.23±0.12
Aldolase	0.42±0.03	0.44±0.02	0.37±0.08	0.46±0.03
G3PDH	0.81±0.05	0.73±0.04	0.95±0.04	0.88±0.08
PK	2.65±0.18	0.82±0.08[b]	1.56±0.24[c]	1.25±0.12
PEPCK	0.66±0.06	0.45±0.03[b]	0.47±0.04[c]	0.35±0.04
LDH	0.37±0.04	0.28±0.04	0.32±0.02	0.18±0.04[b]
FBPase	0.03±0.01	0.04±0.003[b]	0.05±0.01[c]	0.04±0.01
G6PDH	2.97±0.14	2.41±0.12[b]	3.72±0.44	2.95±0.19
6PGDH	0.87±0.08	0.75±0.06	0.75±0.07	0.82±0.06
GPT	10.2±0.68	7.65±0.44[b]	10.1±0.99	7.94±0.79
GOT	5.33±0.44	4.46±0.10	6.04±0.34	4.11±0.27[b]
SDH	0.32±0.05	0.30±0.03	0.28±0.02	0.22±0.04
ME	0.49±0.02	0.40±0.02[b]	0.52±0.02	0.414±0.05
MDH	20.0±1.40	17.8±0.43	16.7±0.43	17.9±0.86
ArgK	85.1±8.09	53.1±3.56[b]	78.3±6.83	70.9±4.48
AK	14.1±1.60	9.61±0.73[b]	14.9±0.94	14.6±1.35

[a] Activities are units/gram wet weight, means±SEM, $n = 3–5$ for PK, PEPCK and SDH and $n = 6–8$ for other enzymes.

[b] Significantly different from the corresponding control value by Student's *t*-test (two-tailed), $P < 0.05$.

[c] Significantly different from the corresponding winter control value, $P < 0.05$.

Table 2
Maximal activities of enzymes from mantle of aerobic, control and 96-h anoxic winter (November) and summer (July) *C. virginica*[a]

Enzyme	Winter		Summer		
	Control	Anoxic	Control	Anoxic	
PK	1.93±0.26	0.83±0.05[b]	3.15±0.72	1.18±0.18[b]	
PEPCK	0.41±0.05	0.62±0.04[b]	0.46±0.08	0.33±0.03	
SDH	0.07±0.01	0.08±0.02	0.11±0.01[c]	0.06±0.01[b]	
	Winter	Summer	Enzyme	Winter	Summer
GP ($a + b$)	1.14±0.06	1.22±0.16	FBPase	0.05±0.006	0.05±0.003
GP*a*	0.94±0.08	0.88±0.12	G6PDH	2.20±0.17	2.35±0.21
GS ($I + D$)	0.60±0.05	1.38±0.11[c]	6PGDH	0.68±0.06	0.79±0.05
GS*I*	0.38±0.06	0.65±0.04	GPT	8.57±0.70	9.30±0.92
HK	0.45±0.03	0.59±0.04[c]	GOT	4.33±0.28	4.83±0.39
PFK	1.18±0.14	1.20±0.14	ME	0.38±0.05	0.39±0.04
Aldolase	0.72±0.05	0.66±0.07	MDH	17.6±0.86	17.2±1.15
G3PDH	0.63±0.06	0.78±0.05	ArgK	87.8±11.8	103.8±13.1
LDH	0.60±0.08	0.40±0.06	AK	20.7±1.89	22.4±2.01

[a] In instances where no significant difference was found between enzyme activities in control versus anoxic individuals, data from the two conditions was combined. Activities are units per gram wet weight, means±SEM, $n = 3–4$ for PK, PEPCK and SDH and $n = 6–8$ for other enzymes.
[b] Significantly different from the corresponding control value by Student's *t*-test (two-tailed), $P < 0.05$.
[c] Significantly different from the corresponding winter value, $P < 0.05$.

