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Effect of hyperosmotic shock on phosphoenolpyruvate carboxykinase gene expression and gluconeogenic activity in the crab muscle

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Abstract ***Chasmagnathus granulata*** **phosphoenolpyruvate carboxykinase (PEPCK) cDNA from jaw muscle was cloned and sequenced, showing a specific domain to bind phosphoenolpyruvate in addition to the kinase-1 and kinase-2 motifs to bind guanosine triphosphate (GTP) and Mg^{2+}, respectively, specific for all PEPCKs. In the kinase-1 motifs the GK was changed to RK. The first 19 amino acids of the putative enzyme contain hydrophobic amino acids and hydroxylated residues specific to a mitochondrial type signal. The PEPCK is expressed in hepatopancreas, muscles, nervous system, heart, and gills. Hyperosmotic stress for 24 h increased the PEPCK mRNA level, gluconeogenic and PEPCK activities in muscle.**
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Key words: Phosphoenolpyruvate carboxykinase; Molecular cloning; Gene expression; Gluconeogenesis; Hyperosmotic shock; Crab muscle

1. Introduction

Apart from differences in the analytical methods utilized by different authors, multiple factors linked to biological and ecological peculiarities of the different species probably contribute to the observed diversity and the controversy about the gluconeogenesis pathway and special phosphoenolpyruvate carboxykinase (PEPCK; E.C. 4.1.1.32) activity in crustaceans. Phillips et al. [1] detected very low levels of PEPCK in two species of crustaceans and rejected the occurrence of gluconeogenesis in the hepatopancreas. Henry et al. [2] did not detect PEPCK activity in mitochondrial or cytosolic fraction in any tissue from three crab species. However, measurable activities of gluconeogenesis enzymes in the muscle, hepatopancreas and gills were detected by Lallier and Walsh [3] in *Callinectes sapidus*. In *Litopenaeus vannamei* the gluconeogenic capacity in the hepatopancreas can be modulated by a diet, with a high enzyme activity in shrimps fed a low carbohydrate concentration [4].

In vertebrates, hepatic carbohydrate metabolism, and more specifically gluconeogenesis, is known to be modulated by liver cell volume changes [5–7]. However, the precise molecular mechanisms involved remain to be identified.

In crustacean euryhaline, as *Chasmagnathus granulata*, it is known that organic solutes participate in the control of intracellular osmoregulation [8]. During hyperosmotic shock, the levels of free amino acids in crustacean tissues increase when compared with animals acclimatized to fresh water, and serve to readjust the osmolarity of the intracellular fluid to that of the extracellular fluid, and thus contribute to volume regulation [8].

In order to obtain more information on the participation of the gluconeogenesis pathway during the acclimation to hyperosmotic conditions in crustaceans, the present study was designed (1) to characterize the sequence of the PEPCK cDNA (AY074922) expressed in *C. granulata* adductor jaw muscle; (2) to estimate the mRNA PEPCK expression in other tissues; (3) to analyze the regulation of expression and activity of PEPCK and measure the in vitro incorporation of [^{14}C]alanine into glucose in jaw muscle from crabs submitted to hyperosmotic conditions for 24 h.

2. Materials and methods

2.1. Experimental procedure

Male crabs from lagoon Tramandaí, RS, Brazil, weighing 15 ± 2 g were placed in aquaria at a salinity of 20‰, temperature of 25°C, and light/dark cycle of 12 h/12 h. They were fed, ad libitum, once a day with meat for 2 weeks before being used in the experiments. Afterwards the crabs were anesthetized, the jaw muscle was removed and used to PEPCK cloning. The PEPCK mRNA expression was performed by semiquantitative reverse transcription-polymerase chain reaction (RT-PCR) in the jaw and limb muscles, gills, hepatopancreas, nervous system, and heart from the control animals (salinity of 20‰). The gluconeogenesis capacity, PEPCK activity and gene expression were determined in jaw muscle from control and hyperosmotic stress animals (24 h at a salinity of 34‰).

2.2. Total RNA extraction

Total RNA extractions were made following the guanidine thiocyanate method [9].

2.3. PEPCK cDNA cloning

Partial cloning of PEPCK cDNA from muscle has been obtained by using degenerate primers defined from consensus coding regions (Table 1). Mitochondrial (M) and cytosolic (C) PEPCK sequences from chicken (J05419 and M14229), human (L12760), rainbow trout (AF246149), *Drosophila melanogaster* (Y00402) and crayfish *Nephrops norvegicus* (AJ132380) were compared using Clustal W multiple alignment algorithm [10]. Degenerate primers were chosen at positions 1383–1401 and 1655–1674 of the PEPCK gene from *N. norvegicus* (5′-TGGGARGAYCCNAARGGNGT-3′/5′-AACCARTTNACRTGRAARAT-3′).

