Characterization of Insulin-Like Peptides and Their Relation to Molt and Metabolism of the Shrimp *Litopenaeus vannamei*

Ana Gutiérrez[1,3], Julie Nieto[2], Francisco Pozo[1], Luc VandenBosch[3], Sofie Van Soest[3], Liliane Schoofs[3]

[1] Centro Nacional de Acuicultura e Investigaciones Marinas, P.O. Box 09-01-45-19, Guayaquil, Ecuador.

[2] OMARSA Aquaculture Group, Processing Plant division. Lotización Industrial Al Río Lote 3, Zona El Recreo, Duran 11495, Ecuador.

[3] Department of Animal Physiology and Neurobiology, Zoological Institute K. U. Leuven, Naamsestraat 59, B-3000 Leuven, Belgium

(Correspondence should be addressed to L. Schoofs, email: Liliane.Schoofs@bio.kuleuven.be)

Abstract

Insulin-like peptides are key regulators of metabolism, reproduction, and senescence in higher eukaryotic organisms. They have been described in different invertebrates, including nematodes, molluscs, and insects. Insulin-like peptides have also been detected in some decapod crustaceans, where they may display typical insulin effects. On the basis that common biological function may be related to a similar structure, we searched for an insulin-related substance in the shrimp *Litopenaeus vannamei* and further investigated its function. An IGF-I-like molecule was detected in neurosecretory cells of the brain and thoracic ganglion in shrimps at different molt stages. Furthermore, we demonstrated that ILP did not differentially stimulate ecdysteroid secretion as it is observed in some insect species. An *in vivo* assay was performed and the results revealed that injections of heterologous insulin/IGF-I increased the glycogen contents of gills and digestive gland from intermolt animals (fasting state), suggesting that an endogenous ILP may display a conserved function in decapods. Moreover, cloning and sequencing of a cDNA from hemocytes revealed that the sequence code for a precursor similar to the Insulin-like Growth Factor binding proteins. A quantitative real-time RT-PCR study revealed the presence of LivIGFBP-related peptide transcript in a variety of tissues, including nervous tissues, gills, and muscle, brain, thoracic ganglion and nerve cord. In addition, the expression of this transcript differs according to the animal's size, suggesting a possible role in growth regulation.

Gutiérrez, A., Nieto, J., Pozo, F., VandenBosch, Van Soest, S y L. Schoofs. 2008. Characterization of Insulin-Like Peptides and Their Relation to Molt and Metabolism of the Shrimp *Litopenaeus vannamei*. 424 - 454 pp. Editores: L. Elizabeth Cruz Suárez, Denis Ricque Marie, Mireya Tapia Salazar, Martha G. Nieto López, David A. Villarreal Cavazos, Juan Pablo Lazo y Ma. Teresa Viana. Avances en Nutrición Acuícola IX. IX Simposio Internacional de Nutrición Acuícola. 24-27 Noviembre. Universidad Autónoma de Nuevo León, Monterrey, Nuevo León, México

Introduction

Insulin is the best-documented peptide hormone and its failure to regulate carbohydrate metabolism in humans results in diabetes. Type II diabetes mellitus is the most common metabolic disorder worldwide. It was first discovered by Banting and Best in 1921. Since then, its structure and function have been described in unicellular eukaryotes as well as in primitive species as insects, tunicates, annelids, and molluscs (LeRoith *et al*., 1980; Smit *et al*., 1993; Duret *et al*., 1998; Claeys *et al*., 2002; Olinski *et al*., 2006). Despite the broad magnitude of functional divergence present within the family, all proteins of the insulin family exhibit a high degree of structural conservation.

In mammals, the insulin gene superfamily includes insulin, insulin-like growth factors (IGF-I and IGF-II), relaxin (1, 2, 3) and insulin-like peptides (INSL 3-7) (Burkhardt *et al*., 1994; Chassin *et al*., 1995; Koman *et al*., 1996; Conklin *et al*., 1999; Lok *et al*., 2000; Bathgate *et al*., 2002). Insulin-like growth factors (IGFs) and insulin are closely related hormones that perform unique roles in the induction of cellular hypertrophy. Depending on the type of stimulation (either by IGF-I or by insulin), this cellular hypertrophy has different consequences. IGF enhances cell hypertrophy that is necessary for cell survival, proliferation and differentiation, while insulin enhances cell hypertrophy in order to increase nutrient stores (McCusker, 1998).

Studies in the model invertebrates *Drosophila melanogaster* and *Caenorhabditis elegans* have shown that the insulin pathway regulates development, longevity, metabolism, and female reproduction (Tatar *et al*., 2001; Gerisch *et al*., 2001; Ikeya *et al*., 2002; Tatar *et al*., 2003; Wu and Brown, 2006).

Increasing evidence suggests that insulin-related peptides and their putative receptors are also present in crustaceans (Sanders, 1983a; Lin *et al*., 1993; Chuang and Wang, 1994; Chang *et al*., 1995; Gallardo *et al*., 2003). It has been demonstrated that they may regulate glucose uptake and glycogenesis in the muscle and hepatopancreas (Davidson *et al.*, 1971; Sanders, 1983b; Richardson *et al.,* 1997; Gallardo *et al*., 2003). Moreover, an insulin-like androgenic gland (IAG) factor was recently identified in the crayfish *Cherax quadricarinatus* by screening a cDNA library generated from the same tissue (Manor *et al*., 2007). The deduced product has a linear and a three-dimensional organization similar to those of members of the insulin/insulin-like growth

factor family and its exclusive expression in the male-specific endocrine gland suggests that insulin may regulate sexual differentiation.

In addition to insulin-like factors, peptides displaying significant similarity with the insulin-like growth factor (IGF) binding proteins have also been identified in insects (Claeys *et al*., 2003; Badisco *et al*., 2007) and mollusks (Weiss *et al*., 2001). The IGFBP is group of binding proteins unique to the IGFs, they serve as transport, store and modulate the bioavailability of the IGFs to their ligands (Shimasaki *et al*., 1991). Two of the related peptides identified in invertebrates, named perlustrin and neuroparsin actually bind to vertebrate insulin/IGF-I (Weiss *et al*., 2001) and to the locust (*Schistocerca gregaria*) insulin-like peptide, respectively (Badisco *et al*., 2008). Furthermore, expressed sequence tags (EST) from shrimp and from other arthropods have been found to code peptides similar to mammalian IGF binding proteins and the insect neuroparsin (Claeys *et al*., 2003; Badisco *et al*., 2007). Hence insulin/IGF binding proteins are likely to be expressed in crustaceans.

Altogether, these previous studies confirm that insulin-like peptides are present in crustaceans and some of their function may be conserved. Therefore, based on our interest in studying the role of insulin-like peptides in the shrimp physiological processes, we were eager to identify the ILP of *Litopenaeus vannamei* and to analyze its effects on ecdysteroidogenesis and carbohydrate metabolism. In addition, we clone an IGFBP-related precursor and analyze possible tissue, size and molt stage-dependent differences in its transcript levels.

Materials and Methods

Animals

L. vannamei juveniles (10 g) were obtained from CENAIM's experimental field station (Palmar, Ecuador). Molt stages of postmolt (AB), intermolt (C), early premolt (D0), middle premolt (D1) and late premolt (D2) were selected for the experiments.

Immunocytochemistry

Brains, thoracic ganglion, abdominal muscle and complete cephalothorax of *L. vannamei* juveniles were dissected and immersed in (or injected with) a Bouin Hollande's (10%) sublimate solution (24 h). Fixed samples were rinsed with distilled water (12 h), dehydrated in an ethanol

series, cleared in xylol and embedded in paraplast. Alternating sections of 4 µm were made with a LKB Historange glass microtome (LKB, Stockholm, Sweden). For cephalothorax samples, sections were made with a Shadon AS325 microtome. Tissue sections were treated with the following primary antibodies: rabbit anti A-chain of DILP (Cao and Brown, 2001) and Lom-IRP (Riehle *et al*., 2006) 1:500 dilution; guinea pig anti-bovine insulin and goat anti recombinant human insulin-like growth factor (rhIGF-I) (Sigma-Aldrich, USA). Samples treated with the insect ILP antiserum were processed using the peroxidase anti-peroxidase (PAP) method (Vandesande and Dierickx, 1976). Tissue sections treated with the commercial antisera were deparaffinized in histosol, hydrated in 100% alcohol and washed in phosphate-buffered saline (PBS) pH 7.4 containing 0.1% Triton X-100. To reduce non-specific reactivity, all tissue sections were incubated with PBS-0.1% Triton containing 20% normal serum and 3% of skimmed milk, for 45 min at room temperature in a humidified container. Sections were rinsed in PBS-0.1% Triton and then incubated overnight at room temperature with anti-bovine insulin (1:200) or anti-rhIGF-I (1:50, 1:100, 1:200, and 1:500). Then, sections were rinsed in PBS-0.1% Triton and incubated for 1 h at room temperature with the secondary antibodies conjugated to horseradish peroxidase (1:100). Sections were then rinsed in PBS as before and followed by 5 min equilibration in a Tris solution (0.05 M Tris-HCl, pH 7.2) prior substrate reaction. Peroxidase activity was demonstrated with DAB (Sigma) and 0.1% H_2O_2 in Tris for 5 min.

Controls for the specificity of the reactions were performed by (1) replacement of the primary antiserum with non-immune serum to act as negative control. Normal serum from rabbit was used instead of insects ILP antiserum, normal serum from guinea pig for the bovine insulin antiserum and normal serum from goat instead of rhIGF-I antiserum. (2) Preabsorption of antisera against purified peptide (1 or 2 µg/ml for 48 h at 4˚C) prior to tests on tissue sections.

Synthetic peptides preparation

Bovine insulin and recombinant human IGF-I (rhIGF-I) were purchased from Sigma (St. Louis, MO). Both peptides were prepared reconstituted following manufacturer's instructions. For *in vitro* assay, peptides were serially diluted in culture medium (0.1 nM –10 µM for insulin and 0.001 – 1 nM for rhIGF-I) and for the *in vivo* assay, peptides were diluted in phosphate buffered saline (pH 7.4).

