Mode of action of retinoic acid in the regulation of glucose metabolism in mud crab, *Scylla serrata*: Evidence for the involvement of crustacean hyperglycemic hormone

Abstract

In the current study, we evaluated the effect of retinoids, 9-*Cis* retinoic acid (9CRA) and all-*trans* retinoic acid (ATRA) on carbohydrate metabolism in the mud crab, *Scylla serrata*. Uninjured male crabs at intermolt stage and eyestalk ablated crabs (n=10 crabs per group) were selected and retinoic acid isomers, 9CRA and ATRA were injected into the crabs. Parallel controls were maintained. Significant reduction in the hemolymph sugar levels was observed in crabs subjected to eyestalk ablation (ESX) over intact crabs. Injection of 9CRA into intact but not in ESX crabs exhibited a dose- and time-dependent manner hyperglycemic response. ATRA administration did not induce hyperglycemia either in intact or ESX crabs. Injection of 9CRA showed a significant decrease in total carbohydrate content and glycogen levels in hepatopancreas and muscle tissues of intact crabs over their respective controls. Further, the activity levels of glycogen phosphorylase were also elevated in selected tissues of intact crabs administered with 9CRA. In addition, intact crabs which received 9CRA resulted in up regulation of expression of CHH mRNA from the eyestalks. Taken together, we postulate that 9CRA-induced hyperglycemia might be ascribed to neurotransmitter effects mediating the release of CHH from the eyestalk. The release of CHH eventually caused glycogenolysis in the selected hepatopancreas and muscle tissues of the mud crab *Scylla serrata* thereby hyperglycemia.

Keywords: Crabs, crustacean hyperglycemic hormone, eyestalks, glucose, retinoic acid, *Scylla serrata*

Introduction

Carbohydrates and lipids are important energy sources and nutritional components to meet the energy demand of an organism including crustaceans (Zhan et al., 2020; Chen et al., 2023). With regards to the carbohydrates, studies have shown that the dietary composition of crustaceans comprised of at least in part 20 % to 30 % carbohydrates (Wang et al., 2016). Moreover, glucose is one of the metabolic fuels that is vital for crustaceans to compensate energy demanding processes like molting/growth and reproduction and hence, regulation of glucose levels is considered important (Jimenez and Stephen, 2015). On the other hand, excess of carbohydrate intake may cause abnormal growth, and increases high mortality in crustaceans (Li et al., 2013). Hence, regulation of glucose metabolism is a key factor and is controlled and coordinated by endocrine factors in crustaceans. Crustacean eyestalks comprised of a major centre linked to nervous system and endocrine system known as the X- organ sinus gland complex (XO-SG) that secretes a range of peptides. Crustacean hyperglycemic hormone (CHH) is one of the eyestalk peptides involved in the regulation of homeostasis of carbohydrate metabolism homeostasis (Fanjal-Moles, 2006; Pillai et al., 2010; Nithya et al., 2013). Despite of thorough understanding of CHH at the molecular level and also mode of action and its target tissues like hepatopancreas and muscle in crustaceans, factors that are involved in the regulation of secretion of CHH from its source is not well understood, (Ohira, 2016; Zhang et al., 2020).

Retinoic acid (RA) a metabolite of vitamin A is popularly known as morphogen and its role in cellular processes including apoptosis, differentiation, and proliferation, physiological events like reproduction in vertebrates is well established (Theodosiou et al., 2010; Clagget-Dame and Knutson, 2011; Andre et al., 2014; Macejova et al., 2016; Nakajima et al., 2016; Persaud et al., 2016). There are two isoforms of RA: 9-cis retinoic acid (9CRA) and all-trans retinoic acid (ATRA) that play key roles in the biological framework of vertebrates including the regulation of carbohydrate metabolism (Rhee and Plutzky, 2012; Olsen and Blomhoff,

2020; Napoli et al., 2022). Studies have shown that the regulation of glucose levels by RA possibly occurs via stimulation of insulin secretion from the beta cells of Langerhans in pancreas and also expression of glucose transporter 2 (GLUT-2) gene and these events may be achieved through RA-retinoid receptor system (Pan et al., 2014; Brun et al., 2016). Surprisingly, studies at the level of RA isomers showed differential effects on insulin secretion and GLUT-2 expression, wherein 9CRA administration enhanced insulin release, while administration of ATRA has been associated with both insulin release and the expression of the glucose transporter 2 (GLUT 2) gene, suggesting differential effects of RA in the regulation of glucose levels (Rhee and Plutzky, 2012). Published have shown that the mode of action of RA isomers on carbohydrate metabolism underlines both genomic and non-genomic actions (Blaner et al., 2019). Genomic actions of RA occurs through retinoid receptors [9CRA binds retinoic acid X receptors (RXRs) and retinoic acid receptor (RARs) and ATRA exerts its genomic action via RARs] (Mangelsdorf et al., 1995; Theodosiou et al., 2010). Published studies have shown that the 9CRA serves as the cognate ligand for RXR which is an obligatory partner for nuclear receptors that are involved in metabolic functions and energy homeostasis, while ATRA executes its actions through RAR only. Based on this notion, studies have indicated that 9CRA induced regulation of insulin secretion and gene expression of genes such as GLUT-2 may be different as compared to ATRA induced effects on insulin secretion (Chertow et al., 1997; Kane et al., 2010). However, such information is not well clarified in invertebrates and in particular crustaceans. Due to the recognition of edible crustaceans as one of the food production aqua-sectors, research towards the identification signalling molecules that that influence biologic events thereby growth is an active area of crustacean endocrinology. Interestingly, the counterparts of vertebrate retinoid system have been identified in protostomes including crustaceans and also non- deuterostome chordates (Mangelsdorf et al., 1995; Hopkins 2001; Hopkins et al., 2008; Theodosiou et al., 2010; Andre et al., 2014), suggesting a cue for

the common retinoid signalling pathways.

