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Circadian oscillations of RPCH gene expression in the eyestalk of the crayfish *Cherax quadricarinatus*

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This work is dedicated to the memory of Dr. Hugo Aréchiga.

Abstract

The RPCH and β-actin cDNAs from the crayfish *Cherax quadricarinatus* were amplified, cloned and sequenced. The primary structure sequences of these cDNAs were compared to other members of the AKH/RPCH family. Fluctuations in the amount of the *C. quadricarinatus* RPCH and β-actin mRNAs, as cDNAs, were quantified every 3 h by RT-PCR. Single cosinor analysis supports the notion of β-actin and RPCH mRNA circadian behavior in animals subjected to 12 h:12 h light/dark regimes. In constant darkness RPCH mRNA concentration changes to ultradian cycles.
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1. Introduction

Migration of crustacean pigment granules in the tegumentary chromatophores and in the retinal cells is modulated by circadian oscillations as a result of the action of classical neurotransmiters like 5-hydroxytryptamine, norepinephrine and dopamine. The former two promote the action of the pigment dispersing hormone (PDH) and the later one promotes the action of the red pigment concentrating hormone (RPCH) [18,32]. The neuropeptides PDH and RPCH are members of the chromatophorotropic hormones and are synthesized by the X organ-sinus gland system located in the optic peduncle of crustaceans [12,35]. RPCH is also synthesized by neural groups of the medulla interna, the medulla externa and the medulla terminalis of the crustacean eyestalk [1,31,35]. The concentration of RPCH in the lamina ganglionaris plus the X organ-sinus gland system, as shown by immunohistochemistry measurements, is the highest [1]. RPCH is an octapeptide [13,20,22] that induces retraction of pigment granules from the pigment tegumentary chromatophores [1,31] and from the distal segment of retinal cells [17]. In contrast, PDH is an octadecapeptide with an opposite functional role since it elicits dispersion of pigment granules in the same target cells [12,32]. Several mechanisms have been proposed to explain granule distribution after RPCH and PDH interaction with their receptors like the intracellular increase of Ca^{2+} concentration [25,30], the regulation of cyclic nucleotide concentration by increased biosynthesis or by degradation and movement along the cytoskeleton

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microtubules [15]. PDH is released also by a neural reflex, triggered by light acting on extra-retinal photoreceptors [32].

The structure of RPCH is closely related to the neuropeptide family known as adipokinetic hormone (AKH). This hormone controls lipid and carbohydrate metabolism in insects [29]. Since AKH induces pigment concentration in crustacean chromatophores, as RPCH does, and RPCH induces hypoglycemia in insects [26], as AKH does, they are considered as a single RPCH/AKH family, probably sharing a common origin [23]. The amino acid alignment of the RPCH/AKH peptides has revealed a common overall architecture. The hormones start at the signal peptide of 19–25 amino acid residues [28], followed by the RPCH or AKH active peptide sequence, then the two basic amino acids Lys-Arg, serving as a recognition site for protease activity [33] and ending at the related peptide, that has 72 amino acid residues in crustaceans and 32 amino acid residues in several insect species. These peptides adopt similar α-helix and random coil secondary structures [23]. The precursors of the propeptides form disulfide bonds between two cysteines: one located in the related peptide and the other one located either in the related peptide, or in a few cases, in the signal peptide. The cystine produced, is essential for dimmer prohormone cleavage at the Lys-Arg peptide bond [14]. In all crustacean species studied, the RPCH primary structure is: pGlu-Leu-Asn-Phe-Ser-Pro-Gly-Trp-NH_2 [13]. It was assumed that RPCH was present exclusively, in crustaceans, but recently a similar peptide, with Ile as the amino residue in position 2, which functions as a lipid-mobilizing hormone, was recently found in the corpora cardiaca of the stinkbug *Nezara viridula* [16].

Numerous members of the RPCH/AKH family are known, but only two cDNAs from crustaceans, those from the crabs *Carcinus maenas* [22] and *Callinectes sapidus* [20] have been reported. In contrast, cDNAs from 10 insect precursor peptides that belong to *Locusta migratoria* [7], *Schistocerca gregaria* [14,38], *Schistocerca nitans* [28], *Blaberus discordalis* [21], *Manduca sexta* [8] and *Drosophila melanogaster* [27] are known.

The movement of retinal pigments is subject to circadian cycles in the crayfish. The pigments are dispersed during the day and they concentrate during the night, even in animals which were kept in constant darkness [2,4]. In the dark, the movement of the distal pigment is toward the corneal end of the compound eye, whereas the proximal pigment moves in the opposite direction, toward the photoreceptor axons in the proximal part of the retinal cells [2,4,17]. As a consequence of both events, the rabdhom area is widely exposed to light. Crustacean neurohormones, like RPCH, play a role in altering the output of rhythmic pattern generators. RPCH concentration in the crayfish shows seasonal changes with maximum values in the summer months and minimum values during winter time [34]. The RPCH synthesizing neurons electrical activity of [41], as well as the RPCH release to the hemolymph, and the amount of this peptidic hormone [36], all change according to a circadian basis. Modulation of retinal pigment distribution in *P. clarkii* related to RPCH has been described also [3,17]; its minimal concentration is detected early during the daytime and the circadian increases to a maximum after 12 h of light exposure, decreasing afterwards throughout the night. Circadian rhythms of this hormone have been detected by immunological measurements in the same experimental animal [36].

In this work we asked whether the oscillations of RPCH detected by immunochemistry, are reflected or not, at more basic molecular levels, like the transcription of genes, and if they are sensed or not as 24 h rhythmic circadian oscillations. To that end 12 h:12 h light/dark experiments were carried out to measure the amount of mRNA for RPCH and for β-actin. The results show relatively constant levels of β-actin mRNA in light/dark or dark/dark schedules and circadian or ultradian oscillations of the mRNA for RPCH in light/dark or dark/dark schedules, respectively.

2. Materials and methods

2.1. Experimental animals

The crayfish *Cherax quadricarinatus* was bought from a local fishery supplier. Adult specimens were used, without distinction of sex, in intermolt at the time of the experiment and with food ad libitum. The animals were kept at constant temperature in a 12 h:12 h light/dark program for at least 2 weeks before the experiment. Light was on from 8.00 to 20.00 h daily. A separate group of animals was kept in darkness from 3 weeks up to 4 months.

