# Determination of Trace Elements levels in Plasma from Larvae in the Course of Baculoviral and Bacterial Infections by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

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by

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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presented by Rui Sun, a candidate for the degree of Master of Science, and hereby certify that, in their opinion, it is worthy of acceptance.

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ABSTRACT

While a number of studies have examined the effect of infections on the trace elements status of mammals, not any similar study in insects are found. In this study, we used inductively coupled plasma-mass spectrometry (ICP-MS) to quantify trace elements of Mg, Mn, Fe, Cu, Zn and Mo levels in the plasma from the tobacco budworm, *Heliothis virescens*, following infection of the insect with a baculovirus (Helicoverpazea single nucleopolyhedrovirus (HzSNPV)) and bacteria (*Micrococcus lysodeikticus*) at different times post infection.

There are no changes in any metal due to bacterial infection and no differences between any 12 hours post infection mocks versus 12 hours post infection bacterial infected samples.

For the larvae with a baculovirus (HzSNPV) infection, in both fourth and fifth instars, all the trace elements of interest (Mg, Mn, Fe, Cu, Zn, Mo) change over the course of the 72 hours of infection in both instars, which is due to the development of larvae. Iron level in plasma from larvae with the HzSNPV infection was elevated in the late $4^{th}$ instar (60 hours post infection) when compared to iron level in the same aged controls. This could be explained by the ability of iron storage protein ferritin to buffer iron, or free iron of damaged tissues leaking intracellular iron into the plasma. Copper level in insect plasma with the HzSNPV infection was lower in the late $4^{th}$ instar (60 hours post infection) when compared to copper level in the same aged controls, which is probably due to the decline of copper-binding protein prophenoloxidases (PPOs) levels during the course of infection.

Keywords: trace elements, insect plasma, viral infection, ICP-MS

# Chapter 1

# Introduction

## 1. Micronutrient and immune system of vertebrates

While a number of studies have examined the effect of infections on the trace elements status of mammals, we are not aware of any similar study in insects. In this work, the concentration levels of trace elements Mg, Cr, Mn, Fe, Co, Ni, Cu, Zn and Mo in the plasma from the tobacco budworm, *Heliothis virescens,* following infection of the insect with a baculovirus (Helicoverpa zea single nucleopolyhedrovirus (HzSNPV)) and bacteria (*Micrococcus lysodeikticus*) at different times post infection were investigated.

Observation of elevated metals could be the response of viral damaged tissues leaking intracellular metals into the plasma. On the other hand, the bacterial infections may cause the metal levels to decline. This work is an exploratory investigation of the changes of trace elements levels in insect plasma following viral and bacterial infections.

It is widely accepted that, in addition to antigens and genetic and environmental factors, macro- and micronutrients also influence the efficiency and activity of the immune system. Micronutrients are nutrients needed by all living organisms in small quantities. The most important micronutrients are vitamins and microelements which are also called microminerals or trace elements. The microelements include iron, cobalt, chromium, copper, iodine, manganese, selenium, zinc and molybdenum.

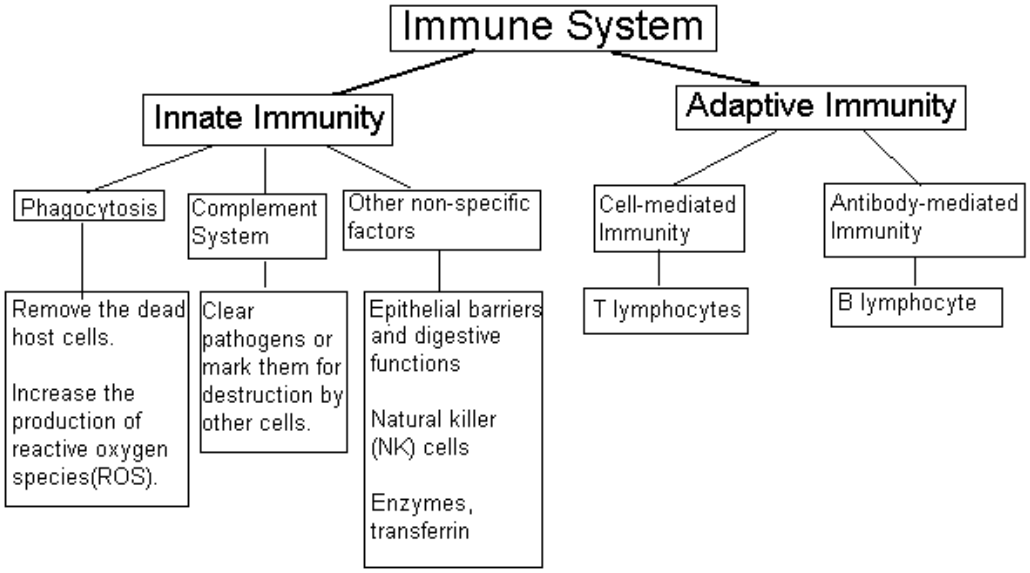
Micronutrients influence immune functions by affecting the innate and adaptive immune response [1]. There are two factors which influence the severity of viral infection: the virus and the health of the host. The host response mainly depends on

the functioning of the immune system which in turn can depend upon the host's nutritional status. Since the micronutrients have important influence on immune system, they influence the host's susceptibility to infections [2-4]. Likewise, infections aggravate micronutrient deficiencies by reducing nutrient intake, increasing nutrient losses, and interfering with utilization of nutrients by altering metabolic pathways [5]. An excellent review on the interaction of viral infections and trace elements is given by Chaturvedi and Shrivastava et al. in reference [6].

### 1.1 Immune system

The immune system consists of many types of cells, proteins, organs and tissues that collectively protect the body from bacterial, parasitic, fungal, and viral infections and from the growth of tumor cells. Figure 1 gives the classification and major components of the human's immune system.

Figure 1. Classification and major components of the human's immune system




The immune system can be classified as innate (non-specific) and adaptive (specific or acquired) immunity. The innate immune system provides immediate defense

against infection whereas the adaptive immune system provides long-lasting immunity to the host.

Innate immunity includes the complement system, phagocytosis, and other non-specific factors such as epithelial barriers, natural killer (NK) cells, enzymes and transferrins. The innate immune system is found in plants, fungi, insects, and in primitive multicellular organisms. The complement system helps antibodies function by clearing pathogens or by marking them for destruction by other cells. This system is composed of many plasma proteins, such as serum proteins, serosal proteins, and cell membrane receptors [1, 7]. The phagocytic cells include neutrophilic granulocyte, eosinophilic granulocyte and macrophage and dendritic cells (DCs) [1]. Phagocytic cells remove the dead host cells induced by programmed cell death processes (apoptosis) or bacterial or viral infection from the affected site. Phagocytic cells also increase the production of reactive oxygen species (ROS), which are involved in host defense. Natural killer cells are a type of cytotoxic lymphocyte which rejects tumor and virus-infected cells by releasing small cytoplasmic granules of proteins. These proteins form pores in the cell membrane of the target cell through which the proteins and associated molecules can enter and induce apoptosis [1].

The adaptive immune system takes over to eliminate or prevent pathogenic challenges if the innate response cannot clear the infection in a short time. The adaptive immune system is divided into two types: cell-mediated and antibody-mediated immunity. T lymphocytes (T cells originating from bone marrow and maturing in the thymus) are the major component of cell-mediated immunity, and B lymphocytes (B cells originating and maturing in the bone marrow) are the major component of antibody-mediated immunity. T lymphocytes are usually divided into two major subsets: the T helper subset, also called the CD4+ T cell and T killer/suppressor subset or CD8+ T cell. The function of T helper subset is to aid or activate B or other T cells by secreting specialized factors. T killer/suppressor subset cells directly kill viral-infected cells and some certain tumor cells. B lymphocytes produce secreted

antibodies in order to fight against foreign proteins of bacteria, viruses, and tumor cells. They function by secreting antibodies which then bind to antigens and signal other cells such as macrophages to attack that substance.

Trace elements are required for various body functions and the well-being of the immune system [7]. The following is a brief summary of the role of select trace elements in the immune systems of vertebrates.

### 1.2 Selenium and the immune system of vertebrates

Selenium is an essential trace element in vertebrates. Selenomethionine and selenocysteine are the two major forms of selenium present in animal tissues. Selenium functions mainly through selenoproteins which are proteins that contain selenium in stoichiometric amounts [8]. The major dietary form of selenium is selenomethionine, 90 percent of which is absorbed by the same mechanism as methionine itself. The other dietary form is selenocysteine. There are two pools of selenium in humans and animals: selenium present as selenomethiomine and selenium present as glutathione peroxidase (GSPX) [9].

Selenomethiomine enters the methionine pool in the body and then is catabolized to form selenocysteine which is further broken down to selenide. Ingested selenite, selenate and selenocysteine are all metabolized directly to selenide. The selenide can be metabolized to selenophosphate, which is the precursor of selenocysteine in the selenoproteins, and the source of the selenium in transfer RNA [10].

The biological functions of selenium include defense against oxidative stress, regulation of thyroid hormone actions, and regulation of the redox status of Vitamin C and other molecules. The antioxidant effect is mediated through glutathione peroxidases (GSPX) that remove an excess of potentially damaging lipid hydroperoxides, hydrogen peroxide, and peroxynitrite produced during oxidative

stress. Thus, selenium plays an important role in balancing the redox state, and helping to protect the host from oxidative stress generated by the microbicidal effects of macrophages and inflammatory reactions. The selenoenzyme thioredoxin reductase affects the redox regulation of several key enzymes, transcription factors and receptors, including ribonucleotide reductase, glucocorticoid receptors, and anti-inflammatory protein AP-1 which binds to DNA and activates expression of gene encoding proteins involved in immune responses (cytokines, adhesion molecules)[11, 12]

There are several studies on the interaction of viral infections and selenium. The results indicate that a deficiency in selenium has effects both on the virus itself and on the host. In a study on selenium deficiency, coxsackie B virus in the infected mice converted to virulence so that its genome closely resembled that of other known virulent coxsackievirus B3 (CVB3) strains [13]. And also, in the course of this infection, the antioxidant selenoenzyme GSPX was genetically deprived [13, 14]. In another study [15], selenium exhibited inhibitory activity on coxsakievirus B5 replication. In Beck and Mattew's study [16], selenium-deficiency influences expression of mRNA for chemokines, which are secreted during an infection of myocarditic strains of Coxsackie B virus (CBV), in order to attract immune cells to the site of injury. Therefore, an adequate selenium status is essential in counteracting certain viral infections [6].

### 1.3 Zinc and the immune system of vertebrates

Zinc is known to be essential for the activity of about 100 enzymes and for all highly proliferating cells, especially cells in the immune system. Zinc concentration has a variety of effects *in vivo* and *in vitro* on immune cells. *In vivo*, the NK cell number and activity are dependent on the serum zinc level [17], and it was shown that with zinc deficiency, the NK cell activity and the relative number of precursors of cytolytic cells decreased [18]. Phagocytosis of macrophages and neutrophils and generation of

the oxidative burst are impaired when zinc levels are low [19, 20]. The number of granulocytes was shown to decrease during zinc deficiency [21]. Zinc also affects the activity of cytolytic T cells [22]. The relative amount of CD8+ CD73+ T lymphocytes is found to decrease during zinc deficiency [21]. On the other hand, zinc is required by human beings and pathogens for proliferation. Thus, decreasing plasma zinc levels during an acute phase of infection is a defense mechanism of the human organism [21].

