Int. J. Aquat. Biol. (2022) 11(1): 76-85
ISSN: 2322-5270; P-ISSN: 2383-0956
Journal homepage: www.ij-aquaticbiology.com

Original Article

Effect of selected retinoids on carbohydrate metabolism in the freshwater monsoon prawn, *Macrobrachium malcolmsonii*

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Abstract: Retinoic acid isomers such as 9 *cis* retinoic acid (9CRA) and all *trans* retinoic acid (ATRA) have been discovered in crustaceans. However, their physiological significance in the biological framework of crustaceans is unclear. The present study evaluates the effect of retinoic acid on the hemolymph glucose levels in the monsoon prawn, *Macrobrachium malcolmsonii*. Injection of 9CRA into intact prawns significantly elevated the hemolymph glucose levels in a dose-dependent and time-dependent manner. However, such hyperglycemic response in 9CRA-injected eyestalk ablated (ESX) prawns was not observed. No changes in the hemolymph glucose levels were noticed in ATRA-injected intact or ESX prawns. Bilateral ESX showed significant elevation in the total carbohydrates and glycogen levels with a significant reduction in the activity levels of phosphorylase activity in the crustacean hyperglycaemic hormone (CHH) target tissues, hepatopancreas and muscle of prawns. Injection of 9CRA into intact prawns showed significant elevation in the activity levels of phosphorylase activity with a concomitant decrease in the total carbohydrates and glycogen levels in the CHH target tissues compared to vehicle-injected prawns. No significant differences were observed in the selected biochemical variables in 9CRA-injected prawns over 9CRA-injected ESX prawns. The expression levels of CHH in the eyestalks of 9CRA-injected prawns were significantly elevated over its respective control. It can be concluded that 9CRA-induced hyperglycemia, at least in part, mediates CHH in *M. malcolmsonii*.

Article history:
Received 22 October 2022
Accepted 1 January 2023
Available online 25 February 2023

Keywords:
Eyestalks
Crustacean
Hyperglycemic hormone
Glucose
Glycogen phosphorylase

Introduction

Among the top global exporters of edible crustaceans, such as crabs and prawns, India occupies the third position globally. Because of their economic importance and high nutritional value, the momentum of culturing edible crustaceans on par with fisheries is happening at a pace in India. However, the limited seed availability is one of the major bottleneck problems associated with the crustacean aquaculture industry (Sainath et al., 2013). Eyestalk ablation (ESX), a classical surgical operation technique, has been followed by aquafarmers to induce ovarian maturation in crustaceans. Though ESX is promising, it often leads to deterioration of seed quality and mortality in the broodstock. Compared to fisheries, technologies to promote reproduction (such as injection of crude extract of the pituitary gland and gonadotropin-releasing hormone) (Hoga et al., 2018) are yet to be developed in the crustacean aquaculture industry. Though clear-cut-picture is not available, many researchers investigated the signalling molecules that can promote ovarian maturation and, thereby seed in the hatchery industry (Sainath et al., 2013). However, to convert successful experiments at the laboratory level to the land/field level, it is important to understand the interplay between endogenous hormones that control and coordinate crustacean reproduction. This provides a suitable platform to understand the crosstalk between various signalling molecules that regulate ovarian growth in crustaceans at multiple levels.

Glucose is one of the substrates for energy metabolism in crustaceans (Chung et al., 2010). Several studies indicated that circulating levels of glucose alter in

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DOI: ttps://doi.org/10.22034/ijab.v11i1.1724
DOR: https://dorl.net/dor/20.1001.1.23830956.2023.11.1.10.0

response to various biotic (parasitic infections) and abiotic (temperature, hypoxia, autotomy and salinity and environmental pollutants) factors (Webster, 1996; Chang et al., 1998; Kuo and Yang, 1999; Stentiford et al., 2001; Wilcockson et al., 2002; Reddy et al., 2011). Changes in circulatory levels of glucose indicate abnormal changes, including a reduction in survival rate, decreased immunity against bacterial infections, and altered behaviour (Wanlem et al., 2011; Sun et al., 2013; Wang et al., 2017).

The X-organ sinus gland complex in crustaceans is a major neuroendocrine system synthesizing peptide hormones, including crustacean hyperglycemic hormone (CHH), a neuropeptide primarily involved in regulating glucose homeostasis (Fanjal-Moles, 2006). At the molecular level, the mature peptide of CHH comprises 72-73 amino acid residues (Soyez, 1997) and the amidation of CHH is critical for its biological activity (Katayama et al., 2002). Though the synthetic sites, chemical nature, mode of action, and target sites for CHH have been demonstrated in several crustaceans, the exact mechanism of CHH release from the XO-SG complex into the circulation is not completely understood (Fanjal-Moles, 2006; Ohira, 2016).