I + inactive D forms) in the hepatopancreas and a 43% decrease in the amount of the G6P-independent, active form (GS*I*); hence, the percentage of active enzyme remained roughly the same, 63% in control and 71% in anoxic animals. By contrast, total GS activity in rose by 29% in anoxic phasic adductor but the %GS*I* again remained constant (36–44%). Anoxia exposure of summer oysters had no effect on total GS or GS*I* in any tissue.

A comparison can also be made of GP and GS activities in winter versus summer animals and here substantial effects were seen. Total GP activity was significantly lower in summer animals in three tissues: hepatopancreas, phasic adductor and catch adductor, reduced to 44, 44, and 57% of the corresponding values in winter animals. By contrast, total GS was higher in summer versus winter animals in three tissues (by 66% in gill, 100% in catch adductor, and 130% in mantle) but was just 25% of the winter value in phasic adductor.

3.2. The effect of anoxia on maximal activities of enzymes from winter and summer oysters

Table 1 shows the maximal activities of enzymes in gill from winter and summer oysters and the effects of anoxia exposure (N_2 atmosphere) for 96 h on these. Anoxia exposure of winter animals had widespread effects on enzyme activities in the gill with activities of nine enzymes decreasing significantly but activity of only one enzyme, FBPase, rising by 33%. Maximal activities of FBPase and ME fell by 19% in anoxia,

Table 3
Maximal activities of enzymes from hepatopancreas of aerobic, control and 96-h anoxic winter (November) and summer (July) *C. virginica*[a]

Enzyme	Winter		Summer	
	Control	Anoxic	Control	Anoxic
GP ($a + b$)	1.12±0.20	0.87±0.08	0.49±0.06[c]	0.64±0.04
GP*a*	0.76±0.07	0.22±0.01[b]	0.09±0.01	0.11±0.02
GS ($I + D$)	1.12±0.08	0.58±0.08[b]	1.40±0.13	1.31±0.11
GS*I*	0.71±0.06	0.41±0.03[b]	0.51±0.06	0.42±0.03
PK	2.87±0.60	0.80±0.03[b]	7.06±1.01[c]	2.66±0.20[b]
LDH	0.67±0.07	0.44±0.07[b]	0.54±0.08	0.50±0.06
FBPase	0.04±0.01	0.11±0.01[b]	0.10±0.01[c]	0.10±0.01
6PGDH	0.79±0.10	0.38±0.03[b]	0.67±0.04	0.59±0.04
GPT	12.8±0.40	11.3±0.892	13.4±0.68	11.3±0.49[b]

	Winter	Summer	Enzyme	Winter	Summer
HK	0.52±0.023	0.69±0.03[c]	GOT	6.64±0.58	7.47±0.79
PFK	1.44±0.09	1.91±0.18	SDH	0.08±0.01	0.10±0.01
Aldolase	0.13±0.02	0.14±0.01	ME	0.44±0.03	0.46±0.04
G3PDH	1.10±0.10	1.52±0.12	MDH	23.8±1.31	23.9±1.29
PEPCK	1.00±0.05	0.78±0.06	ArgK	106±6.9	148±13.5[c]
G6PDH	2.21±0.15	2.98±0.16	AK	8.91±0.77	11.0±1.01

[a] In instances where no significant difference was found between enzyme activities in control versus anoxic individuals, data from the two conditions was combined. Activities are units/gram wet weight, means±SEM, $n = 3$–5 for GP, GS, PK, LDH, FBPase, 6GPDH, and GPT and $n = 6$–10 for other enzymes.
[b] Significantly different from the corresponding control value by Student's *t*-test (two-tailed), $P < 0.05$.
[c] Significantly different from the corresponding winter value, $P < 0.05$.

whereas HK, PFK, PEPCK, GPT, ArgK and AK fell by 26–39%, and PK was most strongly affected, decreasing by 69% in anoxia. Anoxia exposure had fewer effects on the activities of enzymes in the gill of summer oysters with only a 44% decrease in LDH activity and a 32% decrease in GOT. Comparison of winter and summer control oysters showed seasonal differences for only four enzymes in gill: GS (see above), FBPase (62% higher in summer) and two enzymes at the PEP branchpoint: PK and PEPCK (41 and 29% lower in summer).

