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PRELIMINARY STUDIES ON THE EFFECT OF RETINOIDS ON OVARIAN MATURATION IN SELECTED EDIBLE CRUSTACEANS OF AQUACULTURE IMPORTANCE

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ABSTRACT

Eyestalk ablation (removal of eyestalks) is a classical technique performed by aqua-farmers to induce seed in hatchery industry. However, this operational technique often leads to deterioration of seed quality and also leads to mortality in the brood stock. Among several strategies to overcome this problem, endocrine manipulation approach is well acknowledged. The present study was aimed to investigate the effect of retinyl palmitate on ovarian growth in selected fresh water crabs, *Oziotelphusa senex senex* and prawns, *Macrobrachium malcomsonii*. Intermolt stage female crabs were allocated into four groups (n= 15 per group). Crabs in group 1 served as controls (no treatment). Whereas, crabs in groups 2, 3 and 4 were treated as experimental groups and received different doses of retinyl palmitate (1, 5 and 10 µg/g, respectively) on days 1, 7 and 14 over a period of 21 days. Similarly, female prawns were subdivided into controls (no treatment) and experimental groups (n= 20 per group). Prawns in experimental group received retinyl palmitate at a dose of 10 µg/g body weight and followed same experimental regimen as that of crabs. Injection of retinyl palmitate to crabs and prawns showed a significant increase in the weights of ovaries, with a significant increase in the oocyte diameter of treated crabs and prawns over their respective controls. Retinoid-induced reproductive effects in crabs were dose-dependent. Further, histological studies of ovaries revealed that the crabs and prawns injected with retinyl palmitate showed vitellogenic stage as evidenced by accumulation of yolk globules in the occyte, whereas such changes were not observed in untreated crabs and prawns. From these preliminary results, it can be suggested that retinyl palmitate may be used as a tool to promote ovarian maturation in edible crustaceans. However, in-depth studies such as understanding the portfolio of retinoid signalling cascade and its crosstalk with endogenous hormonal factors are needed to develop an alternative strategy against eyestalk ablation to induce seed in hatchery industry.

***Keywords*:** Aquaculture, crustaceans, eyestalk ablation, hormone manipulation, sustainable hatchery industry

1. *Introduction*

Crustaceans such as shrimps, prawns, lobsters, crayfishes and crabs play an important role in the aquaculture industry, gaining popularity day-by-day in recent years. The increasing demand for high-protein food from aquatic sources and also to find an alternate for fisheries has given rise to a worldwide expansion of shellfish culture. The development of intensive aquaculture has opened a new field in the engineering area and would be a challenge for biotechnological research. Animal species important for aquaculture have evolved many complex reproductive strategies, although most of them are still poorly understood. Indeed, reproduction and other physiological aspects have been studied in only a limited number of species.

Therefore, successful development of aquaculture needs the support of detailed research in the field of reproductive biology.

It is well known that reproduction in crustaceans is regulated by endogenous and exogenous factors. Endogenously, the ovarian development in crustaceans is primarily regulated by two antagonistic neuro-endocrine factors; gonad-inhibiting hormone (GIH/VIH) of XO-SG complex located in the eyestalks (Panouse, 1943) and gonad-stimulating hormone (GSH) secreted from the brain and thoracic ganglia (Otsu, 1963). *In vitro* studies in several crustacean species suggest that GIH and GSH may target ovary to influence female reproduction (EastmanReks and Fingerman, 1984).

Eyestalk ablation technique is now practiced in the shrimp farms world-wide (which removes GIH source) to induce ovarian maturation in crustaceans. The eyestalk ablation technique involves - a. simple pinching of the eyestalk at the base, b. enucleation, c. cauterizing the eyestalk with an electrocautery device or a hot forceps and d. ligation by tying of the eyestalk tightly with a surgical thread. The increased use of eyestalk ablation technique in the hatchery industry has brought forth both positive and negative effects on the quality of spawning and the seeds produced (Benzie, 1998). The major criticism leveled against the eyestalk-ablation-induced reproduction is the poor quality of the seed produced. This may be due to incomplete diets which could not provide nutrients quick enough for the consequent accelerated ovarian maturation. To overcome the above problems, one important task for the endocrinologists will be finding the alternatives for eyestalk ablation that can accomplish the same increase in productivity. In this direction, several non-surgical procedures have been tried to induce ovarian maturation to improve crustacean aquaculture, such as double-stranded RNA to knock off GIH mRNA and injections of GIH antibody, hormones, and neurotransmitters (Treerattrakool et al., 2008). Though the results are promising, these methods are limited to a few crustaceans only.

