

Molecular cloning and seasonal expression of oyster glycogen phosphorylase and glycogen synthase genes

H. Bacca^a, A. Huvet^{a,*}, C. Fabioux^a, J.-Y. Daniel^a, M. Delaporte^a, S. Pouvreau^a,
A. Van Wormhoudt^b, J. Moal^a

^aUMR Physiologie et Ecophysiologie des Mollusques Marins, Ifremer, Centre de Brest, B.P. 70, 29280 Plouzané, France

^bUMR 5178, Station de Biologie Marine du Muséum National d'Histoire Naturelle, BP225, 29900 Concarneau, France

Received 5 August 2004; received in revised form 22 December 2004; accepted 2 January 2005

Abstract

To investigate the control at the mRNA level of glycogen metabolism in the cupped oyster *Crassostrea gigas*, we report in the present paper the cloning and characterization of glycogen phosphorylase and synthase cDNAs (*Cg-GPH* and *Cg-GYS*, respectively, transcripts of main enzymes for glycogen use and storage), and their first expression profiles depending on oyster tissues and seasons. A strong expression of both genes was observed in the labial palps and the gonad in accordance with specific cells located in both tissues and ability to store glucose. *Cg-GPH* expression was also found mainly in muscle suggesting ability to use glycogen as readily available glucose to supply its activity. For seasonal examinations, expression of *Cg-GYS* and *Cg-GPH* genes appeared to be regulated according to variation in glycogen content. Relative levels of *Cg-GYS* transcripts appeared highest in October corresponding to glycogen storage and resting period. Relative levels of *Cg-GPH* transcripts were highest in May corresponding to mobilization of glycogen needed for germ cell maturation. Expression of both genes would likely be driven by the oyster's reproductive cycle, reflecting the central role of glycogen in energy storage and gametogenic development in *C. gigas*. Both genes are useful molecular markers in the regulation of glycogen metabolism and reproduction in *C. gigas* but enzymatic regulation of glycogen phosphorylase and synthase remains to be elucidated.

© 2005 Elsevier Inc. All rights reserved.

Keywords: Bivalve; *Crassostrea gigas*; Energy; Gene; Expression; Glycogen; Oyster; Regulation; Reproduction

1. Introduction

Glucose constitutes an important nutrient which is provided either from the diet, from glycogen storage or from amino acids and lactate via gluconeogenesis (Felber and Golay, 1995). In marine bivalves, glycogen is the major source of glucose reserves stored in specific vesicular cells (Berthelin et al., 2000b) and is known to play a central role in providing energy for maintenance and gametogenic development of bivalves (Bayne et al., 1982; Gabbott and Whittle, 1986; Ruiz et al., 1992; Mathieu and Lubet, 1993). A seasonal cycle of storage and mobilization of energetic reserves, especially glycogen but also stored lipids, was previously correlated with the annual reproductive cycle of

bivalves (Berthelin et al., 2000b). Indeed, glycogen content was positively associated with fecundity in oysters and negatively associated with gametogenic rate (Deslous-Paoli et al., 1981). Glycogen content is also positively linked with survival: survival of oysters during summer (Perdue et al., 1981; Berthelin et al., 2000a) or survival of crustacea following anoxic stress (Hervant and Mathieu, 1995; Oliveira and da Silva, 2000).

Glycogen is a polysaccharide with $\alpha(1-4)$ glucosidic bonds plus secondary $\alpha(1-6)$ glucosidic branches spaced every 7–20 residues along the principal chain. In vertebrates, glycogen is mainly found in the liver and in skeletal muscle where it constitutes stores of readily available glucose to supply tissues. Its major role to sustain and regulate available glucose is well-known and changes in glycogen metabolism are associated with important human diseases such as hypoglycemia and myopathy (Blass et al., 1988).

* Corresponding author. Tel.: +33 2 98 22 46 93; fax: +33 2 98 22 46 53.
E-mail address: ahuvet@ifremer.fr (A. Huvet).

The glycogen pathway is directly under the control of two unidirectional enzymes: glycogen phosphorylase (glycogenolysis) and glycogen synthase (glycogenesis). Glycogen phosphorylase is a homodimeric or tetrameric enzyme that removes glucose residues from $\alpha(1-4)$ linkages within glycogen molecules to produce glucose-1-phosphate (Childress and Sacktor, 1970; Morishima and Sakurai, 1985; San Juan et al., 1991; for review see King, 1996). Covalent modifications by phosphorylation (by a phosphorylase kinase) lead to regulation of its activity. Phosphorylation of phosphorylase-b to phosphorylase-a greatly enhances its activity towards glycogen breakdown as observed in immediate adaptive response of mussels to thermal stress (San Juan et al., 1993). The enzyme is activated by AMP and is inhibited by a variety of allosteric effectors that include glucose, ATP (Kasvinsky et al., 1978; San Juan et al., 1998). Glycogen synthase is a tetrameric enzyme that catalyses the transfer of the glucose molecule from UDP-glucose to a terminal branch of the glycogen molecule (Cohen, 1986). The activity of glycogen synthase is inversely regulated by phosphorylation of serine residues. The unphosphorylated and most active form is synthase-a while the phosphorylated glucose-6-phosphate-dependent form is synthase-b. Both synthase forms were reported in molluscs as I and D respectively, in *Mytilus edulis* (Gabbott and Whittle, 1986) and in *Crassostrea virginica* (Swift et al., 1988). This enzymatic regulation controls part of glycogen metabolism, especially short-term regulation. In addition, long-term regulation of carbohydrate metabolism can be influenced by the expression of genes encoding enzymes implicated in the glucose pathway (Nordlie et al., 1999), such as genes encoding glycogen synthase and glycogen phosphorylase (Towle, 1995; Vali et al., 2000). In bivalve molluscs and especially in the oyster, the long term regulation of glycogen metabolism has never been studied yet. Long-term regulation is under the control of exogenous or endogenous factors especially insulin and epinephrine (Reynet et al., 1996). In *Mytilus edulis*, distinct neuro-endocrine factors were reported to stimulate the start of both gametogenesis and glycogen breakdown and feedback from gonad maturation to storage metabolism was suspected (Mathieu et al., 1991). In adult oysters, seasonal variation in glycogen storage correlated with the annual reproductive cycle were not totally explained by changes in the concentration of extracellular glucose (Mathieu and Lubet, 1993; Berthelin et al., 2000a) suggesting the role of some other factors (neuroendocrine, external factors).

To contribute to our knowledge of glucose storage processes in oysters and to develop specific molecular markers of glycogen metabolism, we report in the present paper the cloning and characterization of glycogen phosphorylase and glycogen synthase cDNAs and the assay by real time PCR analysis of their expression in different oyster tissues and seasons. The use of these genes in understanding relationships between glycogen metabolism and reproduction of *C. gigas* is then discussed.

2. Material and methods

2.1. Biological material

In order to take into account the influence of environmental effects and age of oysters, experimental cross was performed at the hatchery at the IFREMER Laboratory in La Tremblade (France) from 30 wild oysters collected in the Marennes-Oléron Bay (Dégremont, 2003). These oysters were then cultured at the IFREMER station in Bouin (France). One-year-old cupped oysters (mean total weight = 17 ± 5 g), of same age and same environmental background, were conditioned in experimental raceways, at the IFREMER Laboratory in Argenton (France), from February 2002 to February 2003 in 300-L raceways with 20 μ m-filtered running seawater. Oysters were fed on a mixed diet of three micro-algal species (33% by weight *Chaetoceros calcitrans* and *Skeletonema costatum*, 33% *Isochrysis galbana* (T-ISO), 33% *Tetraselmis chui*) at a daily ration equal to 8% dry weight algae/dry weight oyster. Temperature and photoperiod were regulated to follow the mean natural cycles recorded in Marennes-Oléron Bay during the last ten years (Soletchnik et al., 1998).

For cloning cDNAs, oysters were randomly collected from raceways in March and November 2002 and immediately dissected. Collected tissues (labial palps, gonad) were lysed in RNA extraction buffer (Chomczynski and Sacchi, 1987).

For gene expression analysis, 15 oysters were randomly collected at eight different times (March 2002, May, June, July, August, October, December and January 2003). Six tissue samples (labial palps, mantle, gonad, digestive gland, muscle and gills) were immediately dissected from each oyster. Collected tissues were lysed in RNA extraction buffer (Chomczynski and Sacchi, 1987).

For biochemical analysis, three pools of five animals were sampled at the same time as samples were taken for gene expression analysis and were frozen in liquid nitrogen and then wholly ground with a Danguomeau homogeniser at -180 °C.

2.2. Total RNA extraction and cDNA synthesis

Total RNA was isolated using the procedure of Chomczynski and Sacchi (1987), then treated with DNase I (Sigma) (1 U/ μ g RNA) and precipitated with absolute ethanol after sodium acetate (3 M, pH 5.2) treatment. RNA concentrations were measured at 260 nm using the conversion factor 1 OD = 40 μ g/mL RNA.

Samples of polyadenylated RNA were reverse-transcribed from 1 μ g of total RNA denatured for 10 min at 70 °C. Reactions were carried out in a total volume of 25 μ L and the concentrations of the reaction components were as follows: 1X M-MLV Reverse Transcriptase buffer, 0.2 mM dNTPs, 4 mM DTT, 0.5 μ g oligo(dT) primer, 25 U ribonuclease inhibitor and 50 U M-MLV Reverse Tran-

scriptase. Reverse transcription was carried out for 10 min at 25 °C, 20 min at 42 °C and then 5 min at 94 °C.