Y-organ in vitro culture and ecdysteroid quantification

In vitro culture of Y-organs was performed following the conditions described previously by Blais *et al.* (1994). In order to determine the effect synthetic insulin-like peptides on ecdysteroid synthesis, shrimp Y-organs were placed in 200 µl of culture medium in 96 microplates and incubated at room temperature. The paired-glands from each animal served as treated and control. First, tissues were pre-incubated for 1 hour in fresh medium. Then, one of the glands was transferred to medium containing different peptide concentrations and the other one to fresh medium for 2 hours. Y-organs were then transferred to fresh medium and incubated for an additional hour. Finally, the tissues were discarded and the culture media were stored at -20 ˚C until analysis. The amount of ecdysteroids was determined from the first and last incubation hours. Values were denominated as $B_{treated}$ - $B_{control}$ and $A_{treated}$ - $A_{control}$. Each treatment had 8 replicates.

Ecdysteroids were quantified using a competitive enzyme immunoassay (EIA) described by Porcheron *et al*., (1989). The antiserum (AS 4919) was obtained from Prof. P. Porcheron (Université Pierre et Marie Curie, France) and the enzymatic tracer (20E coupled to peroxidase) was a gift from Prof. J-P. Delbecque (Université Bordeaux I, Bordeaux, France). 20-hydroxyecdysone (20E) was used as the standard in calibration curves. The results are expressed as a delta value of ecdysteroid concentration between T2 (last incubation hour) and T1 (first incubation hour) for treated and control Y-organs. This calculation was followed to evaluate a post-treatment effect. A factorial analysis of variance (ANOVA) followed by a Duncan's multiple comparison tests was used to determine significant difference between effects on molt stages. The level of significance was set at 95%.

Insulin/IGF-I administration

Animals at intermolt stage were selected by microscopical examination of the edge of a uropod (Robertson *et al*., 1987). Experimental set up, glucose and glycogen determination is described in Gutierrez *et al*. (2007). Animals were injected with insulin/IGF-I/PBS-BSA (0 or 0.1 µg/g BW). Hemolymph and tissue samples were taken at 0 hour (no injection), 1, 3 and 5 hours after injection to determine changes in glucose levels and glycogen content.

Preparation of RNA and cDNA

Animals (reared in the same pond) were separated into two groups according to their weight, 10-12 g as normal-size animals and 6-8 g as small animals. Hemolymph samples were taken ventrally from the first abdominal segment. Sterile insulin (26 ½ Gauge) syringes pre-loaded with 100 µl of 10% sodium citrate (anticoagulant) were used to extract 600-1000 µl per animal. Samples from each size and molt stage groups were pooled and centrifuged for 10 min at 3.500 rpm and 4˚C to eliminate the supernatant. Hemocytes pellets were washed twice with a marine anticoagulant solution (0.45 M NaCl, 0.1 M glucose, 30 mM sodium citrate, 26 mM citric acid, 10 mM EDTA, pH 4.6; Gross *et al*., 2001) and then stored in RNAlater solution (Ambion). Shrimp tissues were dissected and immediately collected in RNAlater solution (Ambion) to prevent degradation. Pooled tissues from 10 animals were incubated overnight at 4˚C, and then stored at -20˚C until further processing. Samples were transferred to reaction tubes containing Green Beads and homogenized in a MagNA Lyser instrument (Roche). Next, total RNA was extracted from the homogenates using the RNeasy Lipid Tissue Mini Kit (Qiagen). During the extraction procedure, a DNase treatment (RNase-free DNase set, Qiagen) was applied to eliminate genomic DNA contamination. RNA samples were quantified and verified for quality via the Agilent 1200 Bioanalyser (Agilent Technologies). For first-strand cDNA synthesis, 1 µg of total RNA was reverse transcribed (ReverAid H minus M-MuLv, Fermentas) using random hexamers as described in the provided protocol.

Cloning of the Liv-IGFBP cDNA

Oligonucleotide primers for PCR were based on the expressed sequence tag BE188516 (PvB 375 L99-22 Litopenaeus vannamei cDNA, NCBI EST database) similar to an Insulin-like Growth Factor Binding Protein as assigned by the submitter (Gross *et al*., 2001). The selected primers flanked the sequence of the putative open reading frame in this EST.

→ 5’ CGGCGAGAGCAACATGAAGAC 3’

← 5’ GTAACATCATCATGAAACCTTGTCC 3’

Aliquots of 1 µl of hemocyte cDNA template (1/10 diluted) was amplified in a 50 µl reaction, containing 5 µl 10 X PCR buffer (provided by the manufacturer), 0.2 µM each dNTP, 0.2 µM

each primer, and 0.5 µl of the Advantage 2 Polymerase mix (Clontech). PCR was performed in a thermocycler (Biometra, Göttingen, Germany) under the following parameters: an initial denaturation (3 min, 95˚C), 30 cycles of denaturation (30 s, 95˚C), annealing gradient (60 s, 50˚C – 64˚C), and extension (60 s, 68˚C). The obtained fragments were analyzed by horizontal agarose gel electrophoresis and visualized by ethidium bromide fluorescence. The resulting products were cloned into the pCR®4-TOPO® vector by employing the TOPO TA Cloning Kit for Sequencing (Invitrogen). DNA from the recombinant plasmids was extracted (Gen Elute Plasmid miniprep kit, Sigma) and sequenced using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) with an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems).

Sequencing of additional cDNA

Four additional expressed sequence tags derived from *L. vannamei* hemocytes were selected from the GenBank database. These ESTs were generated together with those published by Gross *et al*. (2001). Dry samples (purified plasmids) containing these ESTs were provided after request by Dr. Gregory Warr (Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, USA). Briefly, samples were resuspended in miliQ water and 5 microliters of each one was used to transform *E. coli* (50 µl). Independent colonies were diluted in 3ml of LB medium containing 100 µg/ml ampicillin and grown overnight at 36˚C with constant shaking. Plasmids were isolated, using the Gen Elute Plasmid miniprep kit (Sigma). Three clones representing each EST were sequenced unidirectionally using the T7 promotor reverse primer.

Quantitative real-time RT-PCR

Real-time PCR was performed applying the SYBR Green chemistry (Morrison *et al*., 1998) for relative quantification. The primers used for this analysis were designed by the Primer Express Software package (Applied Biosystems). In order to compensate for possible differences in reverse transcription efficiency, *L. vannamei* β-actin (Accession Number CK086617) and EF-1α

transcripts (Accession Number CK086625) were analyzed as endogenous control. The sequences of the primers are listed below:

EF-1α → 5' TGGCTGTGAACAAGATGGACA 3'

← 5' TTGTAGCCCACCTTCTTGACG 3'

β-actin → 5' ACGGAGCGTGGCTACACCTT 3'

← 5' TCTCCTTGATGTCACGAACGAT 3'

Liv-target → 5' GTGTGCAACTGCTGCTACATCTG 3'

← 5' G CCAGGCGCGCACTT 3'

The reactions were run in duplicate on an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The amplifications were carried out in a 96 well plate in a 25 µl reaction volume containing 12.5 µl of SYBR®Green Master Mix (Roche), 0.3 µM each of forward and reverse primers and 5 µl of the 1:1500 diluted cDNA templates. The thermal profile was 50˚C 2 min, 95˚C 10 min followed by 40 cycles of 95˚C 15 s and 60˚C 1 min. After the 40 cycles, the samples were run for dissociation protocol (melting curve), in order to confirm the specificity of the product. For each sample, the relative amount of transcript was normalized against both controls and transcript levels were calculated relative to a calibrator sample (intermolt stage hemocytes). Data were analyzed according to the comparative Ct method by means of the ABI Prism 7000 SDS software (Applied Biosystems, version 1.2.3). The results corresponded to the relative quantification of Liv-(*Litopenaeus vannamei*) target from 1 experimental group of animals (mean ± standard deviation from 2 measurements).

Results

Immunolocalization in the central nervous system

Antiserum raised against mammalian insulin/IGF-I and insect insulin-like peptides (ILP) were tested on tissue slices from cephalothorax, brain, thoracic ganglion and muscle. Faint staining with the mammalian insulin and rhIGF-I like immunoreactivity was detected in the digestive gland and gills. However, this staining was difficult to distinguish from the type of staining obtained using no immune serum (data not shown). This observation was not considered as immunopositive reaction. Brain and thoracic ganglion from animals at different molt stages (postmolt, intermolt, early and late premolt) reacted with the rhIGF-I antiserum (1:50 dilution).

Replacement of antisera with normal goat serum abolished evidence of immunostaining. However, the pre-absorption with rhIGF-I decreased the signal (data not shown). Six groups of cells bodies (3-4) were found in the shrimp brain (postmolt stage). These cells were found within groups of neurosecretory cells localized anterior and posterior parts of the protocerebrum (Fig. 1a). One immunostained cell body with its processes was located in the extreme anterior part of the protocerebrum of the shrimp brain at intermolt or late premolt stage (Fig. 1b).

Different groups of neurosecretory cells situated in the third, fourth and fifth segment of the thoracic ganglion (Fig. 1d,e) and their processes reacted with the rhIGF-I antiserum.