In crustaceans, studies pertaining to vitamin A metabolism gained momentum due to aquaculture activities and most notably, vitamin A is used as one of the dietary components of crustacean feed. The discovery of RA isomers might be suggestive of occurrence of biotransformation cascade of vitamin A in crustaceans (Hopkins et al., 2008; Andre et al., 2014; Venkaiah et al., 2019). Published reports also indicated that retinoid isomers such as 9CRA and ATRA, retinoic acid X receptors and cellular retinoic acid binding proteins have been discovered (Asazuma et al., 2007; Hopkins et al., 2008; Tang et al., 2014; Nagaraju et al.,2011; Cui et al.,2013; Venkaiah et al., 2019). Experimental studies have shown that supplementation of vitamin A caused significant effects on growth performance, lipid metabolism and antioxidant capacity in juvenile chinese mitten crabs, *Eriocheir sinensis* (Huang et al., 2022a and b). Few studies have shown that RA isomers may be involved in the regulation of endocrine mediated processes like ovarian maturation, limb regeneration, carbohydrate metabolism (Zou and Bonvillian 2003; Reddy and Sainath, 2008; Andre et al., 2014; Venkaiah et al., 2019; 2023). However, in order to get a clear picture of vitamin A metabolism, adequate data seems to be of paramount importance.

Mud crab, *S. serrata* is an edible crab and is popular as an "excellent high protein aquatic source". Because of its high protein content and also of its economic importance, it is one of the cultivable aquatic species in this part of country. Among the crustacean models, mud crab *S. serrata* is also widely used experimental models to study a) the biological effects of

pharmacological agents (Girish et al., 2015; Venkaiah et al., 2019) b) the effects of environmental pollutants (Bharathkumar et al., 2022; Yang et al., 2023) infection of biotic factors like viruses (Rajendran et al., 2022) and d) identification and characterization of new proteins (Neelima et al., 2022). This study explores the under examined role of RA isomers in carbohydrate metabolism and their potential regulatory interactions with CHH in crustaceans. To address this notion, in this study, we selected *S. serrata* as an experimental model to study the possible role of RA isomers on carbohydrate metabolism. Further, we did in silico analysis to understand the interactions between RA isomers and tributyltin against Scylla, *RXR*.

Materials and methods

Preparation of test chemicals:

The test chemicals 9-cis-retinoic acid, 9CRA and all trans retinoic acid, ATRA (98% purity) were procured from sigma chemicals (St. Louis, MO, USA). The selected concentrations of retinoic acid isomers were freshly prepared by dissolving first in ethanol and followed by addition of crustacean saline for dilution (Van Harrevald, 1936). Retinoic acid was injected into crabs at the base of the walking legs (2nd pair) using a micro syringe (Hamilton syringe). The selected retinoic acid doses were administered in 10 µL volume per leg per crab.

Housing of animals:

Intact intermolt mud crabs, *Scylla serrata* were collected from the Krishnapatnam coast, Nellore District, Andhra Pradesh, India. After their arrival, the crabs were maintained in large tanks (size: 30 cm x 50 cm x 60 cm) and maintained under the laboratory conditions (temperature 25 ± 3^{o}C, salinity 30 ± 1 ppt, pH 7.3; continuous aeration) for acclimatization over a period of at least 10 days prior the start of experimentation. The crabs were fed with fish flesh *ad libitum* once daily (between 7AM to 8.30 AM) and for every 3 h after feeding, water was changed. Feeding was stopped one day before the commencement of the experiment to avoid changes due to prandial activity. In the current study both intact and eyestalk ablated crabs were

used. Eyestalk ablation is a common procedure to deprive the circulatory eyestalk hormones in crustaceans. In this study, blilateral eyestalk ablation was performed by cutting off the stalks at the base and cauterize the wound after operation to avoid leakage.

Experimental design:

In the present study, both intact and eyestalk ablated (ESX; Venkaiah et al., 2023) crabs were used. The experimental groups (n=10 crabs per group) were as follows: Group 1 and 1a: comprised of intact and ESX crabs, respectively and served as controls (did not received any treatment). Groups 2 and 2a: comprised of intact and ESX crabs and treated as concurrent controls wherein these crabs received crustacean saline (10 µL volume). Groups 3 to 10: comprised of intact carbs which received different concentration of 9CRA at 10^{-12} to 10^{-5} mole/crab, respectively. Groups 3a to 10a: comprised of intact carbs which received different concentration of ATRA at 10^{-12} to 10^{-5} mole/crab, respectively. Groups 11 to 18: comprised of ESX carbs which received different concentration of 9CRA at 10^{-12} to 10^{-5} mole/crab, respectively. Groups 11a to 18a: comprised of intact carbs which received different concentration of ATRA at 10^{-12} to 10^{-5} mole/crab, respectively. In order to circadian changes, the timeline selected for performing experiments were in between 8 AM to 11 AM.

Assay of hemolymph sugar levels

Two hours after the injection of selected RA isomers into intact and ESX crabs, hemolymph was collected to measure the sugar levels (Reddy and Sainath, 2008). Next, we analyzed the effect of 9CRA (10^{-7} mole/crab) on hemolymph sugar levels at different time points i.e. 0, 60, 120, 180, 240 and 300 min in crabs.

Biochemical analysis

After selection of specific concentration and time-course of action of 9CRA, the biochemical variables like total carbohydrates, glycogen and phosphorylase activity levels in the CHH targeted tissues like hepatopancreas and muscle of crabs from control and

experimental groups were determined. To accomplish this task, thirty-six crabs were divided into four equal groups and were as follows: group 1 and 2 comprised of untreated intact and ESX crabs, respectively; groups 2 and 4: comprised of intact and ESX crabs that received 10^{-7} mole 9CRA/crab, respectively. Tissues like hepatopancreas and muscle from crabs were isolated for estimation of total carbohydrates, glycogen and phosphorylase activity levels.