2.3. PCR, cloning and sequencing

Degenerated PCR primers, forward primer GPF (5'-GCN GCN TGY TTY YTN GAY WSN ATG GC-3'), reverse primer GPR (5'-AAV AWR TTR TCR TTN GGR TAN ARN AC-3'), and forward primer GSF (5'-GAR TTY CAR AAY YTN CAY GC-3'), reverse primer GSR (5'-GCN GGN GTR TAN CCC CAN GG-3'), were designed based on conserved glycogen phosphorylase and synthase protein sequences, respectively, from human (Genbank Accession No.: P06737, P11217 and P11216 for glycogen phosphorylase; NP_068776 and NP_002094 for glycogen synthase), rat (P09811 and P09812; NP_037221 and XP_341859), *Drosophila melanogaster* (Q9XTL9; Q9VFC8) and *Caenorhabditis elegans* (Q9N5U1; Q9U2D9). Polymerase chain reaction (PCR) was performed in a total volume of 25 µL with 1 µg of cDNAs, 1 µM of each primer, 1X Taq buffer, 0.25 mM dNTPs, 1.5 mM MgCl₂ and 0.5 U Taq polymerase (Qbiogene). Amplification was performed for 35 cycles at 94 °C for 30 s, 47 °C for 1 min, 72 °C for 2 min 30 s and a final extension step at 72 °C for 5 min. PCR products were run on 1% agarose gels with TAE buffer in 1X TAE buffer (Tris, acetic acid, and 0.5 M EDTA pH8) followed by staining with ethidium bromide. PCR products of expected size were gel-extracted (QIAquick gel extraction kit, Qiagen), cloned with a TOPO-TA cloning kit (Invitrogen) and then sequenced (Qbiogene).

5' and 3' ends of the partial glycogen phosphorylase transcript were isolated from cDNA using gene-specific primers with the Smart Race cDNA Amplification Kit (BD Biosciences). Reactions were performed for each 5' (reverse primer: 5'-CAG GTT CCT CAA CCT GCC AAC CAT C-3') and 3' ends (forward primer: 5'-TAC CGT GTC TCG CTG GCT GAG AAG A-3'). For the partial glycogen synthase transcript, a cDNA library constructed in λ-ZAP II from *C. gigas* mantle-edge mRNA was screened as described by Huvet et al. (2004) using specific nested primers for each 5' (reverse primers: 5'-ACT CAT ATC TCC CAG CCG TGA-3'; 5'-TGT CTT ATC CAG ATC AAA GTC-3' and 5'-GGC GTG AGT CGT GAA GAT GG-3') and 3' ends (forward primers: 5'-TGG CCT TGA CTA CGA GGA CTT-3' and 5'-GGG CTG TCA CCT TGG GGT GT-3'). PCR products were electrophoresed as described above and fragments of largest size were gel-extracted, subcloned into pCR 2.1® TOPO plasmid and sequenced as described above.

2.4. Phylogenetic analysis

Phylogenetic analyses were carried out using a range of glycogen phosphorylase and synthase protein sequences from vertebrates and invertebrates. For most of vertebrates, the three characterised gene sequences (liver, muscle and

brain forms) were included in the analysis. The sequences were aligned using CLUSTAL W (Thompson et al., 1994). An unrooted distance base phylogenetic tree was constructed in PHYLIP (Felsenstein, 1993) using the unweighted pair group method with arithmetic mean (UPGMA). One thousand bootstrap trials were run using the Seqboot program from the PHYLIP package.

2.5. Relative quantification of gene expression

The presence of glycogen phosphorylase and glycogen synthase mRNA was investigated using real time PCR analysis. The detection technique for newly synthesised PCR products used SYBR Green I fluorescence dye (Morisson et al., 1998). Specific primers were designed with the assistance of primer 3 software (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) from glycogen synthase mRNA sequence (forward primer GSaF: 5'-GAC GCC AAC GAC CAA ATC-3'; reverse primer GSbR: 5'-TTC AGG AAC TCG GGG TGA-3') and glycogen phosphorylase mRNA sequence (forward primer GPfR: 5'-AGA CGG TCC CCG ATG ATT-3'; reverse primer GPeF: 5'-GGC AGC TCC TGG CTA TCA-3'). Amplifications of actin and elongation factor I cDNA were performed in order to confirm the steady-state level of expression of housekeeping genes allowing an internal control for gene expression. Actin and elongation factor I primers were those used by Huvet et al. (2003) and Fabioux et al. (2004b), respectively.

The real time PCR amplifications were carried out in triplicates in a total volume of 15 µL with 1X SYBR® Green Master Mix (Qiagen), 10 nM of fluorescein, 0.33 µM each of forward and reverse primers and 5 µl of the 1:5 diluted cDNA using iCycler iQ thermocycler (Biorad). After Taq Polymerase activation at 95 °C for 15 min, amplification was performed for 45 cycles at 95 °C for 30 s, at 60 °C for 1 min with a single fluorescence measurement, and a final melting curve program by decreasing 0.5 °C each 10 s from 95 °C to 55 °C. Each run included a positive cDNA control (one sample of the present experiment analyzed in each amplification plate), negative controls (each total RNA sample with DNase I treatment) and blank controls (water) analyzed for each primer pair.

For gene expression calculation, the threshold value (Ct) was determined for each target as the number of cycles at which the fluorescence rises appreciably above the background fluorescence. PCR efficiency (*E*) was determined for each primer pair by determining the slope of standard curves obtained from serial dilution analysis of cDNA. The individual real time PCR efficiencies (*E*) for target or reference gene were calculated according to: $E=10^{(-1/\text{slope})}$. When efficiencies are equal, the ratio becomes: $R=2^{-(Cp_{\text{target}}-Cp_{\text{reference}})}$. The relative expression ratio (*R*) of a target gene (glycogen synthase, glycogen phosphorylase) was calculated based on the Ct deviation of this target gene versus the reference gene, corresponding to

the copy number of the target gene relative to the copy number of the reference gene.

2.6. Glycogen content analysis

Glycogen was determined enzymatically (Keppler and Decker, 1974). Samples (0.25 g wet weight) were first homogenised in sodium citrate 0.1 M, pH 5.0. Glycogen

was hydrolysed 2 h at 33 °C with 3 U amyloglucosidase (EC 3.2.1.3). Cellular fragments were removed by centrifugation for 30 min at 4 °C. Glucose RTU® procedure was used to determinate glucose concentration. Glycogen concentration (mg glycogen/g wet weight) corresponds to the difference between the glucose concentration of the amyloglucosidase treated and non-treated samples, both being analyzed in duplicate.