Zinc also plays an essential role in viral infections. Copper-zinc-superoxide dismutase is an important zinc dependent enzyme which is required for the formation of T-cells, an essential type of cell in the adaptive immune system [23].

A review article by Fernandez-Pol et al. [24] indicates that viral and cellular zinc finger proteins (ZTP) and iron containing proteins are involved in viral infection. In another study [25], it is proposed that $Zn^{2+}$ complexes with proteins interrupt human rhinovirus (HRV) infection, by interrupting nerve impulses and blocking HRV on intercellular adhesion on somatic cells. A study [25] on coxsackievirus B3 myocarditis in the mouse indicates that the level of Zn in the plasma decreases, and the Cu/Zn ratio in plasma increases in the presence of the infection. Lin et al. [26] report that sindbis virus (SV) infection decreases intracellular zinc-copper superoxide levels and thus potentiates SV-induced apoptosis.

Zn levels were lower in blood cells from patients with viral hepatitis than controls. Some interleukins released or secreted from leukocytes or activated phagocytes may cause Zn to decrease by inhibiting its transport from plasma to the liver [27]. It is also reported that decreased Zn levels result from the production of metallothionein in the liver and other tissues. It is known that metallothionein synthesis is induced by interleukin-1 (IL-1).

There are additional studies [28-32] showing zinc levels in serum decrease in the

course of virus infection. For example, a study of hepatitis B virus [28] indicates that as the severity of infection increases the zinc concentration in serum decreases.

### 1.4 Copper and the immune system of vertebrates

Copper has been shown to play a role in the development and maintenance of the immune system, and a large number of experimental studies have demonstrated that copper status alters several aspects of neutrophil, monocyte, and T-cell function in the immune system [33–35].

Copper is an integral part of many important enzymes involved in biological processes. It functions as a catalytic metalloenzyme, acting as oxidase to achieve the reduction of molecular oxygen. There are many copper metalloenzymes identified in humans, including amine oxidases, diamine oxidases, monoamine oxidases, and ferroxidases [35].

Ferroxidases are found in plasma and they function through ferrous iron oxidation to achieve iron's binding to transferrin. The main ferroxidase of human is the multi-copper ferroxidase, ceruloplasmin (Cp). Several studies suggest that Cp transports copper to tissues and cells [36, 37].

The copper-containing enzyme superoxide dismutase (SOD), working together with catalase and glutathione peroxidases (GSPX) in the cytosolic antioxidant defense against reactive oxygen species (ROS), is essential in the dismutation of superoxide anion to oxygen and $H_2O_2$, and depresses damage to lipids, proteins, and DNA.
It was found that low copper intakes lead to impaired T-cell function (proliferation, IL-2 receptor) in human and animal studies [38].

Copper is also an essential trace element for defense against viral infections. Cupric isonicotinic hydrazide ($Cu^{II}INH$) shows antiviral activity since it inhibits

multiplication of avian myeloblastosis virus by blocking the process of reverse transcription. This inhibition is due to preferential binding of the enzyme reverse transcriptase [39].

Copper shows elevated levels in serum with viral hepatitis (A, B, C, D, and E) cases compared to controls. Increased Cu levels might be related to ceruloplasmin (Cp) synthesis induced by Interleukin-1 (IL-1) [40]. In this work, it is proposed that some cytokines produced as a result of immune defense system in viral hepatitis infection might alter the levels of serum trace elements including copper.

### 1.5 Iron and the immune system of vertebrates

Iron is another essential micronutrient required for a variety of biochemical processes. Iron is essential for electron transfer reactions, gene regulation, binding and transport of oxygen, and regulation of cell differentiation and cell growth.

There are four major types of iron-containing proteins found in mammals: iron-containing heme proteins (hemoglobin, myoglobin, cytochromes), iron-sulfur enzymes (flavoproteins, heme flavoproteins), proteins for iron storage and transport (transferrin, lactoferrin, ferritin, hemosiderin), and other iron-containing or activated enzymes (sulfur, nonheme enzymes). Hemoglobin is important for the transport of oxygen from the environment to the tissues and this movement of oxygen is one of the key functions of iron [41].

The role of iron in oxygen toxicity is paradoxical. The main antioxidant is catalase, which contains iron. Environmental iron levels are usually below the amount that cells need for growth [42] (between 0.4 and 4.0 mM iron) because of the insolubility of ferric phosphates and hydroxides. On the other hand, excess iron is destructive to many cellular processes. When in excess, iron is toxic because it generates superoxide anions and hydroxyl radicals in the Haber-Weiss reactions [43, 44]

$Fe^{3+} + \bullet O_2^- \rightarrow Fe^{2+} + O_2$

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH$

$\bullet O_2^- + H_2O_2 \rightarrow \bullet OH + HO^- + O_2$

These superoxide anions and hydroxyl radicals react readily with biological molecules, including proteins, lipids and DNA. As a result, organisms have evolved mechanisms for stabilizing the availability of iron by controlling its uptake, transport, storage, and excretion. Disruption of these processes causes either iron deficient anemia or iron overload disorders.

Iron is also important for the immune system because it promotes immune cells and interferes with cell-mediated immune effector pathways and cytokine activities [45–48]. It has been demonstrated that iron deficiency as well as iron overload can exert subtle effects on immune status by altering the proliferation and activation of T-, B- or NK-cells [49–52].

### 1.6 Magnesium and the immune system of vertebrates

Magnesium is one of the most important micronutrients for both the innate and adaptive immune system [53]. Magnesium participates in immune responses in various ways. For example, magnesium is a cofactor for immunoglobulin synthesis, immune cell adherence, antibody-dependent cytolysis, immunoglobulin M (IgM) lymphocyte binding, macrophage response to lymphokines, and T helper-B cell adherence.

Magnesium deficiency might be accompanied by the activation of cells such as macrophages, neutrophils and endothelial cells [53-55]. In Mak's study [53], the time course and nature of neutrophil activation in oxyradical production during Mg-deficiency were examined and the results indicate that Mg deficiency activates neutrophils, which contribute, in part, to increased oxidative stress. In

Malpuech-Bruge`re's study [54, 55], the Mg-deficient rats were accompanied by a blood leukocyte response and changes in leukocytes subpopulations. A significant increase in interleukin-6 (IL-6) plasma level was observed in Mg-deficient rats compared to rats fed a control diet. The fact that the inflammatory response was an early consequence of Mg deficiency suggests that reduced extracellular Mg might be responsible for the activated state of immune cells. It has been also observed that dietary Mg-deficiency results in early elevation of neuropeptides (substance P, calcitonin gene-related peptide) followed by later increases in inflammatory cytokines including gamma-interferon (IFN-g), interleukin (IL)-1, IL-6 and tumor necrosis factor-alpha (TNF-a) in mice [56,57].

### 1.7 Manganese and the immune system of vertebrates

Manganese is an antioxidant micronutrient and is important in the breakdown of amino acids and the production of energy. Manganese superoxide dismutase is one of the two groups of the family of superoxide dismutase (SOD). SODs protect cells from oxidative damage and regulate superoxide concentrations. MnSOD functions as an antioxidant molecule that protects mitochondria from damage by superoxides and in response to cellular stress [58-60].

In Wang's study, MnSOD turned out to play a role in the alteration of immune function in the course of infection of B-cells with the measles virus. It was also found that intracellular MnSOD inhibited proliferation of the B-cells. MnSOD also decreased the titer of virus produced from infected cells [61].

In another study [62], two of the viruses containing mutant polymerases were heavily dependent on $Mn^{2+}$ for RNA replication and growth. The mutations in these unusual RNA polymerases resulted in an alteration in the cation utilization of the enzyme.

A study on MnSOD in Japanese encephalitis virus (JEV) infection indicates that

MnSOD level increases during JEV infection. Furthermore, JEV infection also stimulates cellular MnSOD activity, which suggests that a cellular factor, regulating the oxidative pathway, could respond to the infection of JEV [63].

Zhang's study suggests that MnSOD over expression decreases anti-inflammatory protein (AP-1) DNA binding activity by regulating intracellular redox status, with the possible involvement of a nuclear redox factor (Ref-1) in this redox-sensitive pathway [64].

### 1.8 Molybdenum and the immune system of vertebrates

Molybdenum is an important trace element in animals and humans as a component of pterin coenzyme. This coenzyme is essential for the activity of xanthine oxidase, sulfite oxidase, and aldehyde oxidase [6]. In Arthington's study [65], erythrocyte superoxide dismutase (SOD) activity was less in Mo-supplemented heifers. Viral challenge had no effect on SOD activity. Lymphocyte proliferative response to phytohemagglutinin stimulation was greater for Mo-supplemented heifers following BHV-1 challenge. These data indicate that molybdenum-induced Cu deficiency alters the acute-phase protein response to viral infection and may affect lymphocyte responsiveness to mitogen stimulation [65].

## 2. Essentiality trace elements in insects

### 2.1 Selenium

The effects of dietary selenium (Se) supplementation on larval growth and immunocompetence of the lepidopteran pest, the cabbage looper Trichoplusia ni, were examined by Popham et al. [66]. This study suggests that dietary Se is rapidly accumulated or lost during larval development. This study also indicated that dietary Se levels rapidly impact Se assimilation and sequestration and that tissue Se levels are

an important factor in resistance to Autographa californica nucleopolyhedrovirus (AcMNPV) infection in larval *Trichoplusia ni*. In the study [67] on dietary Selenium (in the form of selenite) affecting the growth, development, and Se content of *Heliothis virescens*, the results suggest that dietary Se levels are directly correlated with plasma Se levels, and that plasma Se levels are in turn correlated with baculovirus resistance.

### 2.2 Iron

In insects, iron is both an essential nutrient and a potent toxin. Iron-catalyzed free radical production cause toxicity in insects as it does in vertebrates. Increased dietary iron was shown to cause oxidative stress in *Musca domestica* [68].

Iron movement between cells is primarily conducted via reversible binding of iron to the transport protein, transferrin. Vertebrates' transferrins carry iron for synthesis of red cell hemoglobin, in which 2/3 of iron in the body is found. In insects transferrin [69] acts as the iron transfer agent. Most insects lack hemoglobin and iron transport in insects is to move dietary iron from the gut and distribute it for the synthesis of iron-containing molecules [70].

Iron is stored in the form of ferritin (Ft) both in vertebrates and invertebrates. Ferritin plays a pivotal role in iron homeostasis within vertebrate cells [71]. Ferritins have been isolated and characterized from midgut and hemolymph in insects [72]. Locke & Nichol [72] reviewed the distribution of Fts in insects and noted that in most insects, Fts, though present in the cytoplasm, are predominantly found in the lumen of the endoplasmic reticulum and in the hemolymph.

### 2.3 Zinc

Zinc is also essential for stimulating the activity of enzymes in insects as it is in

vertebrates. Zinc-metalloproteases are key components of peptide-signaling system, being involved in neuronal transmission and endocrine pathways that control some aspects of the behavior and development of insects.

There are two major zinc-metalloproteases in insects, angiotensin converting enzyme (ACE) and endothelin converting enzyme (ECE). In general, ACE cleaves almost every peptide that it is offered as a substrate, with the exception of peptides containing a penultimate proline residue or peptides that have an unusual secondary structure. In insects, the form of ACE, like its vertebrate counterpart, acts as a peptidyl dipeptidase, cleaving at the penultimate *C*-terminal peptide bond to release a dipeptide amide as a major fragment [73]. High levels of ACE activity are found in the hemolymph and in reproductive tissues of insects, where the enzyme is considered to have an important role in the metabolism of bioactive peptides [74].

