The role of starvation on selective immunological parameters in land snail Helix aspersa

Atika AL-Rawadeh

Department of Biological Sciences, College of Science, AL Hussein Bin Talal University

ABSTRACT

*Helix aspersa*, (garden snail), is very common and widespread species in mediterranean region and western Europe. This species is edible in many places around the world and has many applications in traditional medicine. The role of starvation on various factors related to immunological defence in *Helix aspersa* were tested in controlled laboratory experiments. The immunological and biochemical measurements were performed on 30 snails. A various parameters were assessed, including total hemocyte counts, phenoloxidase (PO) and phagocytosis. The phenoloxidase activity and total hemocyte frequencies decreased significantly after three weeks of starvation. However, the present results also showed that granulocytes are more affected by starvation than hyalinocytes, and the activity of phagocytosis depressed after the same period of starvation. These data indicate that starvation compromises immunological activity, and accordingly affect immune responses. So that good nutrition play a major role in maintaining immunological parameters in *Helix aspersa*

**Key words:** *Helix aspersa*, starvation, phenoloxidase, phagocytosis.

Introduction

*Helix aspersa*, (garden snail), is a species of land snail, pulmonate gastropod, and is very common and widespread in mediterranean region and western Europe [1]. This species is edible in many places around the world, such as France, Italy, Greece. In addition to that, it has also been used for centuries in traditional medicine [2].

Invertebrates do not possess an acquired immunity, however, phenomena of specificity has recently been demonstrated in invertebrates [3].

Invertebrates immune systems involve innate cellular and humoral responses asimilar to those that found in vertebrates [4,5,6]. Cellular defenses include hemocyte- or coelomocyte-mediated responses such as phagocytosis, nodulation and encapsulation [7,8]. Also they have humoral effectors including reactive oxygen species, antimicrobial peptides, and coagulation and melanization factors [6,8,9].

Hemocytes have been classified using a variety of criteria based on combinations of morphology, cytochemistry and function. Hemocytes from snails mollusks have most often been divided into two types, hyalinocytes and granulocytes [10,11,12]. Granulocytes are the most observed cells with high phagocytic ability. Hyalinocytes, unlike granulocytes, lack filopodia, include few cytoplasmic granules and have low phagocytic ability. [10]. Phenoloxidase is a key enzyme involved in the immunological defence of invertebrates. It is synthesized as inactive prophenoloxidase, which is activated to phenoloxidase by serine proteases [7,13-15].

There are many environmental stress factors, like salinity and temperature, which reduce immunological activity among invertebrates, and increased Susceptibility to pathogens [16-21]. Starvation is the other environmental stressor that has an effect on many molluscs, such as Megalobulimus oblongus where it increased haemolymph glucose levels and glycogen concentration in hepatopancreas, mantle and muscle [22].

Corresponding Author

Atika AL-Rawadeh, Department of Biological Sciences, College of Science, AL Hussein Bin Talal University
E-mail: atikaalrawadeh@yahoo.com
Starvation also caused a reduction in the synthesis of galactogen and glycogen in the albumen gland and the mantle of the pond snail *Lymnaea stagnalis*, [23].

In *H. aspersa*, starvation caused 60% reduction in body weight and significantly decreased the number of digestive cells [24]. Starvation also decreased the egg laying and the activity of the albumen glands in *Bulinus truncates* [25]. Also starvation decreases the content of ammonia in *Bradybaena similaris* snail [26].

The aim of the present study is to evaluate the effects of starvation on immunological parameters (phagocytosis, phenoloxidase (PO) activity, hemocyte frequencies) in *H. aspersa*.

**Materials and methods**

**Experimental Animals and Design.**

*H. asperse* were collected from Alkarak province is located in south of Jordan (near to The Ded sea). They were transported to Ma’an province (about 180 km from the capture site) and maintained in the laboratory under suitable conditions (10 per plastic box, with wet filter paper in the bottom, and temperature (25°C ±1). They were maintained for two weeks with continuously regular feeding (twice weekly). All the other conditions were controlled according to Armelle et al method [27]. Before starting the starvation period, the size and weight of each adult snail has been determined. A total of 60 adult snails were randomly chosen and divided into two equal groups: control (not starved) and experimental (starved ) snails. The experimental snails were starved for 3 weeks.