The cDNA fragment obtained was cloned according to standard

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Table 1
Oligonucleotides used for *C. granulata* muscle PEPCK cloning

Sequence	Direction	Position	Features
5′-TGG GAR GAY CCN AAR GGN GT-3′	forward	1358–1377	degenerate
5′-AAC CAR TTN ACR TGR AAR AT-3′	reverse	1628–1647	degenerate
5′-TCA ACA ACT GGC CGT GTG ACC-3′	forward	714–734	consensus
5′-TAC AAC TTC GGC CAC TAC CTG-3′	forward	1559–1579	specific
5′-TGG CTC AGC ATG GAG GGC CGC-3′	forward	1586–1606	specific
5′-GGC CGA GAT GGG AAC CCC CTT-3′	reverse	1377–1358	specific
5′-CAA GGA GAT GAT CTC GTT AGT-3′	reverse	734–714	specific
5′-GCG TCA CGA TGG TGC GGG CGG-3′	reverse	755–735	specific
5′-TGG AGT AGG GCA CCA CGT AC-3′	reverse	530–511	specific
5′-GGG TCA CGA TGA AGG TCT TGC-3′	reverse	380–360	specific
5′-GAC CAC GCG TAT CGA TGT CGA C-3′			anchor

techniques [11] using pGEM easy vector systems II kit (Promega). The cloning strategy is represented in Fig. 1 and the primers used are in Table 1.

2.4. PEPCK mRNA expression in crab tissues

The reverse transcribed mRNA and the resulting cDNA were employed as templates for semiquantitative RT-PCR. Tissues from control crabs and jaw muscle from hyperosmotic shock animals were utilized for amplification products.

The PEPCK RT-PCR was accomplished using specific primers PEPsen (5′-GGG GTG CAG GTG ACA GAC TC-3′) and PEPrev (5′-GGG TGT CAT CAT GGC CAG GTT-3′). cDNA (1 μl) was used as template for PCR in a final volume of 50 μl containing 1.5 mM $MgCl_2$, 2 pmol from each primer, and 1 U Ampli*Taq* DNA polymerase (Gibco). PCR was performed in Thermocycler (PTC-100, MJ Research, Inc.) and consisted of 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, and extension at 72°C for 1 min.

The size of the PCR product of PEPCK was 428 bp. The analysis of cDNA fragments in agarose gel was performed using ImageMaster® VDS (Pharmacia Biotech) and *C. granulata* 16S (AJ250640) was chosen as control using 16Ssen (5′-TAG CAT AAT CGT TAG TTT TTT AAT T-3′) and 16Srev (5′-TTA AAT TCA ACA TCG AGG TCG CAA-3′) as probes.

2.5. Gluconeogenesis

Jaw muscles (30–50 mg) from crab exposed to 34‰ salinity for 24 h were incubated (60 min) in buffer (500 μl) containing 400 mM NaCl, 10 mM KCl, 25 mM $CaCl_2$, 10 mM $MgCl_2$, 8.8 mM H_3BO_3, plus 10 mM HEPES and 0.1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.8, 815 mOsm/kg (hemolymph osmolality at 34‰ salinity was 853 ± 12.45 mOsm/kg), equilibrated with O_2:CO_2 (95:5, v/v). The muscles from the control group were incubated in the same buffer but the NaCl concentration was changed to 300 mM with 691 mOsm/kg (hemolymph osmolality at 20‰ salinity was 719 ± 10.41 mOsm/kg). In the incubation media of the two experimental groups 10 mM of unlabelled L-alanine plus 0.2 μCi [U-^{14}C]alanine (Amersham Pharmacia Biotech, 5.85 GBq/mmol, 150 mCi/mmol) were added.

Following the incubations, the gluconeogenic capacity of jaw muscles was determined by measuring the incorporation of [U-^{14}C]-L-alanine into [^{14}C]glucose medium according to [12].

2.6. Enzyme assay

The PEPCK activity in jaw muscle cellular fractions was determined as described by Oliveira and Da Silva [12].

2.7. Chemical analyses

Protein concentration was determined by the method of [13], using bovine serum albumin as standard.

2.8. Statistical analysis

The results were analyzed by one-way analysis of variance (ANOVA); post hoc tests were carried out using Student–Newmann–Keuls (SNK) or by Student's *t*-test. Significant differences were accepted if $P < 0.05$. All tests were performed with the Sigma Stat Statistical Software.