Crustacean aquaculture industry is growing rapidly day-by-day which includes culture of many commercially important organisms. Two edible freshwater crustaceans were selected for this study. *Oziotelphusa senex senex* and *Macrobrachium malcolmsonii*. Only very few studies were conducted on this crab regarding manipulation of ovarian maturation. Studies of Kishori and Reddy (2004), Reddy et al. (2006) and Sainath and Reddy (2011) suggest that administration of opioids, steroids and biogenic amines may induce ovarian maturation in the experimental crab, suggesting the importance of hormones in the regulation of gonad development.

Different methods were employed by various workers in the study of crustacean reproduction. Jegla (1966) used recording of ovigerous females as an index to determine the status of the reproduction. Use of gonad index was employed by many workers for identification of the reproductive cycle (Rehman, 1967; Haley,

1972). Histological observations of gonads were used to determine the vitellogenesis in several cruscteans (Varadarajan and Subramoniam, 1980).

In the present study, changes in ovarian index, oocyte diameter and histology of ovary were considered as the reproductive endpoints. The effect of retinyl palmitate in the regulation of ovarian maturation in relation with ovarian index and oocyte diameter in the crabs and prawns were also studied. In addition, the histological alterations in the ovary of crab and prawns during different treatments were also considered. Recent studies of Sainath and Reddy (2008), suggested that 9-cis retinoic acid but not all-trans retinoic acid controls hemolymph sugar levels in freshwater crab, *Oziotelphusa senex senex*. As a pilot study, and based on our recent report, the precursor of retinoic acid isomers, retinyl palmitate (Andre et al., 2014) was selected as test chemical.

Materials and Methods

Un-injured **c**rabs and prawns (upper panel in figure 1) were used in the present study and retinyl palimitate (RP) was selected as test dose. The test doses of RP used in this study were based on earlier studies (Linan Cabello and Jesus, 2004). Intermolt stage female crabs/prawns were allocated into four groups (n= 6 per group). Crabs in group 1 served as controls (no treatment). Whereas, crabs in groups 2, 3 and 4 were treated as experimental groups and received different doses of retinyl palmitate (1, 5 and 10 µg/g, respectively) in corn oil on days 1, 7 and 14 over a period of 21 days. The selected test doses of RP were administered to intact crabs on days 1, 7, 14 and 21 and were sacrificed on day 21 (the crabs which received injections on days 1, 7 and 14) and the reproductive endpoints like ovarian index, oocyte diameter, histology of the ovary and ovarian vitellogenin levels were determined and all these parameters were compared with their respective control group of crabs. Similarly, female prawns were subdivided into controls (no treatment) and experimental groups (n= 6 per group). Prawns in experimental group received retinyl palmitate at a dose of 10 µg/g body weight and followed same experimental regimen as that of crabs.

Reproduction

Determination of ovarian index

The crabs from control and experimental groups were weighed and the ovaries were excised, cleaned in crustacean saline (Van Harreveld, 1936), blotted on filter paper and weighed wet to the nearest milligram. The ovarian index was determined using the following formula:

Ovarian index = Wet weight of the ovary (g)/ Weight of the crab (g)X 100

Histological studies of the ovary

The ovarian sections were prepared according to the method described by Bancraft and Stevens (1982). The ovaries were isolated intact, dried on filter paper, fixed in Bouin's fluid (picric acid: formaldehyde: acetic acid; 75:25:5). After 24 h, the material was washed thoroughly and dehydrated with ascending alcoholic series. After cleaning in xylene, the tissues were embedded in paraffin wax (m.p. 56-58□C).

The sections were cut at 7mm thickness, stained with hematoxylin and counterstained with eosin. Different reproductive stages were observed under a -BX41TF, Japan) and each stage was photographed.

Measurement of oocyte diameter The diameter of 20 oocytes from each ovary was measured using an ocular micrometer under a compound microscope (Olympus, Model-BX41TF HB, Japan). The measurements were made on the longest and shortest axes of each oocyte, both dimensions were added and the mean was taken as mean oocyte diameter.