The activity of ECE was found to be less susceptible to thiorphan and phosphoramidon compared to the mammalian equivalents of ECE at the detection of neuropeptide-degrading endopeptidase activity in insects [75]. Additional studies on endopeptidase activity in invertebrates showed that these inhibitors are not always found to act as efficiently as in vertebrates [76-78].

### 2.4 Copper

Copper proteins Hemocyanins (Hc) are a dominant form of copper in many invertebrates; it may constitute more than 80% of the hemolymph proteins [79]. Although insects lack Hc, they produce a set of Hc-related proteins, hexamerins. These hexamerins are synthesized and secreted by the fat body of feeding larvae and have a variety of binding sites for ligands and transport capabilities [80].

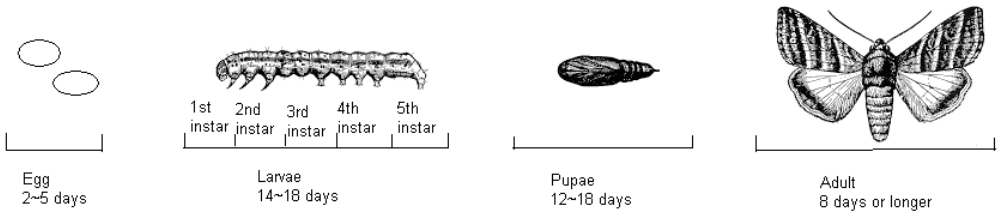
Phenoloxidases (PO) are another group of important copper-binding proteins, which play vital roles in resistance against viral or bacterial challenge in insects [81]. Larval

*Heliothis virescens* plasma PO (HvPO) was found to be virucidal against HIV-1, DNA and RNA viruses such as herpes simplex viruses-1 and -2, vesicular stomatitis, parainfluenza-3, coxsackie B3 and sindbis viruses-2 *in vitro* [82, 83]. Prophenoloxidases (PPOs) subunits are present at a high level in insect plasma and are vital in the immune response against microbes, filariae, and parasitoids [84, 85].

## 3. The tobacco budworm, *Heliothis virescens* life cycle

The insect used in this study is a native species and is found throughout the eastern and southwestern United States. Its common name is tobacco budworm and its scientific name is *Heliothis virescens*. Figure 2 shows the tobacco budworm life cycle of four stages: egg, larvae, pupae and adult. In the first stage, eggs are deposited and hatch in 2-5 days. In the second stage, larvae usually develop in 14-18 days through five to seven instars, with five or six most common. An instar is a developmental stage of insects between each moult. For most insect species the term "instar" is used to denote the developmental stage of the larval or nymphal forms of holometabolous (complete metamorphism) or hemimetabolous (incomplete metamorphism) insects. In this study, larvae develop through 5 instars, and the plasma was collected from larvae in the $4^{th}$ and $5^{th}$ instars. The next pupal stage lasts about 12-18 days. In the last stage, adults are medium-sized moths. Longevity of moths is reported to be 8 days or longer.

Figure 2. The tobacco budworm life cycle



# Chapter 2

# Experimental

## 1. HR-ICP-MS

### 1.1 Introduction to HR-ICP-MS

Inductively coupled plasma (ICP) mass spectrometry (MS) is routinely used in many diverse research fields such as earth, environmental, life and forensic sciences and in food, material, chemical, semiconductor and nuclear industries. The high ion density and the high temperature in plasma provide an ideal atomizer and element ionizer for all types of samples and matrices introduced by a variety of specialized devices. Outstanding properties such as high sensitivity (ppt–ppq), relative salt tolerance, compound-independent element response and accuracy lead to the unchallenged performance of ICP-MS in efficiently detecting, identifying and reliably quantifying trace elements.

In ICP-MS, a plasma consisting of ions, electrons and neutral particles, is formed from argon gas. The plasma is then utilized to atomize and ionize the elements in the sample matrix. The resulting ions are then passed through a series of apertures (cones) into a high vacuum mass analyzer where the isotopes of the elements are identified by their mass-to-charge ratio. The intensity of a specific peak in the mass spectrum is proportional to the amount of the element in the original sample.

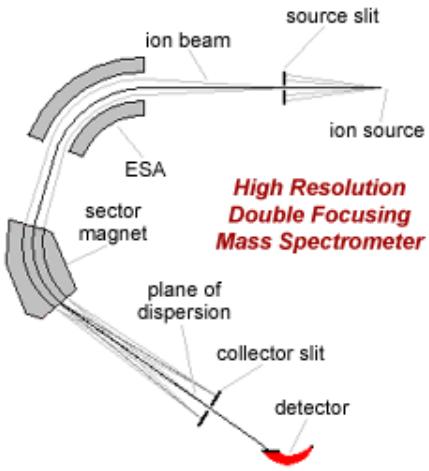
The heart of the ICP-MS is the inductively-coupled plasma ion source. Since the source operates at temperatures of 7000K, virtually all molecules in a sample will be broken into their component atoms. A radio frequency signal (RF) is fed into a tightly wound, water-cooled, coil where it generates an intense magnetic field. Within the center of this coil is a quartz plasma torch where the plasma is formed. The plasma is generated by “seeding” the argon gas with a spark from a Tesla unit (a device similar

to a spark plug). When the spark passes through the argon gas, some of the argon atoms are ionized and the resultant cations and electrons are accelerated within the magnetic field of the RF coil. A stable, high temperature plasma is then generated as the result of the inelastic collisions created between the charged particles and the neutral argon atoms. The concentration of electrons in the plasma reaches equilibrium very quickly, after which the plasma will remain "lit" as long as the RF field is maintained and a constant supply of argon gas is injected into the plasma.

### 1.2 The High-Resolution mass analyzer

The High-Resolution mass analyzer consists of both a magnetic sector and an electric sector to separate and focus the ions and is capable of focusing by both energy and mass/charge ratio. As a result, this arrangement is called a double-focusing high resolution mass spectrometer. Figure 3 gives the schematic of a high resolution mass analyzer.

Figure 3. Schematic of a high resolution mass analyzer




First, the ion beam passes through a narrow slit, which only allows those ions traveling along the correct axial plane of the mass spectrometer to pass through, resulting in a narrow beam of ions all traveling parallel to each other. In the next stage, the electric sector, electro static analyzer (ESA), consisting of two curved plates with

applied direct current voltage, causes the inner plate (negative polarity) attract the positively charged ions, while the outer plate (positive polarity) repels the positive ions. The ion beam passes through the two plates and is both focused and transported through an angle of approximately 40°. Since only ions with a narrow range of kinetic energy are able to pass through the ESA, it forms an effective energy filter. After the ion beam passes through the ESA, the ions then pass into the magnetic field. The magnetic sector is dispersive for both ion energy and mass and focuses the ions with diverging angles of motion. The ion beam then passes through the final part of the system which is a narrow slit situated at the focal point of the magnet known as the collector slit. High resolutions are achieved by making both of the slits very narrow so that the beam reaching the detector has only a very narrow bandwidth of mass at any given time. The resolution of a mass analyzer is described as the ability to separate adjacent mass regions in the mass spectrum. Resolution, R, is defined as: $R= m/\triangle m$, where $\triangle m$ is the mass difference between two separated peaks and m is the nominal mass of the peak.

### 1.3 Advantages and Capabilities of HR-ICP-MS

**Variable Resolution:** The resolution of a mass analyzer is defined as the ability to separate adjacent mass regions in the mass spectrum. The nominal masses of $ArO^+$ and Fe are both 56 amu. The actual mass of $^{40}Ar^{16}O^+$ is 55.934939 amu, and the actual mass of $^{56}Fe$ is 55.957299 amu. Using equation 1 to calculate

R= 56 amu/ (55.957299 amu-55.934939 amu) =2504

So the use of a mass spectrometer with a resolution higher than 2500 would be one of the primary instrumental approaches to distinguish between the two species. The other approaches are use a collusion cell to break apart polyatomic interferences or use of gas phase chemical reactions in a “reaction cell” to eliminate polyatomic interferences.

The High-Resolution ICP is capable of varying its resolving power by means of the two swinging gate slits. By varying the slit width, the analysis can be tailored in such a manner that each element is analyzed at a resolution enabling it to be fully resolved from any interference. The drawback to increased resolution is lower sensitivity.

**High Sensitivity:** High-Resolution systems are known for their extremely high ion transport efficiency which gives rise to very high levels of sensitivity. The VG Axiom typically returns a count rate of greater than 1,000,000 counts per second for a 1 ppb solution of $^{115}$Indium.

**Low Noise:** Due to the design of the High-Resolution mass analyzer there is little opportunity for stray photons to traverse the entire instrument, resulting in an extremely low background noise. This low noise level, combined with high sensitivity capabilities, allows the High-Resolution system to return unparalleled limits of detection. For some applications, the instrument is capable of achieving limits of detection below 1 ppq. Limit of detection (LOD) is a criterion that is defined as $3*SD_0$, where $SD_0$ is the value of the standard deviation as the concentration of the analyte approaches 0.

## 2. Methodology

### 2.1 Sample preparation

2.1.1 Insects and infections

*Heliothis virescens* and *Helicoverpa zea* eggs were received from the North Carolina State Univ. Dept. of Entomology Insectary (Raleigh, NC) and reared individually on an artificial wheat-germ-based diet (BioServe, Frenchtown, NJ) under standard conditions of 14h:10h (L:D) photoperiod, 55% RH, 28°C [86]. For baculoviral response, newly moulted larvae were per os infected with an LC95 (concentration

killing 95% of larvae) at either 5 x $10^8$ ($4^{th}$ instar) or 1 x $10^9$ ($5^{th}$ instar) polyhedra/ml of HzSNPV or mock-infected according to Popham et al. [86]. In short, larvae were infected with virus mixed with food coloring visible through the integument. Additional trays of larvae were per os infected to confirm that the virus killed at the expected rate. To activate the antibacterial immune response, newly moulted $5^{th}$ instar larvae were punctured with a tungsten needle dipped into a suspension of heat-killed *Escherichia coli* and *Micrococcus luteus* and Phosphate buffer solution (PBS) [87]. *Micrococcus luteus* was purchased from Sigma Chemicals as *Micrococcus lysodeikticus* ATCC No. 4698, but is a synonym of Micrococcus luteus.
Mock-infected controls received a sterile puncture. The mock samples were given an oral dose of the same buffer used to deliver the virus so that they have received the same physical disruptions as infected larvae, for example, handling, crushing, fasting, damage, manipulation; but they received no virus.

Plasma from $4^{th}$ and $5^{th}$ instar larvae was collected directly into a chilled 1.5ml microcentrifuge tube containing ice cold, sterile phosphate buffered saline (PBS) (50mM $NaHPO_4$, pH 6.8). Plasma was adjusted to a final dilution of 1:10 by addition of cold PBS after which hemocytes were removed by microcentrifugation at 8000 rpm for three minutes.

2.1.2 Insect plasma sample preparation for ICP-MS analysis

Each plasma sample was weighed into a precleaned trace metal free polypropylene tube (Stockwell Inc., Philadelphia, PA). Three milliliters of Fisher brand Optima grade nitric acid ($HNO_3$) and two milliliters of Fluka brand TraceSelect grade hydrogen peroxide solution ($H_2O_2$) were then added. Blank buffer samples containing only the phosphate buffer solution were prepared in the same way as the samples in order to check for analyte backgrounds. The digestion mixture was then heated in an ultrasonic water bath at 60°C for 2 hours. After digestion, the digestates were cooled to room temperature. The digestates were then diluted to 10 ml with 18.2 MΩ DI

water. In preparation for ICP analysis, 100 uL of 1μg/g internal standards Sc and Y were added to all the diluted solutions in order to compensate for any instrumental variations.

