Chapter

Immune Response of Molluscs

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Abstract

In common with other invertebrates, molluscs are known to have internal immune response against foreign particles and organisms. The innate immunity of molluscs reflects the inherent non-specific response that provides the first line of defense. Anatomic barriers, phagocytic cells, and physiological components are the main elements of the innate immune response in molluscs. It is composed of both cellular and humoral elements. The cellular components are the circulating hemocytes. Small invaders are eliminated by the phagocytic hemocytes, while large invaders are eliminated by encapsulation. The ingested foreign particles are then hemolyzed by the action of certain toxic enzymes that catalyze oxidative burst reactions capable of killing pathogens and foreign invaders. Humoral components of molluscan immunity involve nitric oxide, lysozyme activity, lectins, and the phenyloxidase system. The current chapter sheds light on the elements of the molluscan innate immune system and presents a case study of the immune response of *Lymnaea stagnalis* mollusc against *Chaetogaster limnaei* parasite. The effect of the parasite on some humoral immune response parameters such as nitric oxide, phenol oxidase, and lysozyme production was investigated. In conclusion, the snail *Lymnaea stagnalis* exerts humoral immune response against *Chaetogaster limnaei* parasite. However, this response is insufficient to eliminate the parasite.

Keywords: *Chaetogaster limnaei*, humoral immune response, *Lymnaea stagnalis*, molluscs

1. Introduction

1.1 *Lymnaea stagnalis* in the animal kingdom

Molluscs are invertebrates that form one of the largest groups in the animal kingdom, with more than 100,000 known species. A wide range of molluscs including families of Pulmonata, Gastropoda, Planorbidae, and Lymnaeidae are intermediate hosts of trematodes.

Gastropods or snails are asymmetrical molluscs with a well-developed foot and radula. The visceral mass is spirally coiled. They occur in seas, freshwater, and terrestrial environments. The class Gastropoda is divided into three subclasses: Prosobranchia, Opisthobranchia, and Pulmonata. The snail *Lymnaea stagnalis* belongs to the family Lymnaeidae under the order Basommatophora, class Pulmonata. This snail is a freshwater scavenger that slides along the bottoms of ponds, lakes, and marshes looking for food. It is relatively large species with slender and sharply pointed spires. They can be found in North America, Europe, Asia, Algeria, and Morocco [1]. The head of the snail is on the front

part of the foot that is used to glide on mucus slime produced from its own body. Pulling the foot inside the shell is one way of protection against predators. The shell walls are delicate and fairly transparent. The body is yellowish-gray in color, with a special adaptation of the mantle that allows it to take in air. Eggs are laid from April till the beginning of October and hatch after 10 days. Snails can reproduce when they are 3 months and can live for 6 years. They feed on algae, plants, other snails, and insects. The common name of this snail is the Great Pond Snail in the United Kingdom. This is one of the biggest freshwater snails with a height of 45–60 mm and a width of 20–30 mm [1]. **Plate 1** shows a *Lymnaea stagnalis* snail on vegetation [2].

1.2 Major parasitic infections associated with *Lymnaea stagnalis*

Lymnaea stagnalis is known to be an intermediate host of several parasites. Examples in Europe are *Fasciola hepatica*, *Trichobilharzia ocellata*, *Haplometra cylindracea*, and *Schistosomatium douthitti*.

Trichobilharzia ocellata is a trematode parasite of birds that causes what is known as the cercarial dermatitis of humans (swimmer's itch). This phenomenon has a high prevalence in Europe. The cercariae cannot develop into adults in human, but they cause erythema and urticaria of the invaded area, and a hypersensitivity reaction characterized by intense itching that may last several days up to 3 weeks. It can be caused by dead or dying larvae in the skin. **Plate 2(a)** shows a cercaria of *T. ocellata* and **Plate 2(b)** shows the swimmer's itch caused by penetration of the cercariae [3].

Haplometra cylindracea is a trematode that infects the frogs' lungs. It feeds on the blood that is supplied to the lungs. The xiphidiocercariae encyst in insects. Molluscan intermediate hosts could be: *Lymnaea stagnalis*, *L. ovata*, or *L. palustris*. This parasite is known to be non-pathogenic to man.

Schistosomatium douthitti is known as the rodent schistosome. It is also non-pathogenic to man. The percentage of natural infection with this parasite is only 3–7%. The natural definitive hosts of this parasite in North America are rodents. **Plate 3(a)** shows a cercaria of *H. cylindracea* and **Plate 3(b)** shows a cercaria of *S. douthitti* [4].

In addition, some annelids are found associated with the mantle area of *Lymnaea stagnali*. For example, *Chaetogaster limnaei* is a parasite of freshwater snails including *Lymnaea stagnali*. It belongs to the annelid family, Naididae, which is considered to be an ecologically diverse family of worms common in both running and standing waters. Reproduction occurs predominantly by paratomy, asexual reproduction

Plate 1.
A Lymnaea stagnalis *snail on vegetation.*

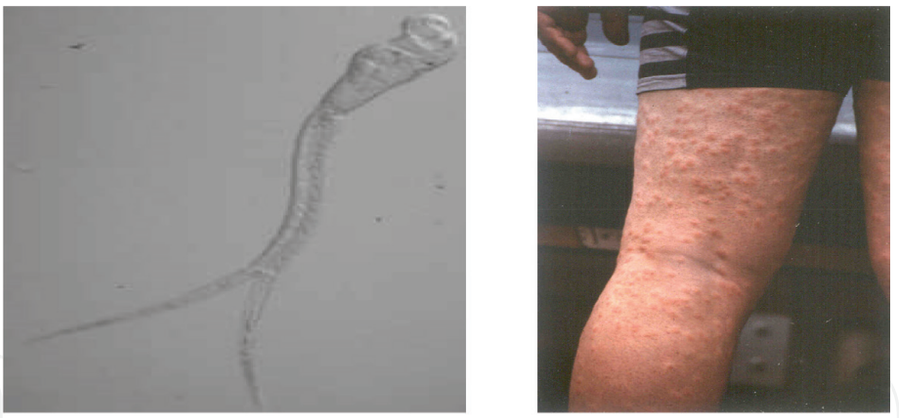

Plate 2.
(a) A cercaria of T. ocellata*, and (b) the swimmer's itch caused by penetration of the cercariae.*

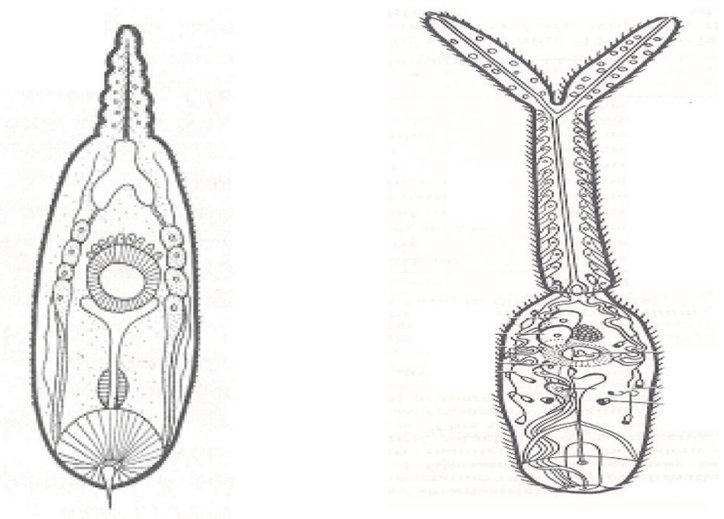

Plate 3.
(a) A cercaria of H. cylindracea*, and (b) a cercaria of* S. douthitti*.*

in which new organs are developed before the animal divides into two or more parts. In most cases, this organism appears to be commensalistic and does not cause high degree of mortality to its host, although it may affect the reproducing capacity by affecting the gonads. The length of the parasite ranges from 7 to 20 mm [5]. **Plate 4** shows *Chaetogaster limnaei* [6].