2.2. Isolation of DNA and RNA from C. quadricarinatus

Genomic DNA was extracted from muscular tissue [42], then purified, firstly using a DNAzol kit (Stratagene; instructions given by the supplier) and secondly by centrifugation through a CsCl density gradient. DNA was dissolved in buffer TE and kept frozen at −70 °C. RNA was extracted from the eyestalks, the hepatopancreas, the gallnuts and the muscle. Every 3 h the organs were obtained and immediately dropped into liquid nitrogen. For each time point 10 organs were pooled. RNA extraction from the eyestalks was problematic since polysaccharides, phenolic compounds, carotenes and retinal pigments interfere with its obtention. Therefore, we followed the modified method here described. Total RNA was extracted from the medulla after removal of the exoskeleton, the fat, the connective tissue and the dissection of the retina [10,39]. The medulla was immersed in 3 ml of lysis buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol, pH 7.0) plus 1 vol.% of water-saturated phenol and was mechanically disrupted, in the presence of 100 mg/ml of glass-beads (0.5 μm in diameter), by five alternate periods of 1 min highest speed vortexing and 1 min cooling in ice-water at 4 °C. The tissue lysate was

mixed with 0.4 vol.% of a chloroform–isoamyl alcohol (24:1) solution and centrifuged at 17 600 × *g* for 45 min at 4 °C. The upper phase layer was saved, mixed with 0.1 vol.%of high salt solution (1.2 M NaCl, 0.8 M sodium acetate), 0.9 vol.%of isopropanol and incubated at −20 °C. The precipitated RNA was centrifuged, dissolved in 700 μl of lysis buffer, precipitated with 1 vol.%of isopropanol at −20 °C for 1 h and washed with 75% ethanol. The purified RNA was dissolved in water and its concentration was calculated by absorbance measurements at 260 nm. Total RNA integrity was tested as the absence of rRNA degradation by electrophoresis in 1% agarose gels [37]. Total RNA was kept in water at −70 °C until use.

The RNA extracted from the eyestalks, the hepatopancreas, the gallnuts and the muscle was tested by RT-PCR (Superscript II; Invitrogen) monitoring the 18S rRNA [11] cDNA synthesis from each organ tissue. The primer numbers, their names, their sequences, and the RNA they belong to are given in Table 1. The 500 ng of primer adaptor #17 were added to 500 ng of total RNA (3:1 ratio of primer to RNA) in a final volume of 20 μl of RT-reaction mixture and incubated at 52 °C for 55 min. The RNA was hydrolyzed with a mixture of 0.25 U of RNAse H, 0.5 U of RNAse T_2 and 0.5 ng of RNase A (Invitrogen) for 20 min at 37 °C. The reaction mixture was diluted to 100 μl with sterile water and stored at −20 °C. The 18S RNA was amplified using 50 ng of cDNA stock, 0.2 mM each of the four dNTPs, 0.5 U of Deep-Vent DNA polymerase (New England Biolabs), 5 U *Taq* DNA polymerase and 100 pM of primers #17 and #18, dissolved in 50 mM KCl, 1.5 mM $MgCl_2$ and 20 mM Tris–HCl (pH 9.4) in 50 μl of reaction mixture and incubated for 27 cycles at 95 °C for 20 s, 62 °C for 20 s and 72 °C for 2 min. The amplified cDNA products were separated by 1% agarose gel electrophoresis and analysed.

2.3. Cloning of the cDNAs for the RPCH precursor and for the β-actin

The cDNAs for RPCH and β-actin were cloned by a two-step procedure. Firstly, partial gene sequences were amplified by PCR to choose a consensus internal DNA stretch fragment. Secondly, 5′ and 3′ RACE were performed to determine the primary sequence of the two ends of the mRNAs.

2.3.1. Design of oligonucleotide primers for RPCH precursor and β-actin clonation

A consensus sequence stretch for RPCH was selected after alignment of the cDNAs for *C. sapidus* [20] and *C. maenas* [22]; the intron gene position for RPCH in *C. sapidus* [24] and other introns for the AKH/RPCH family [23] were taken also into account. The sense primer was #1 containing the last three codons of the signal peptide and the first four codons plus the two first nucleotides of the fifth codon of the active peptide. The two anti-sense primers #2 and #3 were targeted to the exon–exon union of *C. sapidus* and the end of the ORF segment of the two mentioned crabs, respectively. Primers for β-actin cloning were synthesized according to the homologous sequence of the cDNA from *Procambarus clarkii* [19]. The upper primer was #10 and encompassed nucleotides 263–280; the lower primer was #11 and comprised nucleotides 407–426.

2.3.1.1. Polymerase chain reaction: nested PCR. Reaction mixtures in 50 μl contained 100 ng of *C. quadricarinatus* chromosomal DNA, 0.2 mM each of the four dNTPs, 0.5 U of Deep Vent DNA polymerase (New England Biolabs) and 100 pM of sense and anti-sense primers, either for β-actin or for RPCH, dissolved in 50 mM KCl, 1.5 mM $MgCl_2$, 20 mM Tris–HCl, pH 9.4. A nested PCR reaction for RPCH was

Table 1
Primers sequence

Primer	Name	Sequence	mRNA	GenBank
1	upRPCHcs	5′ GTC TCG GCT CAG CTT AAC TTC TC 3′	RPCH	AF031654
2	lpRPCHcs	5′ GGC CTC GCT CCT GAT AAG TCG 3′	RPCH	AF031654
3	lpipRPCHcs	5′ CTC AGC CCA GGT ACT CCT CAT C 3′	RPCH	AF031654
4	lprace5RPCHcq	5′ ACG AAG GAT GGC ATG AGG AAG GGC 3′	RPCH	This work
5	lp5RPCHcq	5′ ACC CCT GCC GGG GGA GAA GTT AAG CTG 3′	RPCH	This work
6	uprace3RPCHcq	5′ CAG CTT AAC TTC TCC CCC GGC TGG GG 3′	RPCH	This work
7	uporfRPCHcq1	5′ CCA AGA ATG GTC CGC GGC AGT 3′	RPCH	This work
8	uporfRPCHcq2	5′ CTG GTG GCC TCC TCC TGC GTC TC 3′	RPCH	This work
9	lporfRPCHcq	5′ CGC ATT TAA CCC ATG TAT TCT TCT TCC 3′	RPCH	This work
10	upACpc	5′ GCT GTG CTG TCC CTG TAC G 3′	β-Actin	D14612
11	lpACpc	5′ GAT CTT CAT GAG GTA GTT GG 3′	β-Actin	D14612
12	uprace5ACcq	5′ ACG AAG GAT GGC ATG AGG AAG GGC 3′	β-Actin	This work
13	uporfACcq	5′ CAT GTG TGA CGA AGA GGA GCT G 3′	β-Actin	This work
14	lporfACcq	5′ TTA GAA GCA CTT GCG GTG GAC G 3′	β-Actin	This work
15	upACcq	5′ GAC TGA GCG TGG CTA CTC CTT CAC 3′	β-Actin	This work
16	lpACcq	5′ GAC TCG TCG TAC TCC TCC TTG GTG 3′	β-Actin	This work
17	uprRNA18	5′ CGA AAC CGC AAA CTG CTC ATT AAA CC 3′	rRNA 18S	AF235966
18	lprRNA18	5′ CTT CCT CTA AAT GAC CAA GTT TGG AC 3′	rRNA 18S	AF235966
19	lprace3polit	5′ GCG GCC GCA TGC TTA TAA TTA ATC $(T)_{24}$ 3′	–	This work
20	lpoltcutrace3	5′ GCG GCC GCA TGC TTA TAA TTA ATC 3′	–	This work