Glycogen phosphorylase

																				M	A	T	Y	K		5
																					ATG	GCC	ACT	TAT	AAA	65
P	T	T	D	H	E	K	R	K	Q	I	<u>S</u>	<u>I</u>	<u>R</u>	G	I	A	P									23
CCC	ACA	ACT	GAC	CAC	GAG	AAG	CGA	AAG	CAA	ATC	AGT	ATT	CGT	GGT	ATT	GCT	CCT									119
V	E	N	V	V	E	F	K	K	A	F	N	R	H	L	H	Y	T									41
GTG	GAA	AAT	GTT	GTC	GAA	TTC	AAA	AAG	GCT	TTT	AAT	CGC	CAT	TTG	CAT	TAC	ACG									173
I	V	K	D	R	N	V	A	<u>T</u>	<u>P</u>	<u>R</u>	D	Y	Y	L	S	L	A									59
ATT	GTG	AAA	GAT	AGA	AAT	GTG	GCG	ACA	CCG	CGA	GAC	TAC	TAC	CTC	TCT	CTT	GCC									227
R	<u>T</u>	<u>V</u>	<u>R</u>	D	Y	L	V	G	R	W	I	R	T	Q	Q	H	Y									77
CGC	ACC	GTG	CGA	GAC	TAT	TTG	GTG	GGG	CGT	TGG	ATC	CGT	ACC	CAG	CAG	CAT	TAC									281
Y	E	K	D	P	K	R	V	Y	Y	L	S	L	E	F	Y	M	G									95
TAT	GAG	AAG	GAC	CCA	AAG	AGA	GTG	TAC	TAT	CTG	TCC	CTG	GAG	TTT	TAC	ATG	GCC									335
R	T	L	S	N	T	M	V	N	L	G	I	Q	S	A	C	D	E									113
AGG	ACC	CTG	TCC	AAC	ACC	ATG	GTG	AAC	CTG	GGC	ATT	CAG	AGC	GCC	TGC	GAT	GAG									389
A	L	Y	Q	I	G	L	D	I	E	E	L	E	E	I	E	E	D									131
GCC	CTC	TAT	CAG	ATT	GGC	CTT	GAC	ATT	GAG	GAG	TTG	GAG	GAG	ATT	GAG	GAG	GAT									443
A	G	L	G	N	G	G	L	G	R	L	A	A	C	F	L	D	S									149
GCT	GGT	CTG	GGT	AAT	GGA	GGA	CTG	GGT	CGG	CTG	GCA	GCC	TGC	TTC	CTT	GAC	TCC									497
M	A	T	L	G	L	A	A	Y	G	Y	G	I	R	Y	D	Y	G									167
ATG	GCA	ACG	CTC	GGA	CTG	GCA	GCA	TAT	GGA	TAT	GGT	ATC	AGA	TAC	GAC	TAT	GGA									551
I	F	A	Q	K	I	E	D	G	W	Q	V	E	E	P	D	E	W									185
ATC	TTT	GCC	CAG	AAA	ATT	GAA	GAT	GGT	TGG	CAG	GTT	GAG	GAA	CCT	GAT	GAG	TGG									605
L	R	Y	G	N	P	W	E	K	S	R	P	E	Y	V	L	P	V									203
CTG	AGA	TAT	GGA	AAT	CCA	TGG	GAG	AAG	TCG	AGA	CCA	GAG	TAC	GTA	CTA	CCG	GTC									659
N	F	Y	G	R	T	E	D	T	G	S	G	V	K	W	V	D	T									221
AAC	TTC	TAT	GGA	CGG	ACA	GAG	GAC	ACT	GGC	TCG	GGG	GTC	AAG	TGG	GTG	GAC	ACT									713
Q	V	V	F	A	M	P	F	D	S	P	I	P	G	Y	G	N	N									239
CAG	GTT	GTG	TTT	GCG	ATG	CCC	TTT	GAC	AGT	CCT	ATA	CCA	GGG	TAC	GGC	AAC	AAC									767
<u>T</u>	<u>V</u>	N	<u>T</u>	<u>M</u>	<u>R</u>	L	W	<u>S</u>	<u>A</u>	<u>K</u>	A	P	N	S	F	N	L									257
ACT	GTC	AAC	ACC	ATG	CGT	CTG	TGG	TCA	GCT	AAA	GCT	CCC	AAC	AGC	TTC	AAT	CTG									821
H	F	F	N	N	G	E	Y	I	N	A	V	C	D	R	N	Q	A									275
CAC	TTC	TTC	AAC	AAT	GGC	GAG	TAT	ATC	AAT	GCT	GTC	TGT	GAC	AGA	AAC	CAG	GCG									875
E	N	I	S	R	V	L	Y	P	N	D	N	F	F	<u>S</u>	<u>G</u>	<u>K</u>	E									293
GAA	AAC	ATC	TCC	AGA	GTC	CTC	TAC	CCC	AAT	GAT	AAC	TTC	TTT	TCT	GGC	AAA	GAG									929
L	R	L	K	Q	E	Y	F	L	V	A	A	T	L	Q	D	I	L									311
CTC	CGC	CTA	AAG	CAG	GAG	TAC	TTC	CTG	GTA	GCA	GCC	ACT	CTC	CAG	GAC	ATC	CTC									983
R	R	F	K	<u>S</u>	<u>S</u>	<u>K</u>	F	G	S	R	D	P	V	R	R	S	F									329
AGG	AGA	TTC	AAG	TCA	TCC	AAG	TTT	GGT	AGT	CGG	GAT	CCG	GTC	CGC	AGG	TCC	TTC									1037
E	S	F	P	D	K	V	A	I	Q	L	N	D	T	H	P	S	M									347
GAG	TCG	TTT	CCA	GAC	AAG	GTG	GCC	ATT	CAG	CTG	AAT	GAT	ACC	CAC	CCG	TCC	ATG									1091
A	I	P	E	L	L	R	I	F	V	D	V	E	G	L	P	W	D									365
GCT	ATC	CCG	GAG	TTA	CTG	AGG	ATC	TTT	GTG	GAC	GTT	GAA	GGA	CTC	CCA	TGG	GAC									1145

Fig. 1. Nucleotide sequence of the cDNA and deduced amino acid sequence of glycogen phosphorylase (above) and synthase (below) enzymes. Grey highlighted sequences are a putative *N*-glycosylation site (consensus: N-{P}-[ST]-{P}). A putative cAMP- and cGMP-dependent protein kinase phosphorylation site ([RK](2)-X-[ST]) is underlined and putative protein kinase phosphorylation sites ([ST]-X-[RK]) are framed. For glycogen phosphorylase, the phosphorylase pyridoxal-phosphate attachment site is bold framed with a dotted line (consensus: E-A-[SC]-G-x-[GS]-x-M-K-x(2)-[LM]-N). For glycogen synthase, the amidation site is bold framed with a dotted line (consensus: x-G-[RK]-[RK]).

K	A	W	G	I	T	V	K	T	F	A	Y	T	N	H	T	V	L	383
AAG	GCC	TGG	GGA	ATC	ACG	GTG	AAG	ACG	TTC	GCC	TAC	ACG	AAC	CAC	ACA	GTC	CTC	1199
P	E	A	L	E	R	W	P	V	S	M	L	E	K	I	L	P	R	401
CCC	GAG	GCC	TTG	GAG	AGG	TGG	CCG	GTG	TCC	ATG	CTG	GAG	AAG	ATC	CTT	CCC	CGC	1253
H	L	Q	I	I	Y	L	I	N	H	N	F	L	Q	E	V	A	K	419
CAC	CTG	CAG	ATT	ATC	TAC	CTG	ATT	AAC	CAC	AAC	TTC	CTC	CAG	GAA	GTA	GCA	AAA	1307
K	Y	P	G	D	A	G	R	M	R	R	M	S	I	V	E	E	D	437
AAA	TAT	CCA	GGT	GAT	GCA	GGC	AGA	ATG	CGA	CGA	ATG	TCC	ATT	GTA	GAG	GAG	GAT	1361
G	E	K	R	I	N	M	A	Y	L	S	I	V	G	S	H	A	V	455
GGA	GAG	AAG	AGG	ATC	AAT	ATG	GCG	TAT	CTC	AGC	ATC	GTC	GGC	TCG	CAC	GCT	GTC	1415
N	G	V	A	A	L	H	S	E	I	I	K	S	E	T	F	R	E	473
AAT	GGA	GTG	GCA	GCG	TTA	CAC	TCA	GAA	ATC	ATC	AAG	AGC	GAA	ACG	TTC	CGT	GAG	1469
F	Y	E	M	Y	P	E	R	F	Q	N	K	T	N	G	I	T	P	491
TTT	TAC	GAG	ATG	TAT	CCA	GAG	CGC	TTC	CAA	AAC	AAA	ACA	AAC	GGG	ATC	ACA	CCA	1523
R	W	L	L	L	C	N	P	G	L	S	D	I	I	A	E	K		509
CGT	CGT	TGG	TTG	TTA	CTG	TGC	AAT	CCT	GGA	CTG	TCT	GAC	ATT	ATC	GCA	GAG	AAA	1577
I	G	E	E	W	V	T	D	L	Y	Q	L	Q	N	L	K	K	F	527
ATC	GGG	GAG	GAA	TGG	GTC	ACA	GAC	TTG	TAC	CAA	CTA	CAG	AAT	CTC	AAA	AAG	TTT	1631
A	D	D	E	N	F	L	R	N	I	I	K	V	K	Q	E	N	K	545
GCT	GAT	GAT	GAA	AAC	TTC	CTG	AGG	AAC	ATC	ATC	AAA	GTC	AAA	CAG	GAA	AAC	AAA	1685
M	K	L	A	E	Y	I	Q	E	N	Y	N	I	K	V	N	T	S	563
ATG	AAG	TTG	GCT	GAG	TAC	ATC	CAG	GAG	AAC	TAC	AAC	ATC	AAG	GTC	AAC	ACG	TCC	1739
S	I	F	D	I	H	V	K	R	I	H	E	Y	K	R	Q	L	L	581
TCC	ATC	TTT	GAC	ATC	CAT	GTC	AAG	CGG	ATC	CAC	GAG	TAC	AAG	AGA	CAG	CTG	CTC	1793
N	C	F	H	I	I	T	L	Y	N	R	L	K	R	D	P	N	Q	599
AAT	TGC	TTC	CAC	ATC	ATC	ACG	CTC	TAC	AAC	CGC	CTC	AAG	CGT	GAC	CCA	AAC	CAG	1847
A	F	V	P	R	T	I	M	V	G	G	K	A	A	P	G	Y	H	617
GCC	TTT	GTT	CCC	AGG	ACT	ATC	ATG	GTC	GGA	GGA	AAG	GCA	GCT	CCT	GGC	TAT	CAC	1901
M	A	K	L	I	I	K	L	I	N	S	V	A	K	V	I	N	N	635
ATG	GCC	AAA	CTG	ATC	ATC	AAG	CTG	ATC	AAC	AGT	GTA	GCC	AAG	GTC	ATC	AAC	AAC	1955
D	P	I	I	G	D	R	L	K	V	V	Y	L	E	N	Y	R	V	653
GAT	CCA	ATC	ATC	GGG	GAC	CGT	CTC	AAG	GTG	GTG	TAT	CTG	GAG	AAC	TAC	CGT	GTC	2009
S	L	A	E	K	I	I	P	A	A	D	L	S	E	Q	I	S	T	671
TCG	CTG	GCT	GAG	AAG	ATC	ATT	CCG	GCA	GCT	GAC	CTC	AGC	GAA	CAG	ATC	TCG	ACA	2063
A	G	T	E	A	S	G	T	G	N	M	K	F	M	L	N	G	A	689
GCA	GGA	ACC	GAG	GCT	TCT	GGA	ACA	GGA	AAC	ATG	AAG	TTC	ATG	TTG	AAC	GGG	GCA	2117
L	T	I	G	T	L	D	G	A	N	V	E	M	R	E	E	M	G	707
CTG	ACT	ATC	GGA	ACT	CTG	GAC	GGT	GCT	AAT	GTA	GAG	ATG	AGA	GAG	GAG	ATG	GGG	2171
D	E	N	I	F	I	F	G	M	K	V	D	E	V	E	E	L	K	725
GAT	GAA	AAT	ATC	TTT	ATC	TTT	GGA	ATG	AAG	GTT	GAT	GAA	GTA	GAG	GAA	CTG	AAG	2225
R	S	G	Y	H	P	Q	E	Y	Y	E	R	N	T	D	L	K	Q	743
CGC	AGT	GGG	TAC	CAC	CCA	CAA	GAA	TAC	TAC	GAG	CGT	AAC	ACG	GAC	TTA	AAG	CAG	2279
V	L	D	Q	I	S	R	G	F	F	S	P	E	E	P	G	M	F	761
GTG	CTT	GAC	CAG	ATC	TCA	AGG	GGC	TTC	TTC	TCT	CCC	GAG	GAG	CCC	GGC	ATG	TTC	2333
T	D	I	Y	N	S	V	M	Y	N	D	R	F	X	P	S	K	D	779
ACT	GAT	ATA	TAC	AAC	TCT	GTG	ATG	TAC	AAC	GAC	AGG	TTC	TNG	CCT	TCT	AAA	GAT	2387