**2.2 Instrumentation**

Measurements were carried out on a VG Axiom HR-ICP-MS. Instrument settings are outlined in Table 1.

Table 1 Instrument settings

| | |
|---|---|
| Instrument | VG Axiom ICP-MS |
| Resolution (m/Δm) | 6000 (for all measured isotopes) |
| Rf power | 1400W |
| Gas flow rates | |
| Plasma gas | 12-131min$^{-1}$ |
| Auxiliary | 0.9-11min$^{-1}$ |
| Sample gas | 1.0-1.21min-1(optimized daily) |
| Torch | Fassel type |
| Nebulizer | PFA MicroFlow |
| Spray chamber | Tracey cyclonic glass |
| Cones | Ni sampler and skimmer |
| Sample uptake | Pumping via a Spetec peristaltic pump |
| Instrument tuning | Performed daily using a 10ng ml$^{-1}$ multi-element solution |

**2.3 Interferences and choice of instrument resolution**

Table 2. gives the major polyatomic interferences on ICP-MS and the resolutions needed to resolve the isotopes of interest from the interferences. R is calculated by the equation 1. R= m/△m, where △m is the mass difference between two separated

peaks and m is the nominal mass of the peak. The isotopes cannot be resolved from some major polyatomic interferences if the resolution is lower than 3000.

The use of high resolution is an effective approach to resolve this problem. Theoretically, 3000 resolution can resolve the major polyatomic interferences in this study, however, for 56Fe, its interference $40Ar12C^+$ has a wide peak in the mass spectra, so the tail of this peak would affect the peak of 56Fe. As a result, a higher resolution is required. Although it is possible to select different mass resolutions for a multi-element analysis to increase the sensitivity (lower resolution yields higher sensitivity), our experience has been that the instrument performs better at a single mass resolution over a multi-element scan. In particular, we have found that rapid, repetitive slit width changes leads to frequent mechanical breakdown in the slit assembly. For this reason, all analytes were measured with a mass resolution of 6000.

Table 2. The major polyatomic interferences on ICP-MS and the resolutions needed to resolve the isotopes of interest from the interferences

| Isotopes (Abundance) | Major interferences | R needed to resolve the isotope of interest |
|---|---|---|
| 52Cr (83.8%) | $40Ar12C^+$ | 2377 |
| 63Cu (69.1%) | $40Ar23Na^+$ | 2794 |
| 65Cu (30.9%) | $32S16O_2H^+$ | 1550 |
| 56Fe (91.7%) | $40Ar16O^+$, $40Ca16O^+$ | 2504 |
| 55Mn (100%) | $40Ar14NH^+$ | 1561 |
| 64Zn (48.9%) | $32S_2^+$, $32S16O_2^+$ | 1642 |

## 2.4 Internal Standard

To select the most appropriate internal standard elements, the atomic mass and first ionization energy are the two factors that need to be considered. For this reason, Sc and Y were tested as internal standards for the multi-element analysis. The first

ionization energy of Sc is 6.54 electron volts, which is close to that of Mg, Cr, Mn, Fe, Co, Ni, and Cu. The atomic mass of Sc is 45, which is relatively close to that of Mg, Cr, Mn, Fe, Co, Ni and Cu. The first ionization energy of Y is 6.38 electron volts, and its atomic mass is 89, so its properties are closer to Zn and Mo. Accurate and precise results were obtained using 45Sc for Mg, Cr, Mn, Fe, Co, Ni, and Cu, and 86Y for Zn, and Mo.

### 2.5 Calibration curves

Mixed standard solutions of Mn, Cr, Fe, Co, Ni, Cu, Zn and Mo were prepared from a 10µg/g multi-element solution (High Purity Standards, Charleston, SC). More concentrated individual Mg standards were also prepared from 1000µg/g single element standards (High Purity Standards, Charleston, SC). The higher Mg concentration was selected to match the samples. All prepared standard solutions were acidified with 2% $HNO_3$. Aqueous standard solutions covering the concentration ranges 0-50ppb were used for external calibration. Eight standard concentrations were used for each element. In every case, the correlation coefficient of the standard response curve exceeded 0.999.

### 2.6 LOD and LOQ

The Limit of Detection (LOD) is defined as $3*SD_0$, where $SD_0$ is the value of the standard deviation of the response function as the concentration of the analyte approaches 0. The value of $SD_0$ was obtained from the standard deviation of 10 runs of the 2% nitric acid blank sample since the determination should be made using a matrix that matches the sample matrix. The limits of detection of isotopes of interest and limits of detection of isotopes in samples are given in Table 3.

Table 3. LOD of isotopes measured by HR-ICP-MS and LOD of isotopes in samples

| Isotope | **24Mg** | **26Mg** | **52Cr** | **55Mn** | **56Fe** | **59Co** | **60Ni** | **63Cu** | **64Zn** | **65Cu** | **95Mo** | **98Mo** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LOD of isotopes on ICP-MS(ppb) | 0.051 | 0.082 | 0.0036 | 0.0027 | 0.0091 | 0.0036 | 0.059 | 0.032 | 0.033 | 0.04 | 0.0065 | 0.0062 |
| LOD of isotopes in samples(in μg/g) | 0.510 | 0.820 | 0.036 | 0.027 | 0.091 | 0.036 | 0.590 | 0.320 | 0.330 | 0.400 | 0.065 | 0.062 |

The lower limits of quantification are determined using ten standard deviations of the single blank as proposed by "American Society Committee on Environmental Improvement" [Jarvis, 1992]. The limits of quantification of isotopes measured by HR-ICP-MS and limits of quantification in samples given in Table 4 are based on 10 separate runs of the nitric acid blank.

Table 4. LOQ of isotopes measured by HR-ICP-MS and LOQ of isotopes in samples

| | **24Mg** | **26Mg** | **52Cr** | **55Mn** | **56Fe** | **59Co** | **60Ni** | **63Cu** | **64Zn** | **65Cu** | **95Mo** | **98Mo** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| LOQ of isotopes on ICP-MS(ppb) | 0.17 | 0.273 | 0.012 | 0.009 | 0.03 | 0.012 | 0.197 | 0.107 | 0.11 | 0.133 | 0.022 | 0.021 |
| LOQ of isotopes in samples(in μg/g) | 1.70 | 2.73 | 0.12 | 0.09 | 0.30 | 0.12 | 1.97 | 1.07 | 1.10 | 1.33 | 0.22 | 0.21 |

It should be noted that the measured levels of 52Cr, 59Co and 60Ni in the insect samples are close to the LOQ values which means the quantitative results of these elements cannot be reported with a high degree of confidence. For this reason, this work focused on Mg, Mn, Fe, Cu, Zn and Mo levels in the samples measured by

HR-ICP-MS.

## 2.7 Accuracy (spike recovery)

To determine the recovery of the sample preparation protocol for individual elements, the samples were spiked with 40µl of a 10µg/g multi-element stock solution and 100µl of a 1000µg/g Mg stock solution before digestion. The higher Mg concentration was selected to match the samples. The spike recovery tests were carried out in one day measurement to determine the multielements levels in a sample without significant matrix suppression and make sure the completeness of the digestion and accuracy of ICP-MS measurement. These spike recoveries are calculated for each element as the ratio of the measured value of spike to the actual value of spike added in the sample. All reported values were corrected for spike recovery. Table 5. gives the spike recoveries of isotopes of interest measured on ICP-MS.

Table 5. Spike recoveries of isotopes measured on ICP-MS

| Isotope | **24Mg** | **26Mg** | **55Mn** | **56Fe** | **63Cu** | **64Zn** | **65Cu** | **95Mo** | **98Mo** |
|---|---|---|---|---|---|---|---|---|---|
| Average percent Spike Recovery | 86.2 | 83.4 | 87.7 | 83.9 | 82.2 | 81.3 | 81.7 | 97.4 | 97.1 |
| % Standard Deviation | 3.4 | 2.3 | 3.7 | 4.8 | 1.6 | 2.7 | 1.2 | 0.8 | 3.2 |

## 2.8 Precision

### 2.8.1 Repeatability of standard QC sample one day

Table 6 gives the results for the quality control standard sample at 5ppb (Mg at 1000 ppb) which was measured on the ICP-MS in between every ten samples during an analytical run. The results are the average of three measurements over the course of a single day.

Table 6. Quality control results of isotopes measured on ICP-MS

| Isotope | **24Mg** | **26Mg** | **55Mn** | **56Fe** | **63Cu** | **64Zn** | **65Cu** | **95Mo** | **98Mo** |
|---|---|---|---|---|---|---|---|---|---|
| Concentration of Standard(ppb) | 1059 | 1059 | 4.49 | 4.67 | 4.67 | 4.67 | 4.67 | 4.67 | 4.67 |
| Average QC concentration measured results | 1143 | 1046 | 4.75 | 4.83 | 4.73 | 4.82 | 4.87 | 4.86 | 5.19 |
| % standard Deviation | 0.37 | 0.37 | 2.86 | 3.84 | 1.17 | 2.72 | 2.01 | 1.32 | 1.81 |

### 2.8.2 Repeatability of samples on different days with different preparations

For the triplicate samples, all of them were treated with different preparations and the third ones were prepared on different days and measured by ICP-MS on different days to test the repeatability of the sample measurement. The average and percent standard deviations repeatable samples are given in Table 7. Except for Mo, the repeatability is less than 20% and in most cases less than 10%.

Table 7. Repeatability of triplicate samples

| Date of Measurement by ICP-MS | 6/20/2008 | 6/20/2008 | 11/07/2008 | Average concentration (in µg/g) | %RSD |
|---|---|---|---|---|---|
| Sample ID/Treatment | HvE5 18/Jan/08 HzSNPV Set 3 (0 hours post infection) | | | | |
| | Concentrations in samples in µg/g | | | | |
| 24Mg | 223 | 187 | 178 | 196 | 12 |
| 26Mg | 211 | 191 | 171 | 191 | 11 |
| 55Mn | 0.191 | 0.196 | 0.177 | 0.188 | 5 |
| 56Fe | 0.203 | 0.233 | 0.179 | 0.205 | 13 |
| 63Cu | 0.1704 | 0.1633 | 0.1374 | 0.1570 | 11 |
| 64Zn | 0.503 | 0.393 | 0.333 | 0.410 | 21 |
| 65Cu | 0.167 | 0.161 | 0.133 | 0.154 | 12 |
| 95Mo | 0.0178 | 0.0154 | 0.0331 | 0.0221 | 44 |
| 98Mo | 0.0182 | 0.0199 | 0.0262 | 0.0214 | 20 |
| | | | | | |
| Date of Measurement by ICP-MS | 6/20/2008 | 6/20/2008 | 11/07/2008 | Average concentration (in µg/g) | %RSD |
| Sample ID/Treatment | HvE5 18/Jan/08 HzSNPV Set 3 (12 hours post infection) | | | | |
| | Concentrations in samples in µg/g | | | | |