performed also. The products of the first PCR reaction were purified by silica-gel columns (Quiagen) as recommended by the supplier. Incubation was with 5% of PCR products and primers #1 and #3. Samples were incubated at 95 °C for 20 s, 50 °C for 20 s and 72 °C for 30 s for 25 cycles.

2.3.1.2. DNA cloning and sequence analysis. Amplification products were separated by agarose gel electrophoresis. They were excised and eluted from the agarose gels and they were subcloned in plasmid PCR 3.1 with TOPO isomerase (Invitrogen) as recommended by the supplier. The products were transformed into *E. coli* competent cells. DNA nucleotide sequences were determined by a deoxi sequencing kit (Biosystem Prysm Dye) using an ABI PRISM 310 Genetic Analyser (Applied Biosystems) automatic sequencer. Primary sequences were compared to other Gene Bank reported sequences by BLAST program.

2.4. RPCH and β-actin 5′ and 3′ end mRNA amplification

The 5′ RACE reactions were performed with a Gene Racer kit (Invitrogen) as recommended by the supplier. mRNA amplification for cDNA synthesis was performed with the Thermosuper Script kit (Invitrogene). Primer adaptors were #4 and #14 for RPCH and β-actin, respectively. The cDNA was RNase treated as above and purified by silica gel columns (Quiagen) as recommended by the supplier. The anti-sense primer #4 for the RPCH precursor was complementary to the cDNA codons and the primer for β-actin was #12. Samples were incubated for 35 cycles at 95 °C for 20 s, 65 °C for 20 s and 72 °C for 30 s. The PCR products were subcloned and sequenced as in Section 2.3.1.2.

For 3′ RACE reactions the anchor primer was #19, an oligonucleotide of 24 thymines with a 5′ poly-linker to restriction enzymes *Not*I, *Sph*I, *Psi*I, *Vsp*I, and *Ase*I. For cDNA synthesis Superscript II (Stratagene) was used, as recommended by the supplier, at 42 °C for 55 min. cDNA purification was as described for 5′ RACE. The PCR mix reaction was the same as for 18S RT-PCR. Single stranded cDNA was purified using silica-gel columns as suggested by the supplier. The sense primers #6 and #10 were used to amplify RPCH and β-actin, respectively. The anti-sense for both reactions was the poly-linker bound to #20. Amplification was for 35 cycles at 96 °C for 30 s, 65 °C for 20 s (RPCH) or 50 °C for 20 s (β-actin) and 72 °C for 1 min. Analysis of PCR products was as described above.

2.5. Semi-quantitative RT-PCR

RT-PCR semi-quantitative measurement of RPCH and β-actin were carried out with their amplified cDNA products. For RT-PCR of RPCH and β-actin, 0.75, 2, and 3 μg of total RNA were added to the reaction mixture. The expected products were monitored every three other cycles beginning at cycle 5. They were visualized in 1% agarose gels and their concentration was measured in pixels (Eagle Eye Stratagene). The RPCH segment amplified by RT-PCR was from position 124 (primer #8) to position 365 (primer #9) and that for β-actin was amplified from position 597 (primer #15) to position 1112 (primer #16). In both cases 2 U of *Taq* polymerase was used and cycles were for 30 s at 96 °C, 20 s at 60 °C and 25 s at 72 °C. 18S rRNA amplification was from position 59 (primer #17) to position 1791 (primer #18) [11]. The cDNAs relative concentration was calculated at exponential phase of synthesis using the β-actin curve as reference. The cDNA sequence was confirmed as in Section 2.3.1.2.

2.6. Phylogenetic analysis of the RPCH precursor and β-actin

The *C. quadricarinatus* RPCH cDNA primary structure sequence alignment was compared with the cDNA sequences of AKH I, II, and III of *L. migratoria* [7], the AKH I and II of *S. gregaria* [14,38], the AKH I and II of *S. nitans* [28] and the HTH from *Blaberus discoidalis* [21], *M. sexta* [8], *D. melanogaster* [27] and with the two available cDNA sequences of RPCH precursors from *C. maenas* [22] and *C. sapidus* [20].

The β-actin gene structures considered were those from cDNA primary structure sequence alignment and were compared with the shrimp *Peneus monodon* 1 and 2 "GenBank accession nos. AAC78682, AAC78681", the lobster *Homarus americanus* "GenBank accession no. AAK84871", *Artemia* sp. "GenBank accession no. X52602" and *Daphnia magna* "GenBank accession no. CAB99474". The precursor alignments were carried out using the program CLUSTAL W [40] with a 0.05 gap station and gap window p 9.

2.7. Statistical analysis

Values from each experimental group were assessed by fitting a single cosine function to the meted least squares; this analysis adjusts the data to a sinusoidal function and provides an objective test of whether the amplitude of the rhythm differs from zero. The cosine function used was: $y = M + A\cos(x + f)$, where y is the variable measured, M is the mesor (mean value of the rhythmic profile), A is the amplitude of the rhythm, x is the time of the day (expressed in radians, where 24 h = p rad) and f is the phase angle. A coefficient of determination (r^2) for nonlinear functions was used as an index of cosinor rhythmicity [5]. Analysis of the data was performed using the software ORIGIN [6].

3. Results

3.1. Product of DNA amplification

The RPCH gene from *C. quadricarinatus* was amplified with primers #1 and #2 (Table 1). The range of sizes covered by the amplification products was between 100 and 1500 bp.

