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Hematological Studies of Freshwater Zooplankton in *Calonoid copepod Sinodiaptomus sarsi*

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Abstract

Crustaceans have developed a mechanism to detect and eliminate harmful microorganisms in a microbe-rich aquatic environment. This research delves into the observation of hemocytes in *Calonoid copepods*, which is a pioneering study according to the literature review. The hemolymph of zooplankton was isolated using centrifugation and homogenization techniques, and hemocytes were identified by staining them with eosin dye and examining them under a trinocular light microscope. Hemocytes were classified into groups based on the presence or absence of cytoplasmic granules, and three types of hemocytes were identified: granulocytes, semi-granulocytes, and hyalinocytes. Hemocyte total and differential counts were conducted using a Neubaur counting device. The results showed that T2 (0.025%) had 90 cells per ml of hemolymph, with 44% of these being hyalinocytes, 30% granulocytes, and 26% semi-granulocytes.

***Key words*:** Zooplankton, Copepods, Cow urine distillate, Hemocytes, Granulocytes semi – granulocytes, Hyalinocytes, *Sinodiaptomus sarsi*

The Zooplankton plays a crucial role in the trophic structure of the aquatic ecosystem, acting as a vital link between phytoplankton and higher-level aquatic organisms [1]. Ecologically speaking, Zooplankton is an essential biotic element that significantly impacts the functional dynamics of the aquatic ecosystem, encompassing food chains, food webs, energy transfer, and nutrient cycling [2]. Copepods, the most abundant metazoans on Earth, are believed to outnumber insects, which are a significant provider of animal protein globally. In aquatic ecosystems, copepods play a vital role in transferring carbon from producers to higher trophic levels. Additionally, they consume substantial amounts of bacteria and phytoplankton, serving as a primary food source for larval and juvenile fishes in pelagic food webs [3-4].

Microbes found in the aquatic ecosystem are widespread and have the ability to cause infections in aquatic organisms. Invertebrates, especially lower aquatic animals, do not have a true adaptive immune system and depend on their innate immune system to protect themselves against invading pathogens. In contrast, fish have both innate and adaptive immunity. Crustaceans, however, usually maintain their well-being by developing a defensive response to potential infections. Crustaceans rely on three main types of hemocytes, namely hyaline (H) cells, semi-granular cells (SGCs), and granular cells (GCs), to mount an effective immune response against invading pathogens. These cells perform a range of functions, including phagocytosis, encapsulation, nodule formation, cytotoxicity mediation, and the production of antimicrobial peptides. H cells, being the most abundant type of hemocyte, are primarily involved in phagocytosis and encapsulation. SGCs, on the other hand, are responsible for nodule formation and cytotoxicity mediation, while GCs are involved in the production of antimicrobial peptides and the release of enzymes that can degrade the cell walls of bacteria. The immune response of crustaceans is initiated when the host defense system encounters a pathogen, triggering a range of mechanisms that are activated to defend the host against infection by various microbes. Hemocytes play a crucial role in this process by recognizing and responding to foreign antigens, thereby mediating the immune response of crustaceans.

To summarize, the immune responses of crustaceans heavily rely on the crucial involvement of hemocytes, which carry out diverse functions including phagocytosis, encapsulation, nodule formation, and cytotoxicity mediation. The immune system of crustaceans is regulated by three primary types of hemocytes: hyaline cells, semi-granular cells, and granular cells. These hemocytes possess the ability to identify and react to foreign antigens, triggering a series of defense mechanisms aimed at safeguarding the host from microbial infections. The host's hemocoel responds to pathogen invasion by activating innate defense mechanisms, which consist of a complex system of cellular and humoral immune components [5]. Although the role of hemocyte-mediated immune mechanisms in microcrustaceans is significant, there has been limited research conducted on this topic specifically in Daphnia [6]. The identified and isolated hemocytes in the cyclopoid copepod, and presented a preliminary technique for isolating hemocytes and hemolymph. The motivation behind the current study stems from the lack of research on copepod hematology [8].

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MATERIALS AND METHODS

Plankton collection

The utilization of Henson's standard plankton net with a mesh size of 150 µm facilitated the procurement of Zooplankton specimens through the towing method. The net was towed horizontally in a zigzag pattern at a depth ranging from 50 to 100 cm for duration of 10 minutes, maintaining a consistent speed. This collection process took place in the freshwater Mahamaham tank, located in the Thanjavur district of Tamil Nadu. Following the collection, *Calonoid copepods* were isolated from the gathered Zooplankton and subsequently cultured.