In common with other invertebrates, molluscs are known to have internal immune response against foreign particles and organisms. The innate immunity of molluscs reflects the inherent non-specific response that provides the first line of defense. Anatomic barriers, phagocytic cells, and physiological components are the main elements of the innate immune response. It is composed of both cellular and humoral elements. The cellular components are the circulating phagocytic hemocytes. Small invaders are eliminated by phagocytic hemocytes, while large invaders are eliminated by encapsulation. The ingested foreign particles are then hemolyzed by the action of certain toxic enzymes that catalyze oxidative burst reactions capable of killing pathogens and foreign invaders. Humoral components of molluscan immunity involve nitric oxide, lysozyme activity, lectins, and the phenyloxidase system [7, 8].

Plate 4.
Chaetogaster limnaei.

2. Innate response of molluscs

2.1 Anatomic barriers

Shells of molluscs act as physical barriers that prevent some pathogens from penetrating into the host's body. Mollusc shells are composed of organominerals with different structures and compositions depending on the species. Most of these shells are composed of an aragonite nacreous and a calcite prismatic layer [9]. The layers of the skin are tightly packed and the acidic environment in these layers is not suitable for the growth of most micro-organisms. The mucous membrane that lines the tissues and tracts contains normal micro-flora that competes with pathogenic micro-organisms for essential nutrients and attachment sites on these layers. The mucosal barrier may also act as a trap for antigens that are then inactivated by phage encapsulation by hemocytes. In addition, the movement of the outer cells expels the trapped pathogens to the outside environment. The flushing action of secretions helps to prevent infection of certain pathogens [10].

2.2 Key cells in innate immunity

There are immune cells in molluscs equivalent to the white blood cells in higher animals that play a role in the non-specific immune response. These cells are called hemocytes. Most of these cells are capable of engulfing extracellular particles by phagocytosis, endocytosis, and encapsulation. Others can produce substances that play a role in the killing process of pathogens. Circulating hemocytes proliferate and differentiate after exposure to pathogens or foreign particles. It was noticed that circulating hemoblasts are present at various phases of mitosis [11, 12].

The role of these phagocytic cells in the hemolymph of gastropods is considered to be the first line of defense system against invading or established organisms and particles. These hemocytes are circulating within the hemolymph as well as residing in the connective tissues. There are also cells lining the hemolymph spaces and are able to trap micro-organisms. Other cells are the fixed phagocytic reticulum cells that are fixed in the tissues by fibrils [13, 14].

A lot of studies have been done to determine the types and numbers of hemocytes in molluscs. This is considered to be a debating question because

these hemocytes are morphologically and functionally heterogeneous. In addition to phagocytosis, these cells participate in digestion, excretion, healing of wounds, shell repair, transport, and encapsulation [14]. Classification of mollusc's hemocytes depends mainly on morphological criteria like cell size, nucleus to cytoplasm ratio, shape of nucleus, and the presence of granules in the cytoplasm using light microscopy. Studies have revealed the presence of two distinct types of hemocytes. These are the granulocytes and the agranulocytes. Acidophilic and basophilic granulocytes and slightly granular cells were also observed [15, 16]. The agranulocytes are relatively small cells and are also called hyalinocytes, they are less than 8 μm in size; while the granulocytes are larger spreading cells with pseudopodia and a polymorphic nucleus. The majority of cells in the hemolymph of molluscs are large spreading cells. Interestingly, there are intermediate stages between the round and the spreading cells in terms of morphology. Some scientists have suggested that the small and round cells are young cells and the larger spreading cells are old cells that are more capable of phagocytosis [17]. On the other hand, the small round cells in *L. stagnalis* do phagocytose, but not as much as the spreading cells. **Plate 5** shows hemocytes of *Lymnaea stagnalis* (A) free in the circulating hemolymph, while (B) shows hemocytes fixed in the tissue. **Plate 6** shows phase-contrast micrograph of *L. stagnalis* hemocytes, R is a round cell and the others are spreading hemocytes [17].

Tripp and Kent [18] have reported that 90% in vitro invading bacteria can be removed by hemocytes from molluscs within 24 h and 99% can be removed within 72 h. The glycolytic pathway in the cells was suggested to supply the hemocytes with the energy that is needed for phagocytosis, this was first proved by the work of Cheng [19] that has shown that hemocytes of the hard clam, *Mercenaria mercenaria*, use glucose/glycogen and produce lactate without an increase in the oxygen consumption.

2.3 Physiological elements of innate immunity in molluscs

In addition to the phagocytic hemocytes that are involved in the innate immunity, there are soluble physiological elements that play a role in defending the host from invading foreign substances and pathogens. Cellular and soluble elements coordinate in a sophisticated network of interactions to mount effective innate immune responses capable of eliminating foreign antigens. These soluble physiological elements include: nitric oxide, lysozyme activity, lectins, and the phenyloxidase system [7, 8].

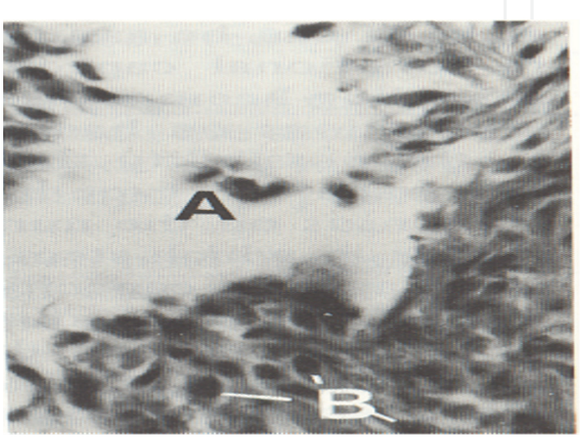

Plate 5.
Hemocytes of Lymnaea stagnalis*, (A) free in the circulating hemolymph and (B) hemocytes fixed in the tissue.*

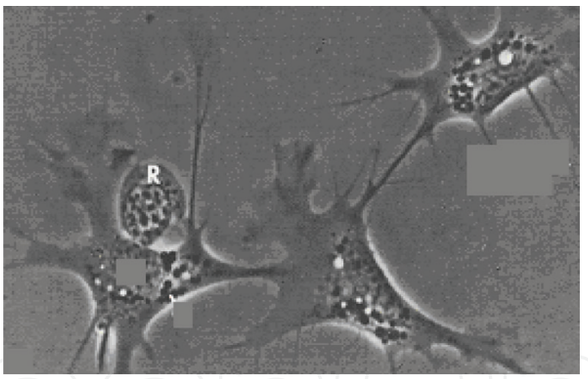

Plate 6.
Phase-contrast micrograph of L. stagnalis hemocyte. R is a round cell and the others are spreading hemocytes.

