

**Sublethal Effects of Methylmercury on the Songbird Immune Response:  
An Experimental Study**

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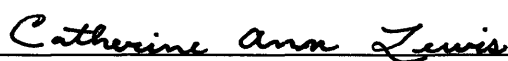
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## APPROVAL PAGE

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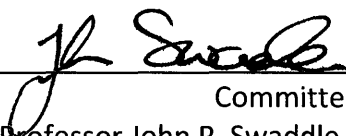
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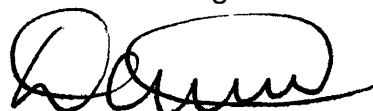
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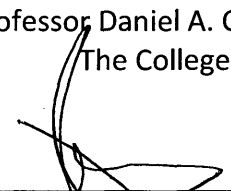
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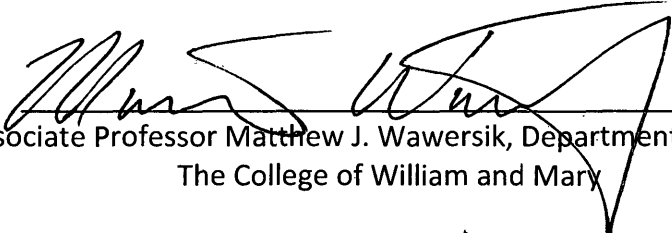
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## COMPLIANCE PAGE

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## ABSTRACT PAGE

Mercury is a ubiquitous contaminant with effects that have been documented in many wildlife species. Studies have predominantly focused on fish, fish-eating birds and mammals. This is because much historical mercury pollution is aquatic, and thus fish and their predators have an obvious route of mercury exposure. Recent studies, however, have found comparable blood-mercury levels in insectivorous songbirds. As a result, research is needed to clarify the effects of mercury on songbirds and other terrestrial organisms. One fundamental endpoint that is still poorly understood is the effect of mercury on the songbird immune response. This poses an important research question because if mercury affects the functioning of the immune system, an exposed organism may be less able to mount an appropriate immune response against invading pathogens. This is the first experimental study to test songbird immune function in response to sublethal doses of mercury. Two tests were developed: 1) a liver macrophage phagocytosis assay to quantify innate immune response; and 2) a splenic B-cell proliferation assay to measure adaptive immune response. The results from the second test suggest that mercury decreases B cell proliferation in songbirds, likely resulting in a slowing of the adaptive immune response. These techniques will help provide a more detailed, mechanistic understanding of how mercury affects immune function, thereby contributing to policies that better protect both wildlife and humans against mercury threats.

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# Chapter I.

## Introduction

### 1.1 History of mercury contamination

In the 1930's and 1940's, an industrial plant in Waynesboro, VA, owned by DuPont, leaked mercuric sulfate into the South River, headwaters of the South Fork Shenandoah River in Virginia. However, it was not until 1977, three decades after the initial contamination had ceased, that DuPont discovered high levels of mercury in both the sediment and fish downstream of the factory (Carter, 1977). In recent years, situations of this nature have become increasingly common, particularly at sites of former chlor-alkali manufacturing plants. While awareness has spread, anthropogenic activity still contributes to the release of 2,000-3,000 tons of mercury each year (Vas and Monestier, 2008) with pollution increasing in some parts of the world (Streets et al., 2005). Natural sources also contribute to mercury dispersal through degassing of the earth's crust, but anthropogenic sources are thought to be responsible for most contamination. Even remote areas such as the Arctic that lack a point source of pollution have been contaminated by mercury due to its ability to move long distances, and an estimated 92% of this mercury derives from anthropogenic sources (Dietz et al., 2009). As a result, mercury contamination has become a global problem. In order to effectively restore the Shenandoah River watershed and other regions of the world facing similar pollution problems, it is important to determine which levels of mercury contamination affect songbird survival and reproduction. This can only be accomplished

through first developing a better understanding of the effects of mercury, especially on a cellular level.

Mercury is a global contaminant that acts as a toxin to wildlife and humans alike (Boening, 2000; Holmes et al., 2009). Mercury pollution from industrial and waste disposal sources usually enters waterways in an inorganic form. Microorganisms in sediment or water can convert inorganic mercury into methylmercury (hereafter MeHg), which has a greater bioavailability to organisms such as fish and birds (Boening, 1999). Furthermore, biomagnification, or increase in concentration of a substance with each step in the food chain, will cause higher mercury concentrations in organisms at higher trophic levels (Evers et al., 2005). This means that birds, which are at relatively high trophic levels, are expected to have a higher concentration of mercury than the fish or invertebrates they eat. It is therefore not surprising that studies have reported reproductive and endocrine disruption in piscivorous songbirds (Boening et al., 2000; Scheuhammer et al., 2007; Frederick and Jayasena, 2011). However, although non-piscivorous birds such as insectivores are also exposed to MeHg, very little is known about effects on this taxa.

## **1.2 Mercury contamination in songbirds**

Recent studies demonstrate that insectivorous birds can have blood-mercury levels comparable to that of piscivorous species (Cristol et al., 2008; Rimmer et al., 2010). Correlations based on field-collected data also suggest that MeHg can have various effects on insectivorous birds including: altered singing behavior (Hallinger et al., 2009); suppressed adrenocortical response (Wada et al., 2009); decreased reproductive

success (Brasso and Cristol, 2007; Hallinger and Cristol, 2011); and suppressed immune function (Kenow et al., 2007; Hawley et al., 2009). In order to determine which contaminated sites require cleanup, it is necessary to find out the lowest levels of mercury exposure at which common biomonitors, such as songbirds, begin to experience observable adverse effects.

One significant way in which birds might be affected by MeHg exposure is through suppressed immune responses (Kenow et al., 2007; Hawley et al., 2009); however, there have not been any direct tests of whether MeHg alters immune functioning. This study intends to address this gap and study changes in immune response of songbirds with a captive dosing experiment. Suppressed immune function leads to increased susceptibility to the many parasites and pathogens to which songbirds are exposed (Kwon et al., 2008), and one study in common loons (*Gavia immer*) found a positive correlation between high mercury exposure and parasite load (Daoust et al., 1998). Immunosuppression is increasingly a danger as habitats are destroyed and songbirds are crowded closer together. Very little research has been performed on this important topic and, due to a lack of songbird immune reagents, most previous research has relied heavily on the use of crude field tests. In this work, I develop new assays of immunocompetence for songbirds so that I can directly examine the effects of mercury on the songbird immune response.

## **2.1 Field assays of immunocompetence in songbirds**

One of the main assays employed in previous research is the Phytohemmagglutinin (PHA) skin test. This test is intended to measure T-lymphocyte

function by injecting PHA, a T-lymphocyte mitogen, intradermally into the bird's wing and measuring subsequent swelling. However, it has been shown that swelling only correlates with local T-lymphocyte levels at certain times after mitogen injection and not necessarily when local T-lymphocyte levels are at their peak (Martin et al., 2006). A PHA swelling response is similar to the human delayed type hypersensitivity (DTH) response, which is distinct from other types of hypersensitivity in that it is an important component of an effective immune response.

A PHA swelling response is a very indirect indicator of immune function that, much like a DTH response, has been demonstrated to stimulate more than one cell type as well as inducing local inflammation. While T cells secrete cytokines, the central protein regulators of immune activation, it is the macrophages and heterophils that are responsible for most of the tissue inflammation associated with a PHA response (Kennedy and Nager, 2006). Macrophages and heterophils can also secrete cytokines that stimulate leukocyte proliferation and migration to the area, suggesting that these cell types also play a critical role in inducing a PHA swelling response. Thus, it is unclear exactly what aspect of the immune response is measured when using the PHA test (Kennedy and Nager, 2006).

Not surprisingly, many studies employing the PHA skin test have found ambiguous or even conflicting results (Kenow et al., 2007; Hawley et al., 2009). For example, one 105-day, experimental, dose-response study in which juvenile common loons were administered rainbow trout containing a gelatin capsule with a MeHg dose of 0 $\mu$ g/g, 0.08 $\mu$ g/g, 0.4 $\mu$ g/g, or 1.2 $\mu$ g/g (ww), found no effect of MeHg on PHA response

(Kenow et al., 2007), while a second study, comparing PHA response in free-living tree swallows (*Tachycineta bicolor*) feeding in contaminated versus reference sites observed a significantly suppressive effect (Hawley et al., 2009). Just as the human DTH response is in some cases beneficial and in others detrimental (due to tissue damage), greater levels of PHA swelling are difficult to interpret in terms of their immunological significance.

A second commonly used field assay is the sheep red blood cell (SRBC) assay, which measures antibody titer as a proxy for humoral, or B cell, immunity. An injection of a novel antigen is used to produce a primary immune response, including T cell-dependent activation, proliferation of B lymphocytes and secretion of antibodies. Often a second injection is used to stimulate a secondary immune response. Antibody secretion during both responses is measured by adding SRBCs to serially diluted blood plasma, and an enzyme-linked immunosorbent assay (ELISA) is used to evaluate relative antibody titers. Much like the PHA skin test, studies using this assay in songbirds have provided ambiguous results that are difficult to interpret on the cellular or organismal level (Saks et al., 2006; Kenow et al., 2007; Hawley et al., 2009). The PHA skin test and SRBC assay exemplify many of the shortcomings of other crude tests used in previous studies such as measures of immune organ cellularity, white blood cell counts, and bacterial killing assays.

## **2.2 Cellular based assay of innate immunity: macrophage phagocytosis**

Recent studies have suggested several new approaches to assessing immune system strength. One aspect of immune function that is a sensitive indicator of the

immunotoxicity of environmental contaminants is phagocytosis (Fournier et al., 2000). This indicator may be of particular importance as a decrease in phagocytosis has been implicated in several human diseases including Alzheimer's disease and Human Immunodeficiency Virus type 1 (HIV-1). *In vitro* tests have shown that Alzheimer's disease may be caused in part by the inability of brain macrophages to clear amyloid plaques through phagocytosis (Zhang et al., 2006). It has also been shown that macrophages in patients with HIV-1 have a decreased ability to phagocytose apoptotic bodies. This could contribute to the disease's characteristic increase in inflammation and incidence of opportunistic infection (Torre et al., 2002). Thus, the abnormal functioning of phagocytic cells suggests a higher likelihood or susceptibility to disease.