Results

Effect of retinyl palmitate on ovarian Index (OI) and oocyte Diameter (OD) in the crab *Oziotelphusa senex senex* **and the prawn,** *Macrobrachium malcolmsonii* **in a 21-day experimental period.**

In the present study the effect of injection of RP on ovarian maturation in both intact and eyestalk (ESX) crabs was investigated using ovarian index (Table 1) and oocyte diameter (Table 2) as reproductive endpoints. The test chemicals were injected on days 1, 7, and 14 and they were sacrificed on days 7, 14 and 21. In the present study, a gradual increase in the selected reproductive endpoints from day 1 to 21 was observed in intact crabs and prawns injected with RP. These endpoints of reproduction were accompanied by histological changes as evidenced by accumulation of yolk globules (starting from day 14 onwards) (Lower panel in figure 1)

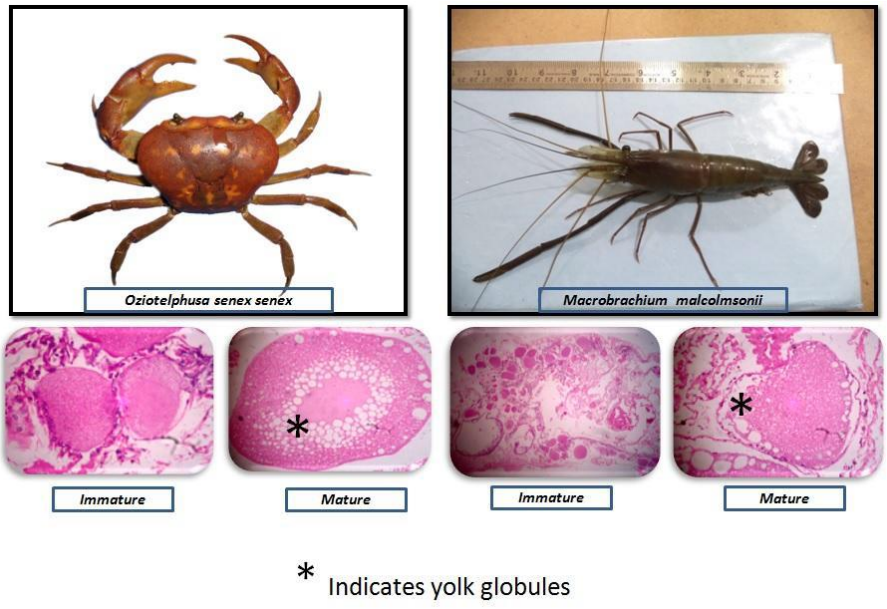

Figure 1: Selected test organisms (upper panel) along with histological changes in their ovary of crabs (left lower panel) and prawns (right lower panel) treated with retinyl palmitate in a 21-day experimental period.

Table 1. Effect of injection of retinyl palmitate (RP) on ovarian index (g %) in selected intact crustaceans at different time points

Days Groups	0	21
Crabs		--
Initial controls	0.371 ± 0.035	
Concurrent controls	--	0.391 ^{ns} ± 0.026
RP- Intact (1µg/g bw)	--	1.667* ± 0.052
RP-intact (5µg/g bw)	--	1.846* ± 0.062
RP- Intact (10µg/g bw)	--	2.125* ± 0.041
Prawns		
Initial controls	0.634 ± 0.131	-
Concurrent controls	-	0.685 ± 0.126
RP- Intact (10µg/g bw)	-	1.121 ± 0.212

Table 2. Effect of injection of retinyl palmitate (RP) on oocyte diameter (µm) in selected intact crustaceans at different time points

Days Groups	0	21
Crabs		--
Initial controls	20.41 ± 2.34	
Concurrent controls	--	19.64^{ns} ± 2.72
RP- Intact (1µg/g bw)	--	51.86* ± 4.38
RP-intact (5µg/g bw)	--	69.81* ± 3.19
RP- Intact (10µg/g bw)	--	79.81* ± 5.19
Prawns		
Initial controls	350 ± 14.21	-
Concurrent controls	-	340 ^{ns} ± 10.35
RP- Intact (10µg/g bw)	-	740* ± 11.26

The values are mean ± S.D. of 6 individuals.
Values in parentheses are percent change from control groups. For computation of percent change and evaluation of 'p' for concurrent controls, initial control crabs/prawns served as controls; for treated intact crabs/prawns, concurrent control crabs/prawns served as controls
*$p<0.001$; ns= not significant

For analysis of data statistically, Student's t-test was performed by using the SPSS software. $p<0.05$ was not considered significant.