Fig. 1 (continued).

2.7. Statistical analysis

Comparisons of levels of transcripts between different tissues or seasons were performed by median comparisons procedure using the Kruskal–Wallis test with STAT-GRAPHICS 5.0 software. Multiple comparisons were made with *t*-distribution test at the 5% level using UNISTAT 5.5 software.

3. Results

3.1. Isolation of the glycogen phosphorylase and glycogen synthase cDNAs from *C. gigas*

For glycogen phosphorylase, a fragment of expected size was amplified from labial palps sampled in March with degenerated primers GPF and GPR. The 5' and 3' ends were

```

Y E D Y I K C Q D S V S E V F K D P 797
TAT GAA GAT TAC ATC AAG TGC CAA GAC AGC GTC AGT GAA GTA TTC AAG GAT CCT 2441

L Q W A K M C V L N I A S S V N F Q 815
CTG CAA TGG GCC AAG ATG TGT GTC CTC AAC ATT GCA TCG TCG GTA AAT TTC CAG 2495

P T E Q F L N M A R D I W G V E P N 833
CCG ACA GAA CAA TTT CTG AAT ATG GCA AGG GAC ATC TGG GGG GTG GAA CCC AAT 2549

D I K L P P P H E G L D S M D S K P 851
GAT ATT AAG CTG CCG CCC CCA CAC GAG GGC CTA GAC TCA ATG GAT AGC AAA CCA 2603

P Q K K * 856
CCA CAG AAG AAA TGA gatctcagctgatagccactagaaacaatataactttttaaactttctgttg
ttgctttgtgtggaatttattaacataggtcttatataaaagtttagcaatattacatgtaactggatgag 2741

gagaattgcagtagagattgacatactgggttttagttgaagtgaaattgttgcaggccattggacatac 2813

tcagctctgtggtagcatcacattgtaagcatcagcttgaattaatatttggctggtctgtagagcatttg 2885

acttgcctgtgctaaagcatttttagtgcctttattgatgtgctgttatgtgtgtaaatcaactatggtat 2957

taaacagtttcattcaaaatctatataaaaatttaagaaaatttgaaaaaaaaaaaaaaaaa 3018

```

Glycogen synthase

```

M R R R N S F Y R 9
tcggcagcaggctgagacagtgaaaattatggct ATG AGA AGA CGA AAC AGT TTT TAC AGA 61

S F K D A C P E F E E M L M D R G A 27
AGT TTT AAA GAT GCA TGT CCG GAA TTT GAA GAG ATG CTT ATG GAC AGA GGG GCC 115

T A A A Q N K W V F E I A W E V A N 45
ACT GCA GCT GCA CAA AAC AAA TGG GTG TTT GAA ATC GCC TGG GAA GTA GCA AAT 169

K V G G I Y T V I K S K A P V S V A 63
AAA GTT GGT GGT ATC TAC ACT GTC ATC AAG TCC AAG GCC CCG GTC AGT GTA GCT 223

E L G E Q Y C L L G P Y N E A C V R 81
GAG TTA GGA GAA CAG TAC TGT CTG CTG GGT CCC TAT AAC GAG GCA TGT GTC AGA 277

T E V E I L E P S H Y V Y R Q T L Q 99
ACG GAG GTG GAG ATC CTG GAG CCC TCC CAC TAT GTC TAC AGA CAG ACG CTA CAG 331

T M R D A G I K V H F G R W L I D G 117
ACC ATG AGG GAC GCA GGT ATC AAG GTT CAT TTT GGT CGC TGG CTG ATA GAC GGA 385

Y P K V I L F D I G S A A W K L D E 135
TAT CCT AAA GTC ATT TTG TTT GAC ATC GGA TCT GCT GCT TGG AAG CTG GAT GAA 439

F K H E L W E K A S I G I P W H D R 153
TTC AAA CAT GAG CTG TGG GAG AAA GCC AGC ATA GGA ATC CCC TGG CAC GAC CGC 493

E S N D A V I F G A L V A W F I G E 171
GAG TCG AAC GAC GCC GTC ATA TTC GGG GCT CTA GTA GCC TGG TTT ATT GGT GAG 547

F R K N L T D Q P I V V T H F H E W 189
TTT CGG AAG AAT CTG ACT GAT CAG CCG ATT GTA GTG ACC CAC TTT CAC GAG TGG 601

L A G A G L M D L R T R K V D C I T 207
TTG GCT GGG GCG GGG CTG ATG GAC CTG CCG ACC AGG AAG GTC GAC TGC ATC ACC 655

```

Fig. 1 (continued).

obtained by RACE-PCR. The total isolated glycogen phosphorylase sequence of 3018 bp (Genbank accession number AY496065) comprised a 5' untranslated region of 50 bp, an open reading frame of 2569 bp, a stop codon (TGA), and a 3' untranslated region of 399 bp. The deduced amino acid sequence is 855 aa long (Fig. 1). It contains the phosphorylase pyridoxal-phosphate attachment site (consensus: E-A-[SC]-G-x-[GS]-x-M-K-x(2)-[LM]-N), six N-glycosylation sites and 12 putative protein kinase phosphorylation sites or which one is cAMP- and cGMP-dependent, determined by homology. The amino acid sequence was 68% similar to that of vertebrates (*Gallus gallus*, *Ovis aries*, *Oryctolagus cuniculus*, *Rattus norvegicus*, *Mus musculus*, *Homo sapi-*

ens) whatever the tissue source (liver, muscle and brain form) and 68% and 66% similar to those of *Drosophila melanogaster* and *Caenorhabditis elegans*, respectively. Analysis of the phylogenetic relationships between glycogen phosphorylase enzymes showed several distinct clusters corresponding to Protozoa (*Giardia intestinalis*, *Dictyostelium discoideum*), Protostomia with Ecdysozoa (*C. elegans*, *D. melanogaster*) and Lophotrochozoa (*C. gigas*) and to Deuterostomia based on three categories corresponding to liver, muscle and brain forms isolated in species (Fig. 2).

For glycogen synthase, a fragment of expected size was amplified from labial palps sampled in November using degenerated primers GSF and GSR. The 5' and 3' ends were