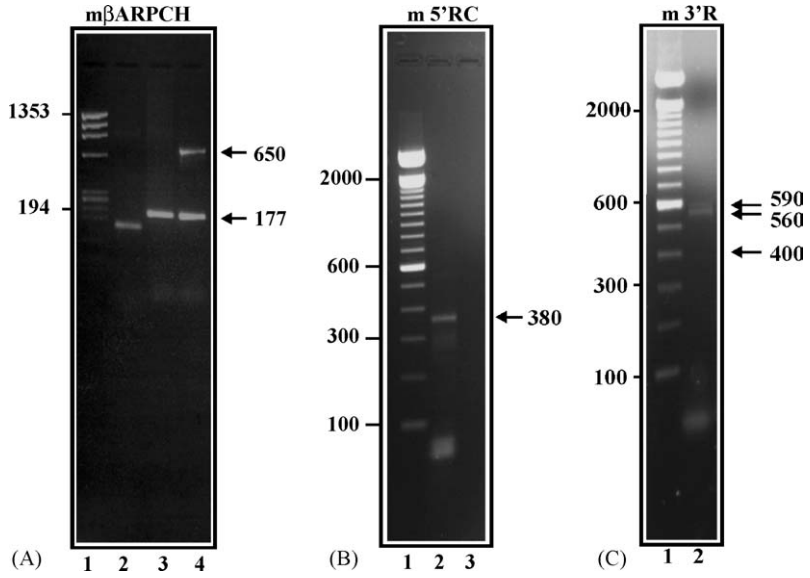

Fig. 1. Agarose gel electrophoresis of DNA products. (A) Control of genomic DNA amplification for β-actin (lane 2) and RPCH from *C. sapidus* (lane 3) and nested DNA stretch for *C. quadricarinatus* (lane 4). (B) 5′ RACE (lane 2) and (C) 3′ RACE (lane 2) reactions of the 177 bp RPCH product. Numbers at left are lambda *Hin*dIII (A) and DNA ladder length markers (B and C) and at right are DNA base pairs (bp). m: marker; βA: β-actin; c: control.

With these substrates, a nested PCR of RPCH was performed utilizing the primers #1 and #3. This new PCR reaction gave two well-defined DNA bands with lengths of 177 bp, for both *C. sapidus* (Fig. 1A, lane 3; control) and *C. quadricarinatus* (Fig. 1A, lane 4) and 650 bp for the cryfish (Fig. 1A, lane 4). The primary sequence of the *C. quadricarinatus* 177 pb RPCH fragment has 98% identity to the *C. sapidus* RPCH DNA "Bankit accession no. AF031654". The 650 bp DNA fragment was non-specific. A sample mixture of total RNA plus primers #4 and #5 was made and an aliquot was used for

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ctctgcaggcagcatccagcgtggtccagcaacaggcagcatcgactgcactcccttcgttaccacacactc    72

                Met Val Arg Gly Ser Val Ala Leu Leu Leu Val Val Leu Val     14
acccagagtccaaga ATG GTC CGC GGC AGT GTA GCC CTC CTG CTG GTG GTA CTG GTG    129

Ala Ser Ser Cys Val Ser Ala Gln Leu Ans Phe Ser Pro Gly Trp Gly Lys Arg     32
GCC TCC TCC TGC GTC TCC GCC CAG CTT AAC TTC TCC CCC GGC TGG GGC AAA CGG    183

Thr Gly Thr Ala Ala Gly Gly Pro Asp Gln Thr Ile Leu His Ser Ser Ser Pro     50
ACC GGT ACC GCG GCT GGG GGA CCT GAC CAG ACC ATC CTC CAC TCA TCC TCT CCC    237

Ala Ala Val Ala Ser Asp Asn Cys Gly Thr Ile Pro Val Ser  Ala Val Met His    68
GCA GCC GTA GCC AGC GAC AAC TGC GGC ACA ATC CCC GTC TCA GCC GTC ATG CAC    291

Ile Tyr Arg Leu Ile Arg Thr Gle Ala Ala Arg Leu Val Gln Cys Gln Glu Glu     86
ATC TAC AGG CTT ATT AGG ACT GAA GCA GCG AGA CTT GTA CAG TGT CAG GAA GAA    345

