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# Effect of Copper Sulfate on Molt and Reproduction in Shrimp *Litopenaeus vannamei*

M. Sreenivasa Rao and N. Anjaneyulu
Department of Chemistry, Chundi Ranganayakulu Post Graduate College,
Chilakaluripet, 522 616, Andhra Pradesh, India

**Abstract:** The effect of copper sulfate in the regulation of molt and gonad development in the shrimp *Litopenaeus vannamei* was investigated. Initially, we determine the copper sulfate safe level for use in culture medium shrimps. Six concentrations of copper sulfate (0, 0.01, 0.05, 0.1, 0.2, 0.5 mg $L^{-1}$) were tested in 10 L glass aquaria for 15 days. After 15 days, survival of the control treatment (0% $CuSO_4$) averaged 100%. The survival in 0.01, 0.05, 0.10 and 0.20 mg $L^{-1}$ (100, 100 and 98%) concentrations of $CuSO_4$ were significantly greater than the higher dose treatment (0.50 mg $L^{-1}$ $CuSO_4$; survival 43%). The copper (0.05 mg $L^{-1}$) exposed shrimps accelerated the molting (premolt stage: female, 100%; males, 90%). The copper (0.10 mg $L^{-1}$) exposed shrimps accelerated the both molting and reproduction (premolt stage: female, 50%; males, 40%). The copper (0.20 mg $L^{-1}$) exposed animals significantly ($p<0.0001$) increased mean oocyte diameter and testicular follicle diameter as well as mean gonad indices from the control as well as other treated shrimps. These results provide strong evidence that trace amount of copper is an essential for both molting and reproduction in shrimps.

**Key words:** Copper sulfate ($CuSO_4.5H_2O$), molt, reproduction, oocytes, testies, shrimp

## INTRODUCTION

The X-organ-sinus gland (XO-SG) complex is the main neuroendocrine organ of crustaceans. It is a part of the medulla terminalis in the eyestalk. This XO-SG synthesize and secreted the several peptide hormones such as Crustacean Hyperglycemic Hormone (CHH), Molt Inhibiting Hormone (MIH), Gonad Inhibiting Hormone (GIH) and several color change hormones (controlling pigment migration). Molting is controlled by MIH acting on the so-called Y-organs, a pair of structures homologous to the prothoracic organs of insects. They are non-neural endocrine organs, which are secreted ecdysone (Huberman, 2000; Nagaraju, 2007; Rodriguez *et al*., 2007). The endocrine control of reproduction is more complex event. In females, the eyestalk hormone GIH directly inhibits the oocyte development, regulating the uptake of vitellogenenin from the hemolymph during secondary vitellogenesis (Charniaux-Cotton and Payen, 1988).

Other, neuroendocrine centers outside the eyestalks also contribute in the regulation of crustacean reproduction. Among them, the brain and thoracic ganglia have been identified as sources of a neuropeptide hormone called Gonad Stimulating Hormone (GSH). Although GSH has not yet been purified, several *in vivo* and *in vitro* studies showed the stimulating effects of the brain and thoracic ganglia on ovarian growth (Nagaraju, 2007; Fingerman, 1997). Reproduction of crustacean males involves a special endocrine gland, the Androgenic Gland (AG). This is a paired structure, one being attached to each vas deferens. The testes are directly controlled by a hormone secreted by the AG, called the Androgenic Gland Hormone (AGH), with GSH and GIH influencing the secretion of

**Corresponding Author:** M. Sreenivasa Rao, Department of Chemistry, Chundi Ranganayakulu Post Graduate College, Chilakaluripet, 522 616, Andhra, Pradesh India Tel: +91-8647-253984 Fax: +91-8647-253859

AGH, instead of directly acting on the testes. AGH determines the normal development of both the male reproductive system and male secondary sexual characters (Fingerman *et al*., 1998).

Copper is an essential trace mineral in animal and human nutrition. It is also a potentially toxic substance. Copper exists in the oxidation states Cu (I) or $Cu^{+}$ (cuprous) and Cu (II) or $Cu^{2+}$ (Cupric) under physiological conditions. Crustaceans can regulate their body copper concentration, which is required for hemocyanin synthesis (White and Rainbow, 1982). Several studies have shown that crustaceans are adversely affected when exposed to high concentrations of copper. Exposure to high copper disrupted respiration in *Carcinus maenas* (Nonnotte *et al*., 1993) and it also affected osmoregulation in *C. maenas* (Hansen *et al*., 1992). But trace amount of copper is essential for the proper functioning of copper-dependent enzymes (Hassall and Dangerfield, 1990). Most crustaceans possess hemocyanin containing much copper as their main oxygen-carrying blood protein (Dallinger, 1977; Rao *et al*., 2007a, b). The limit between the requirement and toxicity of copper is delicate and dependent on a variety of endogenous and exogenous factors (Weber *et al*., 1992). We have studied the changes of copper profiles in different molt and reproductive stages in shrimp *Litopenaeus vannamei* (Rao *et al*., 2007a, b).