It has been suggested that both inorganic and organic forms of mercury may have an effect on the phagocytic ability of immune cells (Christensen et al., 1993; Finkelstein et al, 2008). When an organism is exposed to the particulate form of heavy metals through peritoneal injection, phagocytic cells accumulate these metals in high concentrations due to their function of engulfing foreign particles (Cherdwongcharoensuk et al., 2002). The resultant effect on these phagocytic cells is the subject of current study. In free-living albatrosses (*Phoebastria nigripes*) there is a negative correlation between level of mercury exposure and the number of macrophages that phagocytose intermediate amounts of yeast bioparticles (Finkelstein et al. 2008). Experimental studies suggest a similar relationship between mercury exposure and phagocytosis. Mercury added to primary cultures of peritoneal murine macrophages inhibited phagocytosis and migration (Christensen et al., 1993). In

addition, white blood cells isolated from chickens (*Gallus gallus*) displayed a decrease in phagocytic ability when exposed to mercury (Holloway et al., 2003). However, when whole blood was exposed to mercury, no difference in phagocytic ability was observed. The authors suggest that this may be due to the fact that whole blood contains red blood cells and proteins that may bind and sequester mercury, thus lessening its effects on white blood cells (Holloway et al., 2003).

Even though the data are inconclusive, the ability of macrophages to phagocytose is an important metric as macrophages are the first line of defense against pathogens, and mercury can be taken up by macrophages (Finkelstein et al., 2008; Cunha et al., 2003). One study also found a strong correlation between high phagocytic ability and increased specific antibody production, suggesting that phagocytic ability may be a good indicator of overall disease resistance (Sun et al., 2008).

### **2.3 Cellular based assay of adaptive immunity: Lymphocyte proliferation**

A second common metric of immunocompetence is a lymphocyte proliferation assay, which measures the strength of the humoral immune response (B-cells) or the cell-mediated response (T-cells). The ability of lymphocytes to proliferate rapidly in response to an antigen is a key indicator of immune strength. One study found increased T lymphocyte proliferation in birds with higher organochlorine levels, suggesting that contaminants may induce hypersensitivity to antigens, at potentially high cost to the individual (Finkelstein et al., 2007). However, it is also possible that mercury may act as an immunosuppressant such that increased levels of mercury result in decreased lymphocyte proliferation. For example, when MeHg chloride (0 $\mu$ M,

0.31 $\mu$ M, 0.63 $\mu$ M, 1.25 $\mu$ M, 2.5, 5 $\mu$ M, 10 $\mu$ M, 20 $\mu$ M, and 40 $\mu$ M) was added *in vitro* to cell cultures of peripheral blood leukocytes (PBLs) isolated from harbor seals, mitogen-stimulated T cell proliferation decreased (Das et al., 2008). Interestingly, this study also reported a decrease in Interleukin-2 (IL-2) production and an increase in IL-4, suggesting a shift toward a Type 2 helper T cell response (Das et al., 2008). The Type 2 helper T cell (Th<sub>2</sub>) response is important in fighting bacteria but thought to be ineffective against intracellular pathogens such as viruses, meaning mercury could increase an organism's susceptibility to these pathogens. A Th<sub>2</sub> response is also often indicative of an allergic reaction.

Some sources suggest that mercury might be expected to have a more direct effect on helper T cells than B cells. This is because mercury-exposed macrophages may become activated and in turn activate T cells. Mercury exposure may also modulate cytokine production, which would more indirectly affect B cell survival and proliferation (Vas and Monestier, 2008). Indeed there is also evidence to suggest an effect of mercury on B cells although it is unclear whether this is the direct effect of exposure. For example, one study in loggerhead sea turtles (*Caretta caretta*) found that B cell proliferation decreased in response to both naturally occurring elevated levels of mercury and mercury added to PBLs in culture (Day et al., 2007). In addition, current research demonstrates that teleost and amphibian B cells are phagocytic and mammalian B cells, while not phagocytic, most likely evolved from a phagocytic predecessor (Li et al., 2006). Thus, B cells in these ancestral vertebrates may be directly exposed and affected by especially high levels of mercury, much like other phagocytes.

Therefore, while there have been differences reported between the response to mercury exposure by B and T cells, the molecular mechanisms behind these differences is unclear.

#### **2.4 Interpreting measures of immunocompetence**

Recent reviews of measures of immunity in wildlife stress the importance of conducting multiple assays of immune function (Adamo, 2004; Salvante, 2006). Because the immune system is highly integrative and employs many cell types to eliminate the large diversity of potential invaders, a decrease in one aspect of immune function may not necessarily indicate a significant decrease in disease resistance. Conversely, it is also possible that an investigator examining only one cell type or aspect of immune function may overlook a serious effect of a toxin on a different aspect of immune functioning that affects immune strength. Even when using multiple tests, it is important to interpret the outcome of these tests with caution. The immune system may reallocate resources in order to successfully fight an invader without a significant cost to disease resistance. Only when multiple tests are employed and have indicated a decline can the investigator conclude that the toxin may have immunosuppressive effects (Adamo, 2004).

#### **3.0. Molecular mechanisms underlying putative mercury effects**

Many of the molecular mechanisms by which mercury affects an organism are poorly understood. This is due in part to the fact that as a cation, mercury can bind to a large range of ligands such as sulfhydryl, carboxyl, amino and phosphate groups, thus potentially affecting almost every aspect of cellular functioning and making it difficult to

identify specific mechanisms involved (Clarkson, 1987). While mercury may interact with any of these functional groups, MeHg has an especially high affinity for sulfhydryl groups, thus potentially affecting the function of any sulfhydryl-containing enzyme or compound (Clarkson, 1987). This includes the majority of immunoregulatory proteins as well as important immune cell receptors such as CD4, CD3, and CD45, to which MeHg may bind, causing receptor aggregation. This may affect lymphocyte signal transduction pathways, which could also affect lymphocyte proliferation (Vas and Monestier, 2008). Another well-established effect of mercury is its ability to bind sulfhydryl groups on tubulin subunits, thereby inhibiting polymerization (Miura et al., 1984). Polymerization is required for formation of microtubules, and impaired microtubule formation likely leads to the inhibition of cell division. Microtubules are also critical to cellular trafficking, and thus mercury-exposed macrophages may have a depressed ability to degrade ingested material. Through these mechanisms, mercury may affect lymphocyte signal transduction as well as proliferation and pathogen processing. This could have an especially profound suppressive effect on the organism's ability to mount an immune response.

Mercury may also modulate normal levels of apoptosis. Because the dynamism of microtubules is critical to the equal distribution of genetic material during mitosis, mercury has been indicated in the formation of micronuclei, a common correlate of genotoxicity that leads to cell death (Crespo-López et al., 2009). In addition, one study that used flow cytometry and TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) assays using markers to identify cells with DNA characteristic of apoptosis,

suggested that MeHg may exert immunosuppressive effects by inducing apoptosis in resting T lymphocytes (Shenker et al., 1997).

#### **4.0 Avian immune organs**

The coordinated actions of cells within many organs of the body are necessary for any organism to mount an effective immune response. Not surprisingly, organs and cell types involved in nonspecific innate immunity tend to be conserved across organisms. These organs include epithelial surfaces, the intestine and the liver. The liver will be the focus of discussion because it accumulates high levels of mercury in birds (Boening, 1999; Evers et al., 2005). The liver synthesizes complement proteins, which have many functions including lysis of pathogens, induction of chemotaxis of cells towards the site of injury, and opsonization, or coating, of pathogens to increase the ability of phagocytes to engulf them.

Cell types of the innate immune system include phagocytes such as neutrophils, or heterophils in birds, and macrophages. One critical role these cell types play is to initiate inflammation, an organism's primary response against infection and tissue damage. Characteristics of this response include vasodilation, or dilation of blood vessels in order to increase blood flow and arrival of new leukocytes to the region of injury, as well as extravasation, the exit of leukocytes from the blood stream into tissues. These leukocytes include phagocytes which engulf and lyse foreign invaders, leading to release of the invader's surface molecules into the bloodstream, thus alerting other immune cells. Innate immune cells induce the inflammatory response, in part, by secreting cytokines, the central proteins that regulate activation of the immune system.

Chemokines, a specific class of cytokines, attract leukocytes to the region of infection and aid in extravasation.

In comparison with the innate immune response, the adaptive immune system is not well conserved across vertebrates. Thus avian immune organs differ from those of many other organisms. In most mammals, B cells develop in the bone marrow (Abdou and Abdou, 1972), whereas in birds, B cells develop in the organ where B cells were first discovered, the bursa of Fabricius (Glick, 1956, Cooper et al., 1966). After maturation, B cells migrate to secondary immune organs. Primary among these in all vertebrate species is the spleen, the site where immune cells are activated in response to systemic infection. Unlike primates and rodents, however, chickens do not have organized lymph nodes (although ducks do, see Lundqvist et al., 2006), which is the tissue where immune cells are activated in response to local antigen (John, 1994; Bar-Shira et al., 2002).

Birds' secondary lymphoid organs include the spleen, caecal tonsils and Peyer's patches, both located in the small intestine, and Meckel's diverticulum, a remnant of the connection between the yolk-sac and the small intestine (Bar-Shira et al., 2002; Khan et al., 2007). Both caecal tonsils and Peyer's patches contain organized regions of lymphocytes known as follicles. In mammals and birds, activated primary follicles (but see Yasuda et al., 2003) become larger secondary follicles which surround a germinal center (GC) of activated, proliferating B cells and helper T cells. These germinal centers are necessary for the development of memory cells. Mammals and birds are the only organisms known to have GCs. Peyer's patches and caecal tonsils contain multiple

follicles, which are covered by M cells, phagocytic cells that engulf antigen from the intestinal lumen. Upon exposure to antigen, lymphocytes of Peyer's patches and caecal tonsils are activated and enter the bloodstream.