Glu Tyr Met Gly  *                                                        91
GAA TAC ATG GGT TAA  atgcgacacccccaccactaccactaccagcaaccaccatcaccaccacta   411
ccactaccagcaaccactctcgtcttagctaccagctgtgacagtcacactgctgtgttagttccccaggtc   483
ttcccctgaacacaagagatgtctcccatcgtcttcctctccccttctggcccagtggtgggctggggttta   555
gcctctcctggaattcatatatttttctcgtctttcggaaactcgtggggtggacaactggaatcagaagtg   627
(A) ttggaatgatgaataacat                                                    6
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Fig. 2. (A) RPCH *C. quadricarinatus* cDNA primary structure. The first 87 nucleotides correspond to the 5′-UTR sequence. Signal peptide (nucleotides 88–150), active peptide (nucleotides 151–177) and related peptide (nucleotides 184–357). The underlined letters indicate the upper #8 and lower #9 primers. The italic underlined letters denote the polyadenilation signal. The conceptual translation amino acids are indicated and the neuropeptide hormone is in bold italic letters. (B) β-actin. The first 12 nucleotides are the 5′ UTR sequence. Nucleotides in capital letters show the ATG initiation codon followed by the rest of the primary sequence up to the stop codon in nucleotide 1144. The 3′ non-translated region (nucleotide 1145 and thereafter) is in small letters. The two possible sites of polyadenilation (nucleotides 1159–1164 and 1171–1176) are shown in underlined-italic small letters. The upper #15 and lower #16 primers are in capital letters and underlined. The universal actin proteic signs of the conceptual translation polypeptide: actin1 (amino acids 55–65), actine-related protein sequence (amino acids 96–119) and actin2 (amino acids 357–365), are in bold-underlined letters.

```
                Met Cys Asp Glu Glu Glu Leu Thr Ala Leu Val Val Asp Asn Gly Ser Gly    17
cgaactaccatc ATG TGT GAC GAA GAG GAG CTG ACT GCC CTT GTG GTC GAC AAT GGC TCC GGC    63

Leu Cys Lys Ala Gly Phe Ala Gly Asp Asp Ala Pro Arg Ser Val Phe Pro Ser Ile Val     37
CTT TGC AAG GCC GGC TTC GCC GGA GAC GAC GCT CCC CGT TCT GTC TTC CCC TCC ATC GTC    123

Gly Arg Ala Arg His Gln Gly Val Met Val Gly Met Gly Gln Lys Asp Ala Tyr Val Gly     57
GGC CGC GCC CGT CAC CAG GGT GTG ATG GTC GGT ATG GGT CAG AAG GAC GCC TAT GTT GGC    183

Asp Glu Ala Gln Ser Lys Arg Gly Ile Leu Thr Leu Asn Tyr Pro Ile Glu His Gly Ile     77
GAT GAG GCC CAG AGC AAG CGT GGT ATC CTC ACC CTC AAC TAC CCC ATT GAA CAC GGT ATC    243

Ile Thr Asn Trp Asp Asp Met Glu Lys Ile Trp Tyr His Thr Phe Tyr Asn Glu Leu Arg     97
ATC ACC AAC TGG GAT GAC ATG GAG AAG ATC TGG TAC CAT ACT TTC TAC AAT GAG CTC CGT    303

Val Ala Pro Glu Glu Ser Pro Thr Leu Leu Thr Glu Ala Pro Leu Asn Pro Lys Ala Asn    117
GTT GCC CCC GAG GAG TCC CCC ACA CTT CTC ACT GAG GCT CCC CTC AAC CCC AAG GCC AAC    363

Arg Glu Lys Met Thr Gln Ile Met Phe  Glu  S Phe Asn Val Pro Ala Thr Tyr Ile Thr    137
CGT GAG AAG ATG ACT CAG ATC ATG TTC GAG TCC TTC AAT GTA CCT GCC ACT TAC ATT ACC    423

Ile Gln Ala Val Leu Ser Leu Tyr Ala Ser Gly Arg Thr Thr Val Leu Val Cys Asp Ser    157
ATC CAG GCT GTG CTC TCC CTC TAC GCC TCT GGT CGT ACC ACT GTT CTG GTG TGC GAC TCT    483

Gly Asp Gly Val Thr His Met Val Pro Val Tyr Glu Gly Phe Ala Leu Pro His Ala Ile    177
GGT GAT GGT GTC ACT CAC ATG GTC CCC GTG TAT GAA GGT TTC GCC CTT CCT CAT GCC ATC    543

Leu Arg Leu Asp Leu Ala Gly Arg Asp Leu Thr Gln Tyr Leu Met Lys Ile Met Thr Glu    197
CTT CGT CTT GAC CTT GCT GGT CGT GAC CTG ACC CAG TAT CTC ATG AAG ATC ATG ACT GAG    603

Arg Gly Tyr Ser Phe Thr Thr Thr Ala Glu Arg Glu Ile Val Arg Asp Ile Lys Glu Lys    217
CGT GGC TAC TCC TTC ACC ACC ACA GCT GAG CGT GAG ATT GTC CGT GAC ATC AAG GAG AAG    663

Leu Cys tyr Ile Ala Leu Asp Phe Glu Asn Glu Met Asn Val Ser Ala Ala Ser Ser Ser    237
CTT TGC TAC ATT GCC CTT GAC TTC GAG AAT GAG ATG AAT GTA TCT GCA GCT TCT TCG TCT    723

Ile Asp Lys Ser Tyr Glu Leu Pro Asp Gly Gln Val Ile Thr Ile Gly Asn Glu Arg Phe    257
ATT GAC AAG TCC TAC GAG CTT CCC GAC GGT CAG GTC ATC ACC ATT GGT AAT GAA CGC TTC    783

Arg Cys Pro Glu Ala Leu Phe Gln Pro Ser Phe Leu Gly Met Glu Ser Ala Gly Val His    277
CGT TGC CCC GAG GCT CTG TTC CAG CCT TCC TTC CTT GGT ATG GAA TCT GCT GGT GTT CAT    843

Glu Thr Val His Ser Ser Ile Met Arg Cys Asp Ile Asp Ile Arg Lys Asp Leu Phe Ala    297
GAA ACC GTC CAC AGC TCC ATC ATG AGG TGC GAC ATT GAC ATC AGG AAG GAC CTG TTC GCC    903

Asn Ile Val Met Ser Gly Gly Thr Thr Met Tyr Pro Gly Ile Ala Asp Arg Met Gln Lys    317
AAC ATT GTC ATG TCT GGT GGT ACC ACC ATG TAC CCT GGT ATT GCT GAC CGC ATG CAG AAG    963

Glu Ile Thr Ala Leu Ala Pro Ala Thr Ile Lys Ile Lys Ile Ile Ala Pro Pro Glu Arg    337
GAA ATC ACT GCT CTG GCT CCT GCT ACC ATC AAG ATC AAG ATC ATT GCT CCT CCC GAG CGT   1023

Lys Tyr Ser Val Trp Ile Gly Gly Ser Ile Leu Ala Ser Leu Ser Thr Phe Gln Thr Met    357
AAG TAC TCC GTC TGG ATC GGT GGT TCC ATC CTG GCC TCT CTG TCC ACC TTC CAG ACC ATG   1083