3. Results

The amplification product of the first PCR has a size of 289 bp (data not shown). This fragment was purified, cloned and sequenced, showing a specific domain to bind phosphoenolpyruvate in addition to the kinase-1 and kinase-2 motifs to bind guanosine triphosphate (GTP) and Mg^{2+}, respectively, specific for all PEPCKs (Fig. 2). In the kinase-1 motifs the GK was changed to RK. The full-length cDNA obtained for PEPCK from *C. granulata* crab is 2270 bp. The open reading frame of *C. granulata* PEPCK, which started with ATG and ended with a stop codon (TAA), was 1940 bp, encoding 646 amino acid residues (AY074922) (Fig. 1). The putative signal peptide is underlined in Fig. 2. The predicted mitochondrial target sequence is enriched in hydroxylated residues together with six hydrophobic amino acids on a total of 19 amino

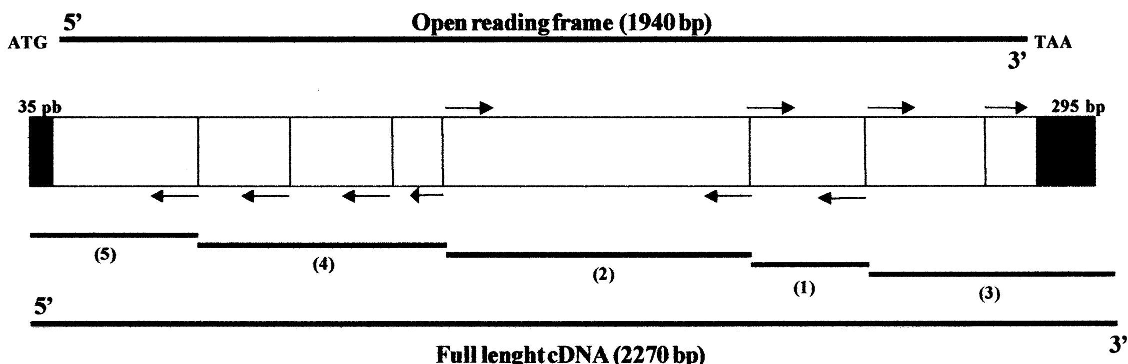

Fig. 1. Strategy of PEPCK cDNA isolation. Five different overlapping PCRs were necessary to get the total PEPCK cDNA from the jaw muscle. The numbers in parentheses indicated sequencing strategy and the arrows indicated the forward or reverse primer positions.

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                                                  Phosphoenolpyruvate-binding site
     10        20        30        40        50        60        70        80        90        100
1  -----MLLFLRLNGVLGEAARVAAEANKLNLAFQARSLATIHGSLSDLKPKVRSFVEEGARLCQPKNVHICDGSERELRDLLNVMQQVGMIEPLPKYTNC   95
2  ------MTEARLSSVLAEASRIASEAAKKNLDFQSRSLSVIHGKLSDLKPKVRTFVEESARLCQPENVHICDGSERELRHLLNVMQQAGMIEYLPKYKNC   94
3  ------MLDKKPSVSSQMHRALAAEAAKKSLNFEHYSFAVSYGKLTSLKPKVRTFVEDTARLCRPDKVHICDGSERDVRHLLNTMQQAGMIESLPKYKNC   94
4  MPELIEQSKIISGNVCGLPQLHKLRQDNCGLYSHIRGIPISYGNVDLLTTGVRAFVEEGIALCQPDQVHICDGSEQENKVLIKSLLEAGTIVPLPKYDNC  100
5  --------MAALYRPGLRLNWHGLSPLGWPSCRSIQTLRVLSGDLGQLPTGIRDFVEHSARLCQPEGIHICDGTEAENTATLTLLEQQGLIRKLPKYNNC   92
6  -------MFWLRGGAQSCRGGETEDRMQRGMWGVGLARRRLSTSLSALPAAARDFVEEAVRLCRPREVLLCDGSEEEGKELLRGLQDDGVLHPLPKYDNC   93
7  -----------------MSCLLLGLIRRRGGVGTSVGVRSLASIPSLPPAVADFVKRAVDECKPANVHVVTGSAEESAHILAGLEKDGMVKRLPKYENC   82
8  --------------------------APELKTEVNIISKVIQGDLESLPPQVREFIESNAKLCQPESIHICDGSEEENKKILDIMVEQGMIKKLSKYENC   74
9  ----------MRCRSLSHFKDDDFAVVSEVVTHKQNHIPVIKGDFVSLPKHVQRFVAEKAELMKPSAIFICDGSQNEADELIARCVERGVLVPLKAYKNN   90