Although it is not considered a secondary lymphoid organ, the Harderian gland (HG), located near the eye, also plays a role in immune defense as it contains high concentrations of antibody-producing plasma cells as well as macrophages. Antibodies secreted by plasma cells in the HG bind with a secretory component produced by epithelial cells. This allows them to be secreted into the lumen of the HG and released into the affiliated duct system (Khan, 2007). Scott (2004) proposed that the Harderian gland may be the source of Interleukins IL-4 and IL-5, two cytokines important in antibody class-switching that have yet to be isolated in birds. This is because, among other reasons, the HG is the source of IL-6, an important mediator of the inflammatory response, and it also supports immunoglobulin class-switching.

### **5.0 Liver immunology: Kupffer cells**

The liver plays a crucial role in metabolism, bile secretion and detoxification. Up to 80% of the mass of the liver is made up of hepatocytes, a cell type critical to metabolizing, storing and biotransforming toxins (Ishibashi et al., 2009). The liver also contains the body's largest population of tissue macrophages and natural killer cells (Nemeth et al., 2009). This indicates that the liver also plays a critical role in the innate immune response. In fact, although it is not their primary function, hepatocytes also make important contributions to the immune response (Ishibashi et al., 2009). Within the adaptive immune system, the liver is important for trapping and removal of

activated T cells by inducing apoptosis through neglect, as the liver lacks the necessary survival signals. Similarly, the liver is important in induction of tolerance to self-antigens through its lack of co-stimulatory signals and low number of helper T cells, which are both necessary for activation (Parker and Picut, 2005). The roles of the liver in the innate immune system, which will be the primary focus here, include nonspecific cell killing by natural killer cells, initiation of the inflammatory response, and phagocytosis by the liver's resident macrophages known as Kupffer cells (Ishibashi et al., 2009).

The location and structure of the liver suggest its important role in the body's immune response. Because the portal vein delivers blood directly to the liver from the kidney, spleen and pancreas, the liver is exposed to a wide range of pathogens (Nemeth et al., 2009). Thus, it is critical that the liver contain cells necessary to recognize and fight pathogens as well as respond to systemic infection. In addition, these cells must be arranged such that they will be exposed to the blood. The liver lobules are made up of rows of hepatocytes, which are separated from sinusoidal blood vessels by the Space of Disse. These blood vessels are characterized by their fenestrated endothelial lining, which allows for exchange of solutes such as sugars and lipids (Ishibashi et al., 2009). In addition, the endothelial tissue contains Kupffer cells, which can take up large particles from the blood, including antigens (McCuskey et al., 1987).

Studies have shown that Kupffer cell populations are heterogeneous in function and size (Parker and Picut, 2005; Nemeth et al., 2009). Intermediate Kupffer cells tend to be the most phagocytic while small Kupffer cells do not have high phagocytic capabilities but tend to be responsible instead for production of  $\text{TNF}\alpha$ , a pro-

inflammatory cytokine, and reactive oxygen species (ROS). Large macrophages, on the other hand, secrete antimicrobial substances as well as anti-inflammatory interleukins such as IL-6 and IL-10 (Nemeth et al., 2009). The location and density of different Kupffer cell types also varies throughout the liver, likely based on local function. For example, the periportal region of the liver, the first region directly exposed to incoming blood, contains a large number of highly phagocytic Kupffer cells (Parker and Picut, 2005).

As evidenced by the fact that Kupffer cells can produce both pro- and anti-inflammatory cytokines, these macrophages play an important role in liver homeostasis. Because the liver is constantly exposed to antigen in the bloodstream, Kupffer cells in a healthy liver usually play an “immunosuppressive role” (McCuskey et al., 1987; Nemeth et al., 2009). For example, when Kupffer cells are exposed to lipopolysaccharide (LPS), a cell membrane component common to all gram-negative bacteria, both the inflammatory cytokine TNF $\alpha$ , and the anti-inflammatory cytokine IL-10, are released. This leads to a decrease in receptor-mediated uptake of antigen by antigen-presenting cells and a subsequent decrease in T cell activation in the liver (Ishibashi et al., 2009). However, in cases where an immune response is appropriate, the release of cytokines such as IL-6 from Kupffer cells can activate neighboring hepatocytes to secrete acute phase proteins, which increase a systemic or local inflammatory response (Ishibashi et al., 2009). Because of this ability of Kupffer cells to signal an inflammatory response as well as release cytotoxic substances, dysregulation of this signaling can lead to serious liver damage.

### **5.1 Liver Immunology: the molecular mechanisms of Kupffer cell phagocytosis**

Phagocytosis, or the cellular uptake of large insoluble particles, is a critical component of development, tissue remodeling, and the immune response. Many cell types, including hepatocytes, endothelial and epithelial cells, can phagocytose at low levels (Hart et al., 2007). However, macrophages are highly specialized phagocytic cells and therefore do so at much higher rates. Elucidating the mechanism of phagocytosis has been difficult in part because macrophages contain multiple receptors, which likely participate in complicated cellular communication. In addition, each receptor uses a slightly different mechanism to effect phagocytosis (Aderem and Underhill, 1999).

Macrophages contain multiple receptors known as pattern recognition receptors (PRRs), which recognize the highly conserved pathogen-associated molecular patterns (PAMPs) on pathogens. For example, Mannose receptors recognize the Mannans found in yeast cell walls, whereas scavenger receptors recognize common components of bacterial membranes. Pathogens may also be opsonized by immune molecules such as antibodies, which enhances recognition and uptake by macrophages. This interaction occurs through contact between the constant, Fc region on antibodies and an Fc receptor on the macrophage. Binding of antibody to Fc receptors leads to phosphorylation of the cytosolic domain (Aderem and Underhill, 1999), which in turn causes recruitment of factors involved in plasma membrane dynamics and actin remodeling (Booth et al., 2001).

The classical model of pseudopod formation relies on actin remodeling in response to ligand binding. As new Fc receptors make contact with antibodies, actin

polymerization allows the membrane around the particle to “zip” closed (Henry et al., 2004). Once the particle is surrounded, it is contained within a vacuole known as the phagosome. Actin is shed and phagosome trafficking is mediated by microtubules (Aderem and Underhill, 2001). This model, however, is far from complete. Plasma membrane dynamics are also critical to pseudopod formation and phagocytosis. Studies showing that macrophages can engulf up to 100% of their surface area, without experiencing a decrease in their own surface area, suggest that exocytosis of membrane from internal compartments could also play an important role in phagocytosis (Booth et al., 2001).

In addition, inhibition of proteins involved in intracellular trafficking and vesicle fusion leads to a decrease in phagocytosis. This model suggests that ligand binding not only leads to actin remodeling, but it also triggers exocytosis directed toward sites of phagocytosis. This would provide new membrane as well as the proteins necessary for phagocytosis and phagosome maturation (Booth et al., 2001). There may be additional motor molecules involved as well. For example, myosin has been shown to co-localize with actin, suggesting it may also play a role in uptake (Aderem and Underhill, 2001). As the best characterized mechanism, Fc receptor-mediated phagocytosis is often used as a model, but it is possible that the relative importance of different molecules varies by receptor. The “zippering” mechanism that requires contact between an Fc receptor and an antibody is clearly not possible for a receptor engulfing a non-opsonized particle, and it has been suggested that myosin may play an important role in these receptors (Swanson et al., 1999).

What is clear is that ligand binding and phagocytosis requires the coordination of multiple proteins and motor molecules. Regardless of the mechanism, uptake of particles leads to phagosome formation. The phagosome likely matures through interaction with different endocytic vesicles leading to eventual fusion with the lysosome where its contents are degraded (Henry et al., 2004). Maturation can include a change in vesicle components as well as membrane composition and is usually driven by the presence of different small GTPases known as Rabs (Aderem and Underhill, 2001).

### **6.0 Bursal immunology: B cell development**

B cells are the central aspect of humoral immunity in all vertebrates. The ability to produce a great diversity of B cell receptors or membrane immunoglobulin is crucial to an organism's ability to recognize and ultimately destroy pathogens. While rodent and primate immunology has been studied extensively, avian immunology differs in some important ways and is less understood. In addition, B cell development and mechanisms for generating receptor diversity may vary greatly even within a class, as exemplified by the different strategies of the class Mammalia (Flajnik, 2002). Thus the current understanding of avian immunology, which is based almost solely on one of the earliest orders of birds to evolve, the *Galliformes*, may differ greatly from that of a taxon that branched off more recently such as songbirds (*Passeriformes*) (Tizard, 2002). In addition, chickens are a precocial species (fast chick development) and thus have developed most of their immune system before hatching whereas altricial species, which includes *Passeriformes*, may have a very different mode of development (Tizard,

2002). New model species are needed to better understand different orders of birds.

The following discussion of avian immunology is based predominantly on chickens.

In rodents and primates, B cells are produced continuously from hematopoietic stem cells in the bone marrow throughout the organism's life. Birds, by contrast, only produce new B cells up through the first few weeks of life (Flajnik, 2002). B cell production begins in the bone marrow, fetal liver, and yolk prior to hatch, with subsequent migration to the bursa (Scott, 2004). Much like the thymus, the site of T cell development, the bursa is known to atrophy after 6 months in chicken. While birds, rodents and primates alike must rearrange segments of the immunoglobulin gene in order to express surface immunoglobulin and develop into a mature B cell, the timing and purpose of rearrangement differs greatly in the two models (Sayegh et al., 2000).