Determination of Hemolymph glucose level

Glucose oxidase kit (Sigma Co. Ltd., USA) was used to determine the hemolymph glucose levels. 100 µl of hemolymph was withdrawn from the base of walking legs using a 1 mL sterile syringe followed by the addition of 10 % (W/V) trichloroacetic acid solution. Centrifugation of the mixture at 4000 rpm for 10 min. resulted in clear supernatant which was sued to determine the glucose.

Tissue carbohydrate (TCHO), glycogen and phosphorylase activity analysis

The tissues like hepatopancreas and muscle were isolated from the crabs and blotted immediately on filter paper. After removal of adhering body fluids, the tissues were then used to measure the selected biochemical variables. Tissue total carbohydrates and glycogen content was determined using the method as described in Carroll et al., (1956). Briefly tissue homogenates were prepared in TCA (10% W/V) using sterilized mortar and pestle. The homogentate was subjected to centrifugation at 3000 rpm for 15 min at 4°C. The resultant supernatant was used to determine total carbohydrates immediately, however, for the determination glycogen content, five volumes of 95% ethanol was added to 1 ml of supernatant and thoroughly mixed followed by incubation of tubes at 4°C overnight. Next day, the mixture was centrifuged (3000 rpm for 15 min) followed by the addition of 0.5 ml distilled water to dissolve the precipitate which was used to determine tissue glycogen content. The methodology includes the addition of 5.0 mL of Anthrone reagent to the 0.5 mL of supernatants and the tubes were boiled for 15 min. After cooling at room temperature, the colour developed

was read at 620 nm spectrophotometrically (Jasco V-750; Mary's Court Easton, MD 21601) against a reagent blank. The amount of total carbohydrates and glycogen content was determined using a standard graph obtained with a known quantity of glucose.

Activity levels of glycogen phosphorylase

The activity of glycogen phosphorylase from CHH targeted tissues was performed in accordance to the method described by Cori et al., (1955). Briefly, 5 g of selected tissues were homogenized in 100 mL of 0.1 M sodium fluoride and 0.037 M ethylenediamine tetra acetic acid (pH 6.8). The homogenate was centrifuged at 3000 rpm for 10 min. After separating supernatant from the tubes, a buffer comprised of 0.03 M cysteine hydrogen chloride and 0.07 M sodium glycerophosphate was added to the enzyme source (supernatant) in a 1:3 ratio. This enzyme source (supernatant + buffer) was taken into another tube (0.4 mL) followed by the addition of 2 % glycogen solution (0.2 mL) and incubated for 20 min. at 35^{o}C. After incubation, the mixture was taken into two tubes of 0.2 mL each. In tube 1, 0.2 mL of 0.016 M glucose-1-phosphate (G-1-P) was added. This was used to determine the activity of phophorylase 'a' (active form of enzyme), whereas in another tube, 0.2 ml of 2% glycogen solution, 0.2 ml of a 1:1 mixture of 0.016 M G-1-P and 0.004 M adenosine-5-monophosphate was added. This (tube 2) was used to determine the activity of phophorylase 'ab' (total enzyme activity). The reaction in both the tubes were stopped by adding, 5 ml of 5 N sulfuric acid. The activity levels of phosphorylase in selected tissues was analyzed the liberation of inorganic phosphate from glucose-1-phosphate using the **method described by [37]. In** a brief, to 1.0 ml of solution (from tube 1 and tube 2), 1.0 ml of 2.5% ammonium molybdate solution was added and mixed thoroughly followed by the addition of 0.4 ml of 0.2 % 1-2-4-amino naphthosulphonic acid reagent. The colour developed was diluted with 10 ml of distilled water and after five minutes, the intensity of colour developed was monitored spectroscopically at 720 nm against the reagent blank. The units were measured as moles of Pi liberated/mg protein/hr. The protein

concentration in the enzyme source was determined using Lowry method using bovine serum albumin as a reference.

CHH from the eyestalks of *S. serrata*: cloning and expression

Briefly, total RNA was isolated from 50 eyestalk ganglia of crabs using Trizol reagent (Invitrogen, California, USA). The quantity and quality of total RNA was analyzed through Nanodrop (Thermo Scientifics) and agarose gel electrophoresis (1 % agarose). For the synthesis of first strand cDNA synthesis, 5 µl or 2 µg of total RNA was used and synthesized as per the manufacturer's instructions of PrimeScript^{TM} cDNA synthesis kit (Takara, Japan). Semi-quantitative PCR (Applied Biosystems, SimpliAmp^{TM}, Thermal cycler) was used to amplify the *CHH* mRNA using the forward (5'->3': GCAGATGGTTTTGGGCGTAT) and reverse (5'->3': GTCCATCAGCAGGAGGTCTT) primers were designed based on the available CHH sequences from various crustaceans. The reaction mixture (50 µl) comprised of 25 µl of PCR master mixture (TaKaRa, Japan), 3 µl of cDNA template, 1 µl of forward primer, 1 µl of reverse primer and 20 µl of nuclease free water. The PCR conditions were: initial denaturation (94^{0}C, for 1min), and final denaturation (98^{0}C for 30 sec), annealing (55^{0}C for 30 sec) and extension (72^{0}C for 5 min). After the extension step reaction mixture was cooled at 4^{0}C. The amplicons were analysed via agarose gel electrophoresis (1.8 %) and visualized under UV transilluminator to determine the molecular weight. The amplicons were purified from the gels using columns and subjected to sequence analysis. The obtained sequence was subjected to BLASTx analysis for confirmation. The sequence characteristics were determined using computational tools. Phylogenetic analysis was performed to analyze the relationship with other crustceans using neighborhood-joining method using an *in silico* tool MEGA (Ver. 11.0) with a bootstrap value of 1000.

The expression of CHH mRNA from the eyestalks of control (n=20) and 9CRA injected (n=20) crabs was analyzed using RT-PCR analysis. The first and second strand cDNA

synthesis, PCR conditions were similar to the protocol mentioned in previous section. RT-PCR was performed using the primers as mentioned above for CHH. The amplified products were run on 1.8% agarose gels in TAE buffer, and the CHH amplicons' relative intensities were normalized against the corresponding β-actin band. β-actin was used as an internal control and the primers (forward primer: 5'->3' CAAGCGAGGTATCCTGACTCT and reverse primer 5'->3' CCACGTTCATCTCACTCTCG) were designed based on the studies of Girish et al. (2015).