Identification of zooplankton

The identification of zooplankton was carried out by referring to standard manuals [8-9] and textbooks [10-12] with the assistance of a compound microscope. Photomicrographs were captured using an inverted Biological Microscope equipped with a camera. The analysis results indicated that *Calonoid copepods* were the most abundant species found in the collected water samples.

Isolation of copepods

The collected water samples underwent a filtration process utilizing superimposed sieves to separate copepods from other zooplankton and to remove nauplii and other larval forms. The copepods were then scrutinized under a dissecting binocular microscope, and viable adult specimens were isolated and identified based on their morphological features observed through the microscope. The separated copepods were then placed in a 500 ml beaker filled with tap water for further observation.

Experimental protocol to study the effect of CUD on copepods

In this investigation, a total of five tanks, each containing twenty liters of uncontaminated water, were employed to introduce copepod populations with a density of 500 individuals per tank. The experimental tanks were subjected to different concentrations of CUD, namely 0.01%, 0.025%, 0.5%, and 0.1%, which were determined based on the LC50 value. One tank was assigned as a control and received an equivalent volume of tap water devoid of CUD. The media remained undisturbed for a duration of seven days, following which the water was replaced. Subsequently, the plankton were provided with all essential culture requirements and adequately aerated for a total of 60 days. Hematological investigations were carried out on both the CUD-treated and untreated plankton groups.

i) *Isolation of hemolymph* [13]

A group of one hundred fully grown individuals were separated and put under microscopic scrutiny. The plankton was then blended uniformly with a tissue homogenizer and a sterile physiological saline solution. The resulting mixture was then spun at 6000 rpm for 10 minutes, after which the tissue fragments were eliminated. The resulting liquid above the sediment was recognized as the hemolymph.

ii) *Isolation of plasma and hemocytes* [13]

The hemolymph of zooplankton species underwent centrifugation at a speed of 3000 revolutions per minute (rpm) for a period of 10 minutes, leading to the division of plasma and packed cells from the hemolymph. Subsequently, the plasma was isolated and carefully transferred into an Eppendorf tube utilizing a micropipette. The packed plasma or hemocytes were then re-suspended in a sterile phosphate buffer saline solution to facilitate further analysis. This innovative methodology was employed to isolate hemolymph, plasma, and hemocytes from zooplankton species.

iii) *Microscopic examination of hemocytes* [13]

To determine the size, shape, and composition of hemocytes, newly obtained samples underwent eosin staining and were subsequently examined under light microscopy at a 100X magnification. The dimensions of the cells were measured using a calibrated micrometer, following the prescribed protocols outlined.

iv) *Differential hemocyte count*

In this investigation, a total of 100 species were gently crushed using a mortar and pestle with physiological saline solution. Once the animals were broken down, the resulting sample was centrifuged with physiological saline at 200rpm for 5 minutes, leading to the separation of broken body parts as sediment. The supernatant, which contained the hemolymph, was collected using a Pasteur pipette. The hemolymph was then subjected to further centrifugation at 3000 rpm for 10 minutes to isolate the cells, which were subsequently washed in physiological saline and preserved in Eppendorf tubes.

To examine the cells, a drop of the sample was taken and spread onto a glass slide, which was then stained with Eosin stain and allowed to air dry. The cells were then viewed under 100X magnification using a trinocular microscope. The cells were counted from one corner of the slide to the other end, and different types of cells were identified and counted separately.

$$\text{DHC} = \frac{\text{Number of specific type of cell counted}}{\text{Total Number of cells counted}} \times 100$$

a) *Observation of hemocytes*

In this investigation, a total of ten samples of plankton were gathered and exposed to a physiological saline solution. Subsequently, a needle was used to gently press the specimens to extract their hemolymph. The deceased organisms were then spread out and treated with Eosin stain for a period of five minutes. Afterward, the Eosin was rinsed off, and the slide was air-dried before being placed under a Trinocular microscope at 100X magnification. The cells were meticulously scrutinized to determine their morphology, with specific attention given to the presence of granules in the cytoplasm. Based on the observed morphology, three distinct types of hemocytes were identified, namely Hyalinocytes (H), semi-granulocytes (SG), and Granulocytes (G). The identification of these hemocytes was based on a range of morphological characteristics, including size, shape, the presence or absence of cytoplasmic granules, the position of the nucleus, and the nucleo-cytoplasmic ratio.

b) *Determination of Total number of hemocyte count*

A micropipette was used to dispense a diluted hemolymph volume of 100µl onto the central platform near the edge of the cover slip. The micropipette was held at an angle of 450 during the dispensing process. Afterward, the sample was allowed to settle for a period of 2 to 3 minutes. Following this, the ruled area was brought into focus under the microscope. By counting the cells within 80 small squares, the number of hemocytes present was determined. Only the cells that made contact with the upper and left lines were included in the count, while those touching the lower and right lines were excluded.