The objectives of this study were: to evaluate the effect of copper on shrimp *L. vannamei* survival and to determine the exposure of copper on molt and reproduction in shrimp *L. vannamei*.

## MATERIALS AND METHODS

The shrimps (*Litopenaeus vannamei*) were collected from fresh market at Chirala (Andhra Pradesh, South India). The shrimps were brought to the laboratory and maintained in the laboratory at 28±1°C in tubs partially filled with aged seawater. They were acclimatized to laboratory conditions (12:12 L:D) for at least 7 days before being used in experiments. The water in the tubs was changed daily.

In all experiments only intact, uninjured shrimps (body weight 19±2 g carapace length between 21.0 and 23.0 mm) were selected. During their sojourn, the shrimps were fed on shrimp pellet *ad libitum* and the medium was changed 2 h after feeding. Only intermolt (stage C4) shrimps were used to study the effect of copper sulfate on survival, molt and reproduction.

### Copper Sulfate Treatment

Copper sulfate penta-hydrate ($CuSO_4.5H_2O$) used in the present investigation were of analytical grade (E. MERCK, Mumbai, India). Stock copper sulfate solution was freshly prepared and added to each glass aquaria containing 10 L aged sea water to achievement the required range of copper concentration of 0.00 (control), 0.01, 0.05, 0.10, 0.20, 0.50 mg $L^{-1}$) for each group of shrimps. The aquaria's were provided with continuous aeration from an air blower One hundred twenty crabs were divided into twelve groups of 10 shrimps each. Groups with odd numbers were males and those with even numbers were females. Groups 1 and 2 served as control and the shrimps in these groups not exposed to copper treatment. Shrimps in group 3 and 4 exposed to copper concentration 0.01 mg $L^{-1}$; group 5 and 6 exposed to copper concentration 0.05 mg $L^{-1}$; group 7 and 8 exposed to copper concentration 0.10 mg $L^{-1}$ group 9 and 10 exposed to copper concentration 0.20 mg $L^{-1}$ group 11 and 12 exposed to copper concentration 0.50 mg $L^{-1}$. The shrimps were sacrificed on day 16. mortality of the shrimps was noted.

### Isolation of Organs

The shrimps were immobilized by chilling on ice for 5 min. The body weights of the shrimps were determined. The reproductive organs were isolated, immediately placed in ice-cold crustacean physiological saline (Van Harreveld, 1936) to scrap off adhesive tissue. The organs were removed from

the saline and lightly blotted with the paper towels, weighed wet on an electronic balance. The reproductive stages in the shrimps were identified according to Nagaraju *et al*. (2004). The gonad indices were determined using the standard formula:

$$\text{Gonad index} = \frac{\text{Wet weight of the gonad (g)}}{\text{Wet weight of the body (g)}} \times 100$$

The diameters of 25 randomly chosen oocytes/ testicular follicles were measured using an ocular micrometer on a microscope.

**Identification of Molt Stages**

There are three molt stages: postmolt (A, B), intermolt © and premolt (D) which can be distinguished by the degree of hardness of the exoskeleton. The molt stages were observed using setal development in the endopodites of the pleopods as described for this shrimp (Chan *et al*., 1988). Microscopic examination of the setae gives a clear indication of the molting stages of *L. vannamei.*

**Statistical Analysis**

The data were analyzed using one-way ANOVA followed by Student-Newman-Keul's test to determine the level of significance.

## RESULTS

**Effect of Copper Sulfate on Survival of Shrimps**

The survival in the 0.0, 0.01, 0.05, 0.10 and 0.20 mg $L^{-1}$ (In males, 100, 100, 100, 100 and 100%; females,100, 100, 100, 100 and 90%, respectively) concentrations of $CuSO_4$ were significantly greater ($p<0.05$) than higher dose treatments (0.50 mg $L^{-1}$); but were not significantly different from each other ($p>0.05$; Fig. 1). Treatment containing 0.50 mg $L^{-1}$ copper sulfate results a dramatic decrease in shrimp survival (males, 50%; females 40%).