I	F	T	T	H	A	T	L	L	G	R	Y	L	C	A	G	S	S	225
ATC	TTC	ACG	ACT	CAC	GCC	ACA	CTG	CTG	GGG	AGA	TAC	CTG	TGT	GCT	GGC	AGC	TCA	709
D	F	Y	N	N	I	D	K	Y	N	L	V	K	E	A	G	D	R	243
GAC	TTC	TAC	AAC	AAT	ATA	GAC	AAG	TAT	AAC	TTA	GTC	AAG	GAG	GCC	GGT	GAC	CGT	763
Q	I	Y	H	C	Y	C	M	E	R	T	A	V	H	S	S	Q	V	261
CAG	ATA	TAC	CAC	TGT	TAC	TGC	ATG	GAG	AGG	ACC	GCT	GTC	CAC	TCT	TCT	CAG	GTG	817
F	I	S	V	S	E	I	T	E	V	E	A	E	H	L	L	K	R	279
TTT	ATC	AGT	GTG	TCC	GAG	ATC	ACT	GAG	GTG	GAG	GCA	GAG	CAC	CTG	CTC	AAA	CGG	871
K	P	N	M	I	V	P	P	N	G	L	N	V	V	K	F	S	T	297
AAG	CCC	AAC	ATG	ATC	GTC	CCA	CCT	AAC	GGA	TTA	AAT	GTG	GTC	AAA	TTT	AGT	ACC	925
I	H	E	F	Q	N	M	H	A	I	C	K	E	K	I	H	D	I	315
ATC	CAT	GAG	TTC	CAG	AAT	ATG	CAT	GCC	ATC	TGC	AAG	GAG	AAG	ATC	CAC	GAC	ATC	979
V	R	G	H	F	Y	G	H	Y	D	F	D	L	D	K	T	L	Y	333
GTC	AGG	GGA	CAT	TTT	TAT	GGG	CAC	TAT	GAC	TTT	GAT	CTG	GAT	AAG	ACA	CTA	TAC	1033
F	F	T	A	G	R	Y	E	F	S	N	K	G	A	D	M	F	I	351
TTT	TTC	ACG	GCT	GGG	AGA	TAT	GAG	TTT	TCC	AAC	AAA	GGA	GCG	GAC	ATG	TTC	ATA	1087
E	S	L	A	R	L	N	F	Y	L	K	Q	A	N	S	E	A	T	369
GAA	TCA	CTG	GCA	AGA	CTA	AAC	TTT	TAT	CTC	AAG	CAA	GCT	AAC	AGT	GAG	GCG	ACA	1141
V	V	A	F	L	I	F	P	T	K	T	N	N	F	N	V	E	S	387
GTG	GTG	GCT	TTC	CTG	ATC	TTC	CCC	ACC	AAG	ACC	AAC	AAC	TTC	AAC	GTG	GAG	TCC	1195
L	R	G	Q	A	I	S	K	Q	L	K	E	T	V	H	H	V	Q	405
CTG	CGG	GGC	CAG	GCC	ATC	TCC	AAA	CAG	CTG	AAG	GAG	ACC	GTG	CAC	CAC	GTA	CAG	1249
T	Q	I	G	K	R	I	F	E	Q	S	L	K	G	K	I	L	T	423
ACA	CAG	ATC	GGC	AAG	AGG	ATC	TTT	GAA	CAG	AGC	CTG	AAG	GGC	AAA	ATT	CTA	ACT	1303
G	D	E	I	L	E	Q	E	D	I	V	K	L	K	R	C	I	Y	441
GGA	GAT	GAA	ATT	CTG	GAA	CAA	GAA	GAT	ATA	GTG	AAA	CTT	AAG	AGG	TGT	ATC	TAC	1357
S	A	Q	R	N	S	L	P	P	I	C	T	H	N	V	N	E	D	459
TCT	GCT	CAG	AGA	AAC	AGC	TTA	CCT	CCT	ATA	TGT	ACG	CAC	AAT	GTT	AAT	GAA	GAC	1411
A	N	D	Q	I	L	N	A	L	R	R	C	Q	L	F	N	R	K	477
GCG	AAC	GAC	CAA	ATC	CTT	AAT	GCC	CTT	CGA	CGA	TGT	CAA	CTT	TTC	AAC	AGA	AAA	1465
E	D	R	V	K	V	V	F	H	P	E	F	L	N	S	T	N	P	495
GAG	GAC	AGA	GTG	AAG	GTG	GTA	TTT	CAC	CCC	GAG	TTC	CTG	AAC	TCC	ACC	AAC	CCT	1519
L	F	G	L	D	Y	E	D	F	V	R	G	C	H	L	G	V	F	513
TTG	TTT	GGC	CTT	GAC	TAC	GAG	GAC	TTT	GTC	CGG	GGC	TGT	CAC	CTT	GGG	GTG	TTC	1573
A	S	Y	Y	E	P	W	G	Y	S	P	A	E	C	T	V	Y	G	531
GCT	TCG	TAC	TAC	GAG	CCC	TGG	GGC	TAT	TCA	CCA	GCT	GAG	TGC	ACC	GTG	TAC	GGG	1627
I	P	S	I	S	T	N	L	S	G	F	G	C	F	M	Q	E	H	549
ATC	CCA	AGT	ATT	TCC	ACG	AAC	CTC	TCA	GGC	TTC	GGC	TGT	TTC	ATG	CAG	GAA	CAC	1681
I	N	D	P	K	S	Y	G	L	Y	I	V	D	R	R	Y	K	S	567
ATC	AAT	GAT	CCC	AAG	TCC	TAT	GGA	CTC	TAT	ATT	GTA	GAC	CGC	CGA	TAT	AAG	AGT	1735
P	D	E	S	I	H	Q	L	T	Q	Y	M	Y	D	F	T	C	L	585
CCA	GAC	GAA	TCA	ATC	CAT	CAG	CTG	ACT	CAG	TAT	ATG	TAT	GAT	TTC	ACC	TGT	TTA	1789
S	R	R	Q	R	I	I	Q	R	N	R	T	E	R	L	S	D	L	603
TCC	CGG	CGA	CAG	CGT	ATC	ATT	CAG	AGA	AAT	CGT	ACG	GAG	CGC	CTC	AGT	GAC	CTC	1843
L	D	W	R	N	L	G	V	Y	Y	R	K	A	R	Q	I	A	V	621
CTA	GAC	TGG	AGG	AAC	CTG	GGA	GTG	TAC	TAC	AGA	AAA	GCC	CGC	CAG	ATT	GCT	GTA	1897

Fig. 1 (continued).

obtained by PCR using a *C. gigas* mantle-edge cDNA library as the template. The total isolated glycogen synthase sequence was 2408 bp long corresponding to a deduced sequence of 695 aa (Genbank accession number AY496064). It comprised a 5' untranslated region of 34 bp, an open reading frame of 2085 bp, a stop codon (TGA), and a 3' untranslated region of 286 bp (Fig. 1). It contains four N-glycosylation sites, nine putative protein kinase phosphorylation sites or which one is cAMP- and cGMP-dependent and one amidation site, determined by homology. The

amino acid sequence was 53% similar to that of vertebrates (*Gallus gallus*, *Oryctolagus cuniculus*, *Rattus norvegicus*, *Mus musculus*, *Homo sapiens*) and 61% and 50% similar to *D. melanogaster* and *C. elegans*, respectively. Analysis of the phylogenetic relationships between glycogen synthase enzymes showed three distinct clusters corresponding to Protozoa (*Giardia lamblia*) and Protostomia (*C. elegans*, *D. melanogaster* and *C. gigas*), and to Deuterostomia in which one cluster corresponded to liver form and the other one to muscle and brain forms (Fig. 2).

A R G Y P D L A A K E E E I L Q E K	639
GCC CGG GGA TAC CCT GAC CTT GCT GCC AAG GAG GAG GAG ATC CTA CAG GAA AAG	1951
R F M Y P R P A S E P S S P S A S R	657
AGG TTC ATG TAC CCT CGG CCG GCC TCC GAA CCT TCC TCA CCC TCG GCC TCG CGC	2005
S S T P A P S E H G D D E D D D I D	675
AGC TCC ACC CCA GCC CCC TCG GAA CAT GGG GAC GAT GAA GAT GAC GAT ATC GAC	2059
E D E E N A E M S S N P E S D M P M	693
GAG GAT GAA GAG AAT GCG GAA ATG AGT TCT AAC CCA GAG TCC GAC ATG CCG ATG	2113
F K *	696
TTT AAG TGA tgaatcagaaggacatccccggtttattgtttacatgttaaaggatttctatcagtttga	2181
ggtaattacctgtgtattgagtagtgaggtgttttgctttataccacagaaaagctttatataactg	2253
agtgttggcgagtgcaactcaaatgagttgacctcatttttaacattttattttgctttctctttttttct	2325
ctcttctagtttagcaatcaaaatgtttcacaacatagtagtgcgttgtaggtaaaaatttaacaaaaaaaa	2397
aaaaaaaaaaaa	2408

Fig. 1 (continued).

3.2. Tissue specific expression of glycogen synthase and phosphorylase genes

Over an annual period, no significant differences in Ct values (related to 1 µg total RNA) were observed for the two house keeping genes (actin, elongation factor I) among sampling dates (Kruskal Wallis test: $P=0.86$ and 0.2 for elongation factor I and actin, respectively) but results of elongation factor I appeared more stable over the period (coefficient of variation=6% against 9.4% for actin). Therefore, the expressions of the *C. gigas* glycogen synthase and phosphorylase genes were expressed relative to the expression of the *C. gigas* elongation factor I gene, and analysed in various tissues by real time PCR in October and in May. Both genes were expressed, relative to elongation factor I, at different levels depending on the tissues (Fig. 3). For glycogen synthase, two distinct groups were statistically evident: during October, transcripts were mainly found in the gonad ($R_{GS}=0.20\pm0.13$) and labial palps ($R_{GS}=0.11\pm0.04$) whereas they ranged from 4.5 to 12 times less in muscle, mantle, gills and digestive gland. For glycogen phosphorylase, level of transcripts, relative to elongation factor I transcripts, was significantly higher during May at the 1% level in palps ($R_{GP}=3.13\pm0.70$) and muscle ($R_{GP}=2.42\pm0.81$) than those observed in other tissues.

3.3. Seasonal expression of glycogen synthase and phosphorylase genes

During the experimental conditioning of *C. gigas* from March 2002 to January 2003, glycogen content showed a strong decrease from March (mean value=23.41±3.32 mg glycogen/g wet weight; Fig. 4) to July where the minimal value was observed (mean value=6.10±0.73 mg glycogen/g wet weight). Thereafter, glycogen content increased until a maximal value in January (mean value=34.65±5.89 mg glycogen/g wet weight).