Many studies have shown that several signalling molecules such as biogenic amines (melatonin, and serotonin), opioids (methyl enkaphalin), and insulin-like factors can promote ovarian maturation in decapods crustaceans and at the same, they influence carbohydrate metabolism via triggered release of eyestalk peptide, CHH. On the other hand, the involvement of eyestalk peptide CHH in the regulation of crustacean reproduction is well recognized. Not all molecules (for example, dopamine) that are involved in the stimulation of eyestalk CHH, thereby hyperglycemia are involved in the promotion of ovarian growth in crustaceans. These studies indicate that the regulation of crustacean reproduction is complex as it is operated by several molecules and their crosstalk at multiple levels. Thus, the identification of signalling molecules that influence physiological functions in crustaceans needs to be verified first. This information at a later stage might be helpful in designing experiments related to the crosstalk of signalling molecules in the regulation of crustacean reproduction (Ohira, 2016).

Retinoic acid (RA) is the biologically active metabolite of vitamin A. The morphogentic effects of RA on organ differentiation during development are well-acknowledged (Theodosiou et al., 2010; Clagget-Dame and Knutson, 2011; Andre et al., 2014; Macejova et al., 2016). RA exerts in two isoforms, 9-cis retinoic acid (9CRA) and all-trans retinoic acid (ATRA) and both exert their genomic actions via 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). RXRs and RARs can form homodimers and/or heterodimers with other members of the nuclear receptor family, peroxisome proliferator activator receptor gamma (Mangelsdorf et al., 1995; Theodosiou et al., 2010). Interestingly, the occurrence of RA across major phyla of metazoans, including invertebrates, at least in part suggests that RA is not a bonafide molecule associated with vertebrates (Theodosiou et al., 2010; Andre et al., 2014). After the ligand is bounded, the RAR/RXR or RXR/RXR specifically binds the retinoid response elements on the DNA and regulates the transcriptional expression of RA target genes (Andre et al., 2014). In major invertebrate phyla (protostomes and non-vertebrate deuterostomes), the discovery of endogenous retinoids and identification of vertebrate-type counterparts of retinoid metabolism might suggest the existence of common signalling pathways (Andre et al., 2014). In crustaceans, RA isomers, 9CRA and ATRA and the counterparts of retinoid metabolism include a cellular retinoic acid binding protein that transports retinoids and retinoic acid signalling proteins retinoic acid X receptors in crustaceans (Asazuma et al., 2007; Hopkins et al., 2008; Tang et al., 2014; Nagaraju et al., 2011; Cui et al., 2013; Venkaiah et al., 2019).

Retinoid counterparts, though discovered in crustaceans, the physiological significance of retinoids in the biological framework is still in its infancy. In vertebrates, the role of RA in regulating carbohydrate metabolism is well-documented (Rhee et al., 2013). RA, through the stimulation of insulin secretion and expression of the glucose transporter 2 gene regulates the glucose levels in vertebrates and these effects of RA are believed to be mediated by retinoid receptors (Chertow et al., 1997; Pan et al., 2014). Interestingly, Zou and Bonvillian (2003) have shown that administration of 9CRA caused a significant elevation in the hemolymph sugar levels, while administration of ATRA caused inhibitory effects on hemolymph glucose levels in the fiddler crab, *Uca pugilator*. On the other hand, exogenous administration of 9CRA induced hyperglycemic effect,

whereas exogenous injection of ATRA did not result in hyperglycemia in intact freshwater crabs, *Oziotelphusa senex senex* (Reddy and Sainath, 2008). Further, studies of Reddy and Srilatha (2015) showed that 13CRA induced hyperglycemia in the same experimental crab, *O. senex senex,* and ascribed to either metabolism of 9CRA to 13CRA or direct action of 9CRA and/or 13CRA. Despite these limited studies, no information is available with respect to the studies linking retinoic acid and hyperglycemic response in crustaceans.

Macrobrachium malcolmsonii is a freshwater monsoon prawn known as poor man protein. The test organism is one of the most widely available prawns in the Penna River, which drains to Bay of Bengal, Nellore District, Andhra Pradesh, Southern part of India. It is one of the important edible prawns with aquaculture importance. It is the second largest palaemonid prawn after the freshwater giant prawn, *M. rosenbergii,* and has a potential for aquaculture development in the inland waters of India (Mishra et al., 2014). Though it has a tremendous ability for culture in ponds and rivers (Hossain et al., 2012), due to the limited availability of seed, the aquaculture of *M. malcolmsonii* is not expanded as expected (Rao et al., 1986; Mishra et al., 2014). Therefore, understanding the physiological significance of signalling molecules that promote ovarian growth is of paramount importance. However, prior development of signalling molecules as tools to promote ovarian growth, it is important to understand their physiological significance in the biological framework of crustaceans. In accordance with this notion, the present study aims to investigate the effect of selected retinoids, 9CRA, and ATRA on hemolymph glucose levels, tissue (hepatopancreas and muscle) carbohydrate metabolism and eyestalk peptide CHH in the freshwater edible monsoon prawn, *M. malcolmsonii.*