RESULTS AND DISCUSSION

Hematological analysis

Freshly collected hemocytes that were stained with eosin were examined using light microscopy (LM) at a magnification of 100X to assess their cell size, shape, and the presence of granules. Cell measurements were taken using a calibrated micrometer. Our findings indicate that hemocytes of the *Calanus* copepod can be categorized into three main groups: hyaline cells, semi-granulocytes, and granulocytes, each displaying distinct morphological characteristics. Throughout history, researchers have commonly acknowledged these three categories and differentiated them based on their morphological features. Eosin staining, the hemocyte cells of *Sinodiaptomus sarsi* were classified according to their size, shape, and the presence or absence of cytoplasmic granules. (i) Hyaline cells: These cells exhibit no signs of cytoplasmic granules and are abundantly present. (ii) Semi-granulocytes: These cells possess a variable number of cytoplasmic granules. (iii) Granulocytes: These cells contain a significant number of cytoplasmic granules.

Plate 1 Light microscopic picture of *Sinosiaptomus sarsi*

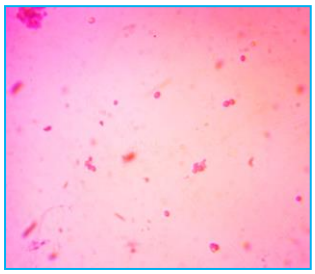

Plate 2 *Calonoid copepod* Hyaline cells stained with Eosin

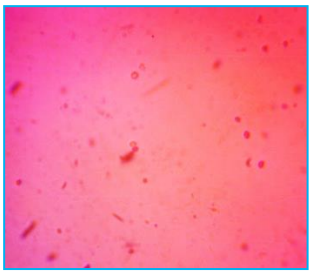

Plate 3 *Calonoid* copepod hemocytes stained with Eosin identified as in semigranulocytes

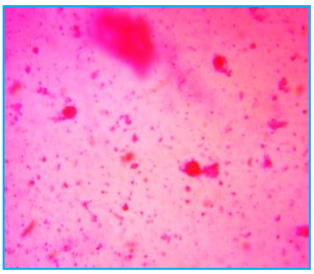

Plate 4 *Calonoid* copepod hemocytes stained with Eosin identified asin granulocytes

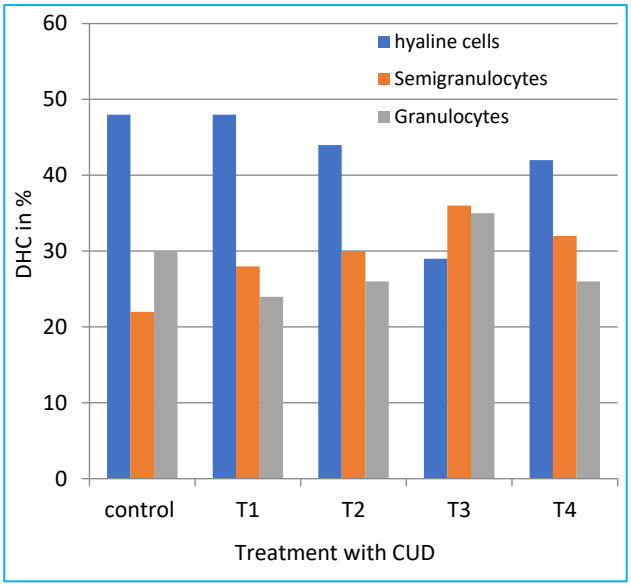

Fig 1 Differential counts of hemocytes in *Sinodiaptomus sarsi*

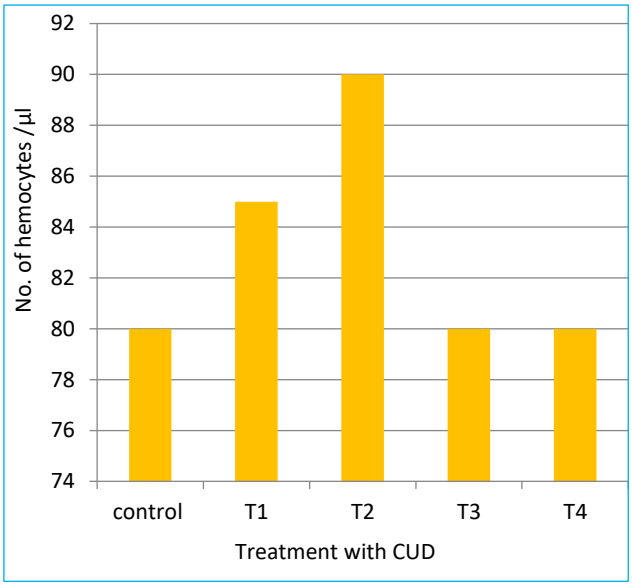

Fig 2 Total number of Hemoytes count

The total hemocyte count of *Sinodiaptomus sarsi* has been recorded as 90 cells per ml. of hemolymph of hemocytes in neubaur chamber.