Anoxia exposure affected the activities of only three enzymes in mantle. PK maximal activity was reduced during anoxia in both winter (by 57%) and summer (by 63%) oysters (Table 2). In addition, PEPCK activity increased in mantle of anoxic winter oysters by 50% whereas SDH activity decreased by 45% in summer mantle. Winter versus summer differences in mantle maximal activities were observed for only 3 enzymes: GS (see above), HK (30% higher in summer), and SDH (62% higher in summer).

Anoxia exposure affected the maximal activities of several enzymes in hepatopancreas (Table 3). Again, many more anoxia-induced changes were seen in the tissue of winter oysters (seven enzymes) than in summer animals (two enzymes). Anoxia exposure strongly reduced PK maximal activity in both winter (by 72%) and summer (by 62%) oysters. In winter oysters, anoxia also led to decreased activities of LDH (by 33%) and

Table 4
Maximal activities of enzymes from phasic adductor of aerobic, control and 96-h anoxic winter (November) and summer (July) *C. virginica*[a]

Enzyme	Winter		Summer	
	Control	Anoxic	Control	Anoxic
GS ($I + D$)	1.66±0.05	2.14±0.07[b]	0.59±0.12[c]	0.57±0.09
GS*I*	0.73±0.14	0.78±0.10	0.19±0.05	0.21±0.02
Aldolase	6.03±0.42	6.75±0.40	5.83±0.33	7.30±0.49[b]
PK	10.9±1.00	2.47±0.34[b]	13.0±0.84	3.61±0.49[b]
FBPase	0.05±0.01	0.08±0.01[b]	0.06±0.01	0.07±0.01
G6PDH	1.70±0.06	1.32±0.10[b]	1.38±0.05	1.99±0.26[b]
MDH	25.4±1.12	25.6±0.96	27.7±1.18	31.2±0.84[b]
AK	31.8±2.0	33.2±2.2	24.7±2.7	48.3±5.1[b]

	Winter	Summer	Enzyme	Winter	Summer
GP ($a + b$)	3.40±0.20	1.50±0.17[c]	LDH	0.89±0.08	0.70±0.08
GP*a*	2.36±0.23	0.63±0.15	6PGDH	0.51±0.03	0.60±0.03
HK	0.36±0.03	0.40±0.04	GPT	7.66±0.92	8.53±0.71
PFK	8.04±0.48	7.95±0.22	GOT	9.77±0.53	10.3±0.45
G3PDH	1.53±0.10	1.31±0.10	ME	0.50±0.06	0.51±0.06
PEPCK	1.18±0.14	1.33±0.04	ArgK	481±29	410±35

[a] In instances where no significant difference was found between enzyme activities in control versus anoxic individuals, data from the two conditions was combined. Activities are units/gram wet weight, means±SEM, $n = 4$–5 for GS, aldolase, PK, FBPase, G6PDH and MDH, and $n = 8$–10 for other enzymes. Serine dehydratase (SDH) activity was below the limit of detection (< 0.04 U/gww).

[b] Significantly different from the corresponding control value by Student's *t*-test (two-tailed), $P < 0.05$.

[c] Significantly different from the corresponding winter control value, $P < 0.05$.

6PGDH (by 50%) but raised FBPase activity by three-fold. During the summer, anoxia altered only PK and GPT (15% reduction) activities. Activities of five enzymes changed seasonally in hepatopancreas. Total GP activity was lower in summer but maximal activities of PK and FBPase were 2.5- and 2.7-fold higher in summer and HK and ArgK were 33% and 40% higher in summer.

In phasic adductor muscle activities several enzymes were affected during anoxia (Table 4). Once again, PK activity was strongly reduced during anoxia in both winter (by 77%) and summer (by 73%) oysters. In winter animals, anoxia exposure also reduced G6PDH activity by 22% but increased GS (see above) and FBPase (by 60%). In summer oysters, activities of four enzymes increased during anoxia: aldolase by 25%, G6PDH by 44%, MDH by 13% and AK by 95%. Seasonal differences in enzyme maximal activities in phasic adductor muscle were noted for GS and GP along with small decreases (by 20%) in G6PDH and LDH in summer, compared with winter.