    110       120       130       140       150       160       170       180       190       200
1  WLARTDPGDVARVESKTFIVTKDRRETIPTPKEGIKGLLGNWMSPEDLKKAVKERFPGCMKGRTMYVVPFSMGPVGSPLSKIGIQLTDSPYVVASMRTMT  195
2  WLARTDPGDVARVESKTFIVTKNRRDTIPTPKEGVKGLLGNWMSYEDLNGTVQERFPGCMSGRTMYVVPYSMGPGSSSLSKIGVEVTDSPYVVASMRTMQ  194
3  WLGPTDPGDVARVESKTFIVTREKRETIPIPKEGWRGLLGNWMSHEDLRRAVQDRFPGCMAGRTMYVVPYSMGPVGSPLSKIGVQVTDSPYVVASMRTMT  194
4  WLARTNPADVARVESRTFICTERREETIPTPVEGVKGTLGNWISPSDMDAAVQQRFPGCMKGRTMYVVPFSMGPVGSPLSKIGIELTDSAYVVASMRIMT  200
5  WLARTDPKDVARVESKTVIVTPSQRDTVQLPPGGARGQLGNWMSPADFQRAVDERFPGCMQGRTMYVLPFSMGPVGSPLSRIGVQLTDSAYVVASMRIMT  192
6  WLARTDPRDVARVQSKTVLVTPEQSDAVPPPPPSGSPQLGNWMSPNAFQAAVQERFPGCMAGRPLYVIPFSMGPPTSPLAKLGVQVTDSPYVVLSMRIMT  193
7  WLARTDPKDVARVESKTVIVTKNQRDTIPIPDGGAKSQLGSWMSEGDFQKARQDRFPGCMSGRTMYVIPFSMGPVGSPLSKFGVQVTDSPYVVASMGIMT  182
8  WLALTNPRDVARIESKTVIITQEQRDTIPIPKTG-SSQLGRWMSEEDFEKAFNTRFPGCMQGRTMYVIPFSMGPIGSPLAKIGIELTDSPYVVASMRMMT  173
9  YLCRTDPRDVARVESKTWMITPEKYDSVCHTPEGVKPMMGQWMSPDEFGKELDDRFPGCMAGRTMYVIPYSMGPVGGPLSKIGIELTDSDYVVLCMRIMT  190

                                                       GTP-phosphoryl binding site
    210       220       230       240       250       260       270       280       290       300
1  RMGKAVLDALAEQ--DFVKCLHSVGCPLPLQRTLVNNWPCDPERTIVTHVPETSEIISFGSGYGGNTLLGKKCFALRIGSTIALREDWLAEHLLILGITN  293
2  RMGGKVLETLDQE--DFVKCLHSVGCPCPQK-TLVNNWPCDPARTIVTHVPDTNEIISFGSGYGGNSLLGKKCFALRIGSNIARREGWLAEHMLILGITN  291
3  RMGSRVLETLGEE--EFVRCLHSVGCPLPLTRPLVNNWPCDPARTIVTHVPESNEIISFGSGYGGNSLLGKKCFALRLGSRLAGREGWLAEHMLILGITN  292
4  RMGAAVLRQLAKK--EEFVRALHSVGAPANGQVEQPSWPCDPERTIILHKPAENLIVSYGSGYGGNSLLGKKCFALRIGSTIAKQEGWLAEHMLILGITD  298
5  RLGTPVLQALGDG--DFVKCLHSVGQPLTGQGEPVSQWPCNPEKTLIGHVPDQREIISFGSGYGGNSLLGKKCFALRIASRLARDEGWLAEHMLILGITS  290
6  RVGPAVLQRLDD---DFVRCLHSVGRPLPLTEPLVSSWPCDRSRVLVAHIPSERRIVSFGSGYGGNSLLGKKCFALAIASRMAQQQGWLAEHMLILGVTS  290
7  RMGTPVMDKLAQGA-EFVRCQHSLGRPLPLKAPLVNSWPCNPEKVLISHLPDTRQILSFGSGYGGNSLLGKKCFALRIASRIAKDEGWLAEHMLILGITN  281
8  RMGTAALKALGNG--EFVKCLHSVGCPLPLKEPLINNWPCNPELTLIAHLPDRREIISFGSGYGGNSLLGKKCFALRIASRIAKEEGWLAEHMLILGITN  271
9  RMGEPVLKALAKNNGEFVRCVHSVGQPKPVATKVINHWPCNPEKTIIAHRPAEREIWSFGSGYGGNSLLGKKCFALRIAMNIGYDEGWMAEHMLIMGVTS  290