After progenitor B cells are produced, rearrangement of the immunoglobulin gene occurs in both the avian and classical mammalian models of B cell development. In rodents and primates, rearrangement generates a large amount of receptor diversity, occurring in two steps (heavy chain and then light chain) and resulting in production of an immature B cell expressing the surface immunoglobulin, IgM. First, negative selection occurs, in which cells that bind too strongly with self-antigen in the bone marrow, or do not express IgM, undergo apoptosis. Then, immature B cells leave the bone marrow and migrate to secondary organs such as the spleen and the lymph nodes. Because some self-antigen may not have access to the bone marrow, further negative selection may occur in the secondary immune organs and the blood. In order for the B

cell to respond to foreign antigen, it must first become a mature B cell, undergoing alternative splicing of RNA to express both IgM and IgD.

In birds, rearrangement of both the heavy and light chain immunoglobulin genes occurs in one step (Pickel et al., 1993), during which B cells begin to colonize the bursa of Fabricius, the site of B cell development and hematopoiesis (Sayegh et al., 2000). In contrast to the rodent and primate model, rearrangement does not generate a significant amount of receptor diversity and is not an ongoing process (Weill et al., 1986). The rearranged immunoglobulin gene merely serves as a substrate upon which gene conversion later acts (Flajnik, 2002).

It is unclear whether rearrangement commits cells to the B cell lineage or whether commitment precedes rearrangement. Research suggests, however, that it is likely that commitment to the B cell lineage occurs prior to entry into the bursa (Koskela et al., 2003). This, taken with the fact that completed gene rearrangement does not appear to be a requirement for entry into the bursa (Ratcliffe, 2006), suggests that B cell commitment occurs before gene rearrangement. The cause of migration of precursor B cells into the bursa is unknown, but one possibility is that dendritic cells enter the bursa first, releasing chemokines that recruit B cells (Ratcliffe, 2006). Once in the bursa, B cells that have completed rearrangement and express IgM on their surface will form follicles of 2-5 cells and begin to proliferate rapidly (Sayegh et al., 2000).

While these precursor B cells are proliferating rapidly, gene conversion begins (Ratcliffe, 2006). Gene conversion is the introduction of new sequences of nucleotides within the immunoglobulin genes using a homologous pseudogene as a template (Scott,

2004). The immunoglobulin genes in birds contain multiple pseudogenes that “donate” sequences of DNA to the coding region downstream (Flajnik, 2002). Once the immunoglobulin in a B cell has undergone gene conversion, the cell is considered an immature B cell. Before hatching, B cells that have differentiated into mature B cells will begin to migrate to the periphery at low levels. The mechanisms involved in B cell maturation and subsequent migration are not well defined (Sayegh et al., 2000). One factor that may be required for B cell maturation is B cell activating factor (BAFF). Studies have suggested that as the cellularity of the bursa increases, BAFF is upregulated in monocytes, dendritic cells, and B cells, acting on B cells in both a paracrine and autocrine fashion to induce maturation of immature B cells and to control migration to the periphery (Koskela et al., 2003; Koskela et al., 2004).

For reasons that are not well understood, after hatching, a massive amount of cell death occurs in the bursa, accompanied by an increase in migration of B cells to the periphery. Only about 5% of cells produced each day leave the bursa (Sayegh et al., 2000; Koskela et al., 2003). One proposed explanation is that gene conversion causes genes to be out of frame, incapable of being transcribed correctly. However, there is strong evidence to suggest that up to 98% of gene conversion events maintain the original reading frame, making this explanation unlikely (Sayegh et al., 2000; Ratcliffe, 2006). A more compelling argument is that gene conversion of the separate heavy and light chains that compose an immunoglobulin creates two components that cannot interact to form a functional receptor. B cells that cannot express immunoglobulin will be deleted from the repertoire (Ratcliffe, 2006).

Another factor that may be important in the final stages of B cell development is exposure to antigen. Pro-apoptotic genes are upregulated during this time (Koskela et al., 2003) likely leading to deletion of self-reactive B cells (Tizard, 2002; Koskela et al., 2003). In addition, studies suggest that after hatching, bursal epithelial cells, which separate B cell precursors from the gut, differentiate into cells similar to the M cells of Peyer's patches (Sayegh et al., 2000). This allows immature B cells of the bursa to be exposed to gut antigen, stimulating B cell maturation through selection of cells expressing immunoglobulin. This could occur either through specific binding of the immunoglobulin to its antigen or non-specific superantigen binding. In order for birds to generate B cells with the ability to recognize non gut-derived antigen, it is likely that gene conversion would continue to diversify receptors after this ligand-binding step (Ratcliffe, 2006).

### **7.0 Spleen immunology: B cell activation**

The spleen is an important secondary lymphoid organ, crucial to the ability of the body to respond to systemic infection. Mammalian spleens are made up of two regions: red pulp, containing a reservoir of erythrocytes and white pulp, made up of white blood cells. Avian spleens tend to be smaller in relation to body size than mammalian spleens, and they lack a reservoir of erythrocytes (John, 1994). Avian spleen size may vary depending on age, sex and time of year. For example, the spleen of willow tit (*Parus montanus*) increases in mass up to age one month, after which the spleen decreases in size. In addition, adult willow tits often have larger spleens during molt in late summer

when the skin is damaged and the organism is particularly susceptible to infection (Silverin et al., 1999).

Because birds do not have lymph nodes, the spleen may be even more important in generating an immune response to blood-borne antigens (John, 1994). The white pulp in both mammalian and avian spleens forms a periarteriolar lymphoid sheath (PALS), made up of mostly T lymphocytes, which surrounds the splenic artery. Much like Peyer's patches and caecal tonsils, the avian spleen also contains germinal centers with proliferating lymphocytes surrounded by secondary follicles (Yasuda, 2003). Germinal centers are usually associated with the development of immunological memory as well as affinity maturation, which is caused by specific mutations that lead to the increased affinity of a B cell receptor for a specific antigen. The presence of germinal centers in birds suggests that avian immunoglobulin undergoes affinity maturation, but the strength of this increase in affinity is disputed (Flajnik, 2002). Gene conversion is thought to continue in germinal centers (Flajnik, 2002), and immunoglobulin class switching from IgM to one of the other two classes of antibody present in birds, IgA and IgY (thought to be the ancestor of mammalian IgG and IgE), has been demonstrated to occur in the avian spleen as well (Yasuda, 2003).

### **8.0 Pax5: a critical regulator of B cell development and function**

Because, in many cases, molecular markers specific to non-mammalian immune cells are unavailable, antibodies against well-conserved transcription factors have also been used. One previously characterized B cell marker is the paired box gene 5 (Pax5), which, within the immune system, is B cell specific (Adams et al., 1992). An antibody

against Pax5 has already been developed (Zwollo et al., 1998), and was used as a B cell marker for this research.

While the transcription factors E2A and early B cell factor (EBF) initiate commitment to the B cell lineage, transcription of Pax5 is required for irreversible commitment to the B cell lineage (Nutt et al., 1999). In fact, in the absence of Pax5 expression, progenitor B cells may instead be able to differentiate into various other cells such as macrophages, dendritic cells or natural killer cells. Restoration of Pax5 expression also restores commitment to the B cell lineage (Nutt et al., 1999). Pax5 continues to be expressed up until the final stage of terminal differentiation and is absent in plasma cells, suggesting that this transcription factor is also involved in maintaining the B cell population. Pax5 is responsible for activation of up to 170 different genes involved in B cell development and function (Hagman and Lukin, 2007). The gene targets of Pax5 encode for a wide range of proteins including cell-surface receptors and signal transducers as well as genes involved in cell-cycle regulation, protein trafficking and possibly cell migration and adhesion (Hagman and Lukin, 2007).

During B cell activation, downregulation of Pax5 is necessary for terminal differentiation into plasma cells. While Pax5 is one of the main inhibitors of terminal differentiation, transcription factors B lymphocyte-induced maturation protein 1 (Blimp-1) and X-box binding protein 1 (XBP-1) are key promoters of plasma cell differentiation (Nera and Lassila, 2006). Interactions between B cell transcription factors have been studied in both mice and the chicken B cell lymphoma line, DT40, which is most similar to the bursal B cell stage in terms of gene expression (Koskela et al., 2003). Results from

these studies suggested that B cell transcription factors in the murine versus avian model may interact differently in order to regulate B cell activation, but both models require Pax5 expression for B cell development and downregulation of Pax5 for terminal differentiation (Nera and Lassila, 2006).

## **9.0 Specific aims**

The overarching goal of this project was to establish the lowest level of MeHg exposure that causes detectable effects on the immune system of European starlings (*Sturnus vulgaris*) and Australian zebra finches (*Taeniopygia guttata*). Starlings were chosen as a model species because they survive well in captivity, and they are insectivorous in the wild, thus potentially accumulating moderate levels of mercury. Zebra finches are a commonly used model species because they breed rapidly in captivity (Burley and Calkins, 1999) and their genome has been sequenced. Both are songbirds in the family Passeriformes.

In order to determine the lowest level of mercury that will have effects on these representative songbirds, it is necessary to identify the possible sublethal effects mercury may have on the immune system so that ultimately it is possible to state which MeHg blood or dietary levels truly have no discernible effects. Thus the primary focus of this project was to identify which aspects of the immune system are affected by MeHg exposure.

The immune system is very complex and has many different types of cells with diverse functions. In order to begin investigating possible effects of MeHg on the

immune response, two tests were developed here. MeHg may differentially affect certain cell types or functions. The two questions that were addressed are as follows:

- (1) Does chronic exposure to sublethal levels of MeHg affect the ability of songbird macrophages to phagocytose bioparticles?
- (2) Does chronic exposure to sublethal levels of MeHg affect the ability of songbird B-cells to proliferate *in vitro* in response to the B cell mitogen LPS?

Each of these questions is a first step in addressing one part of the immune response.