Materials and methods

Collection and maintenance of animals: Monsoon prawns, *M. malcolmsonii* was collected in and around Penna River, Nellore (14°27'52.9"N, 79°58'25.2"E), Andhra Pradesh, India and transferred from the collection site to our laboratory (distance: 14 km) in aerated plastic containers. Without causing stress, they were transferred to aquaria or holding tanks and acclimatized to the controlled laboratory conditions (temperature: 25-32ºC; salinity: 0.5 ppt; dissolved oxygen: 5-7 ppm; total alkalinity: 50-100 ppm). Filtered river water and continuous aeration facility were provided to the prawns, and during their sojourn, the prawns were fed *ad libitum* with a commercially pelleted diet (CP Aquaculture Ltd., Chennai, India). After changing the ambient medium by at least 25 to 35%, prawns were fed pelleted feed once a day. Further, the 1 g biomass to 1 L water ratio was constantly maintained throughout the experimental period. The male prawns at the intermolt stage (C4) with body size and weight of 10±1 cm and 11±2 g, respectively, were used. After acclimatization (10 days), feeding was stopped one day before the commencement of the experiment to avoid changes due to prandial activity. During the experiment period, no feed was provided to the prawns.

Eyestalk ablation: Eyestalk ablation (ESX) is a classical operation to deprive eyestalk peptides that control and coordinate metabolic events in crustaceans. In the present study, ESX prawns were obtained by cutting at the base of the stalks, and post-ablation procedure, cauterization of the wound was performed to prevent fluid loss (Sainz-Hernandez et al., 2008). After 24 hours, ESX prawns were used for the present experiments.

Test chemicals and their preparations: The isomers of retinoic acid, 9-*cis*-retinoic acid and all *trans* retinoic acid (98% purity) were purchased from Cayman Chemicals, Inc., (Ann Arbor, MI). The chemical structures of retinoic acid isomers are shown in Figure 1. Different doses of retinoic acid isomers were prepared freshly. The required concentrations of retinoic acid isomers, 9CRA, and ATRA were prepared by dissolving in ethanol and diluted accordingly in crustacean saline (Van Harreveld, 1936). The concentrations of retinoic acid isomers were prepared freshly on the day of experimentation.

Experimental design: Retinoic acid isomers 9CRA and ATRA or crustacean saline were injected into the prawns through the base of the coxa of the second pair of walking legs using a micro-syringe (Hamilton syringe).

Dose-dependent effects of retinoic acid isomers: Intact and ESX prawns in groups 1 and 1a served as controls, whereas intact and ESX prawns in groups 2 and 2a were treated as concurrent controls and received crustacean saline (10 µl volume). Intact prawns in groups 3, 4, 5, 6, 7, 8, 9 and 10 received injections of 9CRA in 10 µL volume at concentrations 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mole/prawn, respectively. ESX prawns in groups 3a, 4a, 5a, 6a, 7a, 8a, 9a and 10a received

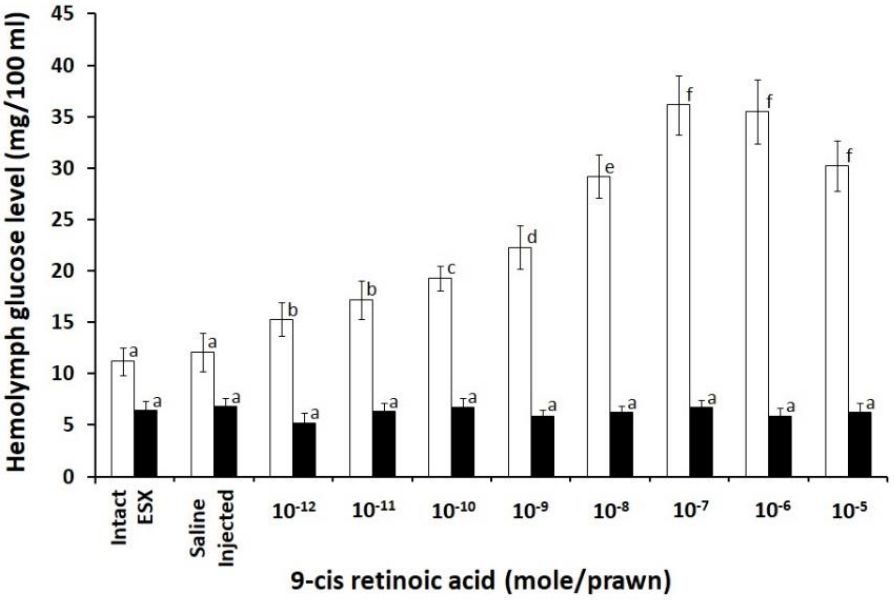

Figure 1. Dose-dependent effects of 9CRA on hemolymph sugar levels in intact (open bars) and eyestalk-ablated (ESX) prawns (solid bars). Hemolymph was collected from animals for sugar quantification 2 hr after injection. Each bar represents a mean ±SD of ten individual prawns. Bars with same superscript do not differ significantly from each other $P<0.001$.

injections of 9CRA in 10 µL volume at concentrations 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mole/prawn, respectively. Similar experiments were designed to evaluate the dose-dependent effects of ATRA. For intact prawns in groups 11, 12, 13, 14, 15, 16, 17 and 18 received injections of ATRA in 10 µL volume at concentrations 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mole/prawn, respectively. For ESX prawns in groups 11a, 12a, 13a, 14a, 15a, 16a, 17a and 18a received injections of ATRA in 10 µL volume at concentrations 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} mole/prawn, respectively. After 2 hrs, hemolymph was collected and analyzed for glucose levels (Reddy and Sainath, 2008), and to avoid circadian variations; all the experiments were carried out between 8 AM and 11 AM.