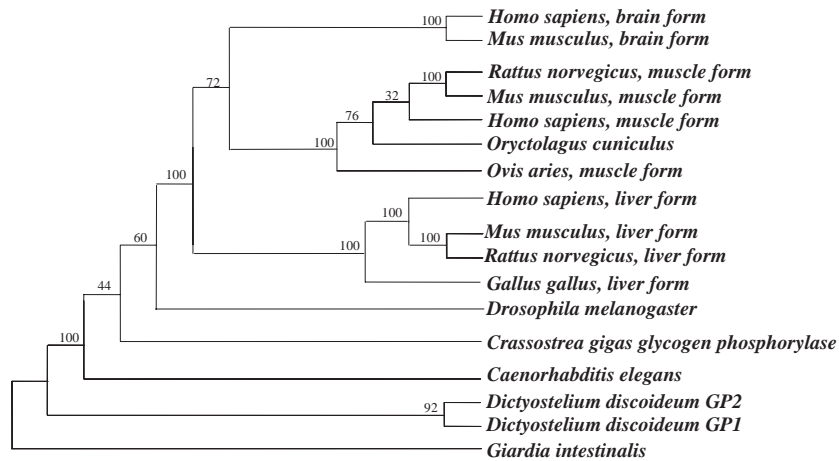
Levels of both mRNAs were assayed by real time PCR in oyster labial palps and the gonad during experimental conditioning. Both mRNA levels showed significant variation in the labial palps and gonad, depending on the month of sampling (Table 1). For both tissues, the level of glycogen synthase was highest in October (R_{GS} palps=0.11±0.05, R_{GS} gonad=0.20±0.13) and not significantly different from values observed during March and May (only during May for labial palps). Its lowest level, close to zero, was observed in July (R_{GS} palps=0.01±0.002, R_{GS} gonad=0.002±0.001; Fig. 4), grouping statistically with values observed in the labial palps during January and in the gonad during August and January (Table 1).

The relative level of the glycogen phosphorylase transcript observed in labial palps (Fig. 4) was high from March to June with a value significantly higher in May compared to all other analysed months (R_{GP} palps=3.13±0.70). In other months, the relative level of glycogen phosphorylase transcript was low with a minimal value observed in July (R_{GP} palps=0.28±0.16) that was not significantly different from values observed in August, October and January (Table 1). In the gonad, glycogen phosphorylase expression was maximum in March (R_{GP} gonad=1.07±0.35, $P<0.001$) but low during the annual cycle. As observed in the labial palps, the minimum value for glycogen phosphorylase mRNA level was observed in July (R_{GP} gonad=0.05±0.04), grouping statistically with values observed during August and January.

Comparing both tissues, mRNA level of glycogen synthase and phosphorylase genes was significantly higher in the labial palps than in the gonad in May and January ($P<0.05$). In June, a significantly higher level of glycogen phosphorylase transcripts ($P<0.05$) was observed in the labial palps compared to the gonad. During other months, no significant differences in transcript levels between the labial palps and the gonad were detected.

Comparing both genes, level of glycogen phosphorylase transcripts appeared significantly higher than level of

Glycogen phosphorylase



Glycogen synthase

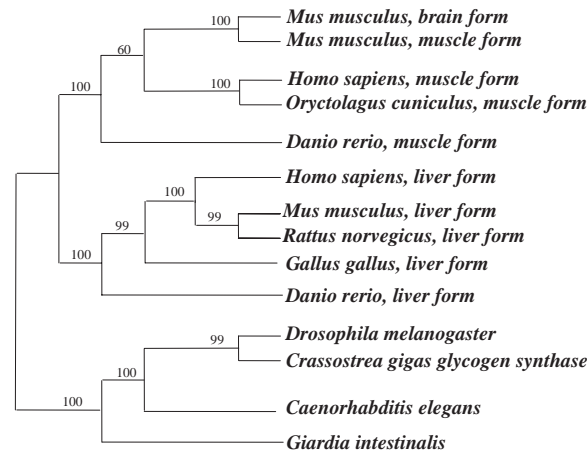


Fig. 2. Graphical representation of phylogenetic analysis of the glycogen phosphorylase (above) and synthase (below) enzymes. Sequence alignment was based on Clustal W (Thompson et al., 1994) and an unrooted distance base phylogenetic tree was constructed by PHYLIP (Felsenstein, 1993) using the unweighted-pair group method with arithmetic means (UPGMA). One thousand bootstrap trials were run using the Seqboot program from the PHYLIP package. Number at each node represents the percentage values given by bootstrap analysis. Genbank accession number of glycogen phosphorylase sequences are: *Giardia intestinalis* (AAK69600), *Dictyostelium discoideum* GPH1 (Q00766) and GPH2 (P34114), *Caenorhabditis elegans* (Q9N5U1), *Drosophila melanogaster* (Q9XTL9), PHS1 *Gallus gallus* liver form (NP_989723), PHS2 *Ovis aries* muscle form (O18751), PHS2 *Oryctolagus cuniculus* muscle form (P00489), PHS1 *Rattus norvegicus* liver form (P09811), PHS2 *R. norvegicus* muscle form (P09812), PHS1 *Homo sapiens* liver form (P06737), PHS2 *H. sapiens* muscle form (P11217), PHS3 *H. sapiens* brain form (P11216), PHS1 *Mus musculus* liver form (Q9ET01), PHS2 *M. musculus* muscle form (Q9WUB3), PHS3 *M. musculus* brain form (Q8CI94). Genbank Accession N^o of glycogen synthase sequences are: *Giardia lamblia* (EAA42246), *Caenorhabditis elegans* (Q9U2D9), *D. melanogaster* (Q9VFC8), *Danio rerio* liver form (CAI20631) and muscle form (NP_957474), *G. gallus* liver form (XP_416432), *O. cuniculus* muscle form (P13834), *R. norvegicus* liver form (NP_037221) and muscle form (XP_341859), *H. sapiens* liver form (NP_068776) and muscle form (NP_002094), *M. musculus* liver form (NP_663547), muscle form (NP_109603) and brain form (P54859).

glycogen synthase transcripts whatever the analyzed sample (mean difference=19.2±10.8; P<0.05).

4. Discussion

4.1. Glycogen synthase and glycogen phosphorylase genes of *C. gigas*

The deduced amino acid sequences of glycogen synthase and phosphorylase of *C. gigas* have high homology with their invertebrate and vertebrate ortho-

logues, suggesting a high degree of conservation through evolution. Phylogenetic analysis showed that *C. gigas* glycogen phosphorylase clustered with other Protostomia glycogen phosphorylase-related enzymes and possessed the phosphorylase pyridoxal-phosphate attachment site considered as a signature pattern of glycogen phosphorylases. In a same manner, the phylogenetic tree showed that *C. gigas* glycogen synthase was closely related to Protostomia glycogen synthase-related enzymes (*D. melanogaster*, *C. elegans*) and that Deuterostomia glycogen synthase-related enzymes clustered into two groups depending on the tissue specific form (liver *versus* muscle

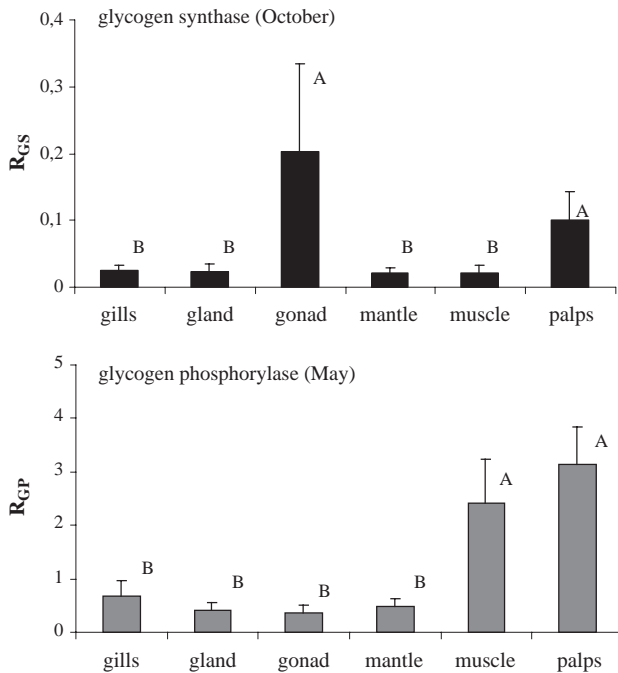


Fig. 3. Ratio of glycogen synthase (R_{GS}) and glycogen phosphorylase (R_{GP}) transcripts relative to elongation factor I transcript in six oyster tissues in October and May, respectively. Data represent mean values for 15 oysters per tissue and are expressed as mean \pm standard deviation. Multiple comparisons were made using a *t*-distribution test at the 5% level and homogenous groups share alphabetic letters.

and brain). These results suggest that our isolated sequences are oyster glycogen phosphorylase and synthase orthologues and can be designated as a *C. gigas* glycogen phosphorylase gene (*Cg-GPH*) and a *C. gigas* glycogen synthase gene (*Cg-GYS*), respectively. These genes are the first glycogen phosphorylase- and synthase-related genes isolated in the phylum Mollusca.