**Effect of Copper Sulfate on Molting of Shrimps**

All control shrimps were in the intermolt stage (C4). The concentration 0.05 mg $L^{-1}$ copper exposed shrimps enter into premolt stage (males 90% and females100%). The copper (0.10 mg $L^{-1}$) exposed shrimps accelerated the both molting and reproduction (premolted; female 50% and males

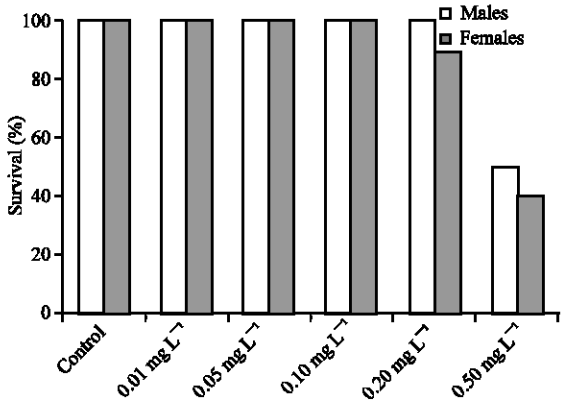


Fig. 1: Effect of copper sulfate on survival of shrimps

Table 1: Exposure of copper sulfate on molting and testicular development in male shrimp, *L. vannamei*

| Copper concentrations (mg $L^{-1}$) | Testicular index | Testicular follicle diameter (μm) | Molt stage (%) |
|---|---|---|---|
| Control (n = 10) | 0.36±0.02 | 4.41± 0.69 | Intermolt (100) |
| (0.01) n = 10 | 0.43±0.01 | 5.33±0.42 | Intermolt (100) |
| (0.05) n = 10 | 0.48±0.06 | 6.14±0.21 | Intermolt (10) Premolt (90) |
| (0.10) n = 10 | 0.62±0.04 | 14.21±0.23 | Intermolt (60) Premolt (40) |
| (0.20) n = 10 | 1.02±0.08 | 18.23±1.5 | Intermolt (100) |
| (0.50) n = 10 | 0.45±0.02 | 0.49±0.29 | Intermolt (100) |
| F-ratio | = 278.98 | = 867.76 | |
| p-value | < 0.0001 | < 0.0001 | |

Values are mean±SD. Values in parentheses are % change from control shrimps

Table 2: Exposure of copper sulfate on molt and ovarian development in female shrimp, *L. vannamei*

| Copper concentration (mg $L^{-1}$) | Gonad stage | Color of the ovary | Body weight (g) | Ovarian index | Oocyte diameter (μm) | Molt stage (%) |
|---|---|---|---|---|---|---|
| Control (n = 10) | Previtellogenic | White | 17.7±1.4 | 0.19±0.03 | 6.3±0.9 | Intermolt |
| (0.01) n =10 | Previtellogenic | White | 19.1±1.7 | 0.21±0.03 | 8.0±1.3 | Intermolt |
| (0.05) n = 10 | Vitellogenic stage 1 | Pale yellow | 17.2±1.7 | 0.33±0.06 | 15.4±1.1 | Premolt (100) |
| (0.10) n =10 | Vitellogenic stage 2 | yellow | 23.7±2.4 | 0.49±0.04 | 16.4±1.1 | Intermolt (50) Premolt (50) |
| (0.20) n = 10 | Vitellogenic stage 3 | Orange | 27.6±5.3 | 0.83±0.09 | 31.2±2.6 | Intermolt (100) |
| (0.50) n = 10 | Vitellogenic stage 1 | Pale yellow | 21.3±2.5 | 0.34±0.1 | 11.7±0.8 | Intermolt (100) |
| F-ratio | | | = 20.022 | = 134.67 | = 389.58 | |
| p-value | | | <0.0001 | <0.0001 | <0.0001 | |

Values are mean±S.D. Values in parentheses are % change from control shrimps

40%). The concentration of copper 0.01, 0.20 and 0.50 mg $L^{-1}$ did not showed any effect on molting. Effect of copper sulfate on reproduction of shrimps: All concentration of copper exposed shrimps significantly ($p<0.0001$) increased the testicular index and mean testicular follicle diameter when compared with the corresponding values for the control (Table 1). The concentration of copper (0.10 and 0.20 mg $L^{-1}$) exposed shrimps mean testicular index and mean testicular follicle diameter significantly ($p<0.0001$) greater than the other copper (0.01, 0.05 and 0.50 mg $L^{-1}$) exposed shrimps (Table 1).