Time-course action of retinoic acid isomers: Based on the dose-dependent effects of retinoic acid isomers, a certain concentration of retinoic acid (which induced hyperglycemia) was injected into intact prawns at different time points (i.e. 0, 60, 120, 180, 240 and 300 min). Hemolymph was collected, and changes in glucose levels at respective time points were determined. Six groups with n = 10 per group were maintained at corresponding time points to determine the time-course effects of selected retinoic acid isomer at selected concentration.

Analysis of selected biochemical variables: Based on the results of time course action of selected retinoic acid isomer and at selected concentration, parameters such as hemolymph glucose levels, total carbohydrate levels, glycogen and glycogen phosphorylase in the CHH target tissues, hepatopancreas and muscle of prawns were determined.

Determination of total hemolymph sugar levels: Total carbohydrates in the hemolymph were quantified as per the method described by Caroll et al. (1956). Briefly, hemolymph was drawn (100 µl) from the arthrodial membrane of the third pair of walking legs using a hypodermal syringe from control and experimental prawns. Hemolymph samples were mixed with trichloroacetic acid (TCA; 10% W/V) and subjected to centrifugation at 4,000 rpm for 10 min. The protein-free samples (supernatant) were used for total carbohydrate analysis using anthrone reagent and the absorbance was measured spectrophotometrically at 620 nm. The concentration of total carbohydrates in the hemolymph was calculated using a standard graph prepared with known glucose concentration (analar grade).

Isolation of CHH target tissues: CHH target tissues like hepatopancreas and muscle were quickly isolated and after removal of adhering material, the tissues were stored at -80°C until further analysis of tissue glycogen levels, total carbohydrates, and glycogen phosphorylase activity.

Determination of total carbohydrates and glycogen levels: The total carbohydrates and glycogen content in hepatopancreas and muscle tissues were analyzed

according to the method described by Caroll et al. (1956) and quantified in terms of anthrone-positive substances. To accomplish this task, the protein-free samples were prepared using tricholoroacetic acid (TCA). Briefly, 5% (W/V) tissue homogenates prepared in TCA (10% W/V) followed by centrifugation (3000 rpm for 15 min at 4°C) were used for the analysis of total carbohydrates. On the other hand, after thoroughly mixing five volumes of 95% ethanol added to 1 ml of TCA supernatant, followed by placing the mixture at 4°C overnight, facilitates precipitation of glycogen. After centrifugation (3000 rpm for 15 min), the resultant supernatant was removed and the obtained precipitate was dried by inverting the tubes at room temperature. After 10 min, the precipitate was dissolved in 0.5 ml of distilled water. Finally, tissue glycogen content was determined from the ethanolic precipitate of TCA supernatant. The methodology includes the following: to the 0.5 ml of supernatants, 5 ml of anthrone reagent was added, followed by boiling for 15 min. After cooling at room temperature, the absorbance of the colour developed was read at 620 nm spectrophotometrically (Model: Jasco V-750; Mary's Court Easton, MD 21601) against a reagent blank. A standard with a known quantity of glucose using the same procedure was prepared in parallel to the experimental samples.

Assay of tissue phophorylase: The activity levels of phosphorylase in hepatopancreas and muscle tissues were estimated in accordance with the method described by Cori et al. (1955). The selected tissues were homogenized (5% W/V) in ethylenediamine tetraacetic acid (0.037 M; pH 6.8) containing sodium fluoride (0.1 M) (Guillory and Mommaerts, 1962). After centrifugation (3000 rpm for 10 min) the resultant supernatant diluted four times (1:3) with a buffer 0.03 M cysteine hydrogen chloride and 0.07 M sodium glycerophosphate (pH 6.8) was used as enzyme source.