2.3.1 Complement system

The complement system is considered to be an important element of the innate immune system that also triggers the adaptive immunity in higher animals in the animal kingdom. Similar hemolytic complement-like activity was also reported against parasites and other pathogens in molluscs [20, 21].

Complement components are proteins and glycoproteins that are mainly synthesized in the liver. Other cells such as the tissue macrophages and the blood monocytes are involved in the complement production. These proteins are circulating in the blood in an inactive form. Once activated upon exposure to an antigen, they enter an enzymatic biochemical cascade that helps in the elimination of antigens by lysis of cells, opsonization, binding to specific complement receptors on cells of the immune system, and/or immune clearance of immune complexes [22–24].

There are three different pathways by which the complement cascade is activated, namely the classical pathway, the alternative pathway, and the mannan-binding lectin pathway. The classical pathway is antibody-dependent, so it is more related to the adaptive immunity. It is initiated by antigen-antibody binding to form immune complexes or when an antibody binds to an antigen on the surface of a pathogen. Activation of complement component 1, often simply called C1 (a protein of the immune system) then occurs when it binds to such antibodies of type IgM and some classes of type IgG. On the other hand, the alternative and the mannan-binding lectin pathways are more related to the innate immunity because they are antibody-independent. The former is initiated by complement component 3 (C3 complement), activation upon exposure of foreign substances on the cell wall of pathogens like bacteria, while mannan-binding lectin pathway is activated when a lectin binds to a mannose residue on the cell wall of pathogens such as *Salmonella*. This lectin is a serum acute phase protein that is produced as a result of the inflammatory response in the site of inflammation. The membrane attack complex (MAC) is produced upon activation of the complement system in the three pathways. This complex mediates lysis of the cell wall of bacterial pathogens. There are serum proteins as well as proteins on the surface of self-cells that control the activity of the complement system to ensure that host cells are not attacked [25–27].

2.3.2 Nitric oxide production

Many evidences have proven the presence of immunological factors in molluscs capable of working against infectious elements. Nitric oxide (NO) is a gaseous intercellular signaling molecule that has several functions in the cardiovascular, reproductive, nervous, and immune systems. Post infection by a pathogen, high levels of NO is synthesized in the immunocytes of the host in order to eliminate the

pathogen or induce inflammation. Recently, NO has been reported to be involved in the immune response of several mollusc species such as scallop, clam, mussel, oyster, and snail. In addition to killing the invasive pathogens, high concentrations of NO can prove fatal to normal host cells as a result of strong cytotoxicity produced during the immune response. Modifying the NO concentration is crucial in order to maintain immune homeostasis and other physiological processes. Also, neuroregulation of NO is of utmost importance so as to prevent prolonged immune response [28].

Nitric oxide synthase was reported to play an important role in the phagocytic activity of hemocytes of molluscs such as *Mytilus galloprovincialis* and *Lymnaea stagnalis* [29–31]. *Lymnaea stagnalis* is a model organism for the study of the effects of environmental pollutants on immunological defense mechanisms, with one of the reasons being that its hemolymph collection is easy and does not require animal sacrifice. Most freshwater snails are dependent on cell-mediated cytotoxicity for the elimination of foreign materials. Phagocytosis plays a key role in the production of reactive oxygen species (ROS) known as the respiratory burst; this eliminates foreign particles and is the major defense mechanism in freshwater snails. Various stressors such as bacteria, viruses, parasites and xenobiotics affect the function of mollusc hemocytes. The elimination of ROS is important in order for the organism to survive. The antioxidant system which helps in this elimination includes superoxide dismutase (SOD), catalase (CAT), selenium-dependent glutathione peroxidase (Se-GPx), and glutathione reductase (GR). Glutathione (GSH) is an important scavenger of free radicals and helps in the maintenance of the redox status of proteinsulfhydryl groups. NO is produced by NO-synthases in hemocytes during phagocytosis and reacts with hydrogen peroxide to form peroxynitrite, a highly potent bactericide. NO may also act as an immunomodulator and mediates the effects of estrogen and opioids on immunity and inflammation [32].

2.3.3 Tyrosinase production

Tyrosinase is also considered to be a defense line system in the primary immune response of molluscs, it is involved in tanning and melanin formation. It is considered to be a non-self-recognition system because this enzymatic reaction can be triggered by minuscule amounts of molecules such as lipopolysaccharide, peptidoglycan and beta-1,3-glucans from micro-organisms. In *Lymnaea stagnalis* infected with the bird's schistosome *T. ocellata*, hemocytes were shown to increase their peroxidase activity between 2 and 8 weeks post infection [33, 34].

2.3.4 Lysosymes production

Lysozymes are enzymes that hydrolyze the 1,4-beta links between N-acetylmuramic acid and N-acetylglucosamine, they are known to destroy the cell walls of certain bacteria. They can be found in tears, saliva, egg whites, some plant tissues, and Sweat. Lysozymes have been recognized as a classic mollusc immune effector in innate immune system. It is a bacteriolytic enzyme produced from a range of organisms such as bacteria and bacteriophages to fungi, plants, and animals. They are characterized by their ability to bacterial peptidoglycan between two amino sugars, N-acetylmuramic acid and N-acetylglucosamine, which results in bacterial cell lysis and also has bactericidal and digestive abilities. In addition to this, they perform numerous other functions such as for growth stimulation, digestion, antiviral, anti-inflammatory, and are even associated with tumors. They are classified into six types based on their structural, catalytic and immunological characteristics. These are chicken-type (c-type), goose-type (g-type), plant, bacteria, T4 phage, and invertebrate type (i-type) lysozymes. The c-, g-, and i-type have been recorded in molluscs. They are the secreting type, which consist of signal

peptide and 8, 6, and 14 cysteine residues, respectively. All three exhibit antibacterial and digestive ability and i-type has antifungi activity [35]. It was reported that the concentration of lysozymes in the hemolymph of molluscs changes as an immune response. Hemocytes of *L. stagnalis* were shown to have a selective bacteriostatic activity that is considered to be a defense factor against foreign materials. In addition of being destroying infectious elements, lysozymes also help in detection of the non-selfagents [33, 36].