           kinase-1                 kinase-2
    310       320       330       340       350       360       370       380       390       400
1  PQEVKKYIAAALPSACGSTNLAMMTRALPRYKVECVGDDIAWIKFDEDGVLRAINPENGFFGVAPGTSMHTNPVAMQTVLSNTIFTNVAKTSDGGVFWEG  393
2  PKGIKKYIAAAFPSACKATNLAMMTTSLPGYKVECVGDDIAWMKYDEDGVLRAINPENGFFGVAPGTSMHTNPVAMKTVLSTTIFTNVAKTSDGGVFWEG  391
3  PAGKKRYIAGAFPSACRKTNLAMMTPSLPGYKVECVGDDIAWMKFDSEGVLRAINPENGFFGLAPGTSMSTNPVAMKTVLSNTIFTNVAKTSDGGVFWEG  392
4  PKGVKKYITAAFPSACGKTNLAMLNPSLANYKVECVGDDIAWMKFDSQGVLRAINPENGFFGVAPGTSMETNPIAMNTVFKNTIFTNVASTSDGGVFWEG  398
5  PAGKKRYVAAAFPSACGKTNLAMMRPALPGWKVECVGDDIAWMRFDSEGRLRAINPENGFFGVAPGTSATTNPNAMATIQSNTIFTNVAETSDGGVYWEG  390
6  PSGEKRYMAAAFPSACGKTNLAMMTPSLPGWRIHCVGDDIAWMKFDDRGRLRAINPERGFFGVAPGTSSRTNPNAMATIARNTIFTNVGLRSDGGVYWDG  390
7  PQGVKRYVAAAFPSACGKTNLAMMKPALPGWTVECVGDDIAWMKFDSQGKLRAINPENGFFGVAPGTSLKTNPHAMATIAKNTVFTNVGETSDGGVWWEG  381
8  PEGEKKYFAAAFPSACGKTNLAMMNPSRPGWKIECVGDDIAWMKFDELGNLRAINPENGFFGVAPGTSIKTNPNAIKTIFKNTIFTNVAETSDGGVYWEG  371
9  PKGEERFVAAAFPSACGKTNLAMLEPTIPGWKVRVIGDDIAWMKFGADGRLYAINPEYGFFGVAPGTSHKTNPMAMASFQENTIFTNVAETADGEYFWEG  390

       guanine-binding site
    410       420       430       440       450       460       470       480       490       500
1  LEKETANDVTITSWLGDTNWSKESGKPAAHPNSLFCTPAGQSPIIDPAWEDPKGVPISAILFGGRRPEGVPLIYEAFSWKHGVLVGGAMRSEATAAAEHK  493
2  LEKEIDNDVTITSWLGDTNWSKESGKPAAHPNSRFCTPAGQCPIIDPSWEDPKGVPISAILFGGRRPQGVPLVYEAFNWKHGVMVGGSMRSEATAAAEHK  491
3  LEKETPNNVSITSWRGETNWSPECRGPAADPNSRFCKPAGQGPIIDPAWEDPKGVPISAILFAGRRPPGLPLVYEAYDWKHGVMVGAAMRSEATAAAEHK  492
4  MESSLAPNVRITDWLGKP-WTKDSGKPAAHPNSRFCTPAAQCPIIDEAWEDPAGVPISAMLFGGRRPAGVPLIYEARDWTHGVFIGAAMRSEATAAAEHK  497
5  IDQPLPPGVTVTSWLGKP-WKPGDKEPCAHPNSRFCAPARQCPIMDPAWEAPEGVPIDAIIFGGRRPKGVPLVYEAFNWRHGVFVGSAMRSESTAAAEHK  489
6  LDEPTEPGVTYTSWLGKP-WKHGDPEPCAHPNSRFCAPADQCPIMDPRWDDPEGVPIDAIIFGGRRPRGVPLVVEAFGWRHGVFMGSAMRSEATAAAEHK  489
7  LDPPAAG-VSLTDWHGKS-WKAGDSGPCAHPNSRFCTPAAQCPIIDPQWESDEGVPIDAIIFGGRRPEGVPLVYESFNWRHGVFVGASMRSEATAAAEYK  479
8  IDEPLPPGVTLTSWKNKD-WTPDNGEPCAHPNSRFCTPASQCPIMDPAWESPEGVPIEGIIFGGRRPAGVPLVYEAFNWQHGVFIGAAMRSEATAAAEHK  470
9  LEHEVKNPKVDMINWLGEPWHIGDESKAAHPNSRFTAPAGQCPIIHPDWEKPEGVPIDAIIFGGRRPEGVPLVFESRSWVHGIFVGACVKSEATAAAEHT  490