Statistical analysis

The results were expressed as mean ± SD. Analysis of variance (one way ANOVA) followed by Tukey's post-test was used to determine statistical significance. SPSS (Version 16.0, SPSS, Inc., Chertsey, UK) was used for the statistical analysis.

Results

Effect of eyestalk ablation on carbohydrate metabolism

Significant reduction in the hemolymph glucose levels was noticed in ESX crabs over intact crabs (Figures 1 and 2). The biochemical variables like total carbohydrates and glycogen content were significantly elevated in muscle and hepatopancreas of ESX crabs over their respective controls. On the other hand, a significant reduction was observed in total phosphorylase activity in selected tissues of ESX crabs (Tables 1 and 2).

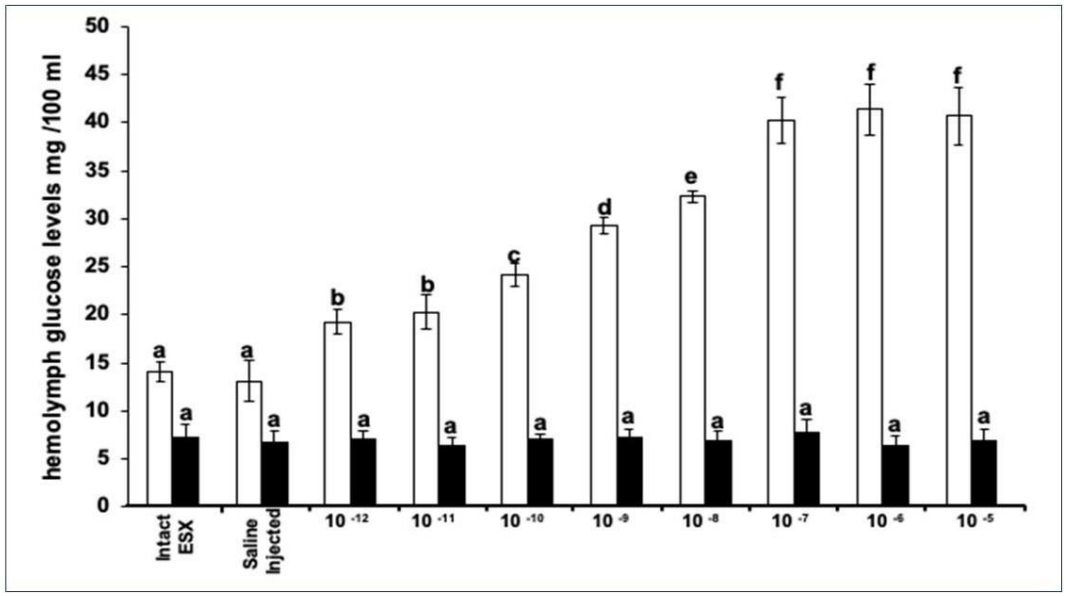

Figure 1: Effect of 9-*cis* retinoic acid on hemolymph glucose levels in intact (open bars) and eyestalk-ablated (ESX) crabs (solid bars).

Hemolymph was collected from animals for glucose quantification 2 hr after injection. Each bar represents a mean ± SD of ten individual crabs. Bars with same superscript do not differ significantly from each other at P<0.001.

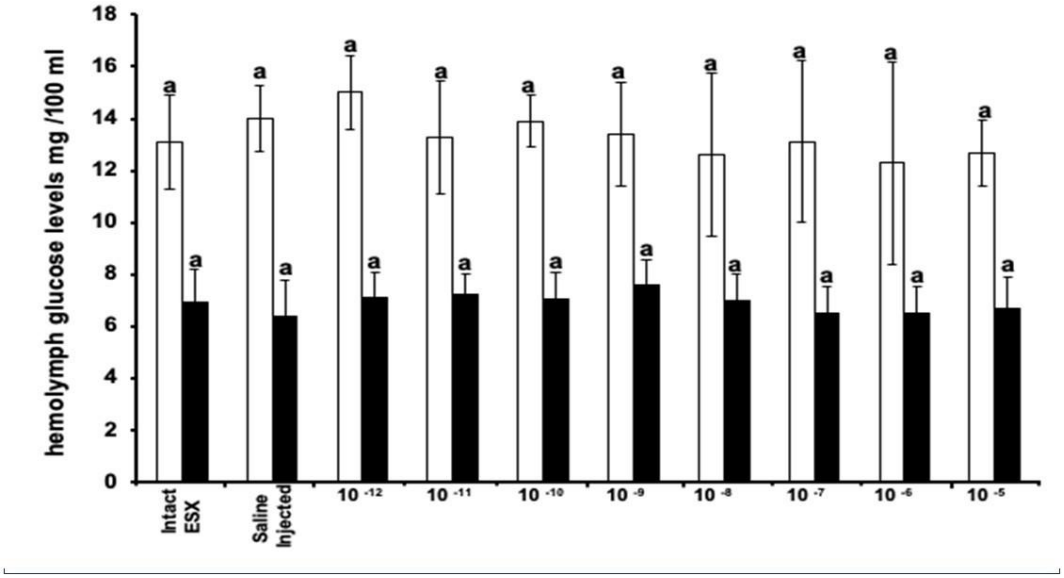

Figure 2: Effect of all-*trans* retinoic acid on hemolymph glucose levels in intact (open bars) and eyestalk-ablated (ESX) crabs (solid bars).
Hemolymph was collected from animals for glucose quantification 2 hr after injection.
Each bar represents a mean ± SD of ten individual crabs.
Bars with same superscript do not differ significantly from each other at P<0.001.