**Haemolymph Collection and Preparation**

After three weeks of treatment, 10 snails from each group were removed and washed with distil water. Hemolymph was then withdrawn by cardiac puncture, directly through a notch in the shell. Hemolymph samples were immediately placed in polypropylene tubes and held on ice.

**Phenoloxidase Assays**

Phenoloxidase activity in whole haemolymph was determined spectrophotometrically according to Peters and Raftos [28]. Assays were performed by recording the diphenolase activity with the substrate, L-3,4-dihydroxyphenylalanine (L-DOPA, ICN, Irvine, CA, USA). The chromogen, 3-methyl-2-benzothiazolimine hydrazone (MBTH, Sigma Aldrich) was added to the substrate. Assays were carried out in 96 well flat bottom microtiter plates. One hundred µl of whole hemolymph were added per well followed by the addition of 100 µl of L-DOPA (4 mg ml⁻¹ in PBS), containing 1 mM MBTH. The absorbance of the reaction mixture was measured at 490 nm immediately after the addition of substrates using a microplate spectrophotometer (SpectroUV. Vis Auto.UV-2602). A second reading was made after the plates had been incubated for one hour at room temperature. Enzyme activities are expressed as the change in optical density at 490 nm (OD₄⁹₀).

**Phenoloxidase Activity Cytology**

Thirty µl of whole hemolymph was immediately placed on acid alcohol washed microscope slides coated with poly-l-lysine and left to adhere for 10 minutes. The hemocytes were then stained for phenoloxidase activity.

The phenoloxidase stain was prepared in PBS and contained 5 µ M L-DOPA and 5 mM MBTH. The adherent hemocytes were overlaid with 30 µl of the stain and allowed to stand for 10 minutes. They were then covered with a clean cover slip and sealed with nail polish. Slides were then incubated for a further 30 min at room temperature. A light compound microscope was used to examine randomly selected fields of view on the slide. A total of 200 hemocytes were examined so that the frequency of phenoloxidase-positive hemocytes (red stained) relative to unstained hemocytes could be determined.

**Phagocytosis Assay**

Phagocytic activity was assessed *In vitro* using *Saccharomyces cerevisiae* (Yeast, Sigma Aldrich), as target cells. Five mg yeast were suspended in 5 ml PBS and mixed with an equal volume of filtered Congo red (Sigma Aldrich; 0.8% in PBS).

The suspension was autoclaved at 120° C for 15 minutes before being washed twice by centrifugation at 1,300g for 5 minutes and resuspended in 10 ml PBS. Forty µl of whole hemolymph were placed on acid alcohol washed microscope slides coated with poly-l-lysine and left to adhere for 20 minutes in a moist chamber at room temperature (25° C).

The supernatants were then removed and the adherent hemocytes were rinsed twice with PBS. The slides were overlaid with 100 µl of Congo red stained yeast (0.7 x 10⁶ ml⁻¹) and incubated for further 30 min at room temperature. The slides were washed 4 times with PBS to remove non-phagocytosed yeast cells. They were then covered with a clean cover slip and sealed with nail polish. A minimum of 200 hemocytes were examined and the number of hemocytes that had phagocytosed one or more yeast was recorded so that the percentage of phagocytic cells could be calculated.
**Total and Differential Hemocyte Counts**

The total hemocyte frequencies in hemolymph was determined by using a Neubauer hemocytometer. Hemocyte monolayers were used to calculate differential hemocyte frequencies for granulocytes and hyalinocytes. Monolayers were prepared by allowing hemocytes to attach to acid alcohol cleaned slides for 25 min at room temperature. Microscope was then used to differentiate between hemocytes types according to the presence or absence of cytoplasmic granules.

**Statistical Analysis**

All experiments were conducted three times. One way analysis of variance (ANOVA) was used to determine the significance of differences between mean values. Differences were considered to be significant if $P < 0.05$.