    510       520       530       540       550       560       570       580       590       600
1  AKVIMHDPFAMRPFFGYNFGHYLQHWLSMETRT-HKALPKIFHVNWFRKD-EKARFIWPGFGENDRVLDWILRRVDGE---DVAEETTRRLLPKPSSLNM  588
2  GKVIMQNPFAMTPFFGYNFGQYLQHWLSMETRT-DKALPKIFHVNWFRKV-KKGRFIWPGFGDNVRVLDWILKRVDGE---DVAEESGRRLLPKDPPSTM  586
3  AQVIMHDPFAMRPFFGYNFGHYLQHWLSMEGRT-TKQMPKIFHINWFRKN-EKGRFKGPGFGENVRVLDWILRRVEGQ---DVAEESAIACLPSPDPSTL  587
4  GKVIMHDPFAMRPFFGYNFGDYVAHWLSMEKR---GQVPKIFHVNWFRKS-AEGKFMWPGYGENSRVLEWILRRVNGE---SCYVDSAIGHIPAEGALNL  590
5  GKIIMHDPFAMRPFFGYNFGHYLEHWLSMEGRK-GAQLPRIFHVNWFRRD-EAGHFLWPGFGENARVLDWICRRLEGE---DSARETPIGLVPKEGALDL  584
6  GGRLMHDPFAMSPFFGYNAGRYLEHWLSTGLRS-NARLPRLFHVNWFLRD-NEGRFVWPGFGHNARVLAWIFGRIQGR---DTARPTPIGWVPKEGDLDL  584
7  GKVIMHDPFAMRPFFGYNFGDYLAHWLSMETRKGATHLPKIFHVNWFRKDPTSGSFLWPGFGDNARVLEWIFKRCSREREDEAAKKSMVGWVPLEGAINL  579
8  GKIIMHDPFAMRPFFGYNFGKYLAHWLSMAHRP-AAKLPRIFHVNWFRKD-SQGKFLWPGYGENSRVLEWMFNRIQGK---ASAKSTAIGYIPADTALNL  565
9  GKQVMHDPMAMRPFMGYNFGRYMRHWMKLGQPP--HKVPKIFHVNWFRQS-ADHKFLWPGYGDNIRVIDWILRRCSGD--ATIAEETPIGFIPKKGTINL  585

       610       620       630       640       650       660
1  AGLE-DQNIDMHELFRLPKGFWQQETQAIAKYFEEQVGDDLPNEIREELKKLDKRVEKM--                                         646
2  AGLE-HENVDMDELFSLPKELWEQEVRDSTKYFAEQVGDDLPNEVREQLKILEKRIEKM--                                         644
3  EGLEDHAHRTPEEAFSLPKDFWSQEIREIRKYFDEQVGSELPNEVRARLKKLEKRVEKM--                                         646
4  DGMK--DKVDVKEIFSLPKEFWSQEVKDIRTYFESQVGADLPASIYQQLDELSSRVDNL--                                         647
5  SGLR---AIDTTQLFSLPKDFWEQEVRDIRSYLTEQVNQDLPKEVLAELEALERRVHKM--                                         640
6  GGLP---GVDYSQLFPMEKGFWEEECRQLREYYGENFGADLPRDVMAELEGLEERVRKM--                                         640
7  QGLG--SKVDMGALFDLPKAFWEKETQELRAYFTQQVGADLPQQVEGELKALEDRIRN---                                         635
8  KGLE---DINLTELFNISKEFWEKEVEEIKQYFEGQVNADLPYEIERELLALEMRIKQL--                                         621
9  EGLP---NVNWDELMSIPKSYWLEDMVETKTFFENQVGSDLPPEIAKELEAQTERIKALKE                                         643
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Fig. 2. Comparison of the *C. granulata* jaw muscle PEPCK (3) (AY074922) and *Penaeus* (1) (AJ250829), *Nephrops* (2) (AJ132380), *Drosophila* (4) (Y00402), human mitochondrial (5) (L12760), chicken mitochondrial (6) (J05419), *Onchorynchus* (7) (AF246149), chicken cytosolic (8) (M14229) and *Ascaris* (9) (L01787) partial amino acid sequences deduced from PEPCK cDNA sequences. The bold characters represent the conserved motifs in all these PEPCKs. The signal sequence is underlined in chicken mitochondrial PEPCK (6). Otherwise a putative signal sequence, determined by signal IP algorithm [25] is underlined in the crab (3) and trout (7).

acids. When compared with sequence databases for proteins (gene databanks NCBI, NIH) using the BLAST algorithm, deduced amino acid residues show that the PEPCK sequence of crab *C. granulata* is very similar to other PEPCK proteins. Homology (%) between *C. granulata* PEPCK and other PEPCKs is showed in Table 2.

PEPCK gene was expressed in muscle (jaw and limb), hepatopancreas, heart, gills and nervous system (Fig. 3) of animals maintained under control conditions.

Fig. 4 shows that the incorporation of [^{14}C]alanine into glucose in jaw muscles from crabs submitted to 34‰ salinity increased ($P < 0.05$) 77% as compared to the control group.