Macrophage phagocytosis is an assay of the nonspecific, innate immune system. The B-cell proliferation assay is a test of the adaptive immune response. While these are not comprehensive tests of the innate or adaptive immune response, any observed effects of mercury would suggest that this branch of the immune system merits further testing.

## Chapter II.

### Methods

#### Animals, treatment groups, and general housing

##### *Zebra finches*

Outbred zebra finches (*Taeniopygia guttata*) were housed in an indoor aviary with one breeding pair per cage. Pairs were formed randomly from the large (> 300 individuals) colony. If repeated breeding attempts did not produce offspring, those birds were re-paired with others. The experimental pairs of zebra finches (N = 38 pairs) were randomly assigned to the following treatment groups, that varied in how much mercury they received in their diet: (a) a control group (N = 10 pairs) that received *ad libitum* “fruity pebbles” (ZuPreem) food with 0ppm of Hg; (b) a group that received the same food with 0.5 ppm Hg mixed in (N = 14 pairs); and (c) a group that received 1.0 ppm Hg (N = 14 pairs). For both of the latter groups (b and c), Hg was delivered in the form of MeHg cysteine (made from a two-fold molar excess of cysteine in a degassed solution) for half of the pairs or in the form of MeHg chloride for the other half. Hence, there were five treatment groups with 7 pairs in each experimental group and 10 pairs in the control group.

Food was prepared by diluting the appropriate concentration of either MeHg chloride or cysteine in 60 ml water and adding half of this stock solution to 0.27 kg of food. Food was then mixed thoroughly. Mercury content of food was measured using a direct mercury analyzer (DMA-80, Milestone), and batches of food were tested weekly

to ensure accurate doses throughout the experiment. The method for mercury analysis has been described previously (Ramos-Varian et al., 2011).

All zebra finches were kept in cages (45 x 45 x 75 cm) with ample perches, *ad libitum* drinking water, a cuttle bone, a plastic nest box and excess nesting material. These birds were housed at approximately 20-23°C on a 14:10 light: dark photoperiod and allowed to breed until they produced a successful clutch (at least 1 fledgling). However, their first clutch had been collected after completion so they produced two clutches. Birds were sacrificed through rapid decapitation, 60 days after the eggs from this second clutch hatched. These sacrifices occurred 24-32 weeks after mercury exposure. Juvenile males were sacrificed 45 days after hatch. Birds that failed to successfully reproduce (N = 4 pairs) were sacrificed after 32 weeks of dietary mercury exposure.

#### *European starlings*

Twelve wild-caught adult European starlings, of undetermined age and sex, were housed in outdoor aviaries (2.5 x 2.5 x 3 m) from December through April and had access to ample perches, a water bath, and *ad libitum* drinking water and food. Starlings were randomly assigned to one of two treatment groups, 0.1 ppm and 0.4 ppm MeHg chloride. Starter-grower crumbles food (Bartlett) was prepared as above with larger volumes. Mercury content of food was measured using the DMA-80, and batches of food were again tested weekly. Each starling was sacrificed through rapid decapitation after 21 weeks of mercury exposure.

## **Blood sampling and dissection**

### *Blood sampling*

Blood samples were taken once every three weeks from all birds. Blood was taken from the brachial vein using 75  $\mu$ l heparinized capillary tubes and 30-gauge needles. In accordance with standards for animal care, no more than 1% of each bird's body weight in blood was drawn (Walberg, 2001). Zebra finches in the aviary range from 13 – 17 g, and therefore no more than 0.13 g or about 130  $\mu$ l of blood was drawn per week. Starlings weighed 80 – 100 g and so up to 500  $\mu$ l could be taken. Blood samples were spun at 250g for 10 minutes, at 4°C, and serum was removed and stored at -20°C for Western blots. Separate samples of whole blood were analyzed for mercury level using the DMA-80 in the same manner as mercury levels were analyzed in food.

### *Spleen and liver dissection*

Lymphocytes and Kupffer cells were isolated from the spleen and liver of zebra finches 60 days after the second clutch was laid. Starling organs were collected 21 weeks after mercury dosing began. Nine non-dosed reference samples were also collected to assess proliferation on day 1 versus day 2 in zebra finches. Dissected organs were immediately placed in 5 ml cold RPMI-1640 (Invitrogen Life Technologies) and macerated with a syringe (Becton Dickinson). The cell suspension was drawn up and released from the syringe 10-15 times before being forced through a 40  $\mu$ m cell strainer (BD Biosciences) into a 50ml conical tube (BD Biosciences) on ice. Cold RPMI-1640 was added up to 10ml in each sample. Cells were spun at 250g for 10 minutes and supernatant was removed. Liver cell pellets from mercury-dosed birds were

resuspended in 200µl fetal bovine serum (FBS) followed by 200µl FBS+20%DMSO and samples immediately stored at -80°C. Cryopreserved liver cells were reconstituted and cultured as described below. Several reference liver samples were also collected and treated as above but were not frozen. Spleen cells were washed twice in sterile HBSS (137 mM NaCl, 5.6 mM d-glucose, 5 mM KCl, 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, and 20 mM Hepes at pH 7.05) and pellets were either frozen at -80C in aliquots of 1 x 10<sup>6</sup> cells for Western blots or washed in medium (RPMI-1640, 10% FBS, 50 µM BME, 100 units/ml of penicillin and streptomycin) at a concentration of 1 x 10<sup>7</sup> cells/ml for culturing.

### **Macrophage phagocytosis assay**

#### *Kupffer cell isolation and culturing*

Cryopreserved liver cells were thawed in a 37°C water bath and immediately placed into 5ml of RPMI with 5%FBS. Cells were spun 5 min at 110g, 4°C and the pellet was resuspended in HBSS. Fresh liver cells from reference samples were resuspended directly in HBSS. Liver cells in HBSS were layered on a 2.5 ml 60% Percoll (Pharmacia) gradient in order to isolate viable leukocytes, as has been described previously (Zwollo et al., 2005). Tubes were spun for 20 minutes at 1000g at 4°C, and the cell layer was removed and resuspended in 10 ml ice cold HBSS. Following a final wash in 5ml HBSS, cells were resuspended at 2.5 x 10<sup>6</sup> cells/ml mouse medium for a final volume of 1-3 mls, and placed in a 6-well plate (Corning). A 22 mm square glass cover slip (Fisher) was added to each well, and cells were incubated at 37°C under 5% CO<sub>2</sub> in a tissue culture incubator for 20 hours to allow for adherence.

### *Addition of fluorescent bioparticles*

Three particles were tested as potential phagocytic targets: 1  $\mu\text{m}$  Fluoresbrite Yellow Green microspheres (Polysciences, Inc.), pHrodo bioparticles (Invitrogen), and fluorescein conjugated *Escherichia coli* Bioparticles (Invitrogen). Ultimately, the *Escherichia coli* Bioparticles were used.

*Escherichia coli* Bioparticles were reconstituted by resuspending particles at 20  $\mu\text{g}/\text{ml}$  in 1X phosphate buffered solution (PBS) (1.9 mM  $\text{NaH}_2\text{PO}_4\cdot\text{H}_2\text{O}$ , 8.1 mM  $\text{Na}_2\text{HPO}_4\cdot 7\text{H}_2\text{O}$ , 137 mM NaCl, and 2.6 mM KCl, pH 7.4) with 2mM azide. Particles were added at a ratio of 10 particles per cell and incubated for 3 hours at 37°C, 5%  $\text{CO}_2$  in accord with previous studies (Li, 2006; Olwell et al., 2006). Cover slips were then rinsed three times with 1X PBS to remove free particles.

### *Macrophage staining*

CellMask Deep Red Plasma Membrane stain (Invitrogen) was used to stain the plasma membrane of adherent cells. The CellMask stain was diluted in warm mouse medium to a concentration of 5  $\mu\text{g}/\text{mL}$ . Slides were submerged in 1 mL of the plasma membrane stain for 5 minutes at 37°, 5%  $\text{CO}_2$ . After rinsing three times in 1X PBS, 0.4% Trypan blue was added for 3 minutes to quench extracellular fluorescence of bioparticles. Control cover slips were not washed with Trypan blue. All slides were then washed twice, for 5 minutes, with 1X PBS containing 0.02% azide. After these washes, cells were fixed with 1ml warm 3.75% paraformaldehyde (16% stock EM-grade, Electron Microscopy Sciences) and incubated for 10 minutes. Following three more PBS rinses, cells were mounted using a gel mount containing DAPI (Electron Microscopy Sciences).

### *Imaging of macrophages*

Samples were analyzed on an Olympus BX51 equipped with a DSU spinning disc system, EXFO2000 light source, and a Q-imaging RETIGA-SRV CCD camera. Images were captured and analyzed using Slidebook 5.0 software by 3I.

### **B cell proliferation assay**

#### *Antibodies*

Three primary antibodies were used for Western blots: a  $\mu$ -chain specific goat anti-chicken IgM (Rockland), a rabbit anti-mouse Pax5 antibody, ED-1 (Zwollo et al., 1998), and an anti-red winged blackbird (*Agelaius phoeniceus*) antibody, a gift from Dr. Dennis Hasselquist (Lund University, Sweden). The polyclonal anti-paired domain antibody ED-1 recognizes Pax5 and has been described previously to recognize the protein in trout, mice and humans (Zwollo et al., 2005; Adams et al., 1992).

For flow cytometry experiments using zebra finch and starling spleen cells, a goat-anti-rabbit IgG conjugated to Alexa 555 (Molecular Probes/Invitrogen) was used as the isotype control. ED-1, hereafter referred to as anti-Pax5, was conjugated to Alexa Fluor 555 (Molecular probes/Invitrogen) and was used to measure different populations of lymphocytes.