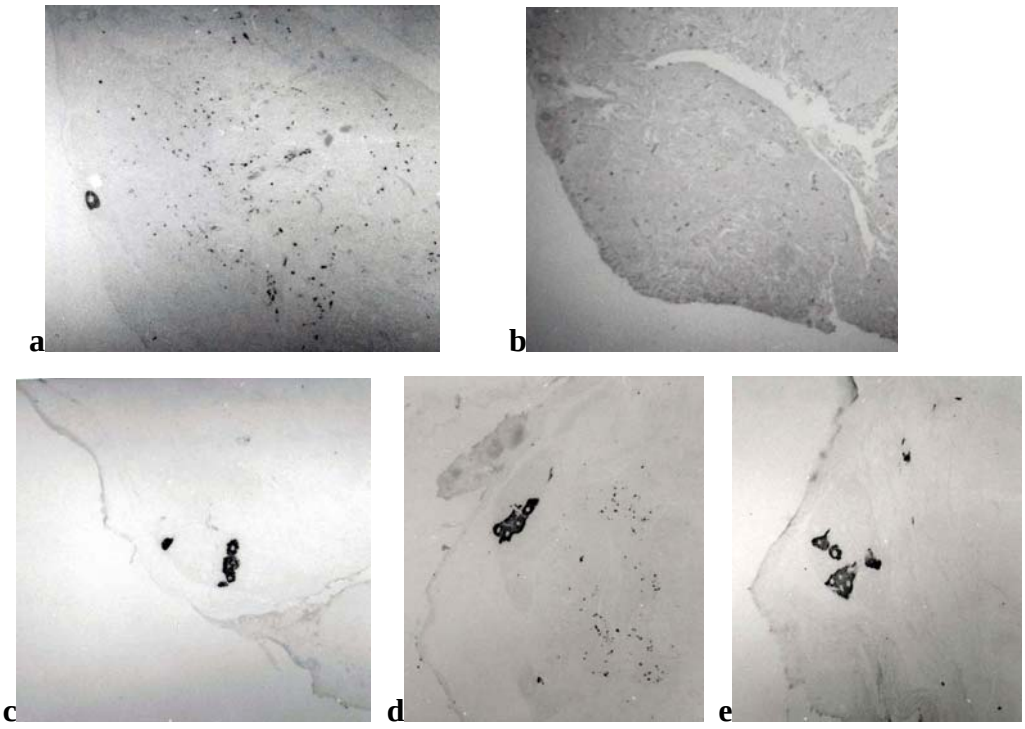
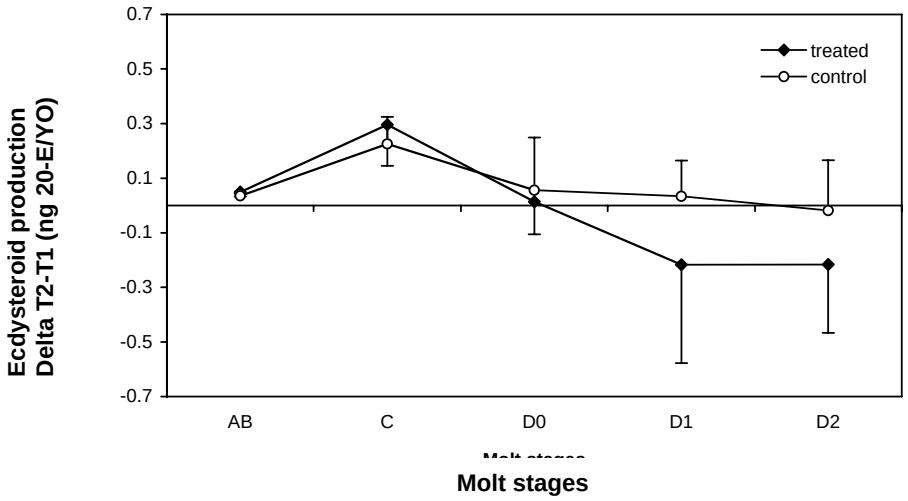


Fig 1. Sagital and horizontal sections through the shrimp brain and thoracic ganglion that immunoreacted with the rhIGF-I antiserum (dilution1/50). (a) One neurosecretory cell body displaying immunoreactivity and its processes (×200) located in the protocerebrum. (b) Alternating tissue section incubated with the non-immune goat serum (×100) as negative control. (c) Four neurosecretory cells laterally located in the protocerebrum (×200). (d) One group of 5 cell bodies with immunoreactive processes located in the fourth segment (×200). (e) Seven neurosecretory cells situated in the fifth segment (×200).

Effects of mammalian insulin-like peptides on Y-organ ecdysteroidogenesis

After *in vitro* incubations with insulin-like peptides we could observe positive and negative effects in the activity of Y-organs at two molt stages. Ecdysteroid secretion of premolt Y-organs (D_1 and D_2) decreased after treatment with 10 μM bovine insulin compared with ecdysteroid secretion of their controls (Fig. 2a). On the contrary, postmolt Y-organs appeared to be slightly stimulated after treatment with 0.001 nM rhIGF-I (Fig. 2b). However, in both cases these responses were not significantly different to the controls due to high interindividual variation.

a



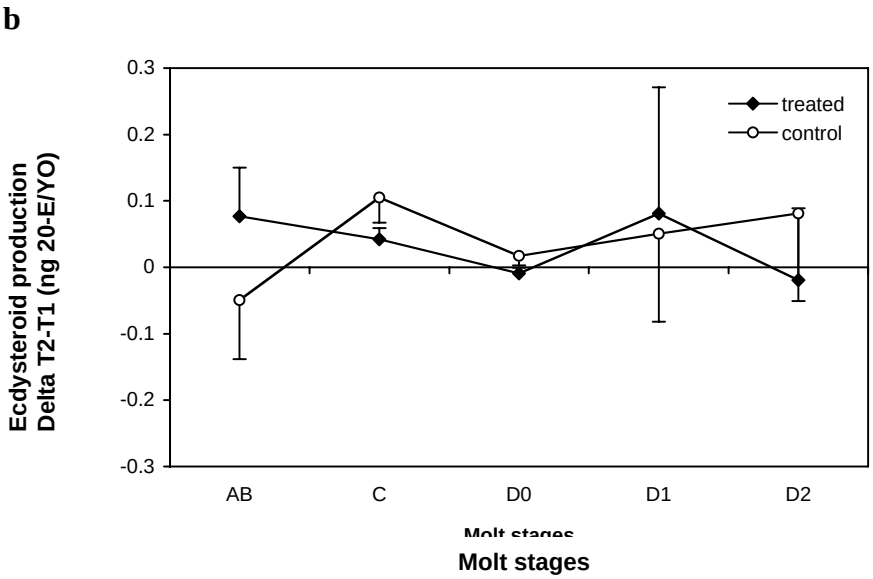


Fig 2. Effect of 10 μM bovine insulin (a) and 0.001 nM rhIGF-I (b) on ecdysteroid production of Y-organs at different molt stages. Each point represents the mean delta value from 8 replicates ± SEM. Molt stages: AB, postmolt; C, intermolt; D_0, early premolt; D_1, middle premolt; D_2, late premolt.

Effects of insulin/IGF-I on glucose levels and glycogen content

The results about the effect of insulin-like peptides on glucose levels and glycogen content in *L. vannamei* were previously presented in Gutiérrez *et al*. (2007). Glucose levels in the hemolymph of rhIGF-I-treated animals changed significantly when compared to the PBS-treated control. However, these values were similar to those registered in non-injected animals. No significant changes in glucose levels were observed in insulin treated shrimps comparable to PBS-injected control.

Shrimp were examined for changes in glycogen content in hepatopancreas, abdominal muscle and gills. A significant increased ($P < 0.05$) was observed in the glycogen content of hepatopancreas and gills from insulin-treated animals. On the contrary, glycogen content of muscle decreased significantly after all treatments.

Cloning of the IGFBP related peptide from Litopenaeus vannamei

Based on the *L. vannamei* EST, PCR amplifications were run with shrimp hemocytes cDNA. This resulted in two fragments of approximately 350 and 384 bp (at 64˚C annealing temperature) when analyzed by gel electrophoresis. The bands were purified from the gel, cloned, and sequenced. Both cDNA fragments include a 285-bp open reading frame (ORF) that predicts a peptide of 94 residues (Fig. 3a). They also contain an incomplete 3' untranslated end, which differed in 34 bp. The N-terminal signal peptide could be predicted using a SignalP 3.0 algorithm (Center for Biological Sequence Analysis, Technical University of Denmark, Lyngby, Denmark); the most probable cleavage site is between residues 19 (Gly) and 20 (Phe) followed by a putative mature peptide of 75 residues that contains 12 cysteine residues. Hydropathy analysis (Fig 3b) shows that half of the sequence is hydrophobic in nature (N-terminal 24 residues and Leu43 to Gly55).

a

```
CGGCGAGAGCAACATGAAGACGCTGCCGCTCCTTCTTCTGCTCGCGTGC 49
             M  K  T  L  P  L  L  L  L  L  A  C   12
                      ⬇
GCATGCGCAACCACGAGCGGCTTCAGCGTCAGGTGTTCCCCGTGCGAC  97
A  C  A  T  T  S  G  F  S  V  R  C  S  P  C  D    28

GAAGTGGACTGCGGACCGCCTCCCGCTGACTGCAAATACGGCCTCGTC 145
E  V  D  C  G  P  P  P  A  D  C  K  Y  G  L  V    44

AAGAATGTGTGCAACTGCTGCTACATCTGCGGGAAGGGTCCTGGTGAA 193
K  N  V  C  N  C  C  Y  I  C  G  K  G  P  G  E    60

GACTGCGACCCCGTGAAAAAGTGCGCGCCTGGCCTTTACTGCAAGCTG 241
D  C  D  P  V  K  K  C  A  P  G  L  Y  C  K  L    76

CACCCCGTCTCCTATGGTAATGGTATCTGCACGGCCACACCGCAGAAA 289
H  P  V  S  Y  G  N  G  I  C  T  A  T  P  Q  K    92

TGGTACTGATTCCTGGGACAAGGTTTCGTGATTCTGGGACAAGGTTTC 337
W  Y  *                                           94

ATGATGATGGTACTGATTCCTGGGACAAGGTTTCATGATGATGTTAC  384
```

b

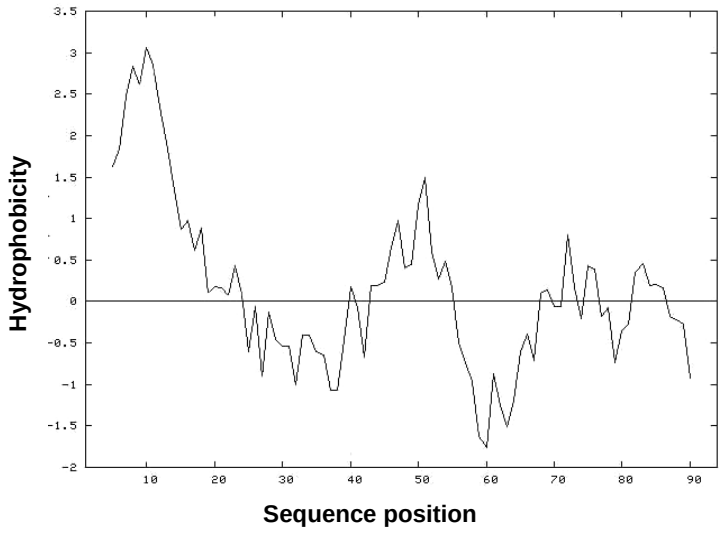


Fig 3. *Litopenaeus vannamei* Insulin-like Growth Factor Binding Protein- related peptide (LivIGFBP-RP). (a) Nucleotide sequence (cDNA) and deduced amino acid sequence of LivIGFBP-RP 1 (384 bp). The computed cleavage site is indicated by an arrowhead. The PCR primers are underlined and Real-time primers are double underlined. The start (ATG) and stop (TGA) codon are shown in bold. (b) Hydropathy profile of LivIGFBP-RP. Method used as described by Kyte and Doolittle (1982).