Table 1: Effect of injection of 9-*cis* retinoic acid (9CRA) into normal and eyestalk

ablated (ESX) crabs on total carbohydrate (TCHO), and glycogen levels in the hepatopancreas and muscle tissues of mud crab *Scylla Serrata*

	TCHO		Glycogen	
Group	**Hepatopancreas**	**Muscle**	**Hepatopancreas**	**Muscle**
Control	15.09 ± 0.48	3.97 ± 0.42	1.17 ± 0.14	0.57 ± 0.002
ESX	23.38*^{A} ± 0.97	8.07*^{A} ± 0.14	2.69*^{A} ± 0.34	1.71*^{A} ± 0.006
9CRA-injected intact	10.09* ± 1.07	2.86* ± 0.21	0.44* ± 0.07	0.38* ± 0.002
9CRA-injected ESX	22.53*^{A} ± 0.89	8.39*^{A} ± 0.31	2.51*^{A} ± 0.68	1.81*^{A} ± 0.008

Values are mean (mg glucose/g tissue) ± SD of ten individual crabs. For evaluation of ‘’P’’ for ESX, and 9CRA injected crabs (normal and ESX), crabs in control group served as controls; for 9CRA injected ESX crabs, ESX crabs served as controls.
*Represents significant value at P<0.0001. Mean values with same alphabets in a row did not differ significantly from each other.

Table 2: Effect of injection of 9-*cis* retinoic acid (9CRA) into normal and eyestalk ablated (ESX) crabs on phosphorylase activity levels in the hepatopancreas and muscle tissue of mud crab, *Scylla Serrata.*

	Hepatopancreas Phosphorylase		**Muscle Phosphorylase**	
Group	**"a"**	**"ab"**	**"a"**	**"ab"**
Control	1.89 ± 0.34	5.01 ± 0.28	2.99 ± 0.24	7.01 ± 0.12
ESX	1.18*^{A} ± 0.18	3.21*^{A} ± 0.14	2.21*^{A} ± 0.17	4.01*^{A} ± 0.23
9CRA-injected intact	3.73* ± 0.51	5.48* ± 0.42	4.82* ± 0.18	7.38* ± 0.41
9CRA-injected ESX	1.21*^{A} ± 0.47	3.16*^{A} ± 0.24	2.21*^{A} ± 0.31	4.13*^{A} ± 0.19

Values are mean (imoles of iP released/mg protein/hr) ± SD of ten individual crabs.
For evaluation of ''P'' for ESX, and Normal+9CRA injected crabs, crabs in control group served as controls; for ESX+9CRA injected crabs, ESX crabs served as controls. P<0.001;
Mean values with same alphabets in a row did not differ significantly from each other.

Effect of RA isomers on hemolymph glucose levels

Intact crabs that received saline did not show hyperglycemic response. Injection of 9CRA but not ATRA into intact crabs resulted in significant hyperglycemia as compared to its respective controls in a dose dependent manner (10^{-12} to 10^{-5} mole 9CRA/crab) (Figs. 1 and 2). However, a saturated response in hemolymph glucose levels were observed in intact crabs that received doses from 10^{-7} mole 9CRA/crab to 10^{-5} mole 9CRA/crab. In the subsequent experiments, 10^{-7} mol/crab was selected as injection dose. Whereas such hyperglycemic response was not observed in crabs without eyestalks that had received 9CRA at any selected doses.

Time course action of 9CRA on hemolymph sugar levels:

Figure 3 represent the effect of 9CRA on hemolymph glucose levels at different time points (o min. to 300 min). Administration of 10^{-7} mol 9CRA/crab resulted in significant elevation in hemolymph glucose levels (P<0.001) and reached a highest peak at 2 hr and a decline in hemolymph glucose levels was observed thereafter. Surprisingly, even after six

hours, significant increase in hemolymph glucose level was observed in crabs that received 10^{-7} mol 9CRA/crab as compared to controls.

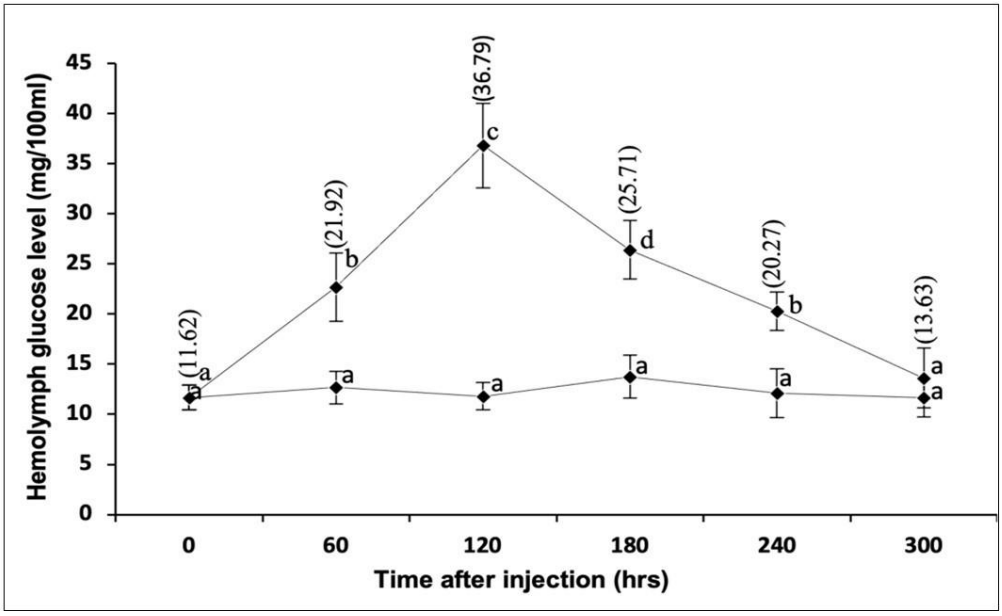

Figure 3: Time course action of 9-*cis* retinoic acid-induced hyperglycemia in intact crabs.

Hemolymph was collected from intact crabs after injection of 9CRA (10^{-7} mol/crab), at the time points indicated for glucose quantification.
Each point represents a mean ± SD of ten individuals. Values in parentheses represent percent change from control (0 hr).