All concentration of copper exposed shrimps significantly ($p<0.0001$) increased the ovarian index and mean oocyte diameter when compared with the corresponding values for the control (Table 2). The concentration of copper 0.10 mg $L^{-1}$ exposed animals entered into vitellogenic stage 2 as well as copper 0.20 mg $L^{-1}$ exposed shrimps entered into vitellogenic stage 3. All other copper concentrations (0.01, 0.05 and 0.50 mg $L^{-1}$) exposed shrimps entered into vitellogenic stage I (Table 2).

The color of the ovary at different reproductive stages in the white shrimp *L. vannamei* is (Table 2). In shrimps, oogonial proliferation and ovarian differentiation takes place when the ovary is translucent to opaque white (previtellogenic ovary). During vitellogenesis the color of the ovary changes from pale yellow (vitellogenic stage 1) to dark yellow (vitellogenic stage 2) and then it become orange (vitellogenic stage 3) to dark orange prior to spawning. Maturation of the ovary also includes an increase in size of ovary as the oocytes proliferate and increase in diameter, due to yolk deposition (Table 2).

## DISCUSSION

It is well-known that eyestalk ablation induces gonad development and oviposition. This is because the source of GIH is removed by the ablation. However, GIH has not yet been identified in

any shrimp (Rodriguez *et al*., 2007). Previously, we observed the increase of copper and protein levels in the hepatopancreas and hemolymph from postmolt to pretmolt stages. Similarly, the hemocyanin concentration also increased significantly in hemolymph from postmolt to premolt stages (Rao *et al*., 2007b). The protein and hemocyanin concentration significantly increased from the previtellogenic stage to vitellogenic stage 3 in the hemolymph. Similarly the protein concentration significantly increased in the both hapatopancreas and ovary from previtellogenic stage to vitellogenic stage 3. These results provide evidence to support the hypothesis that the ovarian vitellin (protein) source is hepatopancreas (Rao *et al*., 2007a). These results also suggest that copper is an important element in ovarian development of crustaceans. During the molt cycle, trace metal concentrations in the hepatopancreas and hemocyanin concentration in the hemolymph of blue crabs changes significantly (Engel and Brouwer, 1987, 1991). The exoskeleton of crustaceans is formed by cells of the hypodermis, but several hemolymph proteins contribute to the synthesis of the new exoskeleton. These hemolymph proteins share a surprising degree of sequence similarity and are members of the hemocyanin gene family (Burmester, 2004). Copper-containing prophenoloxidases of crustaceans are directly involved in cross-linking and hardening of the exoskeleton during molting and repair. The hepatopancreas periodically accumulates and releases copper during molting and starvation (Arumugam, 1989) and it has been shown to be the site of hemocyanin synthesis (Gellissen *et al*., 1991; Spindler *et al*., 1992). Crustacean hemocyanin proteins have been implicated in transport of hormones and phenols and may be used directly as structural components of the new exoskeleton. They are synthesized elsewhere in the body, transported in the hemolymph and probably taken up by the hypodermis via specific receptors. Multiple members of the hemocyanin gene family play vital roles during molting and reproduction (Sellos *et al*., 1997; Terwilliger, 1999). Hemocyanin is a copper containing, multi-subunit protein; it has evolved to carryout the specialized functions of oxygen transport in arthropods and molluscans (Burmester, 2004). Hemolymph concentration of hemocyanin fluctuates during the molt cycle, reaching highest levels just before ecdysis (Rao *et al*., 2007b). In adult crabs that undergo an annual molt, hemocyanin levels fall to undetectable levels in the hemolymph during intermolt. In rapidly growing juveniles with a much shorter molt cycle, hemolymph hemocyanin decreases markedly during ecdysis but does not always totally disappear (Terwilliger, 1999). Hemocyanin, like some of the insect hexamerins, seems to be a molting protein involved in forming a new exoskeleton.

In the present study the exposure of copper stimulated molt and reproduction in shrimp. These observations agree well with changes of copper, hemocyanin and protein concentrations observed in the white shrimp *Litopenaeus vannamei* in relation to size, reproductive and molt stage (Cheng *et al*., 2002; Rao *et al*., 2007a, b). We hypothesized that the copper thus accumulated from the medium and released back in to hemolymph in the form of hemocyanin (copper-bound protein). During premolt, hemocyanins give higher rise to respiratory rather than non-respiratory protein (vitellogenin). On the basis of these results on molt and reproduction in shrimps, we suggest that trace amount of copper may control, at least in part, both molt and reproduction. Furthermore, this work will provide evidence to the hypothesis that copper may be involved hormonal regulation. Further studies are necessary to know the copper role in know copper role in hormonal control.

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