Discussion

In decapods, ovarian maturation is associated with changes in the color of the ovary, histological changes of the ovary, increase in ovarian index and oocyte diameter. These parameters were used as good indicators of female reproductive status in crustaceans by several researchers. For the present study, the above reproductive endpoints were selected to assess the reproductive status in the fresh water edible crab, *Oziotelphusa senex senex* and fresh water edible prawn, *Macrobrachium malcolmsonii*.

Injection of RP into intact crabs and prawns resulted in precocious ovarian maturation in a 21-day experimental period. Stimulation of ovarian growth was evidenced by a significant increase in ovarian index, oocyte diameter and histological changes observed in ovary of crabs and prawns treated with RP as compared to concurrent controls. The histological examination of ovary revealed that in control specimens more number of follicular cells was surrounded by germanium without yolk globules indicating that the ovary was immature, whereas in RP injected crabs the histological architecture of the ovary indicated accumulation of yolk globules, which is a characteristic feature of vitellogenesis. On the other hand, injection of RP to intact prawns also showed significant accumulation of yolk globules suggesting ovarian maturation in prawns over a period of 21 days experimental period. These results are in consonance with previous studies (Paniagua-Michel and Liñan-Cabello, 2000; Liñán-Cabello et al., 2003; Barim-Oz and Yilmaz, 2016).

Vitamin A, also known as retinol (ROL), is an essential nutrient belonging to the retinoid family, which includes molecules structurally and/or functionally related to ROL (Andre et al., 2014). One of the interesting aspects related to retinoid signalling is its occurrence in almost all metazoan phyla, suggesting common signalling pathways between invertebrates and vertebrates. In accordance with the molecular data, endogenous active retinoids were quantified in *Uca pugilator* regenerating limb blastemas (all-trans-RAL, 13-cis-RAL, all-trans-RA and 9-cis-RA) and *Locusta Migratoria* embryos (all-trans- and 9-cis-RA) (Hopkins, 2001; Hopkins et al., 2008; Nowickyj et al., 2008). With regard to reproduction, it has been shown that administration of retinyl palmitate to intact shrimp *Penaeus japoniocus* resulted in ovarian development (Alava et al., 1993). Further, it has been shown that developing occytes of *Litopenaeus vannamei* accumulates beta-carotene, ROL and retinal (Paniagua-Michel and Liñan-Cabello, 2000; Liñán-Cabello et al., 2003).

The crustacean neuro-endocrine system is endowed with many bioactive molecules which include peptides, terpenoids, steroids and biogenic amines. In decapods, the X-organ-sinus gland complex located in the eyestalks is the major neuroendocrine center, which secretes a set of multifunctional regulatory peptide molecules, collectively called as crustacean hyperglycemic hormone (CHH) family peptides. This complex is known to regulate carbohydrate metabolism, molting, and reproduction (Huberman, 2000). However, the exact mechanism of secretion of these hormones during different stress conditions is limited to very few crustaceans. Many

other factors apart from eyestalk hormones like vitellogenesis stimulating hormone secreted from brain and thoracic ganglia, methyl farnasoate (MF) by mandibular organ and ecdysteroids from Y-organs are also primarily involved in the regulation of biological activities in crustaceans. The interplay among these hormones is yet to be resolved. A study by Hopkins et al. (2008). Authenticated that MF and ecdysteroids acts through the retinoid X receptor (RXR) and ecdysteroid receptor (EcR) respectively. Nagaraju et al. (2011) reported that silencing of RXR resulted in decreased vitellogenesis in *Carcinus maenas*. Martin et al. (2001) showed that ecdysteroid signalling plays a key role in induction of vitellogenesis in the mosquito *Aedes aegypti*. The role of these receptors in the regulation of reproduction is well established in crustaceans (Nagaraju et al., 2011). Thus, it is apparent that in crustaceans, ovarian maturation is regulated by several hormones of diverse nature.

Conclusion

Based on experimental data, it is suggested that selective use of RP would be beneficial and this manipulation program seems to be a practical alternative to eyestalk ablation to improve crustacean aquaculture industry; however, further research is required to establish the applicable value of retinyl palmitate. To accomplish this task, there is a need for poly-hormonal approach rather than mono-hormonal approach seems to be a logical necessity and such as approach might be helpful to gain insights into the endocrine control of reproduction in crustaceans.

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