In catch adductor muscle anoxia exposure affected only four enzymes. Again, PK activity was strongly reduced during anoxia in both winter (by 77%) and summer (by 63%). In winter oysters, anoxia also led to a decrease in PFK maximal activity (by 46%) and MDH (by 13%) whereas in summer oysters GOT activity was reduced by 23% during anoxia. Seasonal differences in enzyme maximal activities were noted in five instances. In addition to GP and GS, PFK activity was 43% lower in summer whereas GOT and HK activities were both higher in summer (by 25 and 39%).

Table 5
Maximal activities of enzymes from catch adductor of aerobic, control and 96 h anoxic winter (November) and summer (July) *C. virginica*[a]

Enzyme	Winter		Summer	
	Control	Anoxic	Control	Anoxic
PFK	5.66±0.68	3.06±0.27[b]	3.24±0.42[c]	3.49±0.34
PK	4.34±0.76	0.99±0.08[b]	4.41±0.70	1.63±0.25[b]
GOT	5.80±0.39	5.96±0.36	7.20±0.36[c]	5.57±0.46[b]
MDH	19.5±0.57	16.9±0.84[b]	22.1±2.02	22.2±1.02

	Winter	Summer	Enzyme	Winter	Summer
GP ($a + b$)	2.05±0.17	1.17±0.15[c]	LDH	0.61±0.05	0.52±0.07
GP*a*	1.50±0.13	1.12±0.13	FBPase	0.05±0.01	0.07±0.01
GS ($I + D$)	0.88±0.11	1.72±0.19[c]	G6PDH	3.38±0.36	3.44±0.25
GS*I*	0.40±0.05	0.64±0.14	6PGDH	0.47±0.02	0.60±0.03
HK	0.33±0.016	0.46±0.04[c]	GPT	7.63±0.56	9.55±1.00
Aldolase	2.72±0.21	2.86±0.19	ME	0.38±0.02	0.35±0.03
G3PDH	0.79±0.05	0.97±0.06	ArgK	246±27	248±24
PEPCK	0.81±0.08	0.94±0.07	AK	90.0±9.5	115±11

[a] In instances where no significant difference was found between enzyme activities in control versus anoxic individuals, data from the two conditions was combined. Activities are units per gram wet weight, means±SEM, $n = 3$–5 for PFK, PK, GOT and MDH, and $n = 6$–10 for other enzymes. Serine dehydratase (SDH) activity was below the limit of detection (< 0.04 U/gww).
[b] Significantly different from the corresponding control value by Student's *t*-test (two-tailed), $P < 0.05$.
[c] Significantly different from the corresponding winter value, $P < 0.05$.

4. Discussion

Multiple demands are placed on the metabolism of marine organisms both from the stresses imposed by the external environment (e.g. variation in temperature, salinity, oxygen availability, etc.) and from internal physiological drives including reproduction and growth. Responses to such demands typically involve changes in flux through selected metabolic pathways and this generally translates into changes in the activities of pathway enzymes as they are the machines that run metabolism. Regulation of metabolic enzymes can be achieved in a variety of ways including coarse controls on synthesis and degradation that can alter the total amount of enzyme protein present in cells, post-translational modifications that can make stable but reversible changes to enzyme properties, and kinetic controls via changes in substrate concentrations or the actions of allosteric effectors. In general, these forms of control mediate long-, medium-, and short-term changes in enzyme activities. Hence, seasonal adjustments to metabolic pathways are frequently mediated with changes in the amounts of selected enzymes in tissues or with changes in the type or proportion of isoforms present whereas shorter term responses, such as a switch to anaerobic metabolism, are often most easily accomplished using controls such as reversible protein phosphorylation that can be rapidly initiated when stress is imposed and equally rapidly removed during recovery.