Initially 0.4 ml of enzyme source was incubated with 0.2 ml of 2% glycogen solution at 35ºC for 20 min. After incubation, the reaction was initiated by the addition of 0.2 ml of 0.016 M glucose-1-phosphate (G-1-P) to one tube (tube 1) and in another tube (tube 2), in addition to 0.2 ml of 2% glycogen solution, 0.2 ml of 1:1 ratio of 0.016 M G-1-P and 0.004 M adenosine-5-monophosphate was added. The contents in tubes of 1 and 2 were used to estimate the activity levels of phosphorylase 'a' and phosphorylase 'ab', respectively. The reactions were terminated by the addition of 5 ml of 5 N sulphuric acid. The liberated inorganic phosphate (Pi) was determined based on Subbarow and Fiske (1925). Briefly, the contents 1.0 ml of solution and 1.0 ml of 2.5% ammonium molybdate solution were mixed well, and 0.4 ml of 0.2% 1-2-4-amino naphthosulphonic acid reagent was added. The colour developed was diluted to 10 ml of distilled water. After 5 min, the blue colour developed was measured at 720 nm spectrometrically against the reagent blank. The phosphorylase activity was expressed as µmoles of Pi liberated/mg protein/hr. The protein content in the tissue homogenates of hepatopancreas and muscle was estimated based on Lowry et al. (1951) using bovine serum albumin as standard.

Expression of CHH mRNA levels: Total RNA was isolated from the eyestalks of control and experimental prawns, *M. malcolmsonii*. In this study, 50 eyestalks were pooled and used for the isolation of total RNA from control and 9CRA-injected prawns using Trizol reagent (Invitrogen). The purity of RNA was analyzed using Nanodrop 2000 and by formaldehyde gel electrophoresis. The first strand cDNA synthesis was performed as per the manufacturer's instructions of iscript^{TM} cDNA synthesis kit (Biorad, India) using 1 µg of total RNA. The first strand cDNA was used to determine the expression levels of CHH or internal standard, β-actin using semi-quantitative PCR (Applied Biosystems, SimpliAmp^{TM}, Thermal cycler). The reaction mixture contains 10 µl of Phusion mixture (Thermo Scientifics), 1 µl of forward primer (FP), 1 µl of reverse primer (RP), 2 µl of cDNA and 6 µl of nuclease free water. The PCR cycle conditions were as follows: step 1 includes 1 cycle of 95°C, for 30s followed by 30 cycles of 95°C for 5s, 55°C for 15sec (step 2) and final step includes 1 cycle of 72°C for 10 min. The primers for CHH (Forward primer, FP: 5-CCTTAAGAAAAGGGCCATCC-3; Reverse primer, RP: 5-GTTGGCGTATTCG TCGAGTT-3 was designed based on the CHH sequence from the experimental prawn (Bankit Gene accession no. MN372310). To avoid genomic contamination, the gene-specific primers for CHH were designed from exon 2 and exon 3 (Chen et al., 2004). The primers for β-tubulin, an internal standard/house-keeping gene (FP: 5′-CCCTTCCCTCGTCTCCAC-3′; RP: 5′-GCCAGTGTACCAGTGAAGGGA-3′) was designed from the *M. rosenbergii* (Li and Tsai, 2000). 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-tubulin band

Table 1. Effect of eyestalk ablation (ESX) and injection of 9-cis retinoic acid (9CRA) into normal and ablated prawns on hepatopancreas, muscle total carbohydrate (TCHO), and glycogen levels in the prawn *Macrobrachium malcolmsonii*.

Group	TCHO		Glycogen	
	Hepatopancreas	Muscle	Hepatopancreas	Muscle
Control	15.62±0.68	4.61±0.38	1.08±0.21	0.68±0.003
ESX	20.41±1.09*^{A}	7.28*^{A} ±0.18	2.59*^{A}±0.41	1.51±0.009*^{A}
9CRA-injected intact	11.29±1.27*	3.21*±0.41	0.62*±0.09	0.41*±0.003
9CRA-injected ESX	19.61 ±1.38*^{A}	7.39*^{A}±0.24	2.48*^{A}±0.96	1.61*^{A}±0.009

Values are the mean (mg glucose/g tissue) ± SD of ten individual prawns. For evaluation of "p" for ESX, and 9CRA injected prawns (normal and ESX), prawns in control group served as controls; for 9CRA injected ESX prawns, ESX prawns served as controls. *represents significant value at $P<0.001$. The same capital letters in a row indicate a non-significant difference between ESX and 9CRA injected ESX prawns.