#### *Western blots: testing potential B cell markers*

Samples were prepared by adding a sample buffer containing 5% 2-ME to zebra finch and starling serum (5  $\mu$ l) or cell pellets containing  $1 \times 10^6$  cells, collected as described above, and proteins were separated by size using denaturing 12% SDS-PAGE gels. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane

(Immobilon-P; Millipore). Membranes were incubated in blocking solution of 5% dry milk in 1X PBS for 1 hour on a rotomixer, followed by incubation in blocking solution containing primary Ab (1:1000) for 1 hour. Four 5 minute washes in 1X PBS were then followed by a 1 hour incubation with secondary Ab goat anti-rabbit IgG-horse radish peroxidase (1:5000) conjugate (Zymed Laboratories) in blocking solution, and membranes were washed four more times in 1X PBS and developed using a chemiluminescence kit (ECL; Amersham Biosciences).

*Spleen cell culture and LPS activation*

Before culturing spleen cells, three culture media were tested for maximal proliferation: “chicken” medium (DMEM, 10% FBS, 5% chicken serum, 1.5 g/L sodium bicarbonate, 10% tryptose phosphate broth, and 50 µM 2-ME), “trout” medium (RPMI-1640, 10 mM L-glutamine, 10% FCS, 50 µg/ml gentamicin, 50 µM 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine), and “mouse” medium (RPMI-1640, 10% FBS, 50 µM 2-ME, and 100 units/ml of penicillin and streptomycin).

**Table 1: Comparison of components of 3 media**

	<b>Base medium</b>	<b>Serum</b>	<b>2-ME</b>	<b>Other</b>	<b>Other</b>	<b>other</b>
“chicken” medium	DMEM	10% FBS	50µM 2-ME	5% chicken serum	1.5 g/L NaHCO <sub>3</sub>	10% tryptose phosphate broth
“trout” medium	RPMI-1640	10% FCS	50µM 2-ME	50 µg/ml gentamicin	nucleosides	10mM L-glutamine
“mouse” medium	RPMI-1640	10% FBS	50µM 2-ME	100 units/ml penicillin streptomycin		

Spleen cells were cultured at a concentration of  $1 \times 10^7$  cells/ml culture (mouse) medium and plated in 96-well plates. The minimum volume used was 200  $\mu$ l, and samples with more than  $3 \times 10^6$  were divided into duplicate samples. The B cell mitogen, lipopolysaccharide (LPS) (10mg/ml) was added at 5 $\mu$ l/ml mouse medium to stimulate B cell proliferation, and cells were incubated for a total of 24 hours (37°C, 5% CO<sub>2</sub>). Five of the 9 non-dosed samples were cultured for a total of 24 hours whereas the other 4 were incubated for 48 hours in order to compare proliferation on day 1 and 2.

#### *Adding EdU*

After 16 hours of incubation, a stock solution of culture medium containing 12 $\mu$ l/ml (10mM) EdU was prepared. A volume of stock solution at 1/3 the original volume of each sample was added to each cell culture for a final concentration of 2.5 mM Edu, and plates were returned to incubator for 8 hours. EdU was added to the 5 “day 1” samples after 8 hours, and it was added to the 4 “day 2” samples after 32 hours. EdU was used to measure proliferating cells.

#### *Fixing cells*

After 8 hours of EdU incorporation, cells were collected in 15 ml falcon tubes and spun at 110g for 5 minutes at 4°C and supernatant was removed. After one wash in 1X PBS with 0.02% sodium azide, cells were resuspended in 500  $\mu$ l of a fixative containing 1% paraformaldehyde (Electron Microscopy Sciences) in 1X PBS. Samples were incubated at 4°C in the dark for 15 minutes. Cells were then spun at 250g for 5 minutes at 4°C and washed with 1 ml 1X PBS + 0.02% sodium azide to remove fixative. Cell pellets were vortexed for 8-10 seconds to create a cell suspension and 1 ml ice cold

methanol was added dropwise. Fixed cells were stored at -20°C for at least 16 hours and no more than 2 weeks.

#### *Refixing immune cells for long-term storage*

In order to save cells for later analysis, cells were refixed as follows. Cells were taken from -20°C and placed on ice. Samples were spun at 250g for 5 minutes at 4°C and resuspended in 500 µl 1X PBS. After a second spin, 1% paraformaldehyde fixative was prepared as described above, and 100 µl was added to cells. Samples were incubated in the dark for 10 minutes. 500 µl 1X PBS was added to cell suspension in fixative, and cells were spun at 250g for 5 minutes at 4°C. After removing the supernatant, cells were resuspended in 100 µl FBS and then in 100 µl FBS + 20% DMSO in a CryoTube (NUNC), for a final concentration on 10%DMSO. Samples were then stored at -80°C for later analysis.

#### *Permeabilization of cells for flow cytometry*

Frozen spleen cell samples were thawed partially and immediately resuspended in 1ml 1X PBS + 5% FBS in a 1.5 ml eppendorf tube. Cells were spun in a microcentrifuge at 450g for 3 minutes at 4°C. After removing the supernatant, cells were resuspended in 45 µl Perm/Wash (BD perm wash, BD Biosciences) containing 5% FBS and plated on a 96 well flow cytometry plate. The plate was placed on a nutator for 15 minutes at 4°C in the dark.

#### *Cell staining*

5 µl (0.15mg/ml) of antibody solution was added for a final concentration of 0.015 mg/ml antibody per sample, and cells were incubated for 90 minutes with shaking

at 4°C. 250 µl Perm +2%FBS was added to each sample and cells were spun at 250g for 5 minutes at 4°C. After the plate was tapped to remove supernatant, it was placed on a nutator at room temperature for 10 minutes. Cells were spun and tapped as above, and cells were again washed with Perm and 2% FBS and placed on a nutator.

Click-iT EdU reaction cocktail (Invitrogen) was made according to kit instructions with the exception of the Alexa Fluor 647 azide dye which was added at a ratio of 1:2 instead of 1:8. 250 µl of Click-iT reaction cocktail was added, and plates were incubated 30 minutes in the dark at room temperature. After this incubation, cells were washed twice in 280 µl Perm +2% FBS and resuspended in 100 µl Perm + 2% FBS. Samples were then analyzed using flow cytometry with 50,000 events acquired per sample.

#### *Quantitative and Statistical analyses*

Flow cytometry graphs and plots were generated using WinMDI 2-8 (J. Trotter 1993–1998) software. One color flow cytometry histograms were used to measure total proliferation in starlings (Figure III.2.4a and b), and two color flow cytometry contours were used to define four populations (Figure III.2.4c-f): Pax5+/EdU- (Resting B cells), Pax5+/EdU+ (Proliferating B cells), Pax5-/EdU- (non-proliferating, non-B cells), and Pax5-/EdU+ (proliferating non-B cells).

Principal component analysis (PCA) was used to reduce four variables (muscle, brain, kidney, and liver) of mercury accumulation in tissues of starlings and five in zebra finches (blood, muscle, brain, kidney, and liver) to a single component (Hg PC 1, total body Hg) in each species. Starling blood-mercury values were not included in analysis because samples for three birds were not collected. Hg PC1 accounted for 86.3% and

95.8% of the tissue Hg variation in starlings and zebra finches, respectively. Individual tissue loadings explain the weight each contributes to a given PC score. All of the respective tissue variables loaded highly positively for Hg PC1 of both the zebra finches and starlings (Table 2).

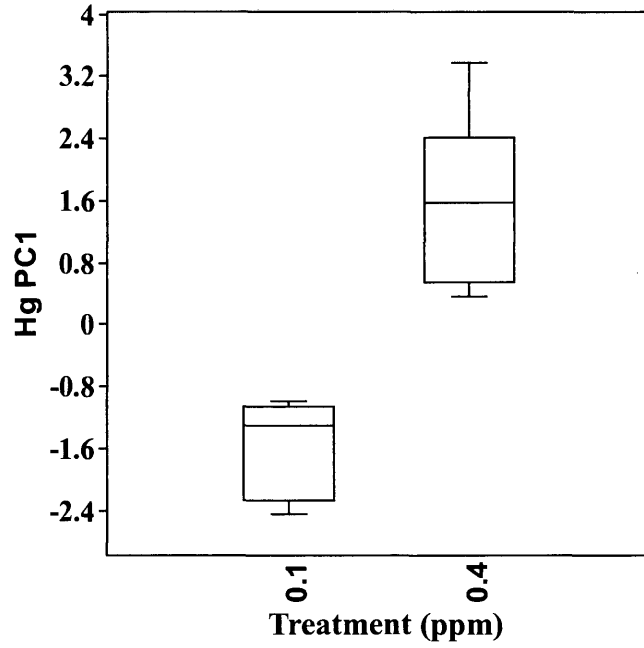
**Table 2: Loading factors of Hg PC1 for both starlings and zebra finches. Starling blood Hg was not used in this analysis. All tissues loaded positively with Hg PC1, for both species.**

	<b>Starling PC1</b>	<b>Zebra finch PC1</b>
<b>Muscle</b>	0.508	0.4538
<b>Brain</b>	0.5265	0.4514
<b>Liver</b>	0.4841	0.4498
<b>Kidney</b>	0.4799	0.4455
<b>Blood</b>	n/a	0.4353