| | | | | | |
|---|---|---|---|---|---|
| 24Mg | 263 | 219 | 202 | 228 | 14 |
| 26Mg | 253 | 218 | 200 | 224 | 12 |
| 55Mn | 0.161 | 0.165 | 0.130 | 0.152 | 12 |
| 56Fe | 0.779 | 0.782 | 0.648 | 0.736 | 10 |
| 63Cu | 0.2506 | 0.2464 | 0.1879 | 0.2283 | 15 |
| 64Zn | 1.178 | 1.037 | 0.846 | 1.020 | 16 |
| 65Cu | 0.251 | 0.252 | 0.186 | 0.230 | 16 |
| 95Mo | 0.0148 | 0.0132 | 0.0235 | 0.0172 | 32 |
| 98Mo | 0.0165 | 0.0165 | 0.0190 | 0.0173 | 9 |
| | | | | | |
| Date of Measurement by ICP-MS | 6/20/2008 | 6/20/2008 | 11/07/2008 | Average concentration (in µg/g) | %RSD |
| Sample ID/Treatment | HvE5 18/Jan/08 HzSNPV Set 3 (24 hours post infection) | | | | |
| Hours post-infection | Concentrations in samples in µg/g | | | | |
| 24Mg | 420 | 380 | 382 | 394 | 6 |
| 26Mg | 364 | 378 | 381 | 374 | 2 |
| 55Mn | 0.193 | 0.286 | 0.224 | 0.235 | 20 |
| 56Fe | 1.788 | 2.018 | 1.635 | 1.813 | 11 |
| 63Cu | 0.5298 | 0.4857 | 0.4182 | 0.4779 | 12 |
| 64Zn | 2.659 | 2.350 | 2.554 | 2.521 | 6 |
| 65Cu | 0.495 | 0.481 | 0.416 | 0.464 | 9 |
| 95Mo | 0.0288 | 0.0244 | 0.0365 | 0.0299 | 20 |
| 98Mo | 0.0270 | 0.0281 | 0.0345 | 0.0299 | 14 |
| | | | | | |
| Date of | 6/20/2008 | 6/20/2008 | 11/07/2008 | Average | %RSD |

| Measurement by ICP-MS | | | | concentration (in µg/g) | |
|---|---|---|---|---|---|
| Sample ID/Treatment | HvE5 18/Jan/08 HzSNPV Set 3 (48 hours post infection) | | | | |
| | Concentrations in samples in µg/g | | | | |
| 24Mg | 299 | 253 | 243 | 265 | 11 |
| 26Mg | 272 | 252 | 213 | 246 | 12 |
| 55Mn | 0.161 | 0.156 | 0.112 | 0.143 | 19 |
| 56Fe | 3.084 | 2.959 | 3.349 | 3.130 | 6 |
| 63Cu | 0.7394 | 0.6867 | 0.7017 | 0.7093 | 4 |
| 64Zn | 2.686 | 2.854 | 2.648 | 2.729 | 4 |
| 65Cu | 0.731 | 0.687 | 0.699 | 0.706 | 3 |
| 95Mo | 0.0202 | 0.0324 | 0.0343 | 0.0290 | 27 |
| 98Mo | 0.0193 | 0.0375 | 0.0320 | 0.0296 | 32 |
| | | | | | |
| Date of Measurement by ICP-MS | 6/20/2008 | 6/20/2008 | 11/07/2008 | Average concentration (in µg/g) | %RSD |
| Sample ID/Treatment | HvE5 18/Jan/08 HzSNPV Set 3 (60 hours post infection) | | | | |
| | Concentrations in samples in µg/g | | | | |
| 24Mg | 200 | 193 | 190 | 194 | 3 |
| 26Mg | 173 | 197 | 177 | 182 | 7 |
| 55Mn | 0.161 | 0.186 | 0.161 | 0.169 | 9 |
| 56Fe | 2.086 | 2.235 | 1.898 | 2.073 | 8 |
| 63Cu | 0.3402 | 0.4821 | 0.3828 | 0.4017 | 18 |
| 64Zn | 2.990 | 2.861 | 3.039 | 2.963 | 3 |
| 65Cu | 0.331 | 0.474 | 0.380 | 0.395 | 18 |

| | | | | | |
|---|---|---|---|---|---|
| 95Mo | 0.0202 | 0.0328 | 0.0425 | 0.0318 | 35 |
| 98Mo | 0.0194 | 0.0358 | 0.0391 | 0.0314 | 34 |

# Chapter 3

# Results

## 1. Description of average values of infected range and average mock range

Concentration levels Mg, Mn, Fe, Cu, Zn and Mo of larvae of Heliothis virescens plasma were determined in the development of 4th instar and 5th instar with Helicoverpa zea single nucleopolyhedrovirus (HzSNPV) treatment and in the 5th instar with Micrococcus lysodeikticus treatment. Tables 8-13 give the concentrations (in µg/g) of the elements of interest in sample with HzSNPV infection in the 4th and 5th instar and the same aged controls.

Table 8. Concentrations (in µg/g) of Mg in plasma from larvae infected with bavulovirus (HzSNPV) in the 4th and 5th instar and the same aged controls

*Average of duplicate samples

**Average of triplicate samples

| 24Mg | 4th instar | | | | | | 5th instar | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 170 | 98 | 143 | 188 | 215 | 355 | 432 | 231 | 626 | 536 | 362 | 305 |
| | 177* | 174 | 166* | 172 | 661 | 589 | 408 | 313 | 517 | 625 | 158 | 239 |
| | 165* | 228 | 155* | 186 | 393* | 634 | 298 | 228** | 493 | 549* | 219 | 194** |
| | 244* | 163* | 246* | 298* | 343* | 658* | | | | | | |
| | 215* | | 449* | | | | | | | | | |
| Average | 194 | 166 | 232 | 211 | 403 | 559 | 379 | 258 | 545 | 570 | 246 | 246 |
| Std Dev | 34 | 53 | 128 | 59 | 187 | 139 | 71 | 48 | 71 | 48 | 105 | 56 |
| P value | 0.365 | | 0.776 | | 0.229 | | 0.070 | | 0.641 | | 0.995 | |
| 26Mg | 4th instar | | | | | | 5th instar | | | | | |
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 172 | 89 | 153 | 192 | 200 | 364 | 466 | 201 | 593 | 508 | 360 | 274 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 182* | 171 | 167* | 167 | 647 | 575 | 390 | 283 | 496 | 599 | 128 | 193 |
| | 174* | 231 | 143* | 152 | 416* | 623 | 267 | 224** | 456 | 515* | 205 | 182** |
| | 255* | 165* | 244* | 281* | 350* | 580* | | | | | | |
| | 211* | | 478* | | | | | | | | | |
| Average | 199 | 164 | 237 | 198 | 403 | 535 | 374 | 236 | 515 | 541 | 231 | 217 |
| Std Dev | 35 | 58 | 140 | 58 | 186 | 116 | 100 | 42 | 70 | 51 | 118 | 50 |
| P value | 0.306 | | 0.624 | | 0.275 | | 0.093 | | 0.637 | | 0.853 | |

Table 9. Concentrations (in µg/g) of Mn in plasma from larvae infected with bavulovirus (HzSNPV) in the 4th and 5th instar and the same aged controls

| 55Mn | 4th instar | | | | | | 5th instar | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.0227 | 0.0589 | 0.085 | 0.099 | 0.040 | 0.305 | 0.252 | 0.211 | 0.109 | 0.061 | 0.049 | 0.090 |
| | 0.0115* | 0.0784 | 0.134* | 0.114 | 0.561 | 0.351 | 0.386 | 0.263 | 0.047 | 0.112 | 0.093 | 0.155 |
| | 0.0151* | 0.1272 | 0.171* | 0.082 | 0.266* | 0.333 | 0.222 | 0.152** | 0.040 | 0.105* | 0.096 | 0.169** |
| | 0.0217* | 0.1122* | 0.186* | 0.165* | 0.202* | 0.362* | | | | | | |
| | 0.0260* | | 0.313* | | | | | | | | | |
| Average | 0.0194 | 0.0942 | 0.1777 | 0.1150 | 0.2674 | 0.3377 | 0.287 | 0.209 | 0.065 | 0.092 | 0.079 | 0.138 |
| Std Dev | 0.0059 | 0.0311 | 0.0852 | 0.0358 | 0.2176 | 0.0248 | 0.087 | 0.056 | 0.038 | 0.028 | 0.027 | 0.042 |
| P value | 0.001 | | 0.215 | | 0.545 | | 0.262 | | 0.380 | | 0.110 | |

Table 10. Concentrations (in µg/g) of Fe in plasma from larvae infected with bavulovirus (HzSNPV) in the 4th and 5th instar and the same aged controls

| 56Fe | 4th instar | | | | | | 5th instar | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.27 | 0.15 | 0.38 | 0.74 | 2.78 | 2.88 | 1.589 | 0.488 | 3.061 | 3.113 | 4.717 | 3.294 |
| | 0.80* | 0.41 | 0.64* | 0.41 | 1.06 | 2.14 | 1.255 | 0.745 | 3.400 | 2.702 | 2.539 | 2.120 |
| | 0.55* | 0.33 | 0.27* | 0.71 | 1.10* | 2.06 | 0.918 | 0.736** | 3.059 | 2.518* | 3.765 | 2.073** |
| | 0.72* | 0.37* | 0.65* | 0.86* | 1.07* | 3.00* | | | | | | |
| | 0.37* | | 0.55* | | | | | | | | | |
| Average | 0.54 | 0.32 | 0.50 | 0.68 | 1.51 | 2.52 | 1.254 | 0.656 | 3.173 | 2.778 | 3.674 | 2.496 |
| Std Dev | 0.22 | 0.11 | 0.17 | 0.19 | 0.85 | 0.49 | 0.335 | 0.146 | 0.197 | 0.305 | 1.092 | 0.692 |
| P value | 0.113 | | 0.171 | | 0.085 | | 0.047 | | 0.131 | | 0.190 | |

Table 11. Concentrations (in µg/g) of Cu in plasma from larvae infected with bavulovirus (HzSNPV) in the 4th and 5th instar and the same aged controls

| 63Cu | 4th instar | | | | | | 5th instar | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.139 | 0.061 | 0.134 | 0.133 | 0.646 | 0.215 | 0.311 | 0.166 | 0.763 | 0.788 | 0.901 | 0.733 |
| | 0.095* | 0.107 | 0.122* | 0.101 | 0.567 | 0.205 | 0.307 | 0.247 | 0.804 | 0.987 | 0.589 | 0.505 |
| | 0.139* | 0.140 | 0.150* | 0.161 | 0.313* | 0.381 | 0.241 | 0.228** | 0.760 | 0.836* | 0.786 | 0.402* |
| | 0.181* | 0.117* | 0.199* | 0.326* | 0.436* | 0.300* | | | | | | |
| | 0.132* | | 0.222* | | | | | | | | | |
| Average | 0.137 | 0.106 | 0.165 | 0.180 | 0.491 | 0.276 | 0.286 | 0.214 | 0.776 | 0.870 | 0.759 | 0.547 |
| Std Dev | 0.030 | 0.033 | 0.043 | 0.100 | 0.147 | 0.082 | 0.039 | 0.043 | 0.025 | 0.104 | 0.158 | 0.170 |
| P value | 0.189 | | 0.775 | | 0.043 | | 0.095 | | 0.200 | | 0.189 | |
| 65Cu | 4th instar | | | | | | 5th instar | | | | | |
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.148 | 0.061 | 0.148 | 0.136 | 0.625 | 0.225 | 0.333 | 0.156 | 0.758 | 0.802 | 0.937 | 0.722 |
| | 0.097* | 0.122 | 0.123* | 0.116 | 0.588 | 0.217 | 0.304 | 0.246 | 0.784 | 0.955 | 0.577 | 0.521 |
| | 0.144* | 0.156 | 0.152* | 0.172 | 0.321* | 0.388 | 0.232 | 0.230** | 0.738 | 0.859* | 0.787 | 0.395** |
| | 0.191* | 0.109* | 0.218* | 0.306* | 0.458* | 0.302* | | | | | | |
| | 0.145* | | 0.247* | | | | | | | | | |
| Average | 0.145 | 0.112 | 0.177 | 0.183 | 0.498 | 0.283 | 0.289 | 0.210 | 0.760 | 0.872 | 0.767 | 0.546 |
| Std Dev | 0.033 | 0.039 | 0.052 | 0.086 | 0.138 | 0.080 | 0.052 | 0.048 | 0.023 | 0.077 | 0.181 | 0.165 |
| P value | 0.215 | | 0.911 | | 0.036 | | 0.124 | | 0.075 | | 0.193 | |