2.3.5 Lectin production

Lectins binding phenomenon have been known since the turn of the nineteenth century, and have been widely used in the field of histochemistry for research and clinical purposes to detect specific carbohydrate structures and derivatives like glycolipids and glycoproteins in cells and tissues at the level of electron and light microscopy. They are also used for purification and isolation of carbohydrates and other specific cells like lymphocytes and bone marrow. In addition, lectins are very important tools in analyzing membrane structures of cells, glycosylation pathways, differentiation, cell division, growth and developmental changes, and mapping neuronal connections [37].

Lectin production is considered as an innate immune parameter against infection. These are non-enzymatic and non-immunoglobulin carbohydrate-binding proteins present in plants, bacteria, fungi, and animals which preferentially bind reversibly to specific carbohydrate structures, either free in solution or on cell surfaces. They are often classified based on saccharide-specificity. This specificity is usually defined by the monosaccharides or oligosaccharides that are best at inhibiting the agglutination or precipitation reactions caused by lectins. Lectins in the same category (e.g., galactose-specific lectins) show considerably different sugar-binding preferences. Interestingly, an increasing number of lectins which never show high affinity to simple saccharides have been found. Lectins can be detected by binding to fluorescent markers such as enzymes, biotin, and FITC and ^{125}Iodine [38–41].

Immune factors that consist of a fibrinogen domain (FBG) are now emerging as important components of the innate immune response of invertebrates. In recent times, some arthropod and mollusc model systems have contributed to the study of the functional role of fibrinogen-related proteins (FREPs) in invertebrate defense mechanism. FREPs have been identified in mussels, scallops, oysters, and gastropods. The FREPs of gastropod molluscs are interesting due to their unique structure, capacity for somatic diversification and their function in the immune system.

Gastropods and Digenean trematodes have shared a parasitic relationship for nearly 200 million years. These trematodes rely heavily on molluscs, mainly on gastropods to complete their larval development. They have developed mechanisms to evade and suppress specific snail immune processes so as to reproduce within the snail host. However, not every nematode can infect the snail species equally; this suggests that snails have developed mechanisms to fight trematode infections. A component of the snail immune response is BgFREPs; they are soluble lectin-like factors. Agglutinins have been discovered in gastropods such as *Helix pomatia* and *B. glbrata* and were involved in the recognition of non-self by binding to carbohydrate targets on hemocyte membranes of other species and pathogen-associated surfaces [42].

2.3.6 Phenoloxidase activity

Phenoloxidase (PO) is an enzyme that oxidizes phenols to produce melanin which plays an important role in egg production in gastropods, sclerotization of a new postmolt exoskeleton, and immunity of invertebrates. PO occurs as inactive

precursors known as prophenoloxidses (proPO). They are activated by a proteolytic cascade system. Studies to investigate PO activity in *L. stagnalis* are limited. Their hemocytes play an important role in internal defense; they are involved in wound healing, encapsulation, and phagocytosis. They also contain several lysosomal enzymes such as peroxidase that are involved in intracellular killing. However, data in this aspect is lacking. Therefore, a study was taken to investigate the PO activity of *L. stagnalis*. The snails were collected from a lake in Russia and maintained under the desired laboratory conditions in 5-l aquaria containing dechlorinated tap water supplemented with mussel shell as a calcium source. Snails were fed lettuce daily and water was replaced once a week. Results showed that no PO activity was found. A low PO activity was seen in hemolymph without cells. On addition of a PO inhibitor, there was no effect on enzyme activity whereas hydrogen peroxide increased it [43].

3. Immune response of the snail *Lymnaea stagnalis* against parasites: study

The current chapter sheds light on some parameters that are associated with the immune response that is resulting from the interaction between the pond snail *Lymnaea stagnalis* mollusc and the parasite *Chaetogaster limnaei*. These parameters include production of nitric oxide, phenol oxidase, and lysozymes as an innate immune response that results from the interaction between the snail and the parasite.

3.1 Material and methods

3.1.1 Collection of snails and parasites

A total of 150 *Lymnaea stagnalis* snails were collected from Ringley, a freshwater pond in Manchester, England. The snails were usually supplied in a batch of 37–50 at a time and were kept in a fresh pond water tank at 4°C in the refrigerator. The water was changed every 5 days to prevent deoxygenation and death of snails. Pesticide-free lettuce was supplied as a source of food. The parasites were collected by scraping the body of the snail with a dissecting needle under a Thomas Scientific binocular dissection microscope and a Petri dish half filled with pond water to avoid drying of the snail. These parasites were then identified using the identification key of British Aquatic Oligochaeta [44].

3.1.2 Bleeding of the snails

Stroking the end of a dissecting pin over the foot of the snail would cause the snail's body to shrink back into the shell. This would expel any excess water outside the shell. Then, a dissecting needle was used to remove a portion of the shell just between the first and the second ring of the shell. Then, the gray membrane was punctured by a dissecting needle. Once punctured, the hemolymph is released and can be collected by using an Eppendorf micropipette set at 100 μl. This hemolymph was then collected in sterile Eppendorf tubes and kept on an ice tray in order to avoid aggregation of the cells.

3.1.3 Staining the hemocytes with Giemsa's stain

Thin smears of the hemolymph were made on microscopic slides and were let to dry for 20 min. 70% ethanol was used to fix the cells. The smears were then washed with distilled water and were stained with Giemsa's stain diluted in

phosphate-buffered saline (PBS) containing sodium chloride, sodium phosphate, potassium chloride, and potassium phosphate at pH 7.3. The purpose of PBS is to maintain the constant pH and osmolarity of the hemocytes. The slides were washed, dried, and examined under light microscope at different powers.

3.1.4 Lysozyme standard curve

2 g of purified agar were added to 200 ml of phosphate-buffered saline (PBS) that has a pH value of 6.2. To this was added 0.15 g of *Micrococcus luteus* bacteria that was crushed prior to addition in order to avoid clumping within the solution. A stirring plate and a magnetic stirrer were used to stir the solution for approximately 15 min. Then, the solution was autoclaved at 121°C and 15 psi. The flask is then cooled in a water bath for 15 min and then poured in to a set of Petri dishes with a thickness of approximately 5 mm. Then, the plates were let to solidify in a biological cabinet. Twelve wells were created in the agar plate using a flame sterilized borer and a suction pipe to remove the agar pieces.

Lysozyme standard solutions were prepared by dissolving 25 mg of frozen-lysozyme in 5 mls of PBS at pH 6.2 to give a concentration of 5 mg/ml. Then, 12 serial two-fold dilutions were produced by removing 2.5 ml from the 5 mg/ml solution and add it to a tube containing 2.5 ml of PBS, this process was repeated until 12 serial dilutions were produced. These known concentrations of the lysozyme were dispensed into the wells that were produced in the agar plates, approximately 30 μl in a well. These plates were then incubated over night at 27°C. Areas of hydrolysis were observed around each well. The diameter of each zone was measured using a ruler and a lysozyme standard curve was plotted with the x-axis representing the lysozyme concentration and the y-axis representing the diameter (squared).