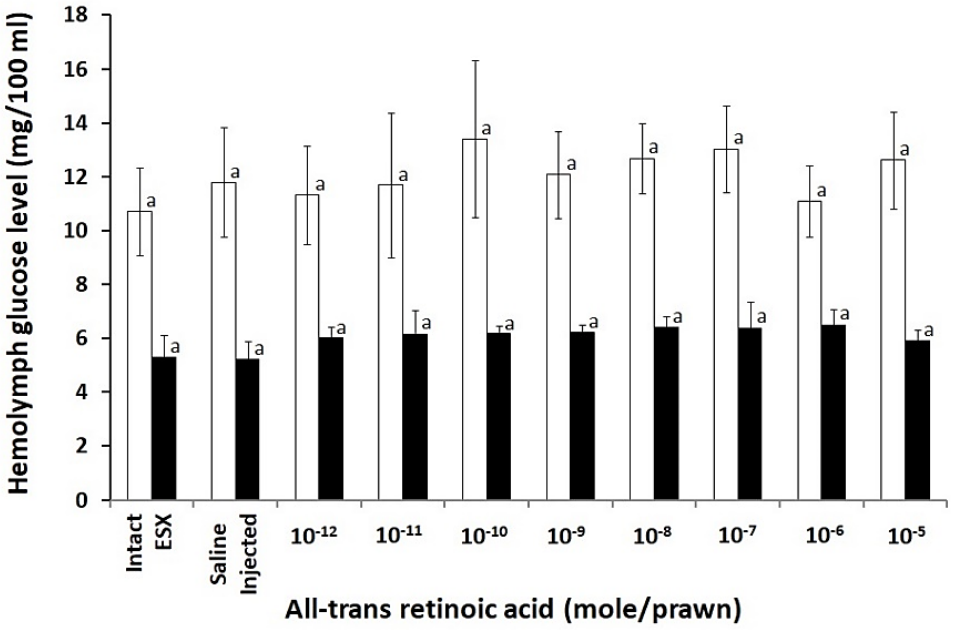

Figure 2. Dose-dependent effects of ATRA on hemolymph sugar levels in intact (open bars) and eyestalk-ablated (ESX) prawns (solid bars). Hemolymph was collected from animals for sugar quantification 2 hr after injection. Each bar represents a mean ±SD of ten individual prawns. Bars with same superscript do not differ significantly from each other $P<0.001$.

intensities.

Statistical analysis: The results were expressed as mean±SD and statistically analyzed using an analysis of variance (one-way ANOVA) followed by Tukey's post-test (SPSS Version 16.0, SPSS Inc, Chertsey., UK). Differences were considered to be significant at $P<0.05$.

Results

Effect of eyestalk ablation on carbohydrate metabolism: Extirpation of both eyestalks resulted in a significant reduction in the total carbohydrates in the prawn, *M. malcolmsonii* (Figs. 1, 2). Total carbohydrates and glycogen content were significantly enhanced, while a significant reduction was observed in total phosphorylase activity in selected tissues of ESX prawns (Tables 1 and 2).

Effect of different concentrations of 9CRA on hemolymph sugar levels: Injection of 9CRA induced hyperglycemia in intact prawns in a dose-dependent manner (Fig. 1). A significant increase was noticed in hemolymph sugar levels as the concentration of 9CRA was increased in intact prawns as compared to untreated, and saline-injected intact prawns. However, a significant elevation in hyperglycemic response was observed up to 10^{-7} mole 9CRA/prawn compared to intact prawns, which received 9CRA at doses 10^{-12}, 10^{-10}, 10^{-9} and 10^{-8} mole/prawn. On the other hand, intact prawns which received doses higher than 10^{-7} mole 9CRA/prawn exhibited a saturated response in inducing hyperglycemia. Hence, in the subsequent experiments,

Table 2. Effect of eyestalk ablation (ESX) and injection of 9-cis retinoic acid (9CRA) into normal and ablated prawns on hepatopancreas and muscle glycogen phosphorylase activity levels of *Macrobrachium malcolmsonii*.

Group	Hepatopancreas Phosphorylase		Muscle Phosphorylase	
	"a"	"ab"	"a"	"ab"
Control	2.69±0.42	4.38±0.31	3.89±0.22	6.62±0.38
ESX	1.21±0.28*^{A}	3.12±0.18*^{A}	2.31±0.24*^{A}	4.18±0.42*^{A}
9CRA-injected intact	3.64±0.62*	5.38±0.82*	4.91±0.28*	7.42±0.61*
9CRA-injected ESX	1.31±0.51*^{A}	3.08±0.21*^{A}	2.38±0.31*^{A}	4.42±0.81*^{A}

Values are mean (imoles of iP released/mg protein/hr)±SD of ten individual prawns. For evaluation of "P" for ESX, and Normal+9CRA injected prawns, prawns in the control group served as controls; for ESX+9CRA injected prawns, ESX prawns served as controls. P<0.001; ns, not significant. The same capital letters in a row indicate a non-significant difference between ESX and 9CRA-injected ESX prawns.