Table 2
Percent homology between PEPCKs of different species of invertebrates and vertebrates

	Shrimp	Crayfish	Crab	Fruit fly	Human (M)	Chicken (M)	Trout	Chicken (C)	Worm
Penaeus vannamei	100.0	86.3	78.9	66.8	67.5	64.4	61.2	64.1	53.1
N. norvegicus		100.0	80.0	65.8	66.9	60.2	61.0	63.8	52.7
C. granulata			100.0	62.8	66.1	59.6	60.4	62.6	51.5
D. melanogaster				100.0	63.3	56.1	56.1	59.3	50.6
Homo sapiens (M)					100.0	69.6	68.0	70.1	57.3
Gallus gallus (M)						100.0	62.6	63.0	53.3
Onchorynchus mykiss							100.0	69.7	51.9
G. gallus (C)								100.0	54.0
Ascaris suum									100.0

(C) cytosolic or (M) mitochondrial form of PEPCK.

Table 3 shows the distribution of PEPCK activity in the mitochondrial and cytosol fractions in jaw muscle. The results indicate that 93% of PEPCK activity in jaw muscles is observed within the mitochondria, and 6.6% in the cytosol.

In the group exposed to 34‰ salinity, PEPCK activity in the mitochondrial fraction was about 4-fold higher than in control crabs. Moreover, the results show a significant ($P < 0.05$) increase in PEPCK mRNA expression in muscles from crabs exposed to 34‰ salinity (Fig. 5A,B).

4. Discussion

The results of the present work are the first to demonstrate in crustacean muscle the gluconeogenic activity, PEPCK molecular cloning, and the regulation of its expression and activity by hyperosmotic shock.

The cDNA nucleotide sequence of *C. granulata* PEPCK possesses about 80% of similarity with PEPCK sequences from other Malacostraca. The high (up to 60%) similarity between the PEPCK sequence from *C. granulata* and PEPCK sequences from other invertebrates and vertebrates, and the maintenance of the PEPCK specific domain suggest that the enzyme cloned is a functional form of PEPCK. *C. granulata* PEPCK seems to possess the PEPCK specific domain to bind phosphoenolpyruvate in addition to the kinase-1 and kinase-2 motifs to bind GTP and Mg^{2+}, respectively, specific for all the known PEPCKs [14–16]. However, in kinase-1 motifs from *C. granulata* PEPCK, the GK was changed to RK.

Chicken-processed mitochondrial PEPCK begins at amino acid 34 by a leucine. The mature protein is 607 amino acids long, and it was noticed by Weldon et al. [17] that the first 12 residues were hydrophobic or hydroxylated amino acids, so those residues may have been involved in import of the fraction into the mitochondrial matrix. It is noteworthy that the first 19 amino acids of the putative crab mature enzyme contain also many hydrophobic amino acids and hydroxylated residues specific to a mitochondrial type signal [18].

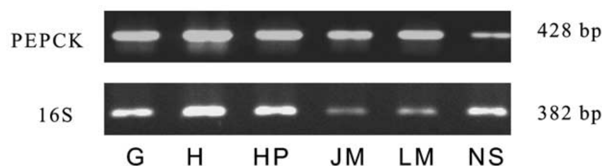

Fig. 3. mRNA expression of PEPCK in different tissues of *C. granulata* demonstrated by RT-PCR analysis. Gills (G), heart (H), hepatopancreas (HP), jaw (JM) and limb muscles (LM) and nervous system (NS).

PEPCK gene is expressed in the hepatopancreas, jaw and limb muscles, nervous system, heart, and gills of the *C. granulata*. Gluconeogenesis has been demonstrated in the hepatopancreas and gills from *C. granulata* [12,19]. Among the vertebrates, the enzyme is expressed mainly in liver and kidney, and is located in cytosol and/or mitochondria fraction [16,20]. In the other tissues the mitochondrial PEPCK form might not have a purely gluconeogenic function, and would serve other functions similar to those in fibroblasts. Mitochondrial PEPCK might operate in the reverse direction from PEP to oxaloacetate by using glycolysis-generated PEP imported from cytosol to replenish the citrate cycle [20].

The present study shows that in *C. granulata* jaw muscle the PEPCK cloned codes for a mitochondrial enzyme. These results were supported by the data that in jaw muscle about 90% of PEPCK activity is found within the mitochondria.

It has been demonstrated that in vertebrates the gene for mitochondrial PEPCK is constitutive in its pattern of expression [14]. However, in *C. granulata* gene expression for mitochondrial PEPCK increased after hyperosmotic stress. The adaptive significance of species-specific differences in PEPCK compartmentalization is not readily apparent. In *C. granulata* the gluconeogenesis is one of the pathways implicated in the metabolic adjustments during hypo- and hyperosmotic stress, anoxia and post-anoxia recovery and seasonal adaptations [21–23]. Thus, an increase in gene expression for mitochondrial PEPCK under the different physiological conditions may be an adaptive metabolic mechanism to the challenges to which the animal is continuously submitted in its estuarine habitat.