Effect of 9CRA on total carbohydrates (TCHO), glycogen levels and phosphorylase activity:

Administration of 9CRA into intact crabs caused a significant decrease in total TCHO and glycogen levels in the hepatopancreas and muscle tissues when compared to controls (Table 1). In addition, the activity levels of phosphorylase 'a' and 'ab' in the hepatopancreas and muscle in 9CRA injected intact crabs showed a significant increase over their respective controls (Table 2). However, ESX crabs injected with 9CRA did not cause significant changes in selected biochemical variables in selected tissues over their respective controls (Tables 1 and 2).

Molecular cloning, and phylogenetic analysis of *S. serrata* CHH

An amplicon with a size of 291 base pairs (equivalent to 97 amino acids) was obtained by RT-PCR (Figure 4A). The CHH sequence from the eyestalk of *S. serrata* revealed an ORF comprised of CHH precursor related peptide (CPRP: 35 amino acid residues), a dibasic cleavage site (KR: 2 amino acid residues) and mature peptide (60 amino acids) (Figure 4B).

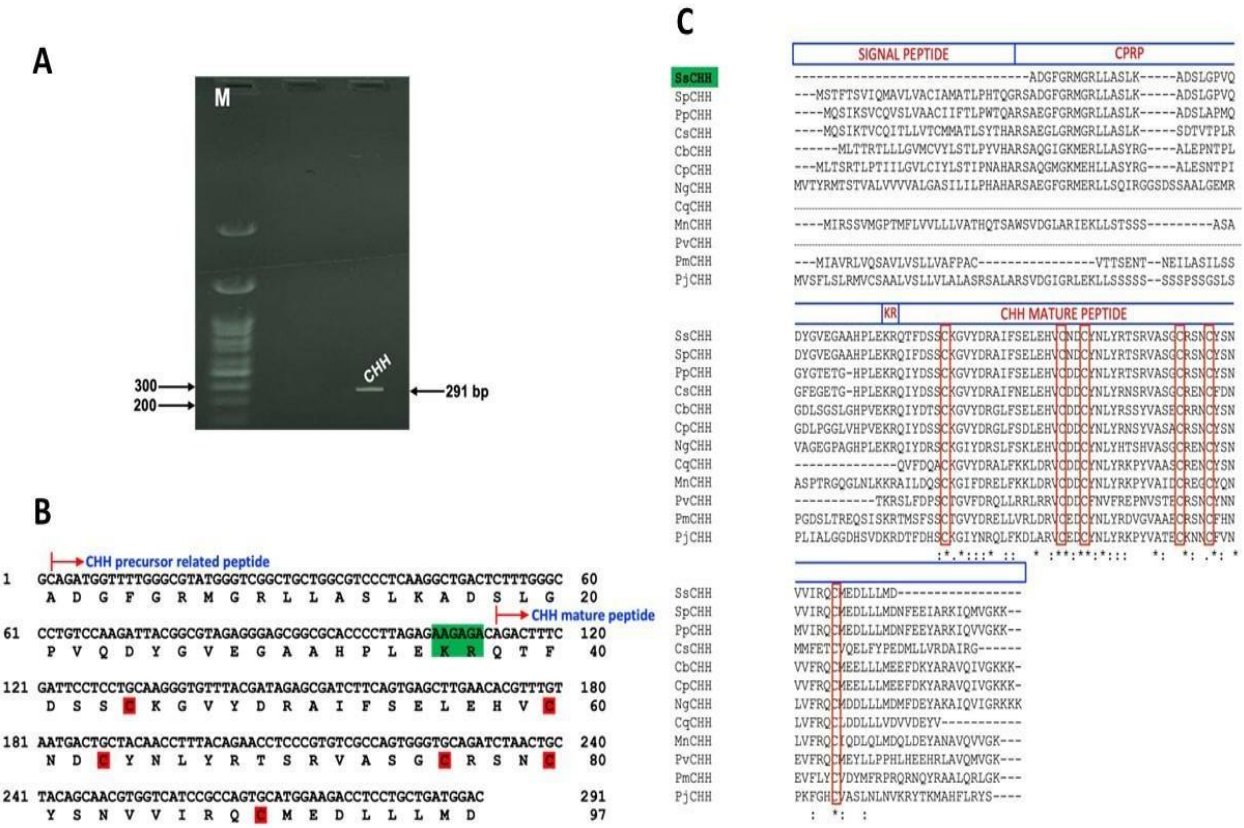

Figure 4: A: PCR amplification fragment for CHH resolved on 1.5% agarose gel electrophoresis;

Lane M = DNA Marker; lane 1 = blank control; lanes 2 = PCR amplification fragment of CHH.
B: Nucleotide and deduced amino acid sequence of a crustacean hyperglycemic hormone cDNA (SsCHH) from eyestalk of *S. serrata*.
The deduced amino acid sequence of the open reading frame is shown in single letter code below the nucleotide sequence (numbers at left). Arrows mark the cleavage site for the CPRP, the dibasic processing site (KR) and CHH mature peptide. Six conserved cysteines are red coloured boxed.
C: Comparison of deduced amino acid sequences of full-length CHH cDNAs from other crustacean species. Amino acid sequence of *Scylla serrata* CHH (Ss CHH) was aligned with CHHs from *Scylla* paramamosain CHH; *Portunus pelagicus* CHH; *Callinectes sapidus* CHH; *Cancer borealis*; *Cancer productus*; *Neohelice granulate* CHH; *Cherax quadricarinatus* CHH; *Macrobrachium nipponense* CHH; *Penaeus japonicus* CHH; *Penaeus monodon* CHH and *Penaeus vannamei* CHH using ClustalW. CPRP, KR and CHH mature peptide

shown in blue colour boxes and six conserved cysteines were shown in red colour boxes.