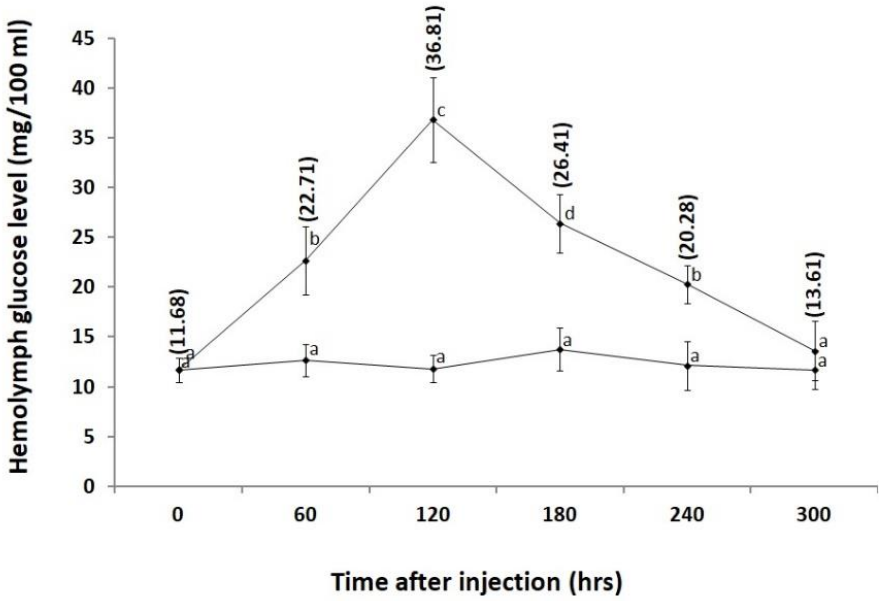

Figure 3. Time course action of 9CRA-induced hyperglycemia in intact prawns. Hemolymph was collected from intact prawns after injection of 9CRA (10-7 mol/prawn), at the time points indicated for sugar quantification. Each point represents a mean ±SD of ten individuals. Values in parentheses represent percent change from control (0 hr).

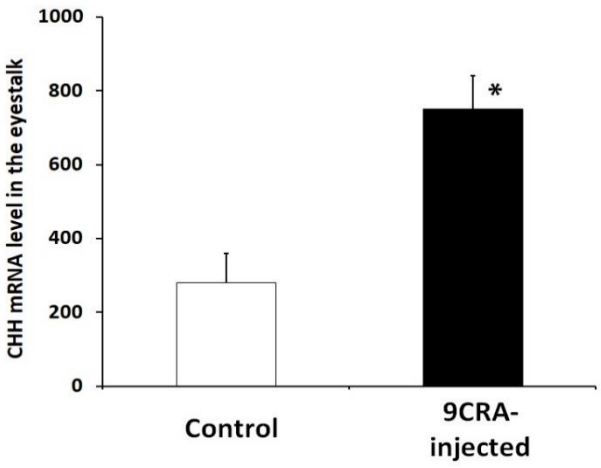

Figure 3. Changes in the expression of CHH mRNA in the eyestalk of intact prawns injected with 9CRA. Each bar represents a mean±SEM of ten individuals. Asterisk (*) represent significant differences between the control and intact prawns injected with 9CRA.

10^{-7} mole/prawn was selected as the test dose. Injection of 9CRA at selected doses did not elicit hyperglycemic response in ESX prawns.

Effect of different concentrations of ATRA on hemolymph sugar levels: Injection of ATRA into intact or ESX prawns did not induce hyperglycemic effect at selected doses (10^{-12} mole/prawn to 10^{-5} mole/prawn) (Fig. 2).

Time course action of 9CRA on hemolymph sugar levels: Figure 3 represents time-course action of 9CRA-induced hyperglycemia in intact prawns. Hemolymph sugar level increased significantly (P<0.001) after 9CRA injection (10^{-7} mole/prawn) and reached the highest peak at 120 minutes, thereafter, a decline in hemolymph sugar levels was observed.

Effect of 9CRA on tissue carbohydrates, glycogen content and activity of phosphorylase: Injection of

9CRA into intact prawns resulted in a significant decrease in the total carbohydrates (TCHO) and glycogen levels in the hepatopancreas and muscle over controls (Table 1). Injection of 9CRA into intact prawns significantly elevated the activity levels of phosphorylase 'a' (active) and 'ab' (total) in both hepatopancreas and muscle (Table 2). Bilateral eyestalk ablation resulted in a significant increase in tissue TCHO and glycogen levels with a decrease in phosphorylase activity (Tables 1, 2). However, injection of 9CRA into ablated prawns did not cause significant changes in the levels of TCHO and glycogen and the activity levels of phosphorylase in both tissues (Tables 1 and 2).

Effect of 9CRA on the expression of CHH mRNA in the eyestalks of prawns: Injection of 9CRA into intact prawns significantly enhanced the expression levels of CHH mRNA in the eyestalks of prawns compared to untreated or saline-injected prawns (Fig. 4).

Discussion

In the present study, we found that injection 9CRA but not ATRA induced hyperglycemia in intact prawns as evidenced by elevated hemolymph sugar levels, reduction in the total carbohydrates, and glycogen content, enhanced phosphorylase activity in hepatopancreas and muscle. Interestingly, 9CRA did not elicit any hyperglycemic response in ESX prawns, suggesting 9CRA could mediate the eyestalk principle in inducing hyperglycemia. This was supported by enhanced expression levels of CHH mRNA (RT-PCR) in the eyestalks of 9CRA-injected intact crabs.