Table 12. Concentrations (in µg/g) of Zn in plasma from larvae infected with bavulovirus (HzSNPV) in the 4th and 5th instar and the same aged controls

| 64Zn | 4th instar | | | | | | 5th instar | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.335 | 0.240 | 0.494 | 0.682 | 3.936 | 1.523 | 1.996 | 0.527 | 4.384 | 5.105 | 5.142 | 4.294 |
| | 0.370* | 0.885 | 0.636* | 0.381 | 3.307 | 2.320 | 1.477 | 1.507 | 4.326 | 5.299 | 3.660 | 3.314 |
| | 0.579* | 0.498 | 0.256* | 0.799 | 2.500* | 2.507 | 1.252 | 1.020** | 4.309 | 3.937* | | 2.963** |
| | 0.474* | 0.455* | 0.764* | 0.806* | 2.336* | 2.378* | | | | | | |
| | 0.298* | | 0.638* | | | | | | | | | |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Average | 0.411 | 0.519 | 0.558 | 0.667 | 3.020 | 2.182 | 1.575 | 1.018 | 4.340 | 4.780 | 4.401 | 3.524 |
| Std Dev | 0.114 | 0.268 | 0.194 | 0.199 | 0.744 | 0.446 | 0.382 | 0.490 | 0.039 | 0.737 | 1.048 | 0.689 |
| P value | 0.437 | | 0.434 | | 0.102 | | 0.195 | | 0.360 | | 0.329 | |

Table 13. Concentrations (in μg/g) of Mo in plasma from larvae infected with bavulovirus (HzSNPV) in the 4th and 5th instar and the same aged controls

| 95Mo | 4th instar | | | | | | 5th instar | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.0399 | 0.0073 | 0.0332 | 0.0089 | 0.0478 | 0.0411 | 0.0366 | 0.0153 | 0.0503 | 0.0379 | 0.0633 | 0.0419 |
| | 0.0296* | 0.0264 | 0.0254* | 0.0195 | 0.0749 | 0.0716 | 0.0259 | 0.0233 | 0.0394 | 0.0567 | 0.0448 | 0.0396 |
| | 0.0369* | 0.0327 | 0.0246* | 0.0240 | 0.0409* | 0.0678 | 0.0217 | 0.0172** | 0.0429 | 0.0488* | 0.0516 | 0.0318*8 |
| | 0.0364* | 0.0173* | 0.0347* | 0.0264* | 0.0372* | 0.0782* | | | | | | |
| | 0.0365* | | 0.0379* | | | | | | | | | |
| Average | 0.0359 | 0.0210 | 0.0312 | 0.0197 | 0.0502 | 0.0646 | 0.0280 | 0.0186 | 0.0442 | 0.0478 | 0.0532 | 0.0378 |
| Std Dev | 0.0038 | 0.0111 | 0.0059 | 0.0077 | 0.0170 | 0.0163 | 0.0077 | 0.0042 | 0.0055 | 0.0094 | 0.0094 | 0.0053 |
| P value | 0.024 | | 0.039 | | 0.266 | | 0.135 | | 0.596 | | 0.068 | |
| 98Mo | 4th instar | | | | | | 5th instar | | | | | |
| | 12hr | | 36hr | | 60hr | | 12hr | | 36hr | | 60hr | |
| | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected | mock | infected |
| | 0.0404 | 0.0090 | 0.0315 | 0.0110 | 0.0450 | 0.0448 | 0.0428 | 0.0177 | 0.0553 | 0.0412 | 0.0692 | 0.0477 |
| | 0.0308* | 0.0323 | 0.0261* | 0.0258 | 0.0759 | 0.0786 | 0.0255 | 0.0256 | 0.0393 | 0.0589 | 0.0511 | 0.0411 |
| | 0.0357* | 0.0397 | 0.0266* | 0.0299 | 0.0429* | 0.0751 | 0.0223 | 0.0173** | 0.0453 | 0.0486* | 0.0603 | 0.0314** |
| | 0.0384* | 0.0203* | 0.0379* | 0.0298* | 0.0371* | 0.0791* | | | | | | |
| | 0.0379* | | 0.0416* | | | | | | | | | |
| Average | 0.0366 | 0.0253 | 0.0327 | 0.0241 | 0.0502 | 0.0694 | 0.0302 | 0.0202 | 0.0466 | 0.0496 | 0.0602 | 0.0401 |
| Std Dev | 0.0037 | 0.0135 | 0.0069 | 0.0089 | 0.0174 | 0.0165 | 0.0111 | 0.0047 | 0.0081 | 0.0089 | 0.0090 | 0.0082 |
| P value | 0.112 | | 0.145 | | 0.161 | | 0.223 | | 0.693 | | 0.046 | |

## 2. Statistical analysis

Subject demographic data were compared using analysis of variance (ANOVA) and the commercially available NCSS software (NCSS, Kaysville, Utah). A general linear model (GLM), univariate, one factor ANOVA was used for comparison of trace

elements levels in plasma from larvae over the 4th instar and 5th instars, respectively. A general linear model (GLM), univariate, two factor ANOVA was used for comparison of trace elements levels in plasma from larvae of time versus treatment at 12, 36, and 60 hours post infection in the 4th and 5th instars, respectively. A two tailed t-test was applied to comparison of trace elements levels in plasma from larvae with infections and the same aged mocks at each time point in the 4th and 5th instars.

**2.1 Comparison of infected time points (statistically difference and trend)**

The general linear model (GLM), univariate, one factor ANOVA statistical analysis of one single element concentration levels at different hours post infection shows that with HzSNPV infection, Mg, Mn, Fe, Cu, Zn and Mo levels in insect plasma and hours post infection interaction were significant ($p<0.05$) both in the 4th instar and 5th instar. And there were increasing trends of these elements levels during the hours post infection. Tables 14 and 15 give the P values by analyzed by one-way ANOVA and changes of levels of trace elements in insect plasma samples with HzSNPV virus infection over the course of 72 hours post infection in 4th and 5th instars.

The ANOVA statistical analysis shows Mg, Mn, Fe, Cu, Zn and Mo levels in mocks in the late 4th in insect plasma and hours post infection interaction were significantly different ($p<0.05$) and the same results were found in the 5th instar. Tables 16 and 17 give the one-way ANOVA analysis results for the mock samples in the 4th and 5th instar over the course of 60 hours post infection.

The same statistical analysis was applied to data of insect plasma in the 5th instar with bacterial (*Micrococcus lysodeikticus*) infection. All the trace elements of interest levels insect plasma and hour post infection interaction were not significant ($P>0.05$). Table 18 gives changes of trace elements concentrations (in μg/g) in insect plasma with bacterial (*Micrococcus lysodeikticus*) infection samples over the course of 24

hours post infection in the 5th instar.

Table 14. Changes of trace elements concentrations (in µg/g) in insect plasma samples with HzSNPV infection over the course of 72 hours post infection in the 4th instar (P values are given by one-way ANOVA)

| **Trace elements** | **Hours post infection** | 0 | 12 | 24 | 36 | 48 | 60 | 72 | P value |
|---|---|---|---|---|---|---|---|---|---|
| **24Mg** | **Average concentration** | 120 | 166 | 208 | 211 | 352 | 559 | 369 | < 0.001 |
| | **Std Dev** | 38 | 53 | 67 | 59 | 49 | 139 | 55 | |
| **26Mg** | **Average concentration** | 115 | 164 | 210 | 198 | 367 | 535 | 373 | < 0.001 |
| | **Std Dev** | 45 | 58 | 72 | 58 | 43 | 116 | 49 | |
| **55Mn** | **Average concentration** | 0.095 | 0.094 | 0.040 | 0.115 | 0.233 | 0.338 | 0.257 | < 0.001 |
| | **Std Dev** | 0.020 | 0.031 | 0.003 | 0.036 | 0.054 | 0.025 | 0.038 | |
| **56Fe** | **Average concentration** | 0.13 | 0.32 | 0.84 | 0.68 | 0.98 | 2.52 | 3.31 | < 0.001 |
| | **Std Dev** | 0.02 | 0.11 | 0.33 | 0.19 | 0.29 | 0.49 | 0.89 | |
| **63Cu** | **Average concentration** | 0.053 | 0.106 | 0.181 | 0.180 | 0.172 | 0.276 | 0.241 | < 0.001 |
| | **Std Dev** | 0.017 | 0.033 | 0.040 | 0.100 | 0.036 | 0.082 | 0.057 | |
| **64Zn** | **Average concentration** | 0.216 | 0.519 | 0.738 | 0.667 | 1.086 | 2.182 | 1.900 | < 0.001 |
| | **Std Dev** | 0.035 | 0.268 | 0.121 | 0.199 | 0.289 | 0.446 | 0.684 | |
| **65Cu** | **Average concentration** | 0.058 | 0.112 | 0.178 | 0.183 | 0.185 | 0.283 | 0.244 | < 0.001 |
| | **Std Dev** | 0.028 | 0.039 | 0.037 | 0.086 | 0.033 | 0.080 | 0.049 | |
| **95Mo** | **Average concentration** | 0.0147 | 0.0210 | 0.0227 | 0.0197 | 0.0403 | 0.0646 | 0.0402 | < 0.001 |
| | **Std Dev** | 0.0116 | 0.0111 | 0.0120 | 0.0077 | 0.0056 | 0.0163 | 0.0029 | |
| **98Mo** | **Average concentration** | 0.0176 | 0.0253 | 0.0269 | 0.0241 | 0.0440 | 0.0694 | 0.0436 | < 0.001 |
| | **Std Dev** | 0.0146 | 0.0135 | 0.0150 | 0.0089 | 0.0066 | 0.0165 | 0.0027 | |