In addition, after nucleotide sequence comparison of the 384 bp fragment against the Marine Genomics EST database four additional *L. vannamei* ESTs (BE188550, CK591994, EE572303, CK592195) were found sharing high sequence similarities with the 384-bp fragment. Three colonies derived from each transformation were sequenced. Nucleotide sequences were only obtained from clones representing EST BE188516 (563 bp) and EST CK592195 (595 bp), and they appeared to include the 3'- and 5'-untranslated ends. These cDNA sequences seem to be two variants (Fig. 4) that differed in their 3' untraslated end and in some single base substitutions. As expected, both cDNA sequences encoded for essentially the same peptide of 94 residues deduced from the 350 bp and 384 bp fragments. However, single base substitutions resulted in one different amino acid in the peptide derived from each EST clone (R51C in the EST CK592195 and P89T in the EST BE188516).

```
384bp       1 -------------------CGGCGAGAGCAACATGAAGACGCTGCCGCTCCTTCTTCTGCTTGCGTGCGCATGCGCAACC  61
BE188516    1 GGGGGTAGTCTTCGACAGTCGGCGAGAGCAACATGAAGACGCTGCCGCTCCTTCTTCTGCTCGCGTGCGCATGCGCAACC  80
CK592195    1 GGGGGTAGTCTTCGACAGTCGGCGAGAGCAACATGAAGACGCTGCCGCTCCTTCTTCTGCTCGCGTGCGCATGCGCAACC  80


384bp      62 ACGAGCGGCTTCAGCGTTAGGTGTTCCCCGTGCGACGAAGTGGACTGCGGACCGCCTCCCGCTGACTGCAAATACGGCCT 141
BE188516   81 ACGAGCGGCTTCAGCGTCAGGTGTTCCCCGTGCGACGAAGTGGACTGCGGACCGCCTCCCGCTGACTGCAAATACGGCCT 160
CK592195   81 ACGAGCGGCTTCAGCGTTAGGTGTTCCCCGTGCGACGAAGTGGACTGCGGACCGCCTCCCGCTGATTGCAAATACGGCCT 160


384bp     142 CGTCAAGAATGTGTGCAACTGCTGCTACATCTGCGGGAAGGGTCCTGGTGAAGACTGCGACCCCGTGAAAAAGTGCGCGC 221
BE188516  161 CGTCAAGAATGTGTGTAACTGCTGCTACATCTGCGGGAAGGGTCCTGGTGAAGACTGCGACCCCGTGAAAAAATGCGCGC 240
CK592195  161 CGTCAAGAATGTGTGCAACTGCCGCTACATCTGCGGGAAGGGTCCTGGTGAAGACTGCGACCCCGTGAAAAAGTGCGCGC 240
                                R51C

384bp     222 CTGGCCTTTACTGCAAGCTGCACCCCGTCTCCTATGGTAATGGTATCTGTACGGCCACACCGCAGAAATGGTACTGATTC 301
BE188516  241 CTGGCCTTTACTGCAAGCTGCACCCCGTCTCCTATGGTAATGGTATCTGTACGGCCCCCCCGCAGAAATGGTACTGATTC 320
CK592195  241 CTGGCCTTTACTGCAAGCTGCACCCCGTCTCCTATGGTAATGGTATCTGTACGGCCACCCCGCAGAAATGGTACTGATTC 320
                                                              P89T

384bp     302 CTGGGACAAGGTTTCGTGATTCTGGGACAAGGTTTCATGATGATGGTACTGATTCCTGGGACAAGGTTTCATGATGATGT 381
BE188516  321 CTGGGACAAGGTTTCGTGATTTTGGGACAAGGTTTCATGATGATGGTACTGATTCCTAGGACAAGGTTTCATGATGATGG 400
CK592195  321 CTGGGACAAGGTTTCGTGATTTTGGGACAAGGTTTCATGATGATGGTACTGATTCCTGGGACAAGGTTTCATGATGATGG 400


384bp     382 TAC---------------------------------------------------------------------------- 384
BE188516  401 TACTGATTCCTAGGACAAGGTTTCATGATGATGGTACTGATTCCTGGGACAGGG------------------------- 454
CK592195  401 TACTGATTCCTGGGACAAGGTTTCATGATGATGGTACTGATTCCTGGGACAAGGTTTCATGATGATGGTACTGATTCCTG 480


384bp         ---------------------------------------------------------------------------
BE188516  455 --------CTTCGTGATGAGTCTCATCAGCATTGTAATGTCGTGATCAGTTTTGTGATTTTATGTTATTGATCAATAAAT 526
CK592195  481 GGATATGGCTTCGTGATGAGTCTCATCAGCATTGTAATGTCGGGATCAGTTTTGTGATTTTATGTTATTGATCAATAAAT 560


384bp         -------------------------------------
BE188516  527 ATGCCCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 563
CK592195  561 ATGCCGAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   595
```

Fig. 4. cDNA sequence alignment of 384 bp fragment, EST BE188516 and CK592195. Nucleotides that differ from the 384 bp fragment sequence are shaded. The triplet, encoding Arg^{51} (EST CK592195) and Pro^{89} (EST BE188516) are underlined. The start codon (ATG) and the stop codon (ATG) are shown in bold. The putative polyadenylation signal is shown in bold and underlined.

Sequence comparisons

The deduced amino acid sequence of 384-bp fragment was compared (Blastp) against the NCBI database for conserved domains (CDD V2.14-24291PSSMs). The resulting hit was the smart00121 domain that corresponds to the Insulin-like growth factor binding protein homologues and high affinity binding partners of insulin-like growth factors (E value =7e-04). The similarity includes Cys residues at conserved positions and other residues within the N-terminal domain, which has the binding capacity. The *L. vannamei* peptide also displayed similarities with the insect neuroparsins, *i.e.* neuroparsin precursors 2, 3 and 4 from the desert locust *Schistocerca gregaria,* which share characteristic features with the N-terminus of IGFBP (Fig. 5). Furthermore, a tBlastn search using the deduced amino acid sequence from the 384-bp fragment as a query- against the GenBank non-human and non-mouse EST database (est_others) revealed that sequences originated from other arthropod and cnidarian species displayed substantial similarities with the *L. vannamei* peptide precursor. The predicted amino acid sequences are represented in Fig. 5. Some of the sequences were already identified as putative neuroparsin precursors by Claeys *et al*. (2003) and Badisco *et al*. (2007). Other peptide-encoding EST sequences are derived from the crustacean *Euphausia superba*, the cephalochordate *Branchiostoma floridae*, and the cnidarians *Hydra magnipapillata* and *Nematostella vectensis*. All these sequences posses a very similar pattern of cysteine residues. Should be noted here that this analysis may contain errors in some of the sequences presented, given the limited reliability of single-pass EST sequences. However, this stipulation does not negate the main conclusion of the sequence comparison, which is the existence of conserved IGFBP-related genes across widely divergent phyla.