The XO-SG complex is a major neuro-endocrine system located in the eyestalks that synthesizes a range of peptides that can control and coordinate physiological functions, including carbohydrate metabolism in decapods (Pillai et al., 2010; Nithya et al., 2013). The findings of the present study demonstrated that bilateral ESX caused a significant reduction in the hemolymph sugar levels in prawns, demonstrating the location and significance of eyestalk peptide, CHH in prawns, *M. malcolmsonii*. The results are in agreement with previous studies (Komali et al., 2005; Reddy and Sainath, 2008; Sainath and Reddy, 2010; Yang et al., 2018). The primary function of CHH is not directly to elevate hemolymph sugar levels but instead, the primary function of CHH is to regulate the intracellular glucose levels through the breakdown of glycogen by stimulating the activity levels of phosphorylase in its target tissues like hepatopancreas and muscle (Fanjal-Moles, 2006). Consequently, the leakage of glucose molecules into hemolymph leads to hyperglycemia, a view supported by Telford (1975). Therefore, reduced hemolymph sugar levels could be ascribed to enhanced levels of total carbohydrates and glycogen content accompanied by reduced activity levels of tissue phosphorylase system in CHH target tissues of ESX prawns (Sainath and Reddy, 2010; Yang et al., 2018).

The current findings indicated that injection of 9CRA into intact prawns induced hyperglycemia in a dose-dependent manner, while the such a response was not observed in 9CRA-injected ESX prawns. These findings support that the 9CRA induced hyperglycemia occurs via eyestalk principle, CHH in intact prawns. RT-PCR studies also revealed that the injection of 9CRA elevates CHH mRNA expression levels in the eyestalks of intact prawns, suggesting 9CRA-induced hyperglycemia could be indirect i.e. via triggering of eyestalk CHH. The results are in agreement with previous studies where 9CRA-induced hyperglycemia in the intact fiddler crab, *U. pugilator* (Zou and Bonvillian, 2003) and also in freshwater rice field crab, *O. senex senex* (Reddy and Sainath, 2008). Whereas, ATRA did not induce hyperglycemic response in intact or ESX prawns, suggesting that 9CRA could be a possible RA isomer associated with the eyestalks (Reddy and Sainath, 2008). Further, Reddy and Srilatha (2015) demonstrated that the administration of 13CRA, a metabolite of 9CRA induces hyperglycemia in intact crabs, *O. senex senex* but not in ESX crabs. Significant reduction in the total carbohydrates and glycogen content associated with elevated activity levels of phosphorylase system in selected tissues of 9CRA injected intact prawns could be ascribed to stimulatory effects of 9CRA at the level of eyestalk peptide, CHH. These events could indicate the mobilization of carbohydrate reserves from its target tissues, thereby elevating hyperglycemic response in 9CRA-injected intact prawns.

The pleiotropic effects of monsoon in vertebrates on various physiological functions vision formation, cell differentiation and proliferation, immune system response, embryonic development and metabolism (Pan et al., 2014). With regard to glucose metabolism, the role RA and its cognate receptors in the regulation of glucose homeostasis is well-studied (Chertow et al., 1997; Rhee et al., 2013; Pan et al., 2014; Zhou et al., 2021). Studies also indicated that RA isomers control and coordinate

glucose homeostasis via insulin secretion from the beta cells of the pancreas and glucose transporters (Clark et al., 1995; Blumentrath et al., 2001; Kane et al., 2010). In crustaceans, retinoic acid isomers (Hopkins et al., 2008; Venkaiah et al., 2019), insulin-related signalling system (Rosen et al., 2013; Li et al., 2015; Huang et al., 2015; 2016; Chandler et al., 2017), and glucose transporter 4 (Li et al., 2017) have been discovered. Indeed, a clear-cut picture of vitamin A metabolism provides a concrete basis for understanding the physiological significance of RA in vertebrates (Chen and Chen, 2014). However, current knowledge of retinoid signalling in crustaceans is far from beyond (Hopkins et al., 2008; Tang et al., 2014; Nagaraju et al., 2011; Cui et al., 2013). Though RA has not been recognized as an endogenous hormone, retinoic acid and RXRs have been detected in crustaceans (Andre et al., 2014). Few studies demonstrated the possible link between the role of RA in the regulation of limb regeneration (Hopkins et al., 2008), ovarian reproduction (Cui et al., 2013; Venkaiah et al., 2019), and carbohydrate metabolism (Zou and Bonvillian, 2003; Reddy and Sainath, 2008). The findings of this study also elaborate on the physiological role of RA in the regulation of carbohydrate metabolism in the monsoon prawn, *M. malcolmsonii*. Further experiments, isolation and characterization of retinoid counterparts such as RXR, CRABP receptors, identification of 9CRA in the eyestalks and their crosstalk with other endogenous molecules provide insights into the regulation of carbohydrate metabolism by RA in the prawns.

Acknowledgements

We thank, the Head, Department of Biotechnology, Vikrama Simhapuri University, Nellore, AP, India for providing laboratory space and instruments. The authors declare that the experiments conducted during these studies comply with the current laws of their country. One of the corresponding authors, SBS thank the DST-SERB for providing financial support to complete part of the current research work.

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