The partial CHH sequence was submitted to NCBI GenBank under the Gene accession Id number OP734461. Clustal W (multiple sequence analysis) was performed using already reported CHH sequences to gain insights into the conserved regions of CHH. Multiple CHH alignments confirmed that all CHH molecules contain six cysteine residues that were conserved (Figure 4C). MEGA (Version 11.0) was used to build a phylogenetic tree using Neighbourhood Joining (NJ) method with a bootstrap value of 1000 to determine the relationship of *S. serrata* CHH to other crustacean CHH sequences (Figure 5) and the results indicated that *S. serrata* CHH was closely associated with CHH sequences of brachyurans (crabs) than astacurans (crayfish), palaemonids (shrimps/prawns).

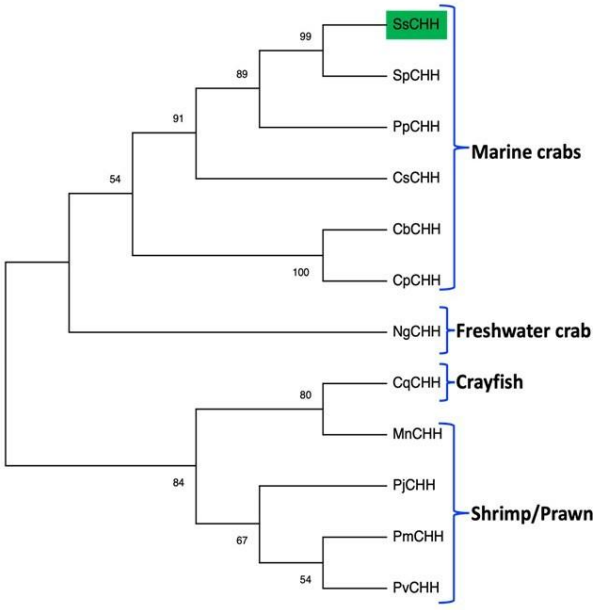

Figure 5: The phylogenetic tree based on protein sequences was constructed using the neighbor-joining method in MEGA 11, with a bootstrap replication number of 1000.

Scylla serrata CHH (OP734461; this study); *Scylla* paramamosain CHH (AFD28273.1); *Portunus pelagicus* CHH (AFM29133.1); *Callinectes sapidus* CHH (ACH85179.1); *Cancer borealis* CHH (QKO41648.1); *Cancer productus* CHH (ABQ41272.1); *Neohelice granulate* CHH (AJD81303.1); *Cherax quadricarinatus* CHH (AAZ03612.1); *Macrobrachium nipponense* CHH (AEJ54624.1); *Penaeus japonicus* CHH (BAE78493.1); *Penaeus monodon* CHH (AAQ24527.1); *Penaeus vannamei* CHH (AAR11295.1).

CHH mRNA levels in intact crabs treated with or without 9CRA

Injection of 9CRA resulted in up regulation of *CHH* mRNA in the eyestalks of intact crabs over their respective controls (Figure 6).

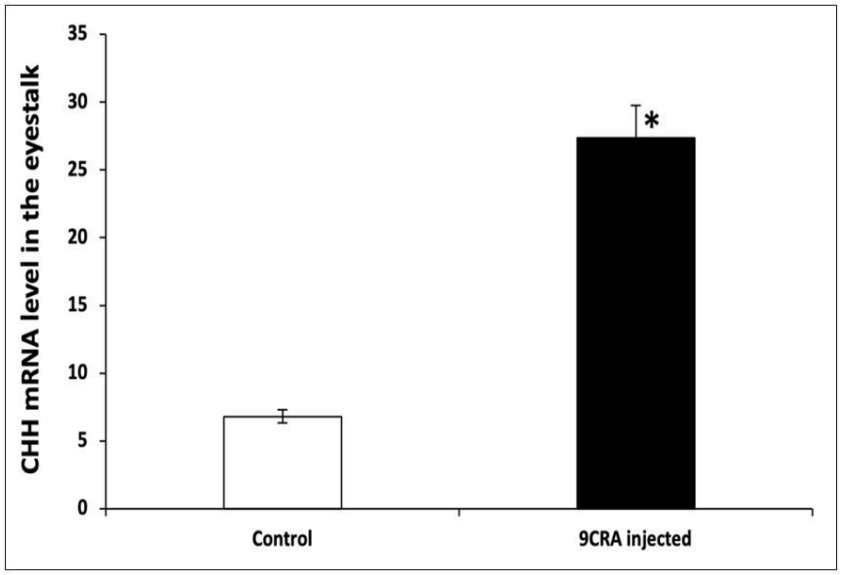

Figure 6: Changes in the expression of *CHH* mRNA in the eyestalk of intact crabs injected with 9-*cis* retinoic acid (9CRA).
Each bar represents a mean ± S.D. of ten individuals. Asterisk (*) represent significant differences between the control and intact crabs injected with 9CRA at $p < 0.001$.

Discussion

The results of present study indicated that the administration of 9CRA resulted in hyperglycemia in intact crabs over its respective controls as evidenced by an increase in a) hemolymph glucose levels, and activity levels of glycogen phosphorylase in CHH targeted tissues like muscle and hepatopancreas and a reduction in b) total carbohydrates and glycogen content from selected CHH targeted tissues, and up regulation of eyestalk *CHH* and *RXR* mRNA levels.