Trp Ile Thr Lys Glu Glu Tyr Asp Glu Ser Gly Pro Gly Ile Val His Arg Lys Cys Phe    377
TGG ATC ACC AAG GAG GAG TAC GAC GAG TCC GGC CCA GGC ATC GTC CAC CGC AAG TGC TTC   1143

 *                                                                                 378
(B) TAA  atactcgatcgg aataaa tgtttt aataaa ctgttttcgaatacga                        1192
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Fig. 2. (*Continued*) .

5′ RACE. The cDNA reaction product has 380 bp in length (Fig. 1B), and it is 92% identical to the *C. sapidus* DNA primary sequence for the pre-pro-peptide of the RPCH endoplasmic reticulum signal structure (Fig. 2A). For 3′ RACE primer #6 was used. Three DNA fragments of 400, 560 and 590 bp were obtained (Fig. 1C). The first two fragments do not correspond to RPCH; the 400 bp cDNA fragment is 98% identical to ribosomal protein L-23 from *H. sapiens* "Bankit accession no. 633162" and the 560 bp cDNA is 60% and 57.3% identical to the PDH mRNA of the shrimps *M. japonicus* "GenBank accession no. AB073367" and *P. vannamei* "GenBank accession no. Y117231", respectively. Surprisingly, the conceptual translation of this fragment is identical to a human DNA sequence located in chromosome 1 "GenBank accession no. 15131847", and 50% homologous to the Hipertrehalosemic hormone of the cockroach *B. discoidalis,*

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AKH2 S.gre  MRQSCALTLMLVVAVCA---ALSAAQLNFSTGWG----------RRYADPNA-------- 39
AKH2 S.nit  MRQGCALTLMLLVVVCA---ALSAAQLNFSTGWG----------RRYADPNA-------- 39
AKH2 L.mig  MTQSCTLTLVLVVAVLA---ALATAQLNFSAGWG----------RRYADPNA-------- 39
AKH  S.gre  MVQRCLVVALLVVVVAA---ALCSAQLNFTPNWGT--------GKRDAADFG-------- 41
AKH  S nit  MVQRCLVVALLVVVVAA---ALCSAQLNFTPNWGT--------GKRDAGDYG-------- 41
AKH  L.mig  MVQRCALVVLLVVAVAA---ALCSAQLNFTPNWGT--------GKRDAADFA-------- 41
RPCH C.mae  MVRRTGVTLLVVALVVVALVSSVSAQLNFSPGWG----------KRAAAGSGSSGGVGEA 50
RPCH C.sap  MVRRSGVTLLVVALLVVTLMSSVSAQLNFSPGWG----------KRAAGASGSNGGVGEA 50
RPCH C.qua  MVRGS-VALLLVVLVAS---SCVSAQLNFSPGWG----------KRTGTAAG-----GPD 41
AKH  M.sex  MYKLT-VFLMFIAFVII-----AEAQLTFTSSWG----------GKRAMTNS-------- 36
AKH3 L.mig  MQVRAVLVLAVVALVAV---ATSRAQLNFTPWWGK--------RALGAPAAG-------- 41
AKH  B.dis  MNHLVKVLIVVVAIALVLC----EAQVNFSPGWG--------TGKRSAVQNS-------- 40
AKH  D.mel  MNPKSEVLIAAVLFMLLACV---QCQLTFSPDWGKRSVGGAGPGTFFETQQG-------- 49
            *                          *  *   **
AKH2 S.gre  ---------------------------------DPMAFLYKLIQIEARKLSGCSN------  61
AKH2 S.nit  ---------------------------------DPMAFLYKLIQIEARKLAGCSN------- 61
AKH2 L.mig  ---------------------------------DPMAFLYRLIQIEARKLAGCSD------- 61
AKH  S.gre  ---------------------------------DPYSFLYRLIQAEARKMSGCSN------- 63
AKH  S.nit  ---------------------------------DPYSFLYRLIQAEARKMSGCSN------- 63
AKH  L.mig  ---------------------------------DPYSFLYRLIQAEARKMSGCSN------- 63
RPCH C.mae  VSALHHSVGGAPGGVVPPGSSSSSGDSCGPIPVSAVMHIYRLIRNEAVRLVQCQDEEYLG- 110
RPCH C.sap  VSGLHPSVGGAPGGVVPPGSSSP-GDSCGPIPVSAVMHIYRLIRSEAVRLVQCQDEEYLG- 109
RPCH C.qua  QTILHSSS---------PAAVAS--DNCGTIPVSAVMHIYRLIRTEAARLVQCQEEEYMG-- 90
AKH  M.sex  -------------------------ISCRND--EAIAAIYKAIQNEAERFIMCQKN------ 65
AKH3 L.mig  -------------------------DCVSASPQALLSILNAAQAEVQKLIDCSRFTSEANS 75
AKH  B.dis  -------------------------PCKGS-AESLMYIYKLVQNEAQKILECEKFSSN--- 72
AKH  D.mel  -------------------------NCKTS-NEMLLEIFRFVQSQAQLFLDCKHRE----- 79
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Fig. 3. The *C. quadricarinatus* RPCH-precursor amino acid composition (in bold letters) and other known AKH/RPCH family members are compared. The cysteine needed for dimmer formation is in italics and underlined. Asterisk (*) stands for conserved amino acids.

"GenBank accession no. Q17128", suggesting that the 560 bp fragment may represent a new member of the RPCH/AKH family "Bankit accession no. 633102". The third 590 bp fragment is 88% identical to the active peptide and to the last portion of the related peptide cDNAs of the RPCH from *C. sapidus* [19] and *C. maenans* [21] (Figs. 2A and 3). Its conceptual translation gives rise to the eight amino acids of the RPCH, followed by the endopeptidase-sensitive KR dipeptide, the RPCH related peptide and the cysteine in position 83 (Fig. 2A) involved in dimerization and common to all precursors of the AKH/RPCH family. When all sequences are joined, an mRNA of 645 bp is obtained, "Bankit accession no. 625501", in which the first 111 bp belong to the 5′-UTR, followed by the 210 bp of the ORF and ending at the 300 bp of the 3′-UTR (Fig. 2A). It should be pointed out that for unclear reasons, amplification of the complete ORF of the RPCH was not possible, but amplification from codon 13 to the stop codon renders the expected 242 bp cDNA product (Fig. 4). The conceptual translation of this cDNA fragment corresponds to the amino acid primary sequence of the *C. quadricarinatus* RPCH precursor which is 70% homologous to that of *C. sapidus* [20] and *C. maenas* [22].