3.1.5 Lysozyme assay

4 agar plates were prepared as mentioned above, each containing 12 wells. 37 snails were bled and each hemolymph from each snail was placed into one of the wells. So, 37 wells in 4 agar plates were occupied. The plates were then incubated at 27°C overnight. The diameters of the hydrolysis zones were measured. The same assay was then repeated for 12 mucous samples and 12 parasite product samples.

3.1.6 Nitric oxide standard curve

A sodium nitrite standard solution was prepared. 100 μl of this solution was then placed in 2 wells at the top of a 96 wells enzymatic plate (ELISA plate) in duplicate. 50 μl of the culture medium was dispensed along the remaining 7 wells in duplicate. Then, serial two-fold dilutions of the standard solution were made going down the plate except for the last set of wells that were used as controls, they only contained culture medium without the standard solution. The culture medium is supplemented with 10% fetal calf serum.

Then, 50 μl of Griess reagent was added to all the wells. Once prepared, the tube should be inverted for 10 min and kept in the refrigerator. After addition of Griess reagent, the absorbance of the solutions in the wells should be read within 30 min using a plate reader with an absorbance filter of 570 nm. A nitric oxide standard curve was then plotted with the x-axis representing the nitrite concentration and the y-axis representing the absorbance.

3.1.7 Nitric oxide assay

Circular glass cover slips were placed in 15 wells of the 24-well tissue/cell culture plates using a pair of fine forceps. Ethanol was used to sterilize the cover slips. Poly-L-lysine solution was used to cover the cover slips in the wells, 100 μl in each well. This solution increases the hemocytes attachment to the cover slips. An incubation period of 1 h at 37°C was allowed in order for the poly-L-lysine to fix on the cover slips. After then, the excess poly-L-lysine solution was removed from the wells by Pasteur pipette and the wells were washed with sterile distilled water. Freshly collected hemolymph was then added to the wells, 50 μl in each well. The plate was then put in the refrigerator for 1 h to allow the hemocytes to attach themselves to the cover slips. The excess hemolymph was then removed by Pasteur pipette and the wells were washed by sterile distilled water.

The culture medium was then added to all the wells, 200 μl to each well. The plate was then incubated at 27°C for 1 h to enhance the growth of the hemocytes that can be observed under the light microscope. Approximately 10 live *C. limnaei* parasites were placed in to 3 wells (triplicate), another 10 dead *C. limnaei* were added in to another 3 wells. Parasite products were placed in to 3 wells. 3 wells containing the monolayers and the medium and another 3 wells containing only the medium were used as the controls. The plate was then incubated for 1 h at 27°C. Then, 200 μl of Griess reagent was added to all the wells. There should be a color change within a minute or so. Approximately 200 μl of the solution in each well was transferred into a 96-well enzymatic assay plate that was read using a plate reader set at 570 nm. This previous procedure was repeated using incubation periods of 3 h, 6 h, and overnight.

3.1.8 Phenol oxidase standard curve

Different concentrations of L-DOPA and fixed concentration of the tyrosinase enzyme were used to measure the initial rate of dopachrome formation at 475 nm wavelength. 5×10^{-3} mol dm^{-3} L-DOPA solution was used to prepare different solutions.

The 5×10^{-3} mol dm^{-3} DOPA solution was prepared by dissolving 0.0493 g of DOPA powder that has a molecular weight of 197.19 in 50 ml of the phosphate buffer saline. Then, 3 ml of each solution (tubes 1–8) were placed in plastic cuvette tubes and 0.1 ml of the tyrosinase solution was added. The enzyme solution was made by dissolving 10 mg of the enzyme (power) in 1 ml of the PBS. The tubes were quickly inverted once the enzyme was added and the absorbance was measured at 475 nm using a spectrophotometer. A graph was plotted with the x-axis representing the concentration of the L-DOPA solution and the y-axis representing the absorbance. From that graph, the most suitable concentration of the L-DOPA substrate to be used in the next step was determined. This concentration was shown to be the 0.1. Different concentrations of the enzyme were then used with the fixed concentration of the L-DOPA substrate that was determined in the previous step. 10 mg of the enzyme powder was dissolved in 1 ml of the phosphate buffer saline and serial dilutions of that stock solution were made. First, 0.2 ml of that stock solution was added into 1.8 ml of PBS to make a dilution of 1/10. Then, 0.2 ml of that solution was added again into 1.8 PBS to make a dilution of 1/100. Another 0.2 ml of the last solution was added into 1.8 of PBS to make 1/1000 dilution and so on until nine serial dilutions were made. Then, 1 ml of each enzyme dilution was added into 1 ml of the 0.1 concentration of the L-DOPA substrate solution. The tubes were then inverted quickly and the absorbance was read at 475 nm. A standard tyrosinase (phenol oxidase) curve was plotted and was used to determine the concentration of the enzyme in the samples.

3.1.9 Phenol oxidase assay

Monolayers of hemocytes were prepared as mentioned previously. Approximately 10 parasites were placed in to 3 wells (triplicate), another 10 dead parasites were added in to another 3 wells. Parasite products were placed in to 3 wells. 3 wells containing the monolayer and the medium and another 3 wells containing only the medium were used as the controls. The plate was then incubated for 1 h at 27°C. This previous procedure was repeated using incubation periods of 3 h, 6 h, and overnight. Then, 200 μl of the substrate solution (L-DOPA), 0.1 concentration of the 5×10^{-3} mol dm^{-3} solution was added to all the wells. The absorbance was then read at 475 nm wavelength using the ELIZA plate reader.

3.2 Results

All the 150 snails collected were found to be infected with the parasite *Chaetogaster limnaei*. The average number of hemocytes in 40 hemolymph samples was 2.489×10^6 cells.

3.2.1 Lysozyme assay

A standard curve of this experiment was first created (**Figure 1**). According to the equation of the standard curve, Y = 1.0158 X + 1.7312, the lysozyme concentration in the 37 hemolymph samples can be estimated. **Figure 2** shows the lysozyme concentration for the 37 hemolymph samples. Then, the lysozyme concentrations of the 12 mucous samples were estimated using the standard curve (**Figure 3**).

3.2.2 Nitric oxide assay

A standard curve was first plotted for the nitric oxide assay (**Figure 4**). Using the equation of the standard curve, y = 0.0014x + 0.0504, the x values that represent the nitrite concentration in the different samples were estimated. **Figure 5** shows the production of nitric oxide in response to live parasites, dead parasites, and parasite products.