In this study, CHH from eyestalks of Scylla was cloned using RT-PCR with an amplicon size of 291 base pairs showing all characteristic features like CPRP, a dibasic cleavage site, and a mature peptide (Liu et al., 2019). Phylogenetic analysis also revealed a close relationship with

marine crabs, while a clear speciation was observed with prawns, shrimps and crayfish (Chen et al., 2005). Our findings showing a substantial reduction in hemolymph sugar levels in ESX crabs, might suggest that the distribution and importance of the CHH, in *S. serrata*. The mechanism of CHH with relevance to glucose metabolism involves the stimulation of glycogen phosphorylase in its target tissues like muscle and hepatopancreas wherein glycogen breakdown into glucose (Fanjal-Moles, 2006). Thus, glycogenolysis in CHH targeted tissues leads to increase in tissue glucose levels which eventually leak into the hemoplymph thereby hyperglycemia. Therefore, it is obvious that in ESX crabs, since eyestalk peptide CHH was completely devoid, significant reduction in activity levels of phosphorylase system associated with elevated levels of total carbohydrates and glycogen content (Reddy and Sainath, 2008; Sainath and Reddy, 2010; Yang et al., 2018). In the present study, we isolated CHH gene from the eyestalks of *S. serrata* and inspection of CHH sequence showed key elements like CPRP site, a dibasic cleavage site, mature peptide and also a highly conserved six cysteine residues (Chen et al., 2020). Phylogenetic analysis revealed that *S. serrata* CHH could be a closely associated to *S. paramamosian* CHH1 isoform (Fu et al., 2014).

Several studies also suggested that during stress mediated hyperglycemia, CHH might be a key factor in the regulation of glucose metabolism (Lorenzon et al., 2004; Swetha et al., 2014; Li et al., 2019). 9CRA injection showed regulatory effects on glucose homeostasis in intact crabs as compared to its respective control and 9CRA-injected ESX crabs, suggesting 9CRA at least in part mediate eyestalk principle, CHH. This was evident by the changes observed at the CHH targeted tissues in 9CRA injected intact crabs. The role of RA in controlling the metabolism of glucose is well known in vertebrates (Theodosiou et al., 2010). In vertebrates, retinoic acid at least in part mediate insulin and glucose transporter 2 gene in the regulation of glucose levels Chertow et al., 1997; Pan et al., 2014). As CHH is fundamentally associated with hemolymph glucose levels, its expression levels under 9CRA treatment were studied to understand, whether

the 9CRA induced hyperglycemia in *S. serrata* could be direct or indirect. The findings indicating that the intact crabs injected with 9CRA showed a significant increase in eyestalk CHH mRNA expression levels over controls might suggest that hyperglycemia induced by 9CRA could be indirect i.e. via triggered secretion of CHH from the eyestalks (Reddy and Sainath, 2008). Piecing these results, we demonstrated that the administration of 9CRA mediated hyperglycemia at least in part involves an eyestalk principle, CHH which eventually cause glycogenolysis in its target tissues. Studies indicated that the glucose in CHH targetted tissues might be leaked into hemolymph thereby hyperglycemia in 9CRA injected intact crabs (Venkaiah et al., 2023).

Experimental studies in rodents show that vitamin A affect liver carbohydrate metabolism and also affect key energy related processes such as glycolysis, gluconeogenesis and glycogenesis (Napoli, 2022). Several genes associated with hepatic enzymes that mediate glycolysis, and gluconeogenesis at least in part regulated by retinoids (Chen and Chen, 2014). Studies also indicated that 9CRA at least in part mediate RXR in the regulation of insulin levels (Chertow et al., 1997; Lenhard et al., 1999; Kane et al., 2010). Taken together, in vertebrates, the regulation of glucose metabolism by vitamin A is wired with genomic and non-genomic actions (Kane et al., 2010; Blaner, 2019; Napoli, 2022; O'Connor et al., 2024). Such specific actions of vitamin A in the regulation of glucose metabolism in crustaceans needs to be clarified. Since administration of either 9CRA induced hyperglycemia in intact crabs but not in ESX crabs might augment the likelihood of interaction of 9CRA with its cognate receptor in the eyestalk of crustaceans. Interestingly, expression of RXR has been demonstrated in the eyestalks of crustaceans (Gong et al., 2016; Kluebsoongnoen et al., 2021). Surprisingly, as amino acids in the ligand binding domain (LBD) of RXR from crustaceans bear a close resemblance to vertebrate RXRs LBD, it is tempted to propose that RA may also have a high affinity to its receptor in eyestalks of the crab. To address this question, studies involving co- treatment of crabs with 9CRA and RXR blocker might provide valuable insights. Studies in these lines are in progress

in our lab. Thus, studies using *in vitro* and *in vivo* studies are warranted.

Further, it is worth mentioning that the role of genomic and non-genomic actions of retinoic acid in the regulation of physiological processes in fishes, amphibians, and reptiles (Bridgham et al., 2012; Petkovich and Chambon, 2022; Hawkins and Wingert, 2023; Maydone and Palvadore, 2023) is well documented. On the other hand, in non-chordate deuterostome i.e. Echinodermata, ancestral RA signaling has been demonstrated (Yamakawa and Wada, 2022). Interestingly, retinoic acid receptors (RAR) was discovered in protostome, molluscs (Gutierrez-Mazariegos et al., 2014) and further heterodimerization between RAR/RXR was also reported in *Nucella lapillus* (Andre et al., 2019). Indubitably, the occurrence of RXR in protostomes like molluscs and crustaceans, non-chordate and chordate deuterostomes is an indication of common signaling mechanisms. However, additional in depth studies are required from protostome perspective to address notion.

Conclusion :

The findings of this study revealed that injection of 9CRA triggered the release of CHH which eventually activated the glycogen phosphorylase machinery in the hepatopancreas and muscle of intact crabs. Consequently, release of glucose into circulation thereby hyperglycemia in 9CRA injected crabs. Such response was not observed in ATRA injected intact crabs. This is one of the important findings as well in the current study. We speculate that the absence of retinoic acid receptors and discovery of RXR in crustaceans may suggest that injection of 9CRA can able to induce its transcriptional effects through RXR homodimers in eyestalk CHH producing neural cells. Such differential effects of 9CRA and ATRA were also demonstrated in human neuroblastoma cells (Redfren et al., 1994). With regards to glucose regulation, differential effects of 9CRA and ATRA on secretion of insulin in RINm5F cell lines have been reported and further, these authors suggested that 9CRA induced effects may occur via RXR (Chertow et al., 1997). However, the effects induced by 9CRA in Scylla needs further authentication studies such as ligand binding assays. This is because,

the endogenous ligand for the crustacean RXR is yet to be addressed.

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