The β-actin primers #10 and #11 (Table 1) amplified a DNA segment of 165 bp (Fig. 1A, lane 2) that is 92% homologous to the *P. clarkii* protein sequence. With the primers for 5′ and 3′ RACE reactions (#12 and #10, respectively) two PCR products were obtained: the first one of 515 bp for the 5′ end, and the second one of 748 bp for the 3′ end (results not shown). The primers #13 and #14 were used to amplify the 1135 bp β-actin ORF (Fig. 4A). The first 13 nucleotides correspond to the 5′-UTR region, the nucleotides 10–12 are the AUG translation initiation codon and the conceptual ORF has 378 codons in length (Fig. 2B). The ORF conceptual translation reveals a protein of 377 amino acids with the three universal actin proteic signs, namely, actin1, the actin-related protein sequence, and actin2, in positions 55–65, 106–118 and 358–366, respectively (Fig. 2B). Two possible sites of polyadenilation are detected, in positions 1159–1165 and 1171–1177 (Fig. 2B), "GenBank accession no. AY430093".

3.2. Changes in RPCH and β-actin mRNA concentration

The presence of RPCH and β-actin mRNAs was searched for by RT-PCR amplification in different organs like the medulla, the hepatopancreas, the gallnuts and the muscle of *C. quadricarinatus*. It was important to verify our method of RNA extraction by detecting those mRNAs in the 3 h RNA samples. Since by chance, at any one time point, the amount of these mRNAs could be normal, low or absent, we first searched for them in a 24 h small pool mixture of the 3 h RNA sample collection of each organ. As expected, the results show cDNA fragments of 242 and 1135 bp for RPCH and β-actin, respectively (Fig. 4A and B). The specificity of the assay is shown in the same figures: the RPCH mRNA is detected, exclusively, in the ocular eyestalk, even after 35

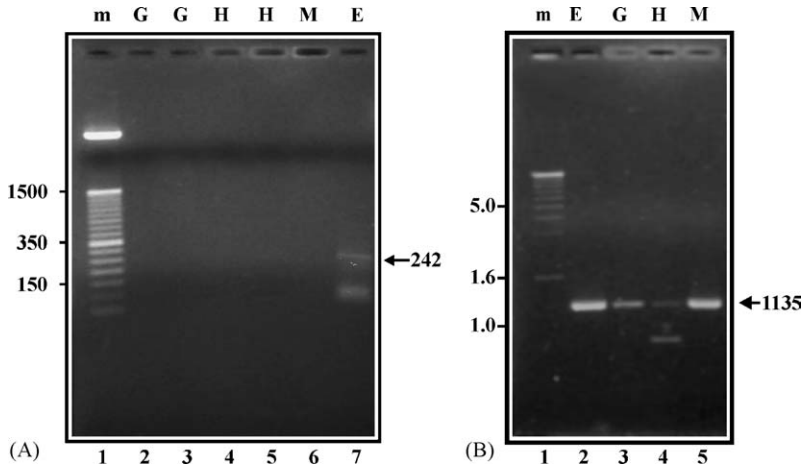

Fig. 4. Agarose gel electrophoresis of the ORF region for the RPCH-precursor and β-actin. (A) *C. quadricarinatus* RT-PCR amplification of the eyestalk (E), the gallnuts (G), the hepatopancreas (H) and the muscle (M) substrates for 27 (lanes 2, 4 and 7) or 35 cycles (lanes 3, 5 and 6) (primers #8 and #9). (B) RT-PCR amplification for 27cycles (primers #13 and #14). Left side numbers are DNA ladder markers (m) and at the right side are base pairs (bp).

cycles of amplification (Fig. 4A), whereas the β-actin mRNA is present in all organs. On the other hand, it was important for the semi-quantitative RT-PCR measurements to determine the number of rounds needed for the quantification curve to reach the midpoint of the exponential phase. They were 27 and 20 for the RPCH and β-actin mRNAs, respectively.

The eyestalk chronograms of the amount of mRNA are shown in Fig. 5. For β-actin, the coefficient of determination of non-linear functions according to the single cosinor method in animals exposed to 12 h:12 h cycles of light/dark, gives the following values for a 24 h time period: mesor = 9.5 pixels/μg wet tissue (S.E. 0.208), amplitude 0.78 pixels/μg wet tissue (S.E. 0.291; *P* value of 0.048), acrophase = 22.4 h and percentage of rhythmicity of 15 (Fig. 5A). A similar analysis of β-actin mRNA for animals kept in continuous darkness could not be made (Fig. 5B) suggesting that more experimental data are needed or that alternate models are to be considered. Single cosinor analysis of the experimental mRNA data for RPCH in animals exposed to 12 h:12 h cycles of light/dark gives the following values for a 24 h time period: mesor = 12.15 pixels/μg wet tissue (S.E. 0.797), amplitude = 3.06 pixels/μg wet tissue (S.E. 1.103; *P* value of 0.22), acrophase = 16.15 h and percentage of rhythmicity of 19 (Fig. 5A). In contrast, when the animals were kept in continuous darkness the mRNA concentration for RPCH was bimodal (Fig. 5D) with periods of 7.26 h in length whose crests were at 9 h (14.00 h real time) and 15 h (20.00 h real time) after the initiation of the cycle.

4. Discussion

Experimental evidence has been gathered on the association between RPCH and its rhythmical concentration oscillations in the eyestalk peduncle of the crayfish [34,36], its circadian clock function [3], its regional distribution and immunological localization [35], its content in the sinus gland from which it is released to the hemolymph [36], and its liberation by single XO peptidergic neurons co-cultured with tegumentary erythrophores [1]. However, little is known on the regulation and biosynthesis of RPCH at the DNA/RNA level, mainly, because primary sequences of these nucleic acids in the crayfish are largely unknown. In fact, the β-actin and RPCH cDNA sequences described in this paper are the first ones, as far as we know, to be reported for the crayfish *C. quadricarinatus* and many other crustacean species.