4.2. Tissue expression of *Cg-GYS* and *Cg-GPH*

In our experiment, both *Cg-GYS* and *Cg-GPH* were transcribed at different apparent rates depending on the tissues. Preferential expression of *Cg-GYS* was observed in the labial palps and the gonad in October. This is in accordance with the high level of glycogen content observed in these two tissues and their glycogen storage ability, previously characterised in oysters (Berthelin et al., 2000a,b). Indeed, specific cells located in the labial palps and in the gonad were reported to be involved in glycogen storage (Berthelin et al., 2000b).

The level of *Cg-GPH* transcripts appeared high in labial palps and in muscle in May corresponding to glycogen degradation and mobilization of glucose and energy. In contrary to labial palps, muscle is not considered as a glycogen storage compartment in oysters since glycogen content does not exceed 5% of its total biochemical content (Berthelin et al., 2000a). However, in many species, muscle is a place of active glycogenolysis to quickly provide ATP to

muscular contractile activity and this activity is known to modulate expression of the glycogen phosphorylase gene in rats (Vali et al., 2000). In oysters, most tissues were capable of slight glycogen hydrolysis and/or glucose formation (Berthelin et al., 2000a,b) such as gills, mantle, digestive gland where we observed a weak expression of both *Cg-GYS* and *Cg-GPH*, and muscle where we observed a weak expression of *Cg-GYS*. Lastly, a weak mRNA level of *Cg-GPH* was observed in the gonad in May. According to Fabioux et al. (2004a), oysters were in maturation stage in May and their gonads were mainly constituted by germ cells. Only few specific storage cells, containing glycogen, remained in the gonad in May which could be at the origin of the low value of *Cg-GPH* transcripts.

4.3. Seasonal expression of *Cg-GYS* and *Cg-GPH*

During our experimental conditioning, translatable mRNA for genes *Cg-GYS* and *Cg-GPH* appeared to be seasonally regulated and correlated to glycogen content suggesting that the expression of genes encoding glycogen synthase and phosphorylase were strongly implicated in regulation of glycogen content as observed in mammals (Towle, 1995; Vali et al., 2000). Since a seasonal cycle of storage and mobilization of glycogen was previously

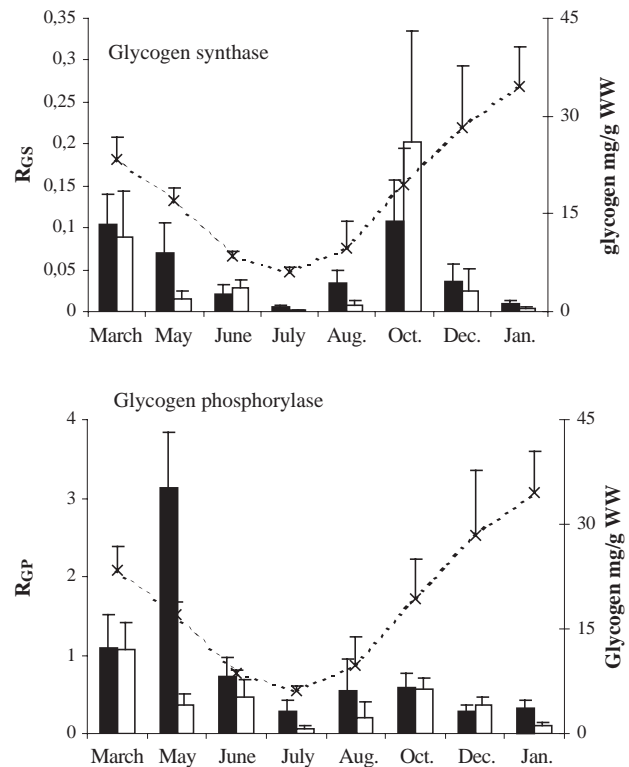


Fig. 4. Ratio of glycogen synthase (R_{GS}) and glycogen phosphorylase (R_{GP}) transcripts relative to elongation factor I transcripts in labial palps (black bar) and gonad (white bar) during experimental conditioning from March to January. Glycogen (mg glycogen/g wet weight of whole animals) levels estimated for tissues are represented by a curve. Data represent mean values for 15 oysters per sample and are expressed as mean \pm standard deviation.

Table 1

Statistical comparison of ratio of glycogen synthase (R_{GS}) and glycogen phosphorylase (R_{GP}) transcripts relative to elongation factor I transcripts in labial palps and gonad during experimental conditioning from March to January

	R_{GS} labial palps	R_{GP} labial palps	R_{GS} gonad	R_{GP} gonad
March	0.10±0.04 (A)	1.08±0.43 (B)	0.09±0.06 (A)	1.07±0.35 (A)
May	0.07±0.04 (AB)	3.13±0.70 (A)	0.02±0.01 (BC)	0.36±0.14 (C)
June	0.02±0.01 (CD)	0.72±0.26 (B)	0.03±0.01 (BC)	0.46±0.21 (BC)
July	0.01±0.001 (E)	0.28±0.16 (C)	0.002±0.001 (E)	0.05±0.04 (D)
Aug.	0.03±0.02 (BC)	0.54±0.40 (BC)	0.01±0.01 (CDE)	0.21±0.20 (CD)
Oct.	0.11±0.04 (A)	0.58±0.19 (BC)	0.20±0.13 (A)	0.57±0.14 (B)
Dec.	0.04±0.02 (BCD)	0.28±0.09 (C)	0.02±0.03 (CD)	0.37±0.09 (BC)
Jan.	0.01±0.002 (DE)	0.32±0.11 (BC)	0.004±0.002 (DE)	0.11±0.04 (D)
<i>K</i>	36.4	36.1	40.5	45
<i>P</i>	<0.001*	<0.001*	<0.001*	<0.001*

Analysis was performed using the Kruskal–Wallis test. Test and probability values were given as *K* and *P*, respectively. Multiple comparisons were made using *t*-distribution test at the 5% level and homogenous groups share similar alphabetic letters.

correlated with the annual reproductive cycle of bivalves (Berthelin et al., 2000b), it can be postulated that the observed seasonal variations of *Cg-GYS* and *Cg-GPH* mRNA levels are closely linked to reproductive stages of oyster. According to histological data obtained with the same sampling (Fabioux et al., 2004a, in press), active gametogenesis began in March in our experiment. First gonadic tubules, composed by active mitotic germ cell, developed among a dense connective conjunctive tissue. Oysters entered maturation stage in April with differentiation of gonia into meiotic germ cell. In the same time, a switch from glycogen storage towards glycogen use seems to occur with a strong increase of *Cg-GPH* and a decrease of *Cg-GYS* and of glycogen content. In females, glycogen would be mobilized for vitellogenesis due to oocytes maturation with the accumulation of yolk and other nutritive substances (Dohmen, 1983) while in males, energy such as glycogen might be mobilized for active production of germ cells. In our conditioning, spawning occurred in July (Fabioux et al., 2004a). The end of reproduction led to very low levels of *Cg-GYS* and *Cg-GPH* transcripts observed in the degenerating gonad during July and August. Thereafter, levels of translatable mRNA for *Cg-GYS* and glycogen content began to increase, especially in October in the labial palps and the gonad. This increase coincided with a change from germinal to somatic development in the gonad. In October, reconstitution of a dense conjunctive tissue, mainly composed by specific storage cells, was observed (Fabioux et al., 2004a, in press).

In the gonad, annual variation of its cellular composition (somatic storage cells vs germinal cells) could be at the origin of the variations observed of the mRNA levels of both genes mainly expressed by a single cell type (i.e. the specific storage cell). In labial palps which are only constituted by storage cells, the observed seasonal variations of *Cg-GYS* and *Cg-GPH* mRNA levels appeared linked to the reproductive cycle, both being putatively controlled by the same exogenous or endogenous factors. During experimental conditioning, the food ration was constant (a mixed diet of three micro-algal species equal to

8% dry weight algae/dry weight oyster per day). Therefore, food availability could not be the parameter that affected seasonal variation in glycogen content and expression of *Cg-GYS* and *Cg-GPH* genes. Temperature and photoperiod were the only environmental parameters that varied during our annual conditioning. These parameters were demonstrated to drive reproductive internal-clock of *C. gigas* (Fabioux et al., in press). The putative role of temperature or photoperiod in the regulation of glycogen pathways, especially for the expression of *Cg-GYS* and *Cg-GPH* genes, via the control of reproductive cycle is therefore questioned.

To conclude, expression of *Cg-GYS* and *Cg-GPH* appeared to be seasonally regulated in oysters and might be strongly implicated in the regulation of glycogen content. Nevertheless, regulation on catalytic parameters also exists (as observed in frog, Scapin and Di Giuseppe, 1994). Molecular and kinetic study of both enzymes are necessary to conclude that *Cg-GYS* and *Cg-GPH* are useful molecular markers to study regulation of glycogen metabolism and reproduction in *C. gigas* as well as to elucidate the physiological significance of the difference of expression between both genes.

Acknowledgements

The authors are grateful to J.F. Samain, M. Mathieu and J.L. Nicolas for their support during the course of this work. The authors are indebted to Chris Langdon (Oregon State University) for helpful comments on the manuscript and for his help for editing the English language. We also acknowledge P. Favrel for his technical advice. We thank all the staff of the Argenton, Bouin and La Tremblade stations for providing experimental oysters under controlled conditions. This work was supported by the MOREST national project funded by IFREMER and by the Région Basse-Normandie, Bretagne, Pays de la Loire and Poitou-Charentes and the conseil général du Calvados.