Table 15. Changes of trace elements concentrations (in µg/g) in insect plasma samples with HzSNPV infection over the course of 72 hours post infection in the 5th instar (P values are given by one-way ANOVA)

| Trace elements | Hours post infection | 0 | 12 | 24 | 36 | 48 | 60 | 72 | P value |
|---|---|---|---|---|---|---|---|---|---|
| **24Mg** | **Average** | 227 | 258 | 393 | 570 | 406 | 246 | 242 | < 0.001 |
| | **Std Dev** | 61 | 48 | 28 | 48 | 134 | 56 | 51 | |
| **26Mg** | **Average** | 214 | 236 | 366 | 541 | 384 | 217 | 212 | < 0.001 |
| | **Std Dev** | 84 | 42 | 27 | 51 | 137 | 50 | 77 | |
| **55Mn** | **Average** | 0.224 | 0.209 | 0.211 | 0.092 | 0.094 | 0.138 | 0.479 | < 0.001 |
| | **Std Dev** | 0.063 | 0.056 | 0.027 | 0.028 | 0.049 | 0.042 | 0.264 | |
| **56Fe** | **Average** | 0.349 | 0.656 | 2.051 | 2.778 | 3.837 | 2.496 | 3.243 | < 0.001 |
| | **Std Dev** | 0.114 | 0.146 | 0.788 | 0.305 | 1.239 | 0.692 | 0.883 | |
| **63Cu** | **Average** | 0.145 | 0.214 | 0.441 | 0.870 | 0.844 | 0.547 | 0.637 | < 0.001 |
| | **Std Dev** | 0.021 | 0.043 | 0.045 | 0.104 | 0.149 | 0.170 | 0.086 | |
| **64Zn** | **Average** | 0.481 | 1.018 | 2.817 | 4.780 | 4.677 | 3.524 | 4.728 | < 0.001 |
| | **Std Dev** | 0.166 | 0.490 | 0.557 | 0.737 | 1.929 | 0.689 | 1.184 | |
| **65Cu** | **Average** | 0.142 | 0.210 | 0.431 | 0.872 | 0.821 | 0.546 | 0.651 | < 0.001 |
| | **Std Dev** | 0.028 | 0.048 | 0.046 | 0.077 | 0.119 | 0.165 | 0.113 | |
| **95Mo** | **Average** | 0.0226 | 0.0186 | 0.0282 | 0.0478 | 0.0466 | 0.0378 | 0.0534 | < 0.001 |
| | **Std Dev** | 0.0092 | 0.0042 | 0.0045 | 0.0094 | 0.0190 | 0.0053 | 0.0125 | |
| **98Mo** | **Average** | 0.0259 | 0.0202 | 0.0316 | 0.0496 | 0.0473 | 0.0401 | 0.0583 | < 0.001 |
| | **Std Dev** | 0.0118 | 0.0047 | 0.0035 | 0.0089 | 0.0182 | 0.0082 | 0.0121 | |

Table 16. Changes of trace elements concentrations (in µg/g) in insect plasma mock samples over the course of 60 hours post most infection in the 4th instar (P values are given by one-way ANOVA)

| Trace elements | Hours post infection | 0 | 12 | 36 | 60 | P value |
|---|---|---|---|---|---|---|
| **24Mg** | **Average concentration** | 120 | 194 | 232 | 403 | 0.059 |
| | **Std Dev** | 38 | 34 | 128 | 187 | |
| **26Mg** | **Average concentration** | 115 | 199 | 237 | 403 | 0.070 |
| | **Std Dev** | 45 | 35 | 140 | 186 | |
| **55Mn** | **Average** | 0.095 | 0.111 | 0.178 | 0.267 | 0.258 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | **concentration** | | | | | |
| | **Std Dev** | 0.020 | 0.026 | 0.085 | 0.218 | |
| **56Fe** | **Average concentration** | 0.13 | 0.54 | 0.50 | 1.51 | 0.011 |
| | **Std Dev** | 0.02 | 0.22 | 0.17 | 0.85 | |
| **63Cu** | **Average concentration** | 0.053 | 0.137 | 0.165 | 0.491 | < 0.001 |
| | **Std Dev** | 0.017 | 0.030 | 0.043 | 0.147 | |
| **64Zn** | **Average concentration** | 0.216 | 0.411 | 0.558 | 3.020 | <0.001 |
| | **Std Dev** | 0.035 | 0.114 | 0.194 | 0.744 | |
| **65Cu** | **Average concentration** | 0.058 | 0.145 | 0.177 | 0.498 | <0.001 |
| | **Std Dev** | 0.028 | 0.033 | 0.052 | 0.138 | |
| **95Mo** | **Average concentration** | 0.0147 | 0.0359 | 0.0312 | 0.0502 | 0.009 |
| | **Std Dev** | 0.0116 | 0.0038 | 0.0059 | 0.0170 | |
| **98Mo** | **Average concentration** | 0.0176 | 0.0366 | 0.0327 | 0.0502 | 0.024 |
| | **Std Dev** | 0.0146 | 0.0037 | 0.0069 | 0.0174 | |

Table 17. Changes of trace elements concentrations (in µg/g) in insect plasma mock samples over the course of 60 hours post mock infection in the 5$^{th}$ instar
(P values are given by one-way ANOVA)

| **Trace elements** | **Hours post infection** | 0 | 12 | 36 | 60 | P value |
|---|---|---|---|---|---|---|
| **24Mg** | **Average** | 227 | 379 | 545 | 246 | 0.002 |
| | **Std Dev** | 61 | 71 | 71 | 105 | |
| **26Mg** | **Average** | 214 | 374 | 515 | 231 | 0.009 |
| | **Std Dev** | 84 | 100 | 70 | 118 | |
| **55Mn** | **Average** | 0.224 | 0.287 | 0.065 | 0.079 | 0.003 |
| | **Std Dev** | 0.063 | 0.087 | 0.038 | 0.027 | |
| **56Fe** | **Average** | 0.349 | 1.254 | 3.173 | 3.674 | <0.001 |
| | **Std Dev** | 0.114 | 0.335 | 0.197 | 1.092 | |
| **63Cu** | **Average** | 0.145 | 0.286 | 0.776 | 0.759 | <0.001 |
| | **Std Dev** | 0.021 | 0.039 | 0.025 | 0.158 | |
| **64Zn** | **Average** | 0.481 | 1.575 | 4.340 | 4.401 | <0.001 |
| | **Std Dev** | 0.166 | 0.382 | 0.039 | 1.048 | |
| **65Cu** | **Average** | 0.142 | 0.289 | 0.760 | 0.767 | <0.001 |

| | | | | | | |
|---|---|---|---|---|---|---|
| | **Std Dev** | 0.028 | 0.052 | 0.023 | 0.181 | |
| **95Mo** | **Average** | 0.0226 | 0.0280 | 0.0442 | 0.0532 | 0.003 |
| | **Std Dev** | 0.0092 | 0.0077 | 0.0055 | 0.0094 | |
| **98Mo** | **Average** | 0.0259 | 0.0302 | 0.0466 | 0.0602 | 0.008 |
| | **Std Dev** | 0.0118 | 0.0111 | 0.0081 | 0.0090 | |

Table 18. Changes of trace elements concentrations (in μg/g) in insect plasma with bacterial (*Micrococcus lysodeikticus*) infection samples over the course of 24 hours post infection in the 5$^{th}$ instar (P values are given by one-way ANOVA)

| Trace elements | Hours post infection | 0 | 3 | 6 | 9 | 12 | 18 | 24 | P value |
|---|---|---|---|---|---|---|---|---|---|
| 24Mg | Average | 219 | 221 | 217 | 212 | 277 | 291 | 239 | 0.737 |
| | Std Dev | 37 | 125 | 66 | 52 | 144 | 45 | 57 | |
| 26Mg | Average | 203 | 203 | 202 | 203 | 292 | 281 | 226 | 0.586 |
| | Std Dev | 45 | 136 | 77 | 49 | 153 | 38 | 70 | |
| 55Mn | Average | 0.181 | 0.201 | 0.174 | 0.148 | 0.168 | 0.183 | 0.163 | 0.968 |
| | Std Dev | 0.057 | 0.088 | 0.066 | 0.029 | 0.097 | 0.025 | 0.042 | |
| 56Fe | Average | 0.323 | 0.460 | 0.463 | 0.432 | 0.917 | 0.543 | 0.409 | 0.539 |
| | Std Dev | 0.111 | 0.364 | 0.135 | 0.158 | 0.940 | 0.044 | 0.101 | |
| 63Cu | Average | 0.139 | 0.153 | 0.151 | 0.153 | 0.333 | 0.228 | 0.186 | 0.487 |
| | Std Dev | 0.018 | 0.075 | 0.047 | 0.049 | 0.327 | 0.068 | 0.026 | |
| 64Zn | Average | 0.604 | 0.871 | 0.622 | 0.840 | 1.680 | 1.505 | 1.063 | 0.241 |
| | Std Dev | 0.179 | 0.846 | 0.303 | 0.348 | 1.367 | 0.685 | 0.120 | |
| 65Cu | Average | 0.131 | 0.149 | 0.143 | 0.144 | 0.327 | 0.225 | 0.184 | 0.412 |
| | Std Dev | 0.020 | 0.073 | 0.041 | 0.048 | 0.316 | 0.069 | 0.040 | |
| 95Mo | Average | 0.0266 | 0.0223 | 0.0261 | 0.0273 | 0.0348 | 0.0352 | 0.0306 | 0.586 |
| | Std Dev | 0.0066 | 0.0205 | 0.0088 | 0.0051 | 0.0163 | 0.0024 | 0.0074 | |
| 98Mo | Average | 0.0286 | 0.0249 | 0.0273 | 0.0296 | 0.0356 | 0.0390 | 0.0326 | 0.532 |
| | Std Dev | 0.0041 | 0.0191 | 0.0102 | 0.0030 | 0.0171 | 0.0014 | 0.0095 | |

### 2.2 Comparison of control VS. infected at each time point

The two tailed t-test statistical analysis shows that in the late 4$^{th}$ instar, Cu level in plasma from larvae with HzSNPV infection was significantly different ($p<0.05$) from that in the same aged mocks at 60 hours post infection. Table 11 gives the concentration levels of Cu in plasma from larvae in the course of HzSNPV infection

in the 4th instar, at 0, 12 and 36 hours post infection, the Cu concentration levels in infected sample were not significantly different from those in the same aged mock samples, however, at 60 hours post infection, the Cu concentration was lower than that in the same aged mocks.

In addition, there was a marginally significant difference between the Fe level in plasma from larvae with HzSNPV infection from that in the same aged mocks at 60 hours post mock infection ($p=0.08$). The concentration levels of 56Fe in the course of HzSNPV treatment in the 4th instar at 0, 12 and 36 hours post infection, the Fe concentration levels in infected sample were not significantly different from those in the same aged mock samples, but at 60 hours post infection, the Fe concentration was higher than that in the same aged mock. In the late 5th instar, Fe level in plasma from larvae with HzSNPV infection was significantly different ($p<0.05$) from that in the same aged mocks at 12 hours post mock infection. Table 10 gives the concentration levels of Fe in plasma from larvae in the course of HzSNPV infection in the 4th and 5th instar.

For all other trace elements, there was no significant difference between the concentration levels with HzSNPV treatment from that in mocks at 60 hours post infection in the 4th instar.

Mg, Mn, Fe, Cu, Zn, Mo levels in insect plasma with *Micrococcus lysodeikticus* infection at 12 hours post infection in the late 5th instar were not significantly different from those in the same aged mocks. Table 19 gives the concentration levels of trace elements of interest in insect plasma samples with bacterial (*Micrococcus lysodeikticus*) infection at 12 hours post infection in the late 5th instar and the same aged controls and P values by two tailed t-test statistical analysis.