The nucleic acid similitude between *C. quadricarinatus* and other crustaceans and insects can be established after comparison of the RPCH and β-actin cDNA sequences to those of the AKH/RPCH family. So, primary cDNA sequence comparison of the β-actin of *C. quadricarinatus* to the same molecule of the shrimp *Penaeus monodon* "GenBank accession no. AAC78682.1", the lobster *H. americanus* "GenBank accession no. AAK84871.1", *D. magna* "GenBank accession no. CAB99474.1" and *Artemia* sp. "BenBank accession no. S11450" gave identities of 94%, 87%, 88%, 86%, respectively (Fig. 3). In the same context, the estimated identity of the *C. quadricarinatus* RPCH precursor to that of the AKH I and II of the crickets *S. gregaria* and *S. nitans*, the AKH I of *L. migratoria* and the AKH III of *L. migratoria* and *D. melanogaste*r, is about 61.7%, 48%, 41% and 36% respectively (Fig. 3). Interestingly, the predicted peptide of the *C. quadricarinatus* 590 bp cDNA RPCH fragment (Fig. 3) has 50% homology to the Hypertrehalosemic hormone of the cockroach *B. discoidalis*, "Bankit accession no. 633102". In contrast, the RPCH translatable product of the 242 bp cDNA fragment from *C. quadricarinatus* shows low homology to the crab RPCH from *C. sapidus* and *C. maenas*, which can be explained by the fact that the former has 20 fewer amino acids, 7 in the signal peptide and 17 in the related peptide (Figs. 2A and 3).

Altogether, the release of RPCH from the sinus gland to the hemolymph [36], the activity of this neurohormone, tested by bioassay, as a means to identify RPCH secreting neurons

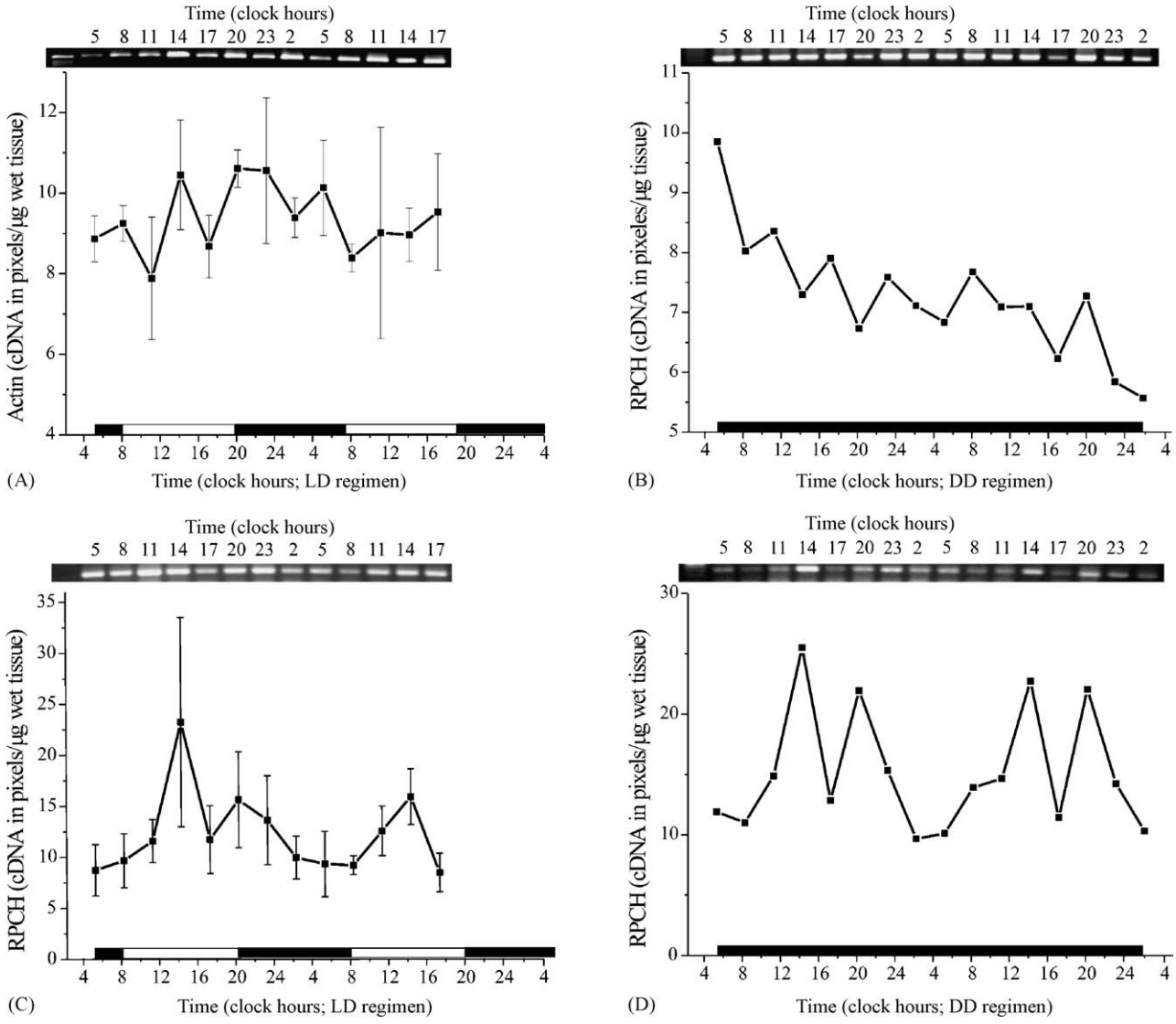

Fig. 5. β-Actin (A and B) and RPCH precursor (C and D) cDNA relative concentration. *C. quadricarinatus* exposed to 12 h:12 h cycles of light/dark for 2 days (A and C) or to absolute darkness for 3 weeks (B) and 16 weeks (D). The intensity and length size (bp) of each one of the experimental cDNA bands in the gel is shown above each graph. Numbers above the gel photograph stand for real time and numbers in the abscissa stand for time after the experiment began. Daytime is depicted by clear rectangles and absolute darkness by black rectangles. Lines between squares are experimental data.

responsible for inducing retraction of erithrophores [1] and the oscillations of the RPCH mRNA (Fig. 5C and D), indicate RPCH secretion. Although we have no experimental evidence on the influence of E boxes in the transcription/translation RPCH mRNA control mechanisms, as evidence for neuropeptide regulation by means of the main clock genes, indirect evidence of this influence is the expression of the RPCH mRNA molecule that changes from light/dark circadian cycles to bimodal dark ultradian cycles (Fig. 5C and D). Therefore, RPCH is entrained by light since the cells have not been exposed to another Zeitgeber. The electroretinogram response is dose-dependent in RPCH injected *P. clarkii* crayfish, where the threshold dose of the neurohormone is five times higher than that for light adapted animals [17]. Accordingly, the results of this work show that the amplitude of this change in *C. quadricarinatus* is 2.45 greater in the dark than in the light, similar to the response of *P. clarkii* whose period length is also larger in the dark [36]. At the molecular level, the RPCH content is around two times higher in animals kept in constant darkness than in animals exposed to light/dark cycles (Fig. 5C and D), similar to previous findings of this laboratory [36,3]. In summary, the role so far demonstrated for RPCH in the circadian cycle is of an output molecule [9].

In the basal membrane of the ocular peduncle of the crayfish *P. clarkii*, immunopositive molecules similar to PER have been recently reported [3]. Although the PER protein itself must be demonstrated, it is very likely that the main pacemakers of the circadian clock are present not only in *P. clarkii* but also in *C. quadricarinatus* and in all crustacean decapods.

The timing schedule for tissue sample collection is important. We found that 4 h schedules for tissue sampling, instead of every 3 h, results in patterns of circadian cycles, both in light/dark schedules and in total dark schedules (results not shown). The RPCH mRNA is not the only one that behaves in a circadian fashion in *C. quadricarinatus*. Modulation of 18S rRNA and G3PDH mRNA, which change from ultradian to circadian cycles, has been recently detected in our laboratory (Martinez-Perez et al., in preparation). It will be interesting to correlate which neurons generate the circadian oscillation and if they are the same ones that control RPCH biosynthesis.

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