b

a

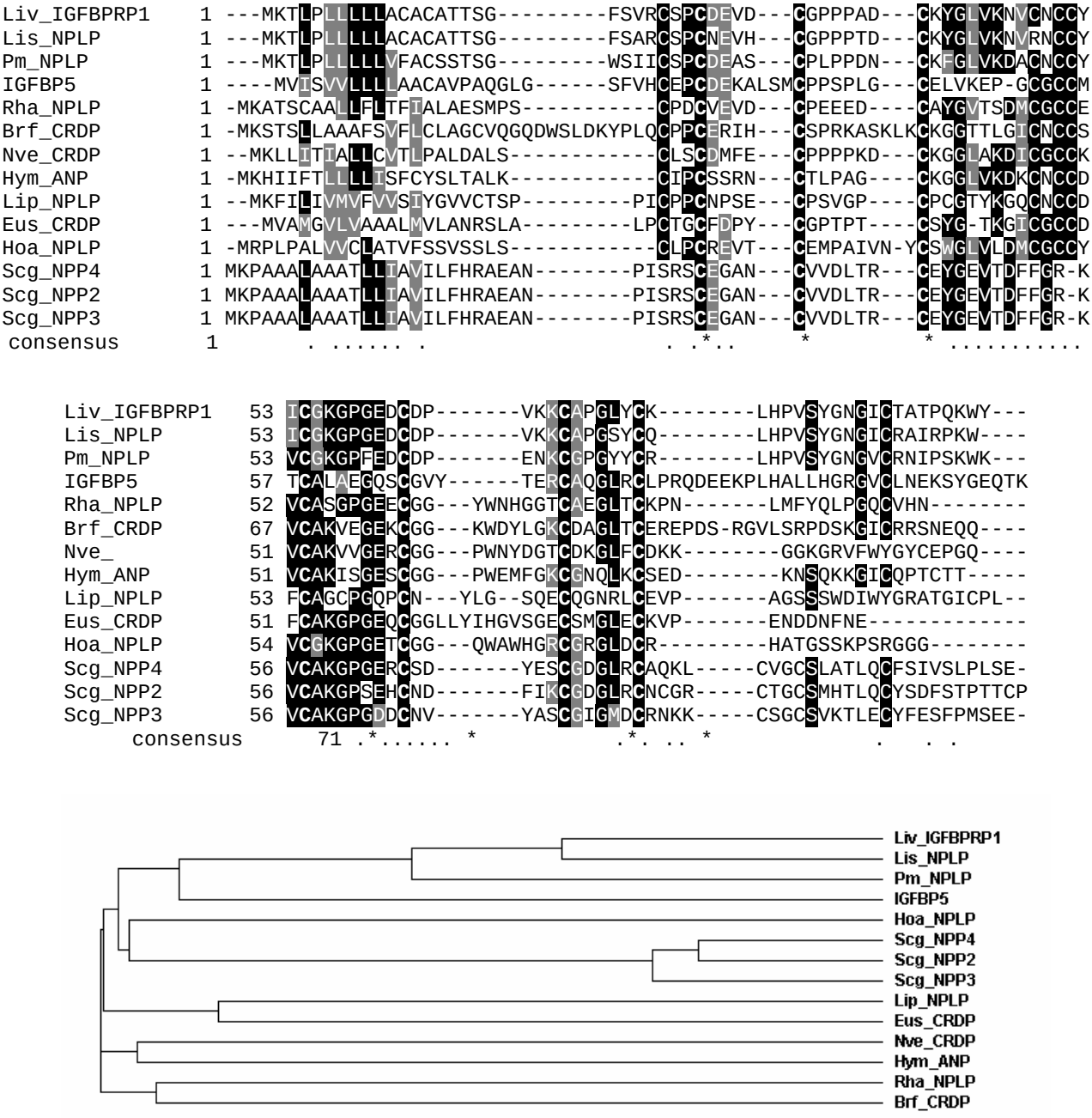


Fig. 5. (a) Multiple sequence alignment (ClustalW, pam matrix) of the shrimp LivIGFBP-RP1 with other arthropod and cnidarian species, and mammalian insulin-like growth factor binding protein 5 (IGFBP5). (b) Dendogram of the alignment (ClustalW analysis). Highly conserved residues are highlighted in black. Residues that are similar to the column's consensus are shaded. The consensus line is represented by symbols: (*) representing a

completely conserved position, (.) representing a position in which not all amino acid are equal to the consensus. Abbreviations: *Lis*-NPLP, *Litopenaeus setiferus* neuroparsin-like precursor (EST-derived, GenBank: BE846730); *Liv*-IGFBPR1-2, *Litopenaeus vannamei* Insulin-like growth factor binding protein related peptide 1 and 2; *Pm*-NLPL, *Penaeus monodon* neuroparsin-like peptide precursor (EST-derived, GenBank: BI784456); Eus-CRDP, *Euphausia superba* (krill) cysteine rich domain peptide (EST-derived, GenBank: ES544423); *Lip*-NPLP, *Limulus polyphemus* (horseshoe crab) neuroparsin-like precursor (EST-derived, GenBank: AAK15535); *Nev*-CRDP, *Nematostella vectensis* cysteine rich domain peptide (derived from genomic fragment, GenBank: EDO43497); *Hym*-Ant, *Hydra magnipapillata* Antistasin precursor (EST-derived, GenBank: CF601707); *Hoa*-NPLP, *Homarus americanus* neuroparsin-like precursor (EST-derived, GenBank: CN854288); *Rha*-NPLP, *Rhipicephalus appendiculatus* neuroparsin-like precursor (EST-derived, GenBank: CD796554); *Scg*-NPP2-4, *Schistocerca gregaria* neuroparsin precursor 2-4 (GenPept: CAC38870, CAC82521, CAC82522); *Brf*-CRDP, *Branchiostoma floridae* (cephalochordate) cysteine rich domain peptide (EST-derived, GenBank: BW859369); IGFBP5, *Rattus norvegicus* insulin-like growth factor binding protein 5 (GenPept: NP_036949).

Distribution of the LivIGFBP-RP transcripts

In order to evaluate whether the LivIGFBP-RP1 expression may be related to growth, a quantitative real-time PCR assay was employed. Relative transcript levels were determined in samples derived from different tissues, two size and 3 molt stages of one group of animals reared in the same pond. Amplification of LivIGFBP-RP1 transcripts was normalized against β-actin and EF-1α controls. LivIGFBP-RP1 transcripts normalized against the β-actin control was detected in all the tissues, (Fig 6a). High expression levels were detected in the hemocyte and gill samples. LivIGFBP-RP1 transcript expression levels tended to be higher in the samples from the small animals than in those samples from the big animals. An equivalent expression pattern was observed when analyzing LivIGFBP-RP1 transcripts normalized against the EF-1α control (Fig. 6b). In both analyses, transcript expression was similar in the samples regardless the molt stage.

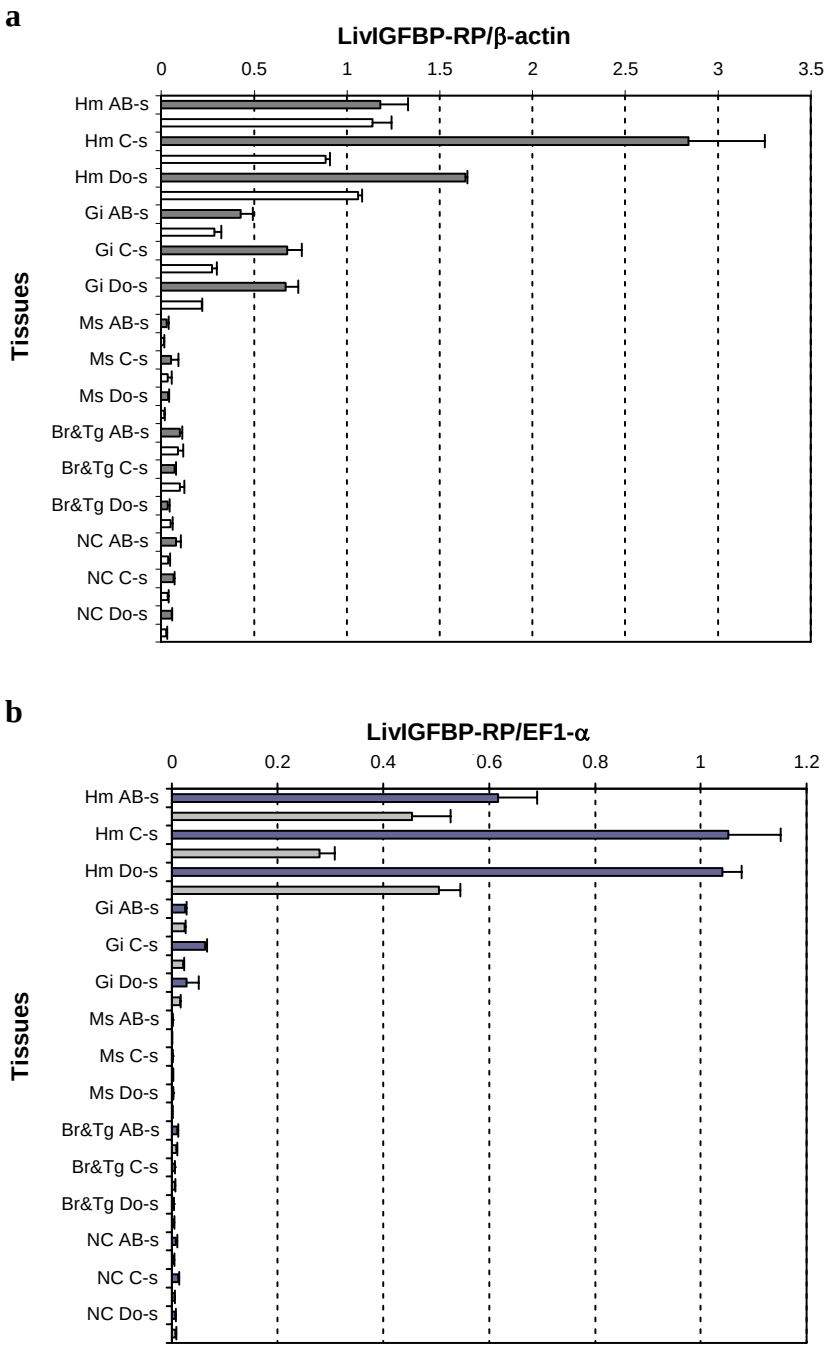


Fig. 6. Quantitative real-time PCR analysis showing relative LivIGFBP-RP1 transcript levels. Transcript were normalized against b-actin (a) and EF-1α (b) in different tissues of small (dark grey bars) and big (light grey bars) juvenile shrimps. The following tissues and molt stages were analyzed, as abbreviated on the x-axis: Hm, hemocytes; Gi, gills; Ms, muscle; Br&Tg, brain and thoracic ganglion; NC, nerve cord; AB, postmolt stages; C, intermolt stage; Do, early premolt stage.

Discussion

In the present study, we describe a partial characterization of insulin-like peptides in *Litopenaeus vannamei* that include it immunolocalization and the evaluation of its possible functions in two important physiological processes. In addition, we identified a precursor related to the IGFBP family in shrimp tissues.