Table 19. Concentrations (in µg/g) of trace elements of interest in insect plasma with bacterial (*Micrococcus lysodeikticus*) infection at 12 hours post infection in the 5$^{th}$ instar and the same aged controls.

| Trace elements | | Individual sample 1 | Individual sample 2 | Individual sample 3 | Average | Std Dev | P value |
|---|---|---|---|---|---|---|---|
| 24Mg | mock | 432 | 298 | 408 | 379 | 71 | 0.64 |
| | infected | 142 | 438 | 399 | 326 | 161 | |
| 26Mg | mock | 466 | 267 | 390 | 374 | 100 | 0.72 |
| | infected | 135 | 472 | 381 | 329 | 174 | |
| 55Mn | mock | 0.252 | 0.222 | 0.386 | 0.287 | 0.087 | 0.11 |
| | infected | 0.106 | 0.084 | 0.240 | 0.143 | 0.084 | |
| 56Fe | mock | 1.59 | 0.92 | 1.26 | 1.25 | 0.34 | 0.97 |
| | infected | 0.32 | 2.51 | 1.01 | 1.28 | 1.12 | |
| 63Cu | mock | 0.311 | 0.241 | 0.307 | 0.286 | 0.039 | 0.53 |
| | infected | 0.091 | 0.871 | 0.406 | 0.456 | 0.392 | |
| 64Zn | mock | 2.00 | 1.25 | 1.48 | 1.58 | 0.38 | 0.56 |
| | infected | 0.61 | 3.75 | 2.28 | 2.21 | 1.57 | |
| 65Cu | mock | 0.333 | 0.232 | 0.304 | 0.289 | 0.052 | 0.55 |
| | infected | 0.086 | 0.843 | 0.408 | 0.446 | 0.380 | |
| 95Mo | mock | 0.0366 | 0.0217 | 0.0259 | 0.0280 | 0.0077 | 0.23 |
| | infected | 0.0258 | 0.0549 | 0.0479 | 0.0429 | 0.0152 | |
| 98Mo | mock | 0.0428 | 0.0223 | 0.0255 | 0.0302 | 0.0111 | 0.19 |
| | infected | 0.0312 | 0.0553 | 0.0499 | 0.0455 | 0.0127 | |

### 2.3 Comparison of controls and infected samples of time vs. treatment at 12, 36 and 60 hours post infection

The analysis as only the matching data in a two factor GLM of time vs. treatment at 12, 36, 60 hours post infection was applied. This analysis indicates there are significant differences of Cu and Zn levels of plasma from larvae with HzSNPV infection and mocks in the 4$^{th}$ instar. Figure 4 and 5 give the Cu and Zn levels in plasma from larvae with HzSNPV infection and mocks in the 4$^{th}$ instar. In these figures, the trace elements levels are given from the average of the concentrations same aged individual samples and the error bars are given of the standard error

deviation ( $\delta_{mean}$), calculated by the equation of $\sigma_{mean} = \frac{\sigma}{\sqrt{N}}$, where $\delta$ is the standard deviation and N is the number of samples used to sample the mean.

Figure 4. Cu levels (in µg/g) in plasma from larvae with HzSNPV infection and mocks in the 4th instar

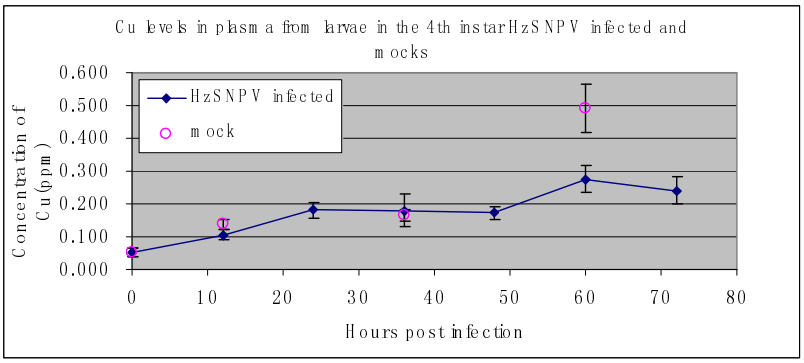
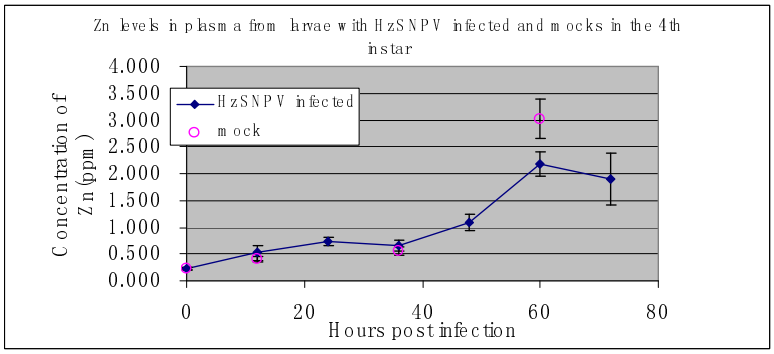



Figure 5. Zn levels (in µg/g) in plasma from larvae with HzSNPV infection and mocks in the 4th instar




The two factor GLM of time vs. treatment at 12, 36, 60 hours post infection also indicate that there are significant differences of Fe and Mo levels in plasma from larvae with HzSNPV infection and mocks in the 5th instar. Figure 6 and 7 give the Fe and Mo levels in plasma from larvae with HzSNPV infection and mocks in the 5th instar.

Figure 6. Fe levels (in µg/g) in plasma from larvae with HzSNPV infection and mocks in the 5th instar

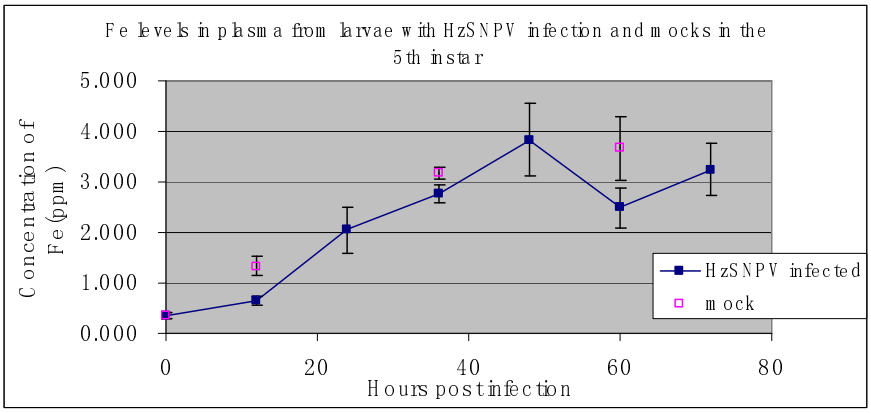
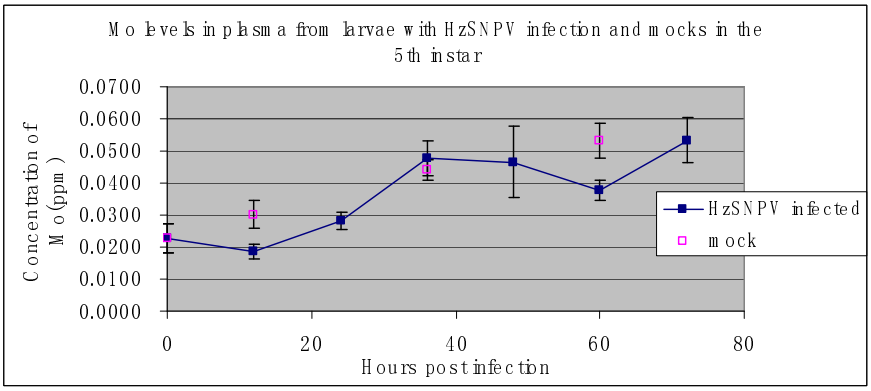



Figure 7. Mo levels (in µg/g) in plasma from larvae with HzSNPV infection and mocks in the 5th instar




In summary, there are no changes in any trace element levels in plasma from larvae due to bacterial infection and no differences between any 12 hours post infection mocks versus 12 hours post infection bacterial infected samples.

For the larvae with HzSNPV infection, in both fourth and fifth instars, all the trace elements of interest (Mg, Mn, Fe, Cu, Zn, Mo) in plasma change over the course of the 72 hours of infection in both instars. There were differences of Fe and Cu levels in plasma from larvae between the 60 hours post infection mock and virus infected larvae in the late $4^{th}$ instar, and differences of Fe levels between the 12 hours post infection mock and virus infected larvae in the late $5^{th}$ instar.

# Chapter 4

# Discussion

## 1．The trends of the concentration levels in insect plasma in the 4th and 5th instar with *Helicoverpa zea* single nucleopolyhedrovirus

Most of trace elements of interest except for iron, showed elevated trends before 60 hours and then began to drop after 60 hours in the HvSNPV infection samples in the 4th instar, which is due to the development of larvae. These larvae didn't pupate for another 2.5 to 3 days though. Uninfected larvae gain a little more weight and then slow down feeding by 84 hours after the start of the 4th instar. On the other hand, *Helicoverpa zea* single nucleopolyhedrovirus is the most effective baculovirus known for killing larval heliothines [88], including *Heliothis virescens,* the insect used for this study. The budded virus (BV) form of this baculovirus transmits infection from cell to cell within the host. At 60 hours, the infection has been transmitted in nearly every cell. As a result, the baculovirus infected larvae were also slowing down feeding around 60 hours post infection for 4th instar as they began to die. The change of trace elements in insect plasma at 60 hours post infection in the 4th instar with HzSNPV virus infection could be evidence of both the feeding habit and the sign of their death.

The insects began to moult from the 4th instar into the 5th instar around 36-48 hours post infection for the 4th instar. So the insects were already in the 5th instar at the time course of 36-72 hours post infection for the 4th instar.

The levels of Mg, Mn, Cu, Zn and Mo in larvae plasma showed elevated trends before 60 hours and then began to drop for the 4th instar with HzSNPV infection. Since the 4th instar ended around 36-48 hours post infection for the 4th instar, 60 hours post infection is actually 12-24 hours after the start of the 5th instar. The levels of Mg, Cu, Zn and Mo showed elevated trends before 36 hours and then began to drop for the 5th

instar with HzSNPV infection, which gave a good agreement for the results in the trends of trace elements in the 4th instar.

2. Discussion about iron

The iron concentration level trend was different from the other trace elements of interest in that the elevated iron levels trend kept increasing over the course of 72 hours post infection in the 4th instar with HzSNPV infection. This could be explained by the ability of ferritin to buffer intracelluar iron. The iron storage protein ferritin shows a power to buffer iron both in vertebrates and invertebrates [88, 90]. Ferritin facilitates the oxidation of Fe (II) Fe (III) at a ferroxidase site on the protein. Insects differ from vertebrates in their iron metabolism in that they rely on vacuolar rather than cytosolic ferritin for intracelluar iron buffering since vacuolar holoferritin is especially noticeable in the midgut [89]. However, the elevated iron level in insect plasma with HzSNPV treatment could be due to the free iron of damaged tissues leaking intracellular iron into the plasma. Since the ICP-MS is not able to differentiate free iron and protein binding iron, this could be my future study on iron in the insect plasma.

3. Discussion about copper

There was a significant difference of Cu levels (for 63Cu, P=0.043; for 65Cu, P=0.036) in insect plasma between HzSNPV virus infected samples and the same aged mocks at 60 hours post infection for the 4th instar. The copper levels in HzSNPV infected larvae plasma samples are lower than those in the same aged mocks, which is probably due to the decline of copper-binding protein prophenoloxidases (PPOs) levels during the course of infection. According to the study [91] by Shelby et. al, per os infection of 4th instar larvae with the HzSNPV had a mild but significant suppression effect upon hemocytic HvPPO-1 expression when compared to

expression in the same aged controls. HvPPO-1 expression was found to be relatively consistent early (0 hours -36 hours post infection) in HzSNPV infection of the 4$^{th}$ instar, but as the larvae began to molt into the 5$^{th}$ instar, at 36 and 60 hours post infection, the amount of expression began to decrease when compared to control levels. Since prophenoloxidases are the major copper binding protein in insects, suppression of their hemocytic expression results in the decline of copper levels in insect plasma at 60 hours post infection for the 4$^{th}$ instar.

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