3.2.3 Phenol oxidase assay

The absorbance of different concentrations of L-DOPA substrate is shown in **Figure 6** and the absorbance of different enzyme concentrations with fixed substrate concentration is shown in **Figure 7**. According to the equation of the standard

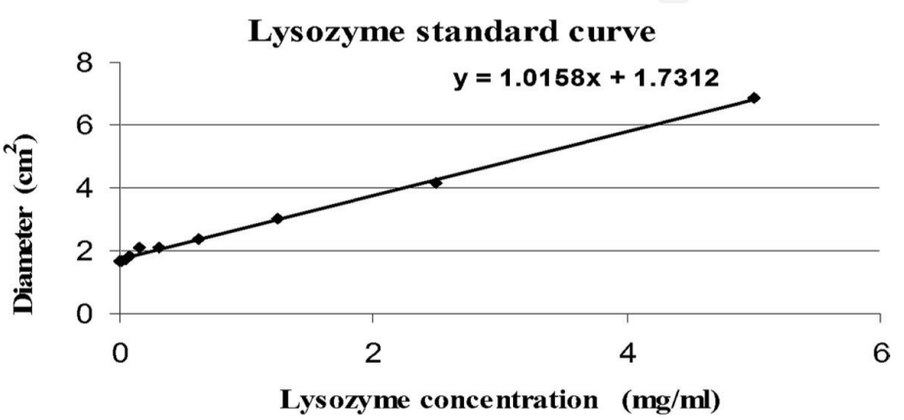

Figure 1.
Lysozyme standard curve.

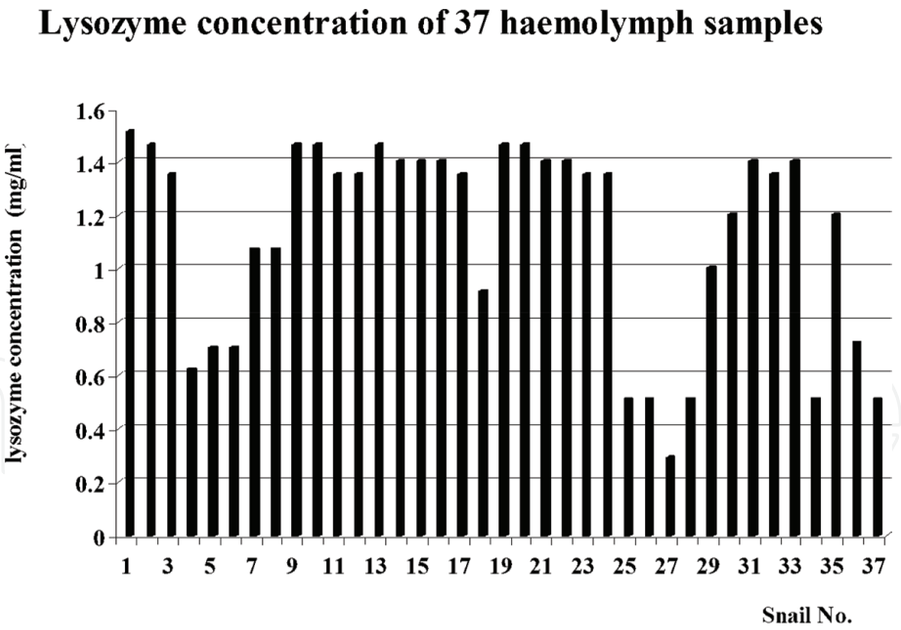

Figure 2.
Lysozyme concentrations of 37 hemolymph samples.

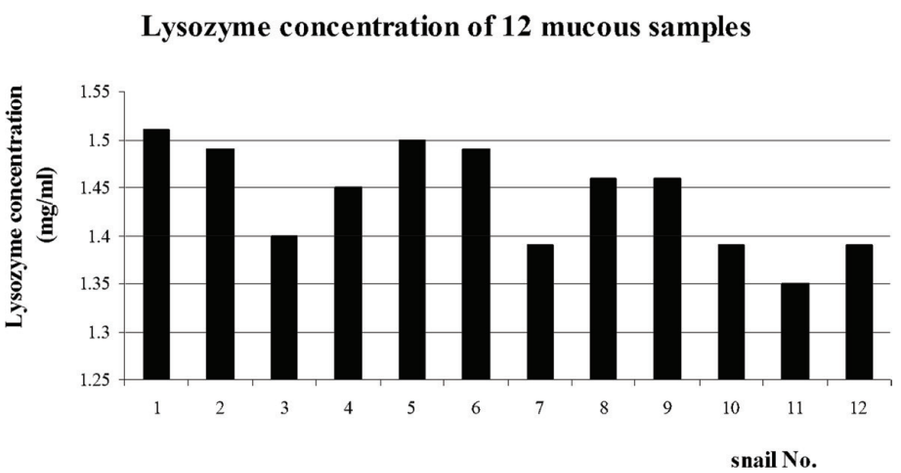

Figure 3.
Lysozyme concentrations of 12 mucous samples.

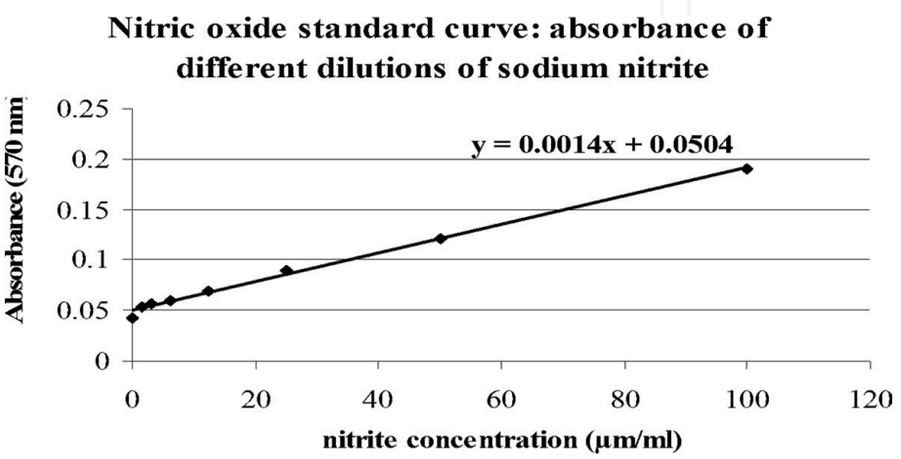

Figure 4.
Nitric oxide standard curve.

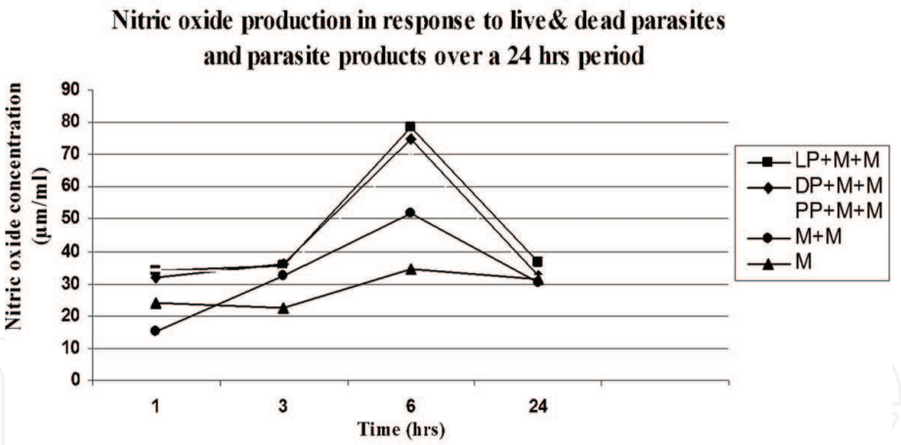

Figure 5.
Production of nitric oxide in response to live parasites, dead parasites, and parasite products. LP + M + M = live parasite, monolayer, and medium; DP + M + M = dead parasite, monolayer, and medium; PP + M + M = parasite product, monolayer, and medium; M + M = monolayer and medium; M = culture medium.