An immunocytochemical study was performed to determine ILP immunoreactivity in shrimp tissues. We localized IGF-I-like material in the central nervous system of *L. vannamei*. Positive staining was observed in neurosecretory cells of the brain and in at least three segments of the thoracic ganglion. This is the first report on insulin-like peptide immunoreactivity in the central nervous system of a crustacean. However, the presence of mRNA homologues to the mouse IGF-I has been previously demonstrated by Chang *et al*. (1995) in the eyestalk and muscle of the lobster *Homarus americanus* and the crab *Cancer magister*. Our results are in agreement with existing reports on insulin-like peptide in insects, which have demonstrated that ILPs are synthesized in neurosecretory cells of the *pars intercerebralis*, an important production site of neurohormones in the insect brain (Mizoguchi *et al*. 1987; Hansen *et al.* 1990; Zitnan *et al*., 1990; Goltzené *et al*., 1992; Sevala *et al*., 1993a; Nogueira *et al*., 1997; Cao and Brown, 2001; Van de Velde *et al*., 2007; Badisco *et al.,* 2008). Opposite to the current reports in crustaceans that have shown the presence of insulin-like material in the lobster digestive gland (Sanders, 1983a; Gallardo *et al*., 2003), we could not differentiate the immunostaining in the shrimp hepatopancreas from the negative control (normal antiserum) when bovine insulin or rhIGF-I antibodies were used. This may suggest that the insulin-like material found in the digestive gland of the lobsters is different from the one we observed in the shrimp nervous system, or that the immunoreactivity observed in lobster is also false positive. The presence of different insulin-like molecules may be plausible since Chang *et al*., (1995) did not observe the presence of mRNA homologous to IGF-I in *Homarus americanus* digestive gland. Pre-absorption tests with the rhIGF-I antiserum resulted in a decrease of the reaction intensity but did not eliminate it. This may indicate that the detected IGF-like material in *Litopenaeus* nervous system differs in part from the human IGF-I, but it still doesn't imply that the reaction was unspecific. The concentrations of the recombinant peptide applied for specificity test in our study were not as high as those used by Reinecke *et al*. (1991 and 1993) to test the specificity of a human IGF-I

antiserum that reacted with brains and digestive tracts of protochordates and cyclostomes. They observed that the immunoreactivity was not eliminated by the pre-absorption of 1:400 or 1:1000 antiserum dilutions with 400 μg/ml of human IGF-I. Nevertheless, it was eliminated with crude extracts from the same tissues, suggesting that the IGF-like material detected in those animals differs in size and in primary structure from the human peptide. The complete purification of that material has not been accomplished. We did not succeed in the purification of the IGF-like material from shrimp CNS extracts using the same serum in a dot blot screening of HPLC fractions, because in subsequent purification steps, more material was needed to allow detection.

Ecdysiotropic effects of insulin-like peptides (ILPs) have been demonstrated in some insect species. For instance, bombyxin was the first ILP characterized in insects and it was isolated from the silkworm *Bombyx mori* based on its ability to stimulate ecdysteroid synthesis in the prothoracic glands (PG) of a closely related moth species (Nagasawa *et al*., 1986). The same molecule was able to stimulate the prothoracic glands of the insect *Rhodnius prolixus* (Vafopoulou and Steel, 1997) as well as the ovary of a blowfly (Manière *et al*., 2004) to produce ecdysone. Under the experimental conditions of our study, the effect of ILP on Y-organs ecdysteroidogenesis appears to be low or it may have no effect at all, since there was no significant difference between ecdysteroid secretions after treatment with synthetic ILP and the contralateral controls. It may be possible that the ILP effect is more relevant in the regulation of other physiological processes such as growth, metabolism or reproduction as it has been demonstrated in several insect species.

The presence of an insulin-like peptide has been reported in some crustacean species that display similar effects to the vertebrate insulin (Sanders 1983b; Davidson *et al*., 1971; Gallardo *et al*., 2003). We evaluated the effect of insulin/IGF on the hemolymphatic glucose levels and glycogen contents in the hepatopancreas, abdominal muscle and gills of the white shrimp *L. vannamei*. We observed that the glycogen content in the hepatopancreas and gills were increased after injections of mammalian insulin when compared to the control and the values registered in non-treated shrimps (Gutierrez *et al*., 2007). Our observations suggested that insulin treatment promoted glycogen synthesis and therefore they indicate that insulin-like peptides may have a conserved function in glucose metabolism of crustaceans.

After a search for insulin-like peptides and related proteins in the database containing ESTs from crustacean cDNA libraries, an EST encoding a precursor similar to mammalian IGF binding

proteins (IGFBP) was found in the hemocytes cDNA library of shrimp (Gross *et al*., 2001). However, this sequence contains many undefined bases, which interfere in primer design destined for a transcript expression analysis. We therefore cloned and sequenced a cDNA fragment based on the published EST (BE188516). We obtained a 384 bp cDNA sequence from *L. vannamei* hemocytes. The deduced amino acid sequence shares substantial similarity to the N-terminal domain of the mammalian IGF binding proteins. The similarity is based on a 38-39% amino acids identity including 7 cysteine residues at conserved position (Fig 4a). Furthermore, we re-sequenced aliquots of the same EST (BE188516) and a very close variant CK592195. The three sequences (384 bp fragment and ESTs) contained a 285 bp ORF encoding for almost the same amino acid sequence, except for two variations due to a single base substitution. Sequence alignment of the clones derived from fragment 350 bp and 384 bp and the EST BE188516 and CK592195 suggested that the re-sequenced clones representing the EST are two variants.

The IGFBP family comprises structurally related proteins with characteristic cysteine-rich N-terminal and C-terminal domains (Jones and Clemmons, 1995). Recent studies have demonstrated that a hydrophobic path located within the IGFBP N-terminal domain is required for IGF binding (Imai *et al*., 2000; Sitar *et al*., 2006). This observation is confirmed with the invertebrate peptides, perlustrin and neuroparsin, which share substantial similarities with the N-terminal domain of the mammalian IGF binding proteins and they have the capacity of binding insulin-related peptides *in vitro* (Weiss *et al*., 2001, Badisco *et al*., 2008). In crustaceans, several reports have demonstrated the presence of a protein from the hepatopancreas and the nervous system that resembles insulin (Sander, 1983a) and displays IGF-typical or insulin-typical effects (Hatt *et al*., 1997; Gallardo *et al*., 2003). In this study, after searching EST databases, several sequence derived from other arthropods were found to display pronounced similarities to the LivIGFBP-RP. Some of sequences derived from crustaceans were previously identified as neuroparsin-like and IGFBP-like peptides (Claeys *et al*., 2003; Badisco *et al*., 2007). We therefore expect that the LivIGFBP precursor may conserve the function of interacting with insulin-related peptides, because a hydrophobic path in its N-terminal domain was identified (Fig. 3b). Moreover, new EST encoding for peptides homologous to the IGFBP in organisms belonging to other animal phyla such as Cnidaria and Chordates were identified after the tBlastn analysis (Fig. 4a).

In order to investigate the physiological relevance of the LivIGFBP-related peptide precursor, we

evaluated transcript expression levels in different tissues, sizes and molt stages from a group of *Litopenaeus vannamei* juveniles. Quantitative RT-PCR analysis revealed that the LivIGFBP-RP1 transcripts occur in all the analyzed tissues (central nervous system, muscle, gills and hemocytes). Part of this result is supported by new information recently submitted (De La Vega *et al* unpublished 2008) in the GenBank database (NCBI). New cDNA sequences (EST) derived from other *Litopenaeus* tissues not included in our experiment share 98% nucleotide sequence similarities with the 384 bp cloned fragment and they encoded for exactly the same peptide that we described in this study. The ESTs are derived from gills (GenBank FE095318), lymphoid organ (GenBankFE149225) and eyestalk (GenBankFE046135). These observations indicate that the IGFBP transcript may be expressed in many different *Litopenaeus* tissues.

Furthermore, LivIGFBP transcript levels tended to be higher in hemocytes compared to other tissues. Hemocytes are a major effector of immune response in arthropods (Söderhäll and Cerenius, 1992; Vargas-Albores *et al*., 1998; Iwanaga and Lee, 2005). Several studies have established the function of IGF-I/GH axis in the mammalian immune system (Clark, 1997; Van Buul-Offers and Kooijman, 1998). In addition, lymphocytes secrete IGFBPs and express IGF type I&II receptors (Nyman and Pekonen, 1993; Kooijman *et al*., 1995). However, these cells express IGFs (I-II) only after stimulation with lymphocyte mitogens (Nyman and Pekonen, 1993; Auernhammer *et al*., 2002). The apparent high expression of the LivIGFBP precursor in the hemocytes may suggest a possible role in the immune response. The experimental animals were obtained from a natural environment (shrimp farms), which are not free from viral particles. These animals may be under a low but constant immune activation. The data presented in this chapter are the result of an exploratory study and an analysis of viral load was not taken into account. The quantification of viral copies in the same samples is necessary first to establish whether a correlation between viral load and the peptide expression may exist. Moreover, no marked changes in transcript expression were observed between molt stages, however LivIGFBP transcript levels tended to be higher in the tissues samples from small animals compared to those from big animals. There is evidence that the shrimp nervous system contains potential growth factors (Hatt *et al*., 1997). IGF-binding proteins modulate IGF access to the IGF-I receptor, inhibiting IGF-stimulated events (Baxter, 2000). If the potential growth factors in the shrimp are biologically active and could interact with the LivIGFBP, the expression of LivIGFBP precursor in the small shrimps suggests that it could be related to growth inhibition in this group of animals.

However, one would expect expression differences between molt stages, since crustaceans undergo several physiological and biochemical changes during the molt cycle (Chang, 1995). Another possibility is that the high transcript levels in small animals are linked to the immune response. Shrimp resistance to diseases caused by at least two viruses has been associated with low growth rates (Castille *et al*., 1993; Argue *et al*., 2002). Based on field observations, Rodriguez and co-workers (personal communication, CENAIM-ESPOL Foundation) have found the presence of WSSV (white spot syndrome virus) in animals exhibiting low growth rates. It has also been suggested that an inverse relation between virus resistance and growth rates may occur in pond reared *L. vannamei*, as demonstrated by Argue *et al.* (2002). Nevertheless, it would be premature to assume that our observations apply to any other shrimp population, since only one group of animals was evaluated. An analysis of the LivIGFBP expression in more groups of animals belonging to pond reared and specific pathogen free (SPF) populations is necessary in order to further support our suggestion.