**Results**

**Phenoloxidase Activity:**

Hemolymph phenoloxidase activities decreased after the 3 weeks of starvation. Fig. 1 shows that whole hemolymph phenoloxidase activities (monophenolase and diphenolase) were reduced significantly ($P < 0.05$) after starvation.

![Fig. 1: The effect of starvation on the PO activity](image)

**Phenoloxidase Activity - positive:**

Fig. 2 demonstrates that the frequency of Phenoloxidase activity - positive hemocytes decreased significantly ($P < 0.05$) after 3 weeks of starvation.

![Fig 2: The effect of starvation on the number of the PO positive cells](image)

**Phagocytic Activity and Starvation:**

Figure 4 reveals that starvation decreased the phagocytic ability of hemocytes. After 3 weeks of starvation, the frequency of phagocytic hemocytes fall, significantly ($P < 0.05$) as compared to control snails.

![Fig. 4: The effect of starvation on the phagocytic ability](image)

**Total and Differential Hemocyte Counts**

The starvation of snails caused a significant decrease in the total number of hemocytes in whole hemolymph as compared to normal snails. Fig. 3A and shows that the number of hemocytes in starved snails decreased significantly ($P < 0.05$) after 3 weeks of starvation. Also there was a significant decrease in the frequency of the granulocytes.

Fig. 3B in hemolymph after the starvation, in comparison with control snails. This decreased significant at ($P < 0.05$). However, the decrease in the frequency of the hyalinocytes was not significant at ($P < 0.05$).

![Fig. 3A: The effect of starvation on the THC](image)

![Fig 3B: The effect of starvation on the total number of granulocytes and hyalinocytes](image)
**Discussion**

Land snails can enter dormancy under many unfavorable conditions, including low humidity [29], low concentration of nutrition [30,31], and high temperatures [32]. They may remain dormant for many months or even years and during these long periods of starvation, all the stored materials (glucose, lipid and protein) are metabolized and consumed [33]. The reduction of all of these sources of nutrients in invertebrates caused a reduction in the body weight, changes in the histologic structure of digestive gland, oxygen consumption rate, and changes in the structure and composition of the shell [34,35,24].

Before discussing the role of starvation on immune system of invertebrate, some consideration must be given to the question of whether stress factors played any significant part in determining immunity in invertebrates. The biochemical and immunological changes recorded here were consistent with previous studies on that some stress factors like altered temperature, hypoxia, pollutants, altered salinity and mechanical disturbance, have negative impacts on invertebrate immune system leading to increased disease susceptibility [36-40]. Furthermore the results of the present experiment reveal that short term starvation (three weeks) inhibited all the immunological parameters tested. After 3 weeks of starvation, phenoloxidase activity (PO) decreased significantly compared to the control snails. This is in agreement with those of Butt et al [41], who found that starvation caused a significant decreases in PO, in *Saccostrea glomerata*. However, Yang et al [42], found that starvation caused a decrease in the melanotic encapsulation rate but not in the PO activity in the *Epirrita autumntata*. In addition to phenoloxidase activity, total hemocyte frequencies also decreased significantly after the same period of starvation. However, the results showed that granulocytes are more affected by starvation than hyalinocytes. These results are partially in agreement with those in the Sydney rock oyster [41], and Zhikong scallop (*Chlamys farreri*) [43]. Starvation also decreased significantly the phagocytic activity. Many studies had showed in invertebrates the granulocytes are more phagocytic than hyalinocytes [44]. The decrease in the phagocytic ability can be explained by the significant decrease in the granulocytes after the three weeks starvation period. These results support the earlier findings reported in *Chlamys farreri* and in *Saccostrea glomerata* [41,43].

Finally, the results of this study showed that good nutrition play a major role in maintaining immunological parameters in *Helix aspersa*.

**Acknowledgements**

Many thanks to the head department of biological sciences Dr Saleem Aladaileh, Prof. Khalil AL-taif’, the technical staff and Dept. of biology.

**References**