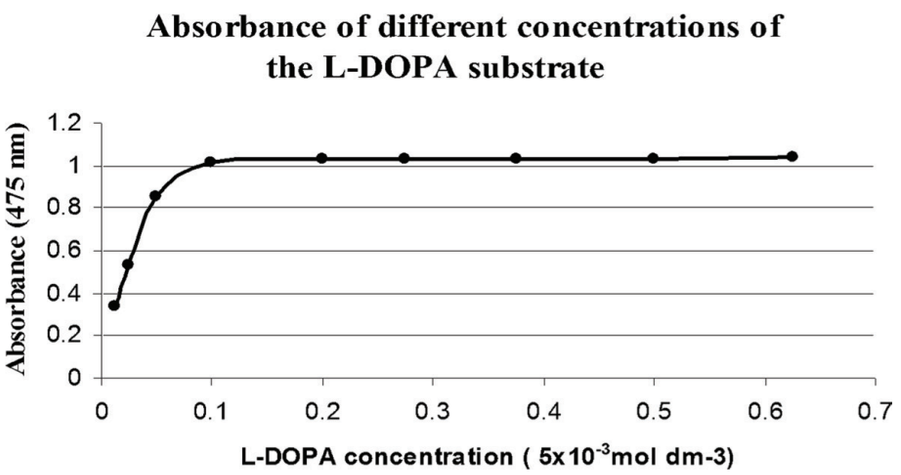

Figure 6.
Absorbance of different concentrations of the L-DOPA substrate.

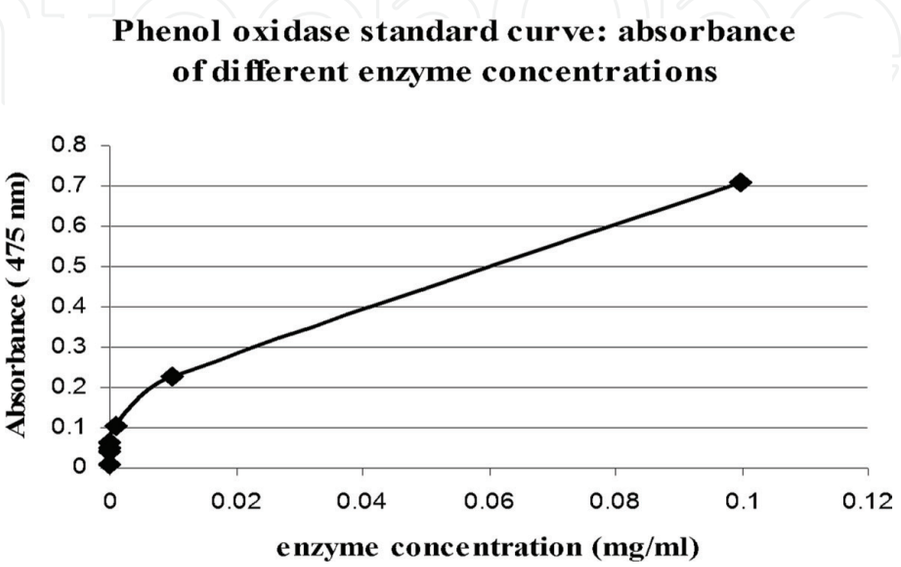

Figure 7.
Phenol oxidase standard curve: absorbance of different enzyme concentration.

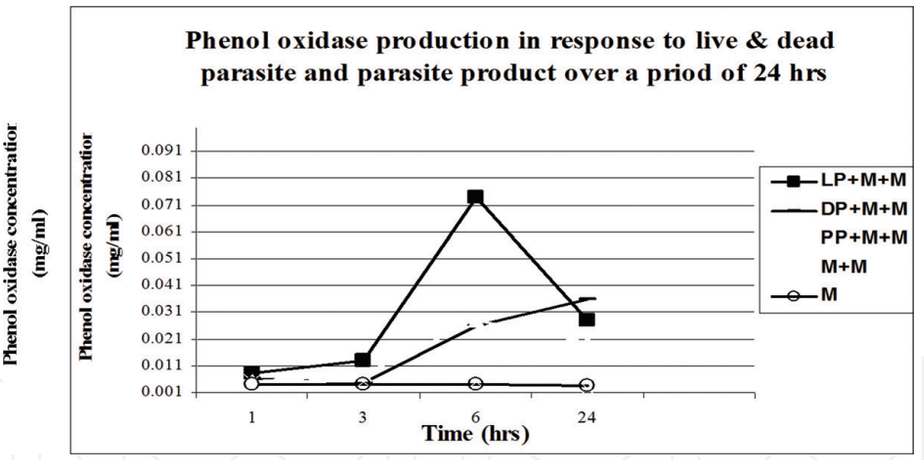

Figure 8.
Phenol oxidase production in response to live, dead parasites, and parasite products over a period of 24 h. LP + M + M = live parasite, monolayer, and medium; DP + M + M = dead parasite, monolayer, and medium; PP + M + M = parasite product, monolayer, and medium; M + M = monolayer and medium; M = culture medium.

curve, Y = 6.5223X + 0.0677, the x values that represent the phenol oxidase concentration in the different samples were estimated. **Figure 8** illustrates the previous results graphically.

4. Conclusion and discussion

The current work was conducted to shed light on some parameters that are associated with the immune response that is resulting from the interaction between the pond snail *Lymnaea stagnalis* and the parasite *Chaetogaster limnaei*. These parameters include production of nitric oxide, phenol oxidase, and lysozymes of the snail *L. stagnalis* as an innate immune response.

Lysozyme assay was carried out for hemolymph, mucous, and parasite products. Care was taken when mucous was collected in order not to contaminate mucous with hemolymph. The lysozyme standard curve was plotted on linear and logarithmic scale and the equation of that curve was used to estimate the lysozyme concentration in the unknown samples. In case of parasite product, zones of hydrolysis were not formed on the agar plates. This indicates that the parasite products did not contain any lysozymes. On the other hand, zones of hydrolysis were observed in the case of hemolymph and mucous samples. Accordingly, hemolymph and mucous of *Lymnaea stagnalis* did contain lysozymes that could have a role in hydrolysis of foreign particles invading the snail.