This study has shown that different insulin-like molecules may exist in crustaceans displaying different functions. IGF-like material was identified in nervous tissues, where its role may be related to other than endocrine function. On the contrary, a molecule close to the mammalian insulin promoted glycogen synthesis. Contradictory results have been found in other decapods as well. Recently, an insulin-like androgenic gland (IAG) factor has been identified in the crayfish *Cherax quadricarinatus* (Manor *et al*., 2007) and its expression may regulate sexual differentiation. It is still unknown whether this factor is the only insulin-like molecule present in crustaceans and if this peptide is responsible for glycogenesis promoting effects observed in other decapod species. Insulin-like peptides also contribute to the regulation of reproduction in several invertebrate species. The relation of insulin-like peptide with sexual differentiation or reproduction of the shrimp was not integrated in this study; therefore the possibility that occurs in *Litopenaeus vannamei* cannot be excluded. Furthermore, an IGFBP related precursor was cloned, which seems to be expressed ubiquitously in the shrimp. This precursor share significant amino acid sequence with those of IGFBP-related or neuroparsin related peptide precursors that are present in other arthropod species. Neuroparsins and an IGFBP-related peptide of *Drosophila melanogaster* maintain the conserve function of binding their endogenous insulin-like peptide (Badisco *et al*., 2008; Honegger *et al*., 2008). It would not be surprising if the LivIGFBP-related peptide conserved also the binding capacity.

In the future, it will be of interest to examine the *in vitro* interaction of the LivIGFBP precursor with insulin-like peptides and to analyze whether the LivIGFBP-RP exerts inhibitory or stimulatory effect on the insulin-like peptide activities.

Acknowledgements

The authors especially thank Inge Bongaers for technical assistance and to Liesbeth Badisco for her suggestions on this manuscript. Research was supported by the Flemish Interuniversity Council (VLIR-UOS) for financial support. A. G. obtained a PhD fellowship from the VLIR-ESPOL Project.

References

Argue, B.J., Arce, S.M., Lotz, J.M., Moss, S.M. 2002. Selective breeding of Pacific white shrimp (*Litopenaeus vannamei*) for growth and resistance to Taura Syndrome Virus. Aquaculture 204, 447-460.

Auernhammer, C.J., Fottner, C., Engelhardt, D., Bidlingmaier, M, Strasburger, C.J. Weber, M.M. 2002. Differential regulation of insulin-like growth factor- (IGF) I and IGF-binding protein (IGFBP) secretion by human peripheral blood mononuclear cells. Hormone Research 57, 15-21.

Badisco, L., Claeys, I., Van Hiel, M., Clynen, E., Huybrechts, J., Vandermissen, T., Van Soest, S., Vanden Bosch, L., Simonet, G., Vanden Broeck, J. 2008. Purification and characterization of an insulin-related peptide in the desert locust, *Schistocerca gregaria*: immunolocalization, cDNA cloning, transcript profiling and interaction with neuroparsin. Journal of Molelucar Endocrinology 40, 137-150.

Badisco, L., Claeys, I., Van Loy, T., Van Hiel, M., Franssens, V., Simonet, G., Vanden Broeck, J. 2007. Neuroparsins, a family of conserved arthropods neuropeptides. General and Comparative Endocrinology 153, 64-71.

Bathgate, R.A., Samuel, C.S., Burazin, T.C., Layfield, S., Claasz, A.A., Reytomas, I.G., Dawson, N.F., Zhao, C., Bond, C., Summers, R.J., Parry, L.J., Wade, J.D., Tregear, G.W. 2002. Human relaxin gene 3 (H3) and the equivalent mouse relaxin (M3) gene. Novel members of the relaxin peptide family. Journal of Biological Chemistry 277, 1148-1157.

Baxter, R.C. 2000. Insulin-like growth factor (IGF) binding proteins: Interactions with IGFs and intrinsic bioactivities. American Journal of Physiology 278, E967-E976.

Blais, C., Sefiani, M., Toullec, J-Y., Soyez, D. 1994. *In vitro* production of ecdysteroids by Y-organs of *Penaeus vannamei* (Crustacea, Decapoda). Correlation with hemolymph titers. Invertebrate and Reproduction Development 26, 3-12.

Burkhardt, E., Adham, I.M. Brosig, B., Gastmana, A., Mattei, M.G., Engel, W. 1994. Structural organization of the porcine and human genes coding for a Leydig cell-specific insulin-like peptide (LEY I-L) and chromosomal localization of the human gene (INSL3). Genomics 20, 13-19.

Cao C., Brown M.R. 2001. Localization of an insulin-like peptide in brains of two flies. Cell and Tissue Research 304, 317-321.

Castille, F.L., Samocha, R.M., Lawrence, A.L., He, H., Frelier, P., Jaenike, F. 1993. Variability in growth and survival of early postlarval shrimp (*Penaeus vannamei* Boone 1931). Aquaculture 113, 65-81.

Chang, E.S. 1995. Physiological and biochemical changes during the molt cycle in decapod crustaceans: an overview. Journal of Experimental Marine Biology and Ecology 193, 1-14.

Chang, E.S., Tamone, S.L., Lin, W.-W., Oberbauer, A.M. 1995. Modifications to the paradigm of the hormonal control of the crustacean molting: effects of metabolic inhibitors on the ecdysiotropic action of methyl farnesoate and evidence for an insulin-like growth factor. In Proceedings of the international symposium on biotechnology applications in aquaculture. Asian Fisheries Society 10, 183-195.

Chassin, D., Laurent, A., Janneuau, J.L., Berger, R., Bellet, D. 1995. Clonning of a new member of the insulin gene superfamily (INSL4) expressed in human placenta. Genomics 29, 465-470.

Chuang, N-N., Wang, P-C. 1994. Characterization of insulin receptor from the muscle of the shrimp *Penaeus japonicus* (crustacea: decapoda). Comparative Biochemical Physiology 108C, 289-297.

Claeys, I., Simonet, G., Poels, J., Van Loy, T., Vercammen, L., De Loof, A., Vanden Broeck, J. 2002. Insulin-related peptides and their conserved signal transduction pathway. Peptides 23, 807-816.

Claeys, I., Simonet, G., Van Loy, T. De Loof, A., Vanden Broeck, J. 2003. cDNA cloning and transcript distribution of two novel members of the neuroparsin family in the desert locust, *Schistocerca gregaria*. Insect Molecular Biology 12, 473-481.

Clark, R. 1997. The somatogenic hormones and insulin-like growth factor-1: stimulators of lymphopoiesis and immune function. Endocrine Reviews 18, 157-179.

Conklin, D., Lofton-Day, C.E., Haldeman, B.A., Ching, A., Whitmore, T.E., Lok, S., Jaspers, S. 1999. Identification of INSL5, a new member of the insulin superfamily. Genomics 60, 50-56.

Davidson, J.K., Falkmer, S., Mehratra, B.K., Wilson, S., 1971. Insulin assay and light microscopial studies of digestive organs in protostomian and deuterostomian species and coelenterates. General and Comparative Endocrinology 17, 388-401.

Duret, L., Guex, N., Peitsch, M.C., Bairoch, A. 1998. New insulin-like proteins with atypical disulfide bond pattern characterized in *Caenorhabditis elegans* by comparative sequence analysis and homology modelling. Genome Research 8, 348-353.

Gallardo, N., Carrillo, O., Moltó, E., Deás, M., González-Suárez, R., Carrascosa JM, Ros M, Andrés A. 2003. Isolation and biological characterization of a 6-kDa protein from hepatopancreas of lobster *Panulirus argus* with insulin-like effects. General and Comparative Endocrinology 131: 284-290.

Gerisch, B., Weitzel, C., Kober-Eisermann, C., Rottiers, V., Antebi, A. 2001. A hormonal signaling pathway influencing *C. elegans* metabolism, reproductive development, and life span. Developmental Cell 6, 841-851.

Goltzené, F., Holder, F., Charlet, M., Meister, M., Oka, T. 1992. Immunocytochemical localization of *Bombyx*-PTTH-like molecules in neurosecretory cells of the brain of the migratory locust, *Locusta migratoria*. A comparison with neuroparsin and insulin-related peptide. Cell and Tissue Research 269, 133-140.

Gross, P.S., Bartlett, T.C., Browdy, C.L., Chapman, R.W. and Warr, G.W. 2001. Immune gene discovery by expressed sequence tag analysis of hemocytes and hepatopancreas in the Pacific White Shrimp, *Litopenaeus vannamei*, and the Atlantic White Shrimp, *L. setiferus*. Developmental and Comparative Immunology 25, 565-577.

Gutiérrez, A., Nieto, J., Pozo, F., Stern, S., Schoofs, L. 2007. Effects of insulin/IGF-I like peptides on glucose metabolism in the white shrimp *Penaeus vannamei*. General and Comparative Endocrinology 153, 170-175.

Hansen, G.N., Hansen, B.L., Jorgensen, P.N., Scharrer, B. 1990. Immunocytochemical localization and immunochemical characterization of an insulin-related peptide in the insect *Leucophaea maderae*. Cell and Tissue Research 259, 265-273.

Hatt, P.J., Liebon, C., Moriniére, M., Oberlander, H., Porcheron, P. 1997. Activity of insulin growth factors and shrimp neurosecretory organ extracts on a lepidopteran cell line. Archive of Insect Biochemistry and Physiology 34, 313-328.

Honegger, B., Galic, M., Köhler, K., Wittwer, F., Brogiolo, W., Hafen, E., Stocker, H. 2008. Imp-L2, a putative homolog of vertebrate IGF-binding protein 7, counteracts insulin signaling in *Drosophila* and is essential for starvation resistance. Journal of Biology 7, 1-10.

Ikeya, T., Galic, M., Belawat, P., Nairz, K., Hafen, E. 2002. Nutrient-dependent expression of insulin-like peptides from neuroendocrine cells in the CNS contributes to growth regulation in *Drosophila*. Current Biology 12, 1293-300.