The aquaculture industry is currently facing two major challenges: feed economics cost-benefit analysis and diseases. The increase in infections among fish populations can be attributed to the rapid proliferation of microorganisms, including bacteria, viruses, protozoans, and fungi. These microorganisms have the ability to develop resistance to synthetic chemotherapeutic drugs, leading to detrimental effects on the host. Moreover, these drugs also have an impact on non-target organisms, such as beneficial plankton, which play a crucial role in the freshwater food chain. As a result, researchers are focusing their efforts on identifying naturally occurring products that can effectively address this abnormal and severe situation.

Cow urine

The study conducted [14] documented that the combination of cow urine and cow dung as a pond fertilizer resulted in an improved growth of plankton and subsequently led to an increase in fish production. In a separate study, [15] reported that even human urine can act as a nourishing agent for plankton. Furthermore, [16] demonstrated that the utilization of cattle urine, specifically Gir cow urine distillate, significantly enhanced the population density of cyclopoid copepods in culture and enriched the biochemical composition of these organisms. Additionally, provided evidence of the positive effects of cow urine distillate on the population density, biochemical, cytological, and anti-microbial properties of hemolymph in *Mesocyclops leuckarti*. Taking into consideration the findings from these scientific studies, cow urine distillate was investigated as a potential promoter for plankton culture in terms of nutrient levels and overall health status.

The demonstrated that [17], cow urine had positive effects on growth promotion and immune stimulation when administered at lower doses. However, the researchers also found that a concentration of 5% cow urine was toxic to *Labeo rohita* fish. Similarly, [18] reported that a concentration of 1%

cow urine resulted in 100% mortality in Mesocyclops leuckarti, while 0.6% caused 50% mortality. The researchers hypothesized that the smaller and less complex nature of microcrustaceans may explain why cow urine caused mortality at a lower concentration in this species compared to fish. This finding is consistent with the study [19] which quantified the impact of size-dependent toxicants. Therefore, based on [18], further testing of cow urine was conducted at a lower concentration of 0.1%.

Cytological studies

Copepods, a group of microcrustaceans, have been found to serve as hosts for various species of parasites. In order to better understand this relationship, scientific research has investigated the potential use of cow urine distillate as a promoter for plankton culture, focusing on nutrient levels and the health status of copepods. Despite the prevalence of copepod infections, there is still limited knowledge regarding the mechanisms of infection and potential defenses against parasites [20]. Copepods play a crucial role as intermediate hosts for different parasites, including *Dracunculus medinesis*, a parasite that affects humans, as well as fish-parasitic nematodes such as *Anguillicola crassus* and *Camallanus* sp. They also act as transport hosts for nematodes [21]. Demonstrated that the immune mechanism of the copepod *Macrocyphines albidus* exhibits specificity and memory against nematode parasites, resulting in a reduced reinfection rate for related parasites compared to prior exposure to unrelated species [22]. The possibility of specific protection being transferred maternally [23]. While copepods primarily rely on innate immunity, there may be an alternative mechanism that allows for specific recognition within the innate immune system. The innate immunity of Drosophila, [24] the changes in metabolism and behavior of the freshwater copepod Cyclops *Strenuus alyssourm* when infected with *Diphyllobothrium* sp.

The current knowledge about the immune system in micro crustaceans is limited. However, the transcriptional levels of dorsal and dorsal-like genes increased in the *Cyclopoid* copepod and *Paracylop inaniana* when exposed to immune modulators. While some research has explored the hemocyte-mediated immune mechanisms in Daphnia, there is a lack of information regarding hemocytes in copepods [6]. Nonetheless, Praveena and Venkatalakshmi were the first to observe hemocytes in the freshwater copepod *Mesocyclops leukarti* in 2019 [13]. Building upon their findings, the present investigation focused on *Sinodiaptomus sarsi* and yielded similar results.

CONCLUSION

The current investigation has revealed that the hemocytes of the *Calanus* copepod can be divided into three primary groups: hyaline cells, semi-granulocytes, and granulocytes. Each group exhibits distinct morphological characteristics. In the case of *Sinodiaptomus sarsi*, the hemocyte cells were classified based on their size, shape, and the presence or absence of cytoplasmic granules using eosin staining. Furthermore, the potential of cow urine distillate as a promoter for plankton culture was examined in terms of nutrient levels and overall health status.

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