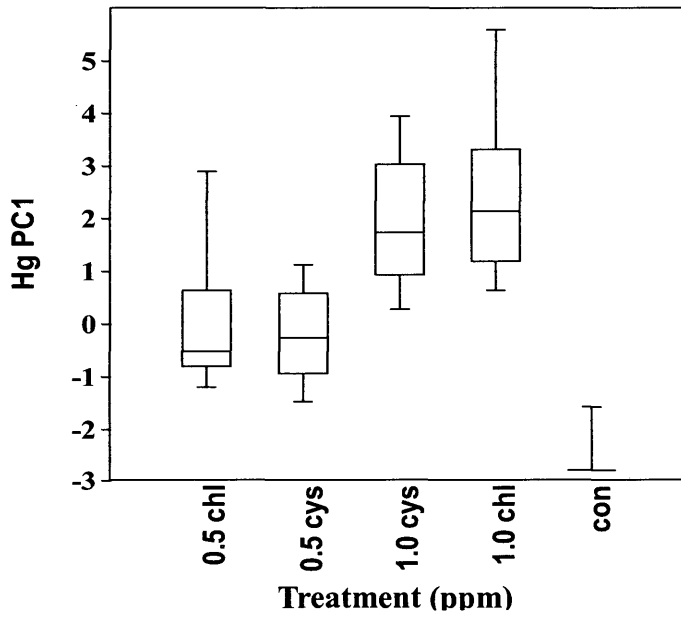
Preliminary data inspection indicated that Hg PC1 varied greatly within treatment groups (Figure II.1.1) hence I decided that it was more appropriate to primarily analyze the effects of Hg on immune system functioning by individual Hg PC1 values rather than by treatment group designations. In addition, general linear analysis indicated that there was no difference between MeHg chloride and MeHg cysteine in terms of mercury accumulation in the tissues measured in zebra finches (Muscle:  $F_{1,51} = 0.29$ ,  $p = 0.59$ ,  $N = 56$ ; Brain:  $F_{1,51} = 0.02$ ,  $p = 0.89$ ,  $N = 56$ ; Liver:  $F_{1,51} = 0.05$ ,  $p = 0.83$ ,  $N =$

56; Kidney:  $F_{1,50} = 0.03$ ,  $p = 0.87$ ,  $N = 55$ ; Blood:  $F_{1,51} = 0.45$ ,  $p = 0.51$ ,  $N = 56$ ). Therefore the five treatment groups were reduced to three during analyses (Varian-Ramos, 2011). To evaluate the relationships between metrics of immune function and Hg PC 1, I employed reduced-major axis (RMA) regression, as there was error associated with both the dependent and independent variables in these analyses. All statistical analyses were performed with PAST Statistics (Hammer et al., 2001) employing two-tailed tests of probability throughout.

**a) Variation in MeHg accumulation in starlings**



**b) Variation in MeHg accumulation in zebra finches**



**Figure II.1.** Individual Hg PC1 values within each treatment group in a) starlings and b) zebra finches

## Chapter III.

### Results

The studies presented here sought to determine the effects of sublethal levels of MeHg on two components of the passerine immune response. Due to a lack of established immune reagents for songbirds, two new assays of immunocompetence were developed. This necessitated the establishment of new reagents and antibody markers. A macrophage phagocytosis assay using confocal microscopy was developed in order to measure one critical component of the innate immune response (Section 1), whereas a B lymphocyte proliferation assay using flow cytometry measures one aspect of adaptive immunity (Section 2). No data were collected for macrophage phagocytosis for reasons described below. Results from the lymphocyte proliferation test in songbirds exposed to mercury in their diet (Section 3), are reported for zebra finches and European starlings.

#### **Section 1. Development of a macrophage phagocytosis assay in songbirds.**

##### *Overview*

In order to develop an assay of macrophage phagocytosis, multiple reagents and methods were tested including: phagocytic targets, plasma membrane stains, and methods for determining whether phagocytic targets are ingested vs. adherent to the outer membrane. In addition, I worked with both fresh and cryopreserved liver cells to compare viability.

### *Phagocytic targets*

One of the greatest challenges in measuring macrophage phagocytosis is distinguishing between ingested and adherent bioparticles. Therefore several particles were tested as potential phagocytic targets with the requirement that the location of the particle with relation to the macrophage plasma membrane could be easily confirmed. One 1  $\mu\text{m}$  Fluoresbrite Yellow Green microspheres (Polysciences, Inc.) were used initially due to their small size, which has been demonstrated to support higher levels of phagocytosis (Li et al., 2006). However, their fluorescence overpowered any membrane stain tested. pHrodo bioparticles (Invitrogen), which fluoresce at low pHs, for example the endosome or lysosome of a macrophage, were also tested as a more biologically relevant and less fluorescent alternative to the fluoresbrite microspheres. However, too much background fluorescence was observed. Ultimately, *E. coli* BioParticles were used and the viability stain, 0.4% Trypan blue was added in order to quench fluorescence of any particles not taken up by cells. This method has been used previously (Wan et al., 1993) with a murine macrophage cell line. My data with freshly isolated songbird macrophages indicate successful quenching of most, but not all, extracellular fluorescence (Figure III.1.1).

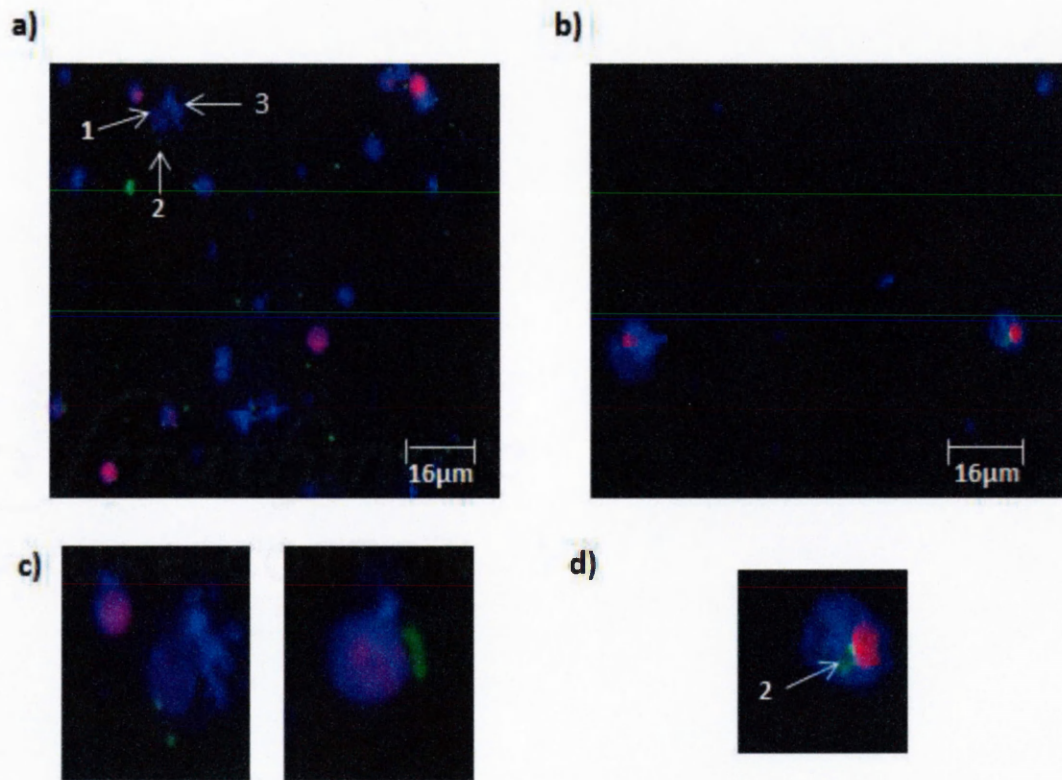
### *Plasma membrane markers*

Because trypan blue was very successful but not 100% effective at quenching extracellular bioparticle fluorescence, a plasma membrane stain should also be used. Combined with confocal microscopy, the use of a cell surface marker could help confirm whether a bioparticle is ingested or adherent. Macrophage specific cell markers have

yet to be characterized in songbirds. Therefore, instead of using a macrophage-specific cell marker, the method described herein relies on a general plasma membrane stain, the CellMask plasma membrane stain (Invitrogen). The current study also used a gel mount containing DAPI (Electron Microscopy Sciences) in order to identify cells with viable nuclei. While the plasma membrane stain is not specific to macrophages, macrophages may be selected by their ability to adhere. Heterophils are also known to be phagocytic and adherent, however heterophils are largely absent in the healthy liver (Nemeth et al., 2009). In addition, this study primarily seeks to determine effects of mercury on the immune system functioning, and it is therefore not critical to distinguish between macrophages and heterophils as the proper functioning of both is crucial for immune system functioning. Data showing successful DAPI and CellMask plasma membrane staining using fluorescence microscopy are shown in Figure III.1.1.

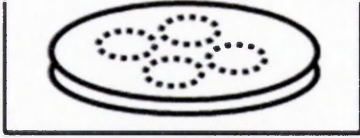
#### *Sample preparation and cryopreservation*

Initially, liver samples from mercury-dosed birds were collected and frozen at -80°C for future analysis. A previous study successfully assessed the effects of contaminants on phagocytosis using macrophages isolated from chicken blood and cryopreserved in 10% DMSO (Finkelstein et al., 2003). However, in the current experiment, liver cell viability decreased too much after cryopreservation to assess macrophage phagocytosis. Therefore no data on the effects of MeHg were collected for this endpoint, and the use of cryopreserved liver cells is not recommended for assaying macrophage function. However, based on preliminary work, a flow chart for a potential method using freshly isolated cells is described (Figure III.1.2).



**Figure III.1.1** Macrophages with DAPI nuclear stain in red (1), TRITC Bioparticles in green (2), and CellMask plasma membrane stain in blue (3). Samples were prepared in the absence of Trypan blue quencher (a,c) or after addition of 0.4% Trypan blue (a,d). Images at low magnification (a,b) show the impact of Trypan blue quencher, while high magnification images (c,d) reveal TRITC Bioparticles inside adherent cells after addition of Trypan blue (d; arrow 2), and both inside and outside adherent cells in the absence of quencher. Approximate scale bar is included (a,b).