Imai, Y., Moralez, A., Andag, U., Clarke, J.B. Busby, W.H. Clemmons, D.R. 2000. Substitutions for hydrophobic amino acids in the N-terminal domains of IGFBP-3 and -5 markedly reduce IGF-I binding and alter their biological actions. Journal of Biological Chemistry 275, 18188-18194.

Iwanaga, S. and Lee, B.L. 2005. Recent Advances in the innate immunity of invertebrate animals. Journal of Biochemistry and Molecular Biology 38, 128-150.

Jones, J.I., Clemmons D.R. 1995. Insulin like growth factor and their binding proteins: Biologic actions. Endocrine Reviews 16, 3-34.

Koman, A., Cazaubon, S., Couraud, P.O., Ullrich, A., Strosberg, A.D. 1996. Molecular characterization and *in vitro* biological activity of placentin, a new member of the insulin gene family. Journal of Biological Chemistry 271, 20238-20241.

Kooijman R., Scholtens L. E., Rijkers G. T. and Zegers B. J.M. 1995. Type I insulin-like growth factor receptor expresion in different developmental stages of human thymocytes. Journal of Endocrinology 147, 203–209.

Kyte, J., Doolittle, R.F. 1982. A simple method for displaying the hydropathic character of a protein. Journal of Molecular Biology 157, 105-132.

LeRoith, D., Shiloach, J., Roth, J., Lesniak, M.A. 1980. Evolutionary origins of vertebrate hormones: substances similar to mammalian insulins are native to unicellular eukaryotes. Proceedings of the National Academy of Sciences of the United States of America 77, 6184- 6188.

Lin, C.L., Wang, P.C., Chuang, N.N. 1993. Specific phosphorylation of membrane proteins of Mr 44,000 and Mr 32,000 by the autophosphorylated insulin receptor from the hepatopancreas of the shrimp *Penaeus monodon* (Crustacea: Decapoda). Journal of Experimental Zoology 267, 104-112.

Lok, S., Johnston, D.S., Conklin, D., Lofton-Day, C.E., Adams, R.L., Jelmberg, A.C., Whitmone, T.E., Schrader, S., Griswold, M.D., Jaspers, S.R. 2000. Identification of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat. Biology of Reproduction 62, 1593- 1599.

Manière G, Rondot I, Büllesbach EE, Gautron F, Vanhems E, Delbecque JP. 2004. Control of ovarian steroidogenesis by insulin-like peptides in the blowfly (Phormia regina). Journal of Endocrinology 181: 147-156.

Manor, R., Weil, S., Oren, S., Glazer, L., Aflalo, E.D., Ventura, T., Chalifa-Caspi, V., Lapidot, M., Sagi, A. 2007. Insulin and gender: An insulin-like gene expressed exclusively in the androgenic gland of the male crayfish. General and Comparative Endocrinology 150, 326-336.

McCusker, R.H. 1998. Controlling Insulin-like growth factor activity and the modulation of insulin-like growth factor binding proteins and receptor binding. Journal of Diary Science 81, 1790-1800.

Mizoguchi, A., Ishizaki, H., Nagasawa, H., Kataoka, H., Isogai, A. 1987. A monoclonal-antibody against a synthetic fragment of bombyxin (4K-prothoracicotropic hormone) from the silkmoth, *Bombyx mori*: characterization and immunohistochemistry. Molecular and Cellular Endocrinology 51, 227-235.

Morrison, T.B., Weis, J.J., Wittwer, C.T. 1998. Quantification of low-copy transcripts by continuos SYBR Green I monitoring during amplification. Biotechniques 24, 954-960.

Nagasawa H, Kataoka H, Isogai A, Tamura S, Suzuki A, Mizoguchi A, Fujiwara Y, Suzuki A, Takahashi SY, Ishizaki H. 1986. Amino acid sequence of prothoracicotropic hormone of the silkworm *Bombyx mori*. Proceedings of the National Academy of Sciences of the United States of America 83: 5840-5843.

Nogueira, B.V., Muehleisen, D.P., Whisenton, L.R., Gray, R.S., Bollenbacher, W.E. 1997. Life cycle expression of a bombyxin-like neuropeptide in the tobacco hornworm, *Manduca sexta*. Journal of Insect Physiology 43, 47-53.

Nyman, T. and Pekonen, F. 1993. The expression of insulin-likegrowth factors and their binding proteins in normal human lymphocytes. Acta Endocrinológica 128: 168–172.

Olinski, R.P., Lundin, L-G., Hallbook, F. 2006. Conserved synteny between the *Ciona* genome and human paralogons identifies large duplication events in the molecular evolution of the insulin-relaxin gene family. Molecular Biology and Evolution 23, 10-22.

Porcheron, P., Morinières, M., Grasi, J., Pradelles, P. 1989. Development of an enzyme immunoassay for ecdysteroids using acetyl-cholinesterase as label. Insect Biochemistry 19, 117-122.

Reinecke, M., Betzler, D., Drankenberg, K., Falkmer, S., Sara, V.R. 1993. Occurrence of members of the insulin superfamily in central nervous system and digestive tract of protochordates. Histochemistry 99, 277-285.

Reinecke, M., Drakenberg, K., Falkmer, S., Sara, V.R. 1991. Presence of IGF-1-like peptides in the neuroendocrine system of the atlantic hagfish, *Myxine glutinosa* (cyclostomata): evidence derived by chromatography, radioimmunoassay and immunohistochemistry. Histochemistry 96, 191-196.

Richardson, N.A., Anderson, A.J., Sara, V.R., 1997. The effect of insulin/IGF on glucose and leucine metabolism in the redclaw crayfish (*Cherax quadricarinatus*). General and Comparative Endocrinology 105, 287-229.

Riehle, M.A., Fan, Y., Cao, C., Brown, M.R. 2006. Molecular characterization of insulin-like peptides in the yellow fever mosquito, *Aedes aegypti*: Expression, cellular localization, and phylogeny. Peptides 27, 2547-2560.

Robertson, L., Bray, W., Leung-Trujillo, J., Lawrence, A. 1987. Practical molt staging of *Penaeus setiferus* and *Penaeus stylirostris*. Journal of World Aquaculture Society 18, 180-185.

Sanders, B. 1983a. Insulin-like peptides in the lobster *Homarus americanus*. I. Insulin immunoreactivity. General and Comparative Endocrinology 50, 366-373.

Sanders, B. 1983b. Insulin-like peptides in the lobster *Homarus americanus*. II Insulin-like Biological activity. General and Comparative Endocrinology 50, 374-377.

Sevala, V.M., Sevala, V.L., Loughton, B.G. 1993. Insulin-like molecules in the beetle *Tenebrio molitor*. Cell and Tissue Research 273, 71-77.

Shimasaki, S., Shimonaka, M., Zhang, H.P., Ling, N. 1991. Identification of five different insulin-like growth factor binding proteins (IGFBPs) from adult rat serum and molecular cloning of a novel IGFBP in rat and human. Journal of Biological Chemistry 266, 10646-10653.

Sitar, T., Popowicz, G.M., Siwanowicz, I., Huber, R., Holak, T.A. 2006. Structural basis for the inhibition of insulin-like growth factors by insulin-like growth factor-binding proteins. Proceedings of the National Academy of Sciences of the United States of America 103, 13028- 13033.

Smit, A.B., Van Kesteren, R.E., Li, K.W., Van Minnen, J., Spijker, S., Van Heerikhuizen, H., Geraerts, W.P.M. 1998. Towards understanding the role of insulin in the brain: lessons from insulin-related signaling systems in the invertebrate brain. Progress in Neurobiology 54, 35-54.

Söderhäll, K. and Cerenius, L. 1992. Crustacean immunity. Annual Review of Fish Diseases 2, 3-23.

Tatar, M., Bartke, A., Antebi, A. 2003. The endocrine regulation of aging by insulin-like signals. Science 299, 1346-1351.

Tatar, M., Kopelman, A., Epstein, D., Tu, M.P., Garofalo, R.S. 2001. A mutant *Drosophila* insulin receptor homolog that extends life-span and impairs neuroendocrine function. Science 292, 107-110.

Vafopoulou X, Steel CGH. 1997. Ecdysteroidogenic action of Bombyx prothoracicotropic hormone and bombyxin on the prothoracic glands of *Rhodnius prolixus* in vitro. Journal of Insect Physiology 43: 651-656.

Van Buul-Offers, S.C. and Kooijman, R. 1998. The role of growth hormone and insulin-like growth factors in the immune system. Cellular and Molecular Life Science 54, 1083-1094.

Vandesande, F, Dierickx, K. 1976. Immunocytochemical demostration of separate vasotocinergic neurons in the amphibian magnocellular neurosecretory system. Cell and Tissue Research 179, 289-296.

Van de Velde, S., Badisco, L., Claeys, I., Verleyen, P., Chen, X., Vanden Bosch, L., Vanden Broeck, J., Smagghe, G. 2007. Insulin-like peptides in *Spodoptera littoralis* (Lepidoptera): Detection, localization and identification. General and Comparative Endocrinology 153, 72-79.

Vargas-Albores, F., Yepiz-Plascencia, G. Shrimp immunity. 1998. Trends in Comparative and Biochemical Physiology 5, 195-210.

Weiss, I.M., Göhring, W., Fritz, M., Mann, K. 2001. Perlustrin, a *Haliotis laevigata* (abalone) nacre protein, is homologous to the insulin-like growth factor binding protein N-terminal module of vertebrates. Biochemical and Biophysical Research Communication 285, 244-249.

Wu, Q., Brown, M. R., 2006. Signaling and functions of Insulin-like peptides in insects. Annual Review of Entomology 51, 1-24.

Zitnan, D., Sehnal, F., Mizoguchi, A., Ishizaki, H., Nagasawa, H., Suzuki, A. 1990. Developmental changes in the bombyxin- and insulin-like immunoreactive neurosecretory system in the wax moth, *Galleria mellonella*. Development Growth and Differentiation 32, 637-645.