The present study focuses on the changes in activities of enzymes of intermediary energy metabolism in oyster tissues that support both seasonal adjustments in metabolism and shorter term responses to environmental anoxia.

Anoxia exposure led to selective changes in the maximal activities of enzymes in *C. virginica* tissues. Given the short time frame in which metabolic adjustments have to be made during the transition from the aerobic to anoxic state and the energy limitation of the anoxic state which would make it unrealistic to expend large amounts of ATP on protein biosynthesis, it is not surprising that anoxia-induced changes to enzyme activities were limited in scope. The one highly-consistent and major effect of anoxia exposure was on the maximal activity of PK which was strongly reduced in every tissue during anoxia (by 57–77%) and in both seasons with the single exception of gill PK activity in summer oysters which did not change significantly. The molecular basis of this suppression of PK activity, as also occurs in response to anoxia in many other species of marine molluscs (Storey, 1993), is probably anoxia-induced covalent modification via phosphorylation or dephosphorylation. In many marine mollusc species, anoxia-induced phosphorylation of PK lowers V_{max}, reduces affinity for PEP, reduces sensitivity to the allosteric activator, fructose-1,6-bisphosphate, and greatly increases enzyme inhibition by L-alanine, one of the products of anaerobic metabolism (Plaxton and Storey, 1984; Storey, 1993). Similar anoxia-induced effects on the kinetics of oyster PK were also found in four tissues (Greenway, 1995). The net effect of covalent modification is strong PK inhibition which has two consequences: (1) aids an overall suppression of glycolytic rate as part of a general anoxia-induced metabolic rate suppression, and (2) diverts glycolytic carbon into the PEPCK reaction and onwards into the pathway of anaerobic fermentation that leads to succinate and/or propionate accumulation. In mantle of winter oysters, a metabolic shift to favour the succinate pathway is further enhanced by a rise in the maximal activity of PEPCK during anoxia such that the ratio of PK:PEPCK activities changed from 4.71 in aerobic controls to 1.34 in anoxic animals. In all other tissues in both summer and winter the ratio PK:PEPCK activities also reduced by at least one-half in anoxia (except summer gill where the ratio was unchanged) showing that this was a general phenomenon.

Anoxia-induced changes in the activities of enzymes of glycogen metabolism, GP and GS, can also most likely be traced to reversible phosphorylation of the proteins. Both of these enzymes exist in active and inactive forms that are interconverted by phosphorylation and dephosphorylation reactions with the active form of phosphorylase being the phosphoprotein (GP*a*) whereas the active form of synthetase (GS*I*) is the dephosphorylated form. Due to the opposing effects of phosphorylation on the enzymes, their activities are typically oppositely regulated. Despite the fact that glycogen is a major substrate for anaerobic metabolism in molluscs, GP*a* activity was elevated during anoxia only in gill of winter animals. In three other tissues, GP*a* did not change during anoxia whereas in hepatopancreas activity decreased to about one-third of the aerobic value. Reduced GP*a* has been noted in tissues of other marine molluscs during anoxia and has been linked with metabolic rate depression (Storey, 1993; Russell and Storey, 1995); thus, despite the reliance on glycogen as a major fuel in anoxia, the very much lower metabolic rates (often only ~10% of aerobic) necessitate little or no increase in GP*a*. Glycogen synthetase activity was also strongly suppressed in hepatopancreas of winter

animals during anoxia affecting both total activity and GS*I* so that both glycogen synthesis and catabolism are suppressed in winter animals.

Apart from PK, PEPCK, GP and GS, anoxia-induced changes in the activities of other enzymes in oyster tissues, both winter and summer, were all (mantle, catch adductor) or primarily (nine out of ten in gill, three out of four in hepatopancreas) reductions in enzyme activity in the anoxic state. The exception to this was phasic adductor muscle where activities of three enzymes in summer and two enzymes in winter rose significantly during anoxia. Since the enzymes involved (except for gill PFK) are all non-regulatory, equilibrium enzymes (of a type often called 'housekeeping' enzymes) that are not known to be subject to reversible protein phosphorylation, the generally reduced maximal activities of these enzymes in four tissues after the 4 days of continuous anoxia exposure would be consistent with reduced rates of protein synthesis (an energy-expensive process) in the anoxic state. One enzyme whose activity was frequently elevated in anoxia was FBPase whose activity rose in winter gill, hepatopancreas and phasic adductor. This enzyme has a regulatory role in gluconeogenesis and hence, its elevation might suggest that gluconeogenesis from amino acids is enhanced in some tissues under anoxia, perhaps in tissues with low endogenous glycogen levels.