References

- Bayne, B.L., Bubel, A., Gabbott, P.A., Livingstone, D.R., Lowe, D.M., Moore, M.N., 1982. Glycogen utilisation and gametogenesis in *Mytilus edulis* (L.). *Mar. Biol. Lett.* 3, 89–105.
- Berthelin, C., Kellner, K., Mathieu, M., 2000a. Storage metabolism in the Pacific oyster (*Crassostrea gigas*) in relation to summer mortalities and reproductive cycle (west coast of France). *Comp. Biochem. Physiol.*, B 125, 359–369.
- Berthelin, C., Kellner, K., Mathieu, M., 2000b. Histological characterization and glucose incorporation into glycogen of the Pacific oyster *Crassostrea gigas* storage cells. *Mar. Biotechnol.* 2, 136–145.
- Blass, J.P., Sheu, R.K., Cedarbaum, J.M., 1988. Energy metabolism in disorders of the nervous system. *Rev. Neurol.* 144, 543–563.
- Childress, C.C., Sacktor, B., 1970. Regulation of glycogen metabolism in insect flight muscle. Purification and properties of phosphorylases in vitro and in vivo. *J. Biol. Chem.* 245, 2927–2936.
- Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162, 156–159.
- Cohen, P., 1986. In: Boyer, P.D., Krebs, E.G. (Eds.), *The Enzymes*, vol. 17A. Academic Press, San Diego, CA, pp. 461–497.
- Dégremont, L., 2003. Genetic basis of summer mortality and relationship with growth in juvenile Pacific cupped oysters *Crassostrea gigas*. Thèse de l'Université de Caen. 322 pp.
- Deslous-Paoli, J.M., Héral, M., Berthome, J.P., Razet, D., Garnier, J., 1981. Natural reproduction of *Crassostrea gigas* thunberg in Marennes-Oleron basin in 1979 and 1981: biochemical and energetic aspects. *Rev. Trav. Inst. Pêches Marit. Nantes* 45, 319–327.
- Dohmen, M.R., 1983. Gametogenesis. In: Verdonk, N.H., Van den Biggelaar, J.A.M., Tompa, A.S. (Eds.), *The mollusca*, Development, vol. 3. Academic Press, New York, pp. 1–48.
- Fabioux, C., Pouvreau, S., Le Roux, F., Huvet, A., 2004a. The oyster vasa-like gene: a specific marker of the germ cell lineage in *Crassostrea gigas*. *Biochem. Biophys. Res. Commun.* 315, 897–904.
- Fabioux, C., Huvet, A., Lelong, C., Robert, R., Pouvreau, S., Daniel, J.Y., Minguant, C., Le Pennec, M., 2004b. Oyster vasa-like gene as a marker of the germline cell development in *Crassostrea gigas*. *Biochem. Biophys. Res. Commun.* 320, 592–598.
- Fabioux, C., Huvet, A., Le Souchu, P., Le Pennec, M., Pouvreau, S., in press. Temperature and photoperiod drive *Crassostrea gigas* reproductive internal clock. *Aquaculture*.
- Felber, J.P., Golay, A., 1995. Regulation of nutrient metabolism and energy expenditure. *Metabolism* 44, 4–9.
- Felsenstein, J., 1993. PHYLIP (Phylogenetic Inference Package, version 3.5c). Department of Genetics, SK, University of Washington, Seattle, WA.
- Gabbott, P.A., Whittle, M.A., 1986. Glycogen synthetase in the sea mussel *Mytilus edulis* (L.). II: seasonal changes in the glycogen content and glycogen synthetase activity in the mantle tissue. *Comp. Biochem. Physiol.* 83B, 197–207.
- Hervant, F., Mathieu, J., 1995. Ventilatory and locomotory activities in anoxia and subsequent recovery of epigeal and hypogeal crustaceans. *C. R. Acad. Sci.*, III 318, 585–592.
- Huvet, A., Dubois, S., Daniel, J.Y., Quéré, C., Prudence, M., Van Wormhoudt, A., Sellos, D., Samain, J.F., Moal, J., 2003. Tissue expression of two amylase genes in the Pacific oyster *Crassostrea gigas*, effects of two different food rations. *Aquaculture* 228, 321–333.
- Huvet, A., Herpin, A., Dégremont, L., Labreuche, Y., Samain, J.F., Cunningham, C., 2004. The identification of genes from the oyster *Crassostrea gigas* that are differentially expressed in progenies exhibiting opposed susceptibility to summer mortality. *Gene* 343, 211–220.
- Kasvinsky, P.J., Shechosky, S., Fletterick, R.J., 1978. Synergistic regulation of phosphorylase a by glucose and caffeine. *J. Biol. Chem.* 253, 9102–9106.
- Kepler, D., Decker, K., 1974. Glycogen determination with amyloglucosidase. In: Bergmeyer, H.U. (Ed.), *Methods of enzymatic analysis*, pp. 11–17.
- King, M.W., 1996. Chapter 3: Biochemistry pp. 77–112 and Practice Test pp. 247–330; in Review for the USMLE Step I. 2nd edition: ed. Thomas K. Barton; Appleton and Lange, Stamford, CT.
- Mathieu, M., Lubet, P., 1993. Storage tissue metabolism and reproduction in marine bivalves—a brief review. *Invertebr. Reprod. Dev.* 23, 123–129.
- Mathieu, M., Robbins, I., Lubet, P., 1991. The neuroendocrinology of *Mytilus edulis*. *Aquaculture* 94, 213–233.
- Morishima, I., Sakurai, S., 1985. Purification and characterization of glycogen phosphorylase b from fat body of the silkworm, *Bombyx mori*. *Comp. Biochem. Physiol.*, B 81, 453–458.
- Morisson, T.B., Weiss, J.J., Wittwer, C.T., 1998. Quantification of low-copy transcripts by continuous SYBR Green I monitoring during amplification. *BioTechniques* 24, 954–962.
- Nordlie, R.C., Foster, J.D., Lange, A.J., 1999. Regulation of glucose production by the liver. *Annu. Rev. Nutr.* 19, 379–406.
- Oliveira, G.T., da Silva, R.S., 2000. Hepatopancreas gluconeogenesis during hyposmotic stress in crabs *Chasmagnathus granulata* maintained on high-protein or carbohydrate-rich diets. *Comp. Biochem. Physiol.*, B 127, 375–381.
- Perdue, J.A., Beattie, J.H., Chew, K.K., 1981. Some relationships between gametogenetic cycle and summer mortality phenomenon in the Pacific oyster *Crassostrea gigas* in Washington State. *J. Shellfish Res.* 1, 9–16.
- Reynet, C., Kahn, C.R., Loeken, M.R., 1996. Expression of the gene encoding glycogen phosphorylase is elevated in diabetic rat skeletal muscle and is regulated by insulin and cyclic AMP. *Diabetologia* 39, 183–189.
- Ruiz, C., Martinez, D., Mosquera, G., Abad, M., Sanchez, J.L., 1992. Seasonal variations in condition, reproductive activity and biochemical composition of the flat oyster, *Ostrea edulis*, from San Cibrán (Galicia, Spain). *Mar. Biol.* 112, 67–74.
- San Juan Serrano, F., Fernandez Gonzalez, M., Sanchez Lopez, J.L., Garcia Martin, L.O., 1991. Purification and molecular properties of glycogen phosphorylase b from mantle tissue of mussel, *Mytilus galloprovincialis*. *Comp. Biochem. Physiol.*, B 98, 33–39.
- San Juan Serrano, F., Fernandez Gonzalez, M., Garcia Martin, L.O., 1993. Effect of temperature on glycogen phosphorylase activity of *Mytilus galloprovincialis* mantle tissue. *Actas del IV congreso nacional de acuicultura, centro de investigaciones marinas, Pontevedra (Spain)*, pp. 425–430.
- San Juan Serrano, F., Suarez Alonso, P., Blanco Lopez, S.L., Garcia Martin, L.O., 1998. Regulation of *Mytilus galloprovincialis* glycogen phosphorylase by glucose and glucose-6-phosphate. *J. Shellfish Res.* 17, 159–163.
- Scapin, S., Di Giuseppe, G., 1994. Seasonal variations of glycogen synthase and phosphorylase activities in the liver of the frog *Rana esculenta*. *Comp. Biochem. Physiol.*, B 107, 189–195.
- Soletchnik, P., Faury, N., Razet, D., Goulletquer, P., 1998. Hydrobiology of the Marennes-Oleron Bay. Seasonal indices and analysis of trends from 1978 to 1995. *Hydrobiologia* 386, 1–3.
- Swift, M.L., Akosah, K.O., Thomas, T.P., Humphrey, C.L., 1988. Characteristics of glycogen synthase activity in the digestive diverticula of the oyster, *Crassostrea virginica* Gmelin. *J. Shellfish Res.* 7, 177–178.
- Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680.
- Towle, H.C., 1995. Metabolic regulation of gene transcription in mammals. *J. Biol. Chem.* 270, 23235–23238.
- Vali, S., Carlsen, R., Pessah, I., Gorin, F., 2000. Role of the sarcoplasmic reticulum in regulating the activity-dependent expression of the glycogen phosphorylase gene in contractile skeletal muscle cells. *J. Cell. Physiol.* 185, 184–199.