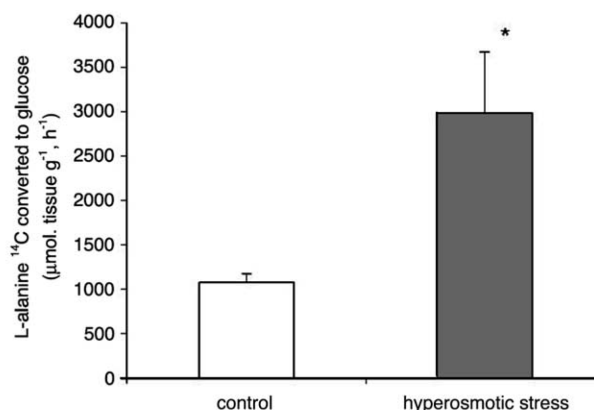

Fig. 4. Effect of hyperosmotic stress on gluconeogenesis activity from [^{14}C]alanine in jaw muscle. Data are given as mean ± S.E.M., $N = 7$–14 animals. *$P < 0.05$.

Table 3
PEPCK activity (nmol $H^{14}CO_3^-$ per mg of protein per min) on jaw muscle of *C. granulata* crab submitted to 24 h of hyperosmotic stress

Fraction	Control	Hyperosmotic stress
Cytosol	7.26 ± 4.32	24.81 ± 12.74
Mitochondrial	47.95 ± 14.03	180 ± 72.18[a]

Values are given as mean ± S.E.M. of seven to 14 observations.
[a]Mean values are significantly different from the control group ($P < 0.05$).

In this work, the increase in the PEPCK mRNA level after 24 h of the hyperosmotic stress led to the increase of the gluconeogenic and PEPCK activities as consequence of the metabolism of amino acids in muscle. In mammals it was suggested that glutamine may regulate the PEPCK gene expression through two different mechanisms: the glutamine-induced cell swelling, decreasing the mRNA level, and another unidentified mechanism, depending on its metabolism, that increases the mRNA level [7,24].

Unpublished data from our laboratory show that in *C. granulata* exposed to hyperosmotic stress for 9 and 12 h the amino acids uptake in jaw muscle increased significantly and the gluconeogenesis and PEPCK activities did not change. The lag phase of about 24 h required to detect hyperosmotic stress effects on PEPCK mRNA levels and activity may be indicative of the formation of transcription factors. The PEPCK induction, the increase in the amino acid uptake and proteolysis-derived amino acids after the hydration could favor gluconeogenesis from the amino acids. This may be seen as another example of coordination of the physiological process in response to changes of cellular volume with different homeostatic mechanisms according to the time that the crabs were exposed to hyperosmotic stress. On the other hand, in crab muscle PEPCK may play a role in the integration of multiple pathways of energy metabolism as suggested by She et al. [25] for mammal liver.

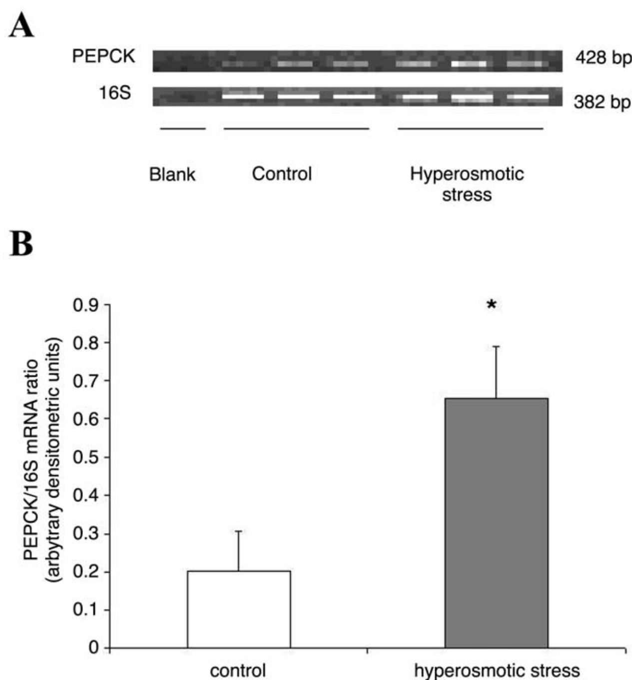

Fig. 5. A: Representative gel of crab PEPCK expression in jaw muscle of control, hyperosmotic stress (24 h) crabs and blank. The 16S rRNA served as an internal control for loading. B: Analysis, by densitometry, of PEPCK mRNAs levels for three crabs from each of the groups normalized by 16S rRNA values. Data are given as mean ± S.E.M., *$P < 0.05$.

In conclusion, the present study provides evidence in support of a role of cellular volume control in regulating PEPCK gene expression and activity in the muscle, as well as the involvement of the gluconeogenic pathway in the acclimation to hyperosmotic shock.

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