The current data also show seasonal differences in the number and identity of enzymes whose maximal activities change under anoxia. With the exception of PK in all tissues and G6PDH in phasic adductor (which decreases in winter and rises in summer under anoxia), there were no other enzymes that showed significant changes in enzyme activity under anoxia in both winter and summer. Hence, of the 19 enzymes tested, there were no other consistent patterns of enzyme response other than PK suppression that occurred in both seasons and in multiple tissues. Furthermore, the effects of anoxia on enzyme maximal activities differed strongly between the seasons in two tissues. In both gill and hepatopancreas, anoxia exposure led to significant changes in the activities of many more enzymes in winter (11 and six, respectively) than in summer (two in both cases).

In addition to the differential effects of anoxia in the two seasons, a number of significant differences were noted in the maximal activities of enzymes in aerobic control tissues between winter and summer. Seasonal changes in metabolism are often closely linked to the reproductive cycle in marine molluscs and a correlation has been demonstrated between the rate of respiration and the cycle of gametogenesis. The gametogenic resting phase of *C. virginica* occurs during the winter (Bayne et al., 1976) and, accordingly, respiration rate is lowest in the active tissues at this time (Galtsoff, 1964; Percy et al., 1971). The gametogenic cycle, directly or indirectly, may determine the seasonal metabolic pattern (Bayne et al., 1976) and since metabolic changes can be quite large (e.g. in *Mytilus edulis* mantle glycogen reserves can change by 10-fold over the year) there may be large seasonal changes in flux through various metabolic pathways (Livingstone, 1981). Comparing summer to winter, total GS activity was higher in thee tissues (gill, mantle, catch adductor) of summer oysters whereas total GP was lower in three tissues (hepatopancreas, phasic and catch adductor). These changes altered the ratio of total GP:GS activities from a situation that generally favoured glycogen breakdown in winter (ratios were 1.72, 1.90, 1.00, 2.05, and 2.33 in gill, mantle, hepatopancreas, phasic and catch adductor, respectively), to a summer situation

where the tissues (except phasic adductor) appeared poised for glycogen synthesis (GP:GS ratios were 0.91, 0.88, 0.35, 2.54, and 0.68, respectively). This would be consistent with a situation of feeding, growth and storage of fuel reserves during the summer. Hexokinase was also higher in mantle, hepatopancreas and catch adductor in summer versus winter which could also favour glucose uptake for use in glycogen synthesis. Furthermore, FBPase was higher in two organs (gill, hepatopancreas) in summer which could favour higher rates of gluconeogenesis (using carbon derived from dietary amino acids) leading to glycogen deposition in the summer.

Overall, then, the present data demonstrate that the enzymatic make-up of oyster intermediary metabolism is influenced by both long-term seasonal change and by shorter-term environmental insult (anoxia). Of the enzymes analyzed, consistent seasonal changes were concentrated among enzymes involved in glycogen metabolism whereas the prominent response to anoxia was suppression of PK activity to regulate anaerobic carbohydrate dismutation. Prolonged anoxia exposure induced tissue-specific changes in enzyme activities and, as suggested by the number of significant changes observed in this study, anoxia induces a substantial metabolic reorganization that can involve both coarse controls on enzyme amount and reversible covalent modification. In addition, the effects of anoxia on enzymes of intermediary metabolism were seasonally dependent and more widespread in the winter. These results demonstrate the interaction of two variables (season, anoxia) and suggest the importance of season as a modifying factor in determining organism response to anoxia.

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