It can be noticed from **Figure 2** that there is a considerable fluctuation in the lysozyme concentration among the 37 hemolymph samples. This is due to variations in response to the presence of the bacteria in the agar. This may be due to the fact that some samples contain more hemolymph than hemocytes.

Figure 3 shows the lysozyme concentration in 12 mucous samples. As noticed, there is a little variation in the lysozyme concentrations among the 12 samples, indicating that different mucous samples contain different amounts of lysosomal enzymes. This is maybe due to differences in ages and sizes of the snails. Accordingly, it seems that the mucous represents the first defense line that prevents the establishment of the parasite in the snail's body.

Nitric oxide production results in response to live, dead parasites, and parasite products (**Figure 5**) do show considerable increase in nitric oxide production due to these inoculations. **Figures 5** and **6** are the same except for the control results (medium) that are shown in **Figure 5** and not in **Figure 6** in order for the reader to view the difference

graphically. **Figure 5** shows that the culture medium does contain some nitric oxide, so, this value should be subtracted from the results of the samples to ensure that the resulting values are representing nitric oxide production from the hemocytes upon inoculation and not from the culture medium as shown in **Figure 5**. **Figures 5** and **6** have shown that there is some nitric oxide produced in the case of cultured hemocytes monolayer and that the concentration of nitric oxide has increased upon inoculation of live, dead parasites, and parasite products. As shown in **Figure 5**, the optimum period of time for production of nitric oxide is 6 h postexposure to live parasites, followed by dead parasite, and then parasite products. In other words, live parasites induce more nitric oxide production than did dead parasites and parasite products. This indicates that viable parasites may produce antigens that are more efficient in initiating the production of nitric oxide. These antigens are less, in terms of efficiency or amount, in the case of dead parasites and parasite products. Another possibility is that some of the measured nitric oxide may be originated from the live parasite it's self.

It is noticed from **Figure 5** that the trend of the graphs in the case of different inoculations (live, dead parasites, and parasite products) is almost the same. After 1 and 3 h, there was little nitric oxide production, and then the concentration increased drastically after 6 h. The concentration of the nitric oxide decreased drastically after 24 h. This drop of nitric oxide concentration after 6 h may be due to the possibility that the hemocytes were viable and were able to produce high amounts of nitric oxide after 6 h. Then, the cells began to lose their viability and they were unable to produce the same amounts of that material. In addition, since different culture plates were used for different time intervals, the concentration of the hemocytes in the 6-h plates may be much more than the concentration in the overnight plate. Accordingly, more nitric oxide was measured in the 6-h plate than in the overnight plate. Moreover, this result may represent what is happening naturally in the snails' body. The concentration of nitric oxide at 6 h may be sufficient to get rid of the infection. After then, the concentration drops away because it already reaches the optimum and there is no need for more amounts. Another possibility is that some contaminants like detritus may be present in high amounts in the 6 h wells.

Results of the phenol oxidase assay are shown in **Figure 8**. The control results (medium and monolayer and medium by its self) were shown in the same figure and were not subtracted in order for the reader to view the difference graphically. It is noticed that the results of the controls, medium and monolayer, and medium alone are showing trace levels of phenol oxidase production (0.0040 mg/ml for the medium and 0.005 mg/ml for the medium and monolayer). It is also noticed that the production rate is steady throughout the experimental period. This indicates that there was no contamination with any other material that may induce sudden increase in the phenol oxidase production. It is shown in **Figure 8** that the optimum period of time for production of phenol oxidase is 6 h postexposure to the live parasites, followed by the dead parasites, and then the parasite products. In other words, live parasites induce more enzyme production than do dead parasites and parasite products. This indicates that viable parasites may produce antigens that are more efficient in initiating the production of phenol oxidase. These antigens are less in the case of dead parasites and parasite products or are not that efficient in stimulating the production of the enzyme. In the case of live parasites, there was little increase in the phenol oxidase production until 3 h. Then the production rate increased drastically to reach its maximum at 6 h postinoculation. After then, it decreased throughout the experimental period. This peak may be due to some contaminants that stimulate the hemocytes to produce more phenol oxidase in the 6 h wells. Also, the hemocytes maybe more viable and were able to produce high amounts of phenol oxidase at 6 h. Then, the cells began to loose their viability and they were unable to produce the same amounts of that enzyme at 24 h. In addition,

since different culture plates were used for different time intervals, the concentration of the hemocytes in the 6-h plates may be much more than the concentration in the other plates. Accordingly, more phenol oxidase was measured in the 6-h plate than in the other plates. Another possibility is that this happens naturally in the snails' body. The concentration of phenol oxidase at 6 h may be sufficient and enough to get rid of the infection. After then, the concentration drops away because it already reaches the optimum and there is no need for more amounts.

It is shown in **Figure 8** that the phenol oxidase production in the case of the dead parasites increases until it reaches its maximum at 24 h. However, the amount produced is much more less than in the case of live parasites. This indicates the antigenic nature of the materials produced by the dead parasites is much less than those produced by the live organisms. The maximum amount produced did not drop away, this may be due to the possibility that this amount is still not sufficient to get rid of the dead parasites and more amounts are needed to be produced. If these samples are kept more than 24 h, a decrease in phenol oxidase production maybe observed.

The production of phenol oxidase in the case of parasite products has almost the same trend as in the case of dead parasites until 6 h postinoculation. After then, the enzyme production decreased. So, there is a slight peak for phenol oxidase production in the case of parasite products, as can be noticed in **Figure 8**. This peak maybe due to the same possibilities discussed earlier. So, phenol oxidase production results in response to live, dead parasites, and parasite products (**Figure 8**) do show a noticeable increase in enzyme production due to these inoculations.

In a similar study, in the first experiment, snails were randomly assigned into six different treatment groups. There was no difference in their sizes. The first group was not exposed to any immune elicitor, the second group was injected with 100 μl of snail saline, the third and fourth groups were injected with lyophilized *E. coli*. The fifth and sixth groups were injected with *Plagiochis* sp. Six hours post injections, the snails hemolymph was sampled for immunocompetence. Samples were also collected to measure PO and antibacterial activity. A second experiment was conducted by exposing the snails to opportunistic micro-organisms into two different water qualities. Statistical analyses were done using multivariate analysis of variance (MANOVA). Results showed that both, PO and antibacterial activity of snail hemolymph differed among the treatment groups. Wounding of the snail by injecting with snail saline increased PO activity of the hemolymph as compared to snails that did not receive any injection. Injecting with immune elicitors did not further increase the PO activity. Levels of PO activity decreased in those injected with *E. coli* wounding did not affect antibacterial activity. For the second experiment, the level of PO activity in snails maintained n micro-organism-rich water was higher compared to snails maintained in clean water [45].

5. Conclusion

In conclusion, lysozymes, nitric oxide, and phenol oxidase play an important role in the non-specific immune response against invading parasites and foreign particle in *Lymnaea stagnalis* snail. However, this immunity was not efficient to eliminate the parasite in the case study mentioned above.

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Conflict of interest

There is no conflict of interest related to the current work.

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