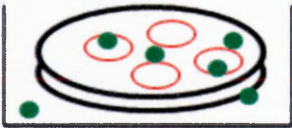
Isolate WBC's using 60% Percoll gradient and allow to adhere to coverslip (20 hrs)



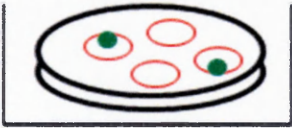
Incubate cells with fluorescent BioParticles (4-6 hrs)



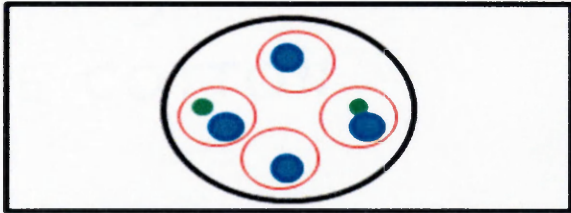
Incubate with CellMask plasma membrane stain



Wash with 0.4% Trypan blue



Mount on slide with DAPI gel mount



Count # cells phagocytosing 0,1, 2,3,4, >5 BioParticles

Figure III.1.2. Flow chart of suggested method for assessing macrophage phagocytosis.

## Section 2. Lymphocyte proliferation assay method

### *Avian B cell antibodies*

The current study tested multiple possible anti-passerine antibodies for use as a B cell specific antibody in flow cytometry. These included an anti-chicken IgM (Rockland) an anti-red-winged blackbird IgG (gift from Dr. Hasselquist), and an anti-Pax5 antibody (Zwollo et al., 2005).

Western blots were conducted in order to determine whether the anti-chicken-IgM, anti-red-winged blackbird Ig, and anti-Pax5 antibodies could bind to songbird spleen cell lysates and blood serum. The chicken anti-IgM antibody was purchased already conjugated to a fluorochrome for use in flow cytometry, and unfortunately was not recognized with high affinity by the secondary antibody. When zebra finch and starling spleen cell lysates were probed with anti-Pax5 antibody, a band was detected around 53kDa, the expected molecular weight of vertebrate Pax5 (Zwollo et al., 1998; Figure III.2.2a). This band appeared in lanes containing cellular lysates but not serum. A second experiment was conducted using the spleen cell fraction of zebra finches and starlings and two dilutions of blood serum (1:10 and undiluted) from both (Figure III.2.2b). This blot was probed with anti-red-winged blackbird Ig to determine if it could recognize songbird Ig. In addition to a certain amount of non-specific binding, bands appeared around 13, 25, and 60kDa.

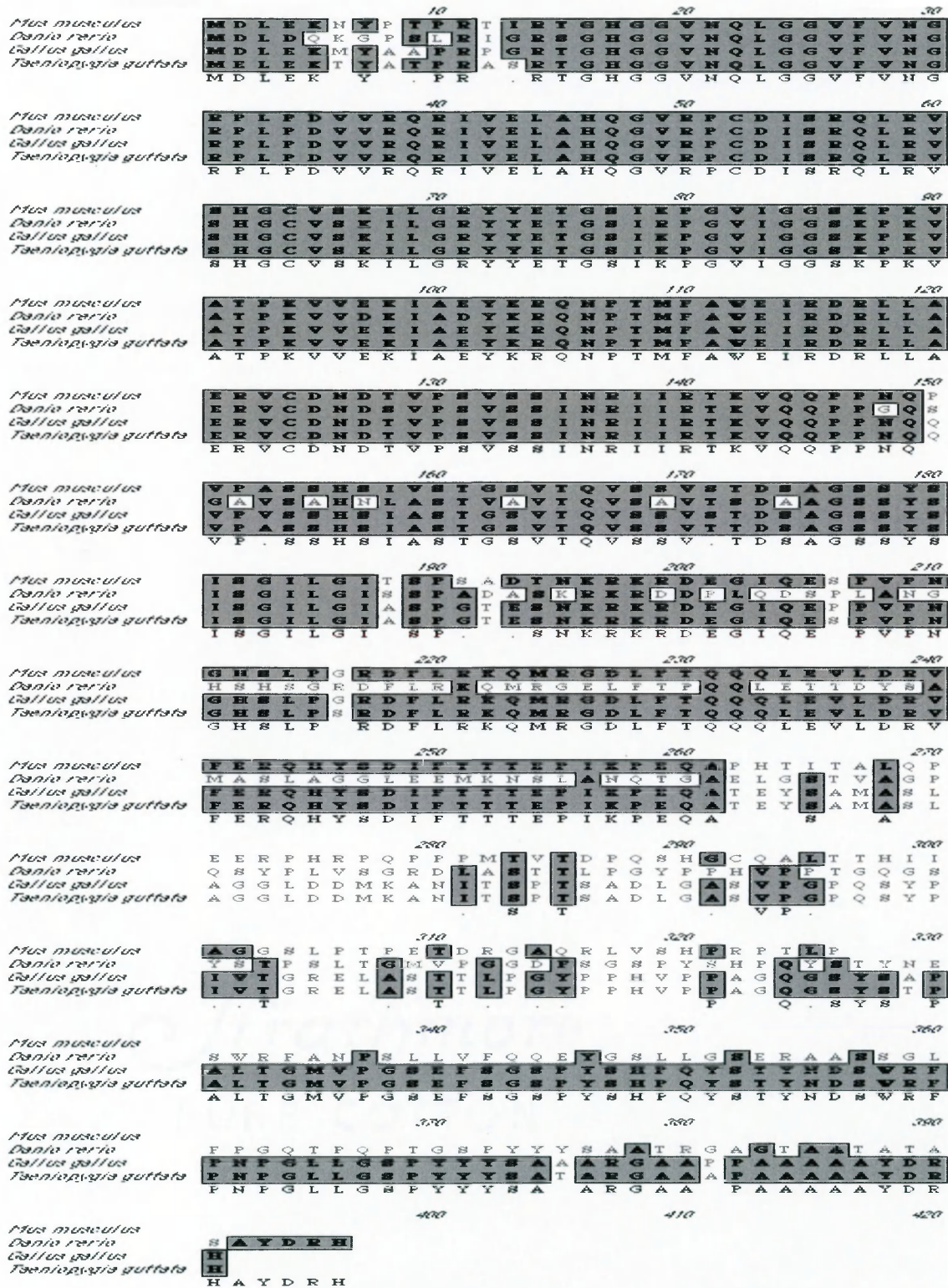
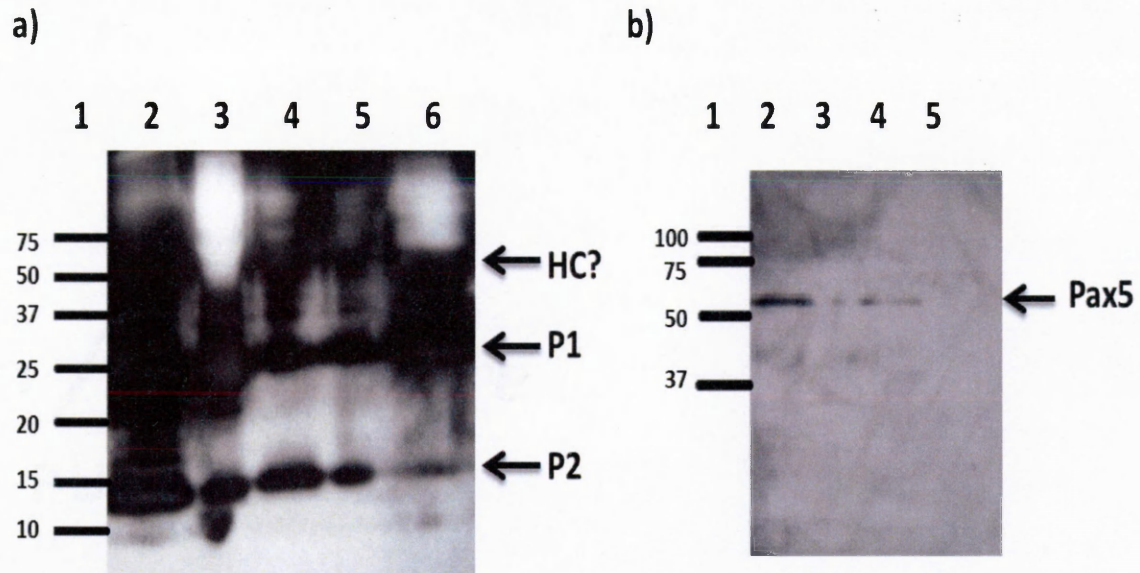


Figure III.2.1. a) Protein sequence for paired box gene 5 in mouse, zebra fish, chicken, zebra finch. Paired domain where anti-Pax5 antibody binds is amino acids 16-142.



**Figure III.2.2.** Molecular weight markers were run in lane 1 (Bio-Rad): a) anti-red-winged blackbird antibody was tested for specificity with zebra finch spleen cells and serum at 1:10, (lanes 2 and 3) and starling spleen cells and serum at 1:10 and undiluted (lanes 5 and 6). P1 and P2 indicate the two bands that appeared representing unknown proteins and HC? indicates the band that may represent an immunoglobulin heavy chain. b) Anti-Pax5 antibody was tested for B cell specific binding with zebra finch spleen cells and serum (lanes 2 and 3), and starling cells and serum (lanes 4 and 5).

### *Proliferation medium*

In order to select a medium that would support maximal B cell proliferation, three culture media, commonly used to culture cells from other organisms, were tested on starling spleen cells: “chicken” medium, “trout” medium and “mouse” medium. On D1 (Figure III.2.3 a and b), the highest levels of proliferation were observed consistently in trout and mouse media cultures (trout mean = 2.88%, mouse mean = 2.47%), and these samples were highly correlated (Spearman’s rank correlation coefficient,  $r_s = 0.83$ ,  $N = 12$ ,  $p < 0.001$ ). Proliferation in trout and chicken ( $r_s = 0.57$ ,  $N = 12$ ,  $p = 0.05$ ) and mouse and chicken ( $r_s = 0.59$ ,  $N = 12$ ,  $p = 0.04$ ) were positively correlated as well. Only trout and mouse medium were tested on D2 (Figure III.2.3c and d). Mouse medium supported slightly higher levels of proliferation (mouse mean = 3.17% and trout mean = 2.62%), and levels of proliferation were again highly correlated ( $r_s = 0.79$ ,  $N = 12$ ,  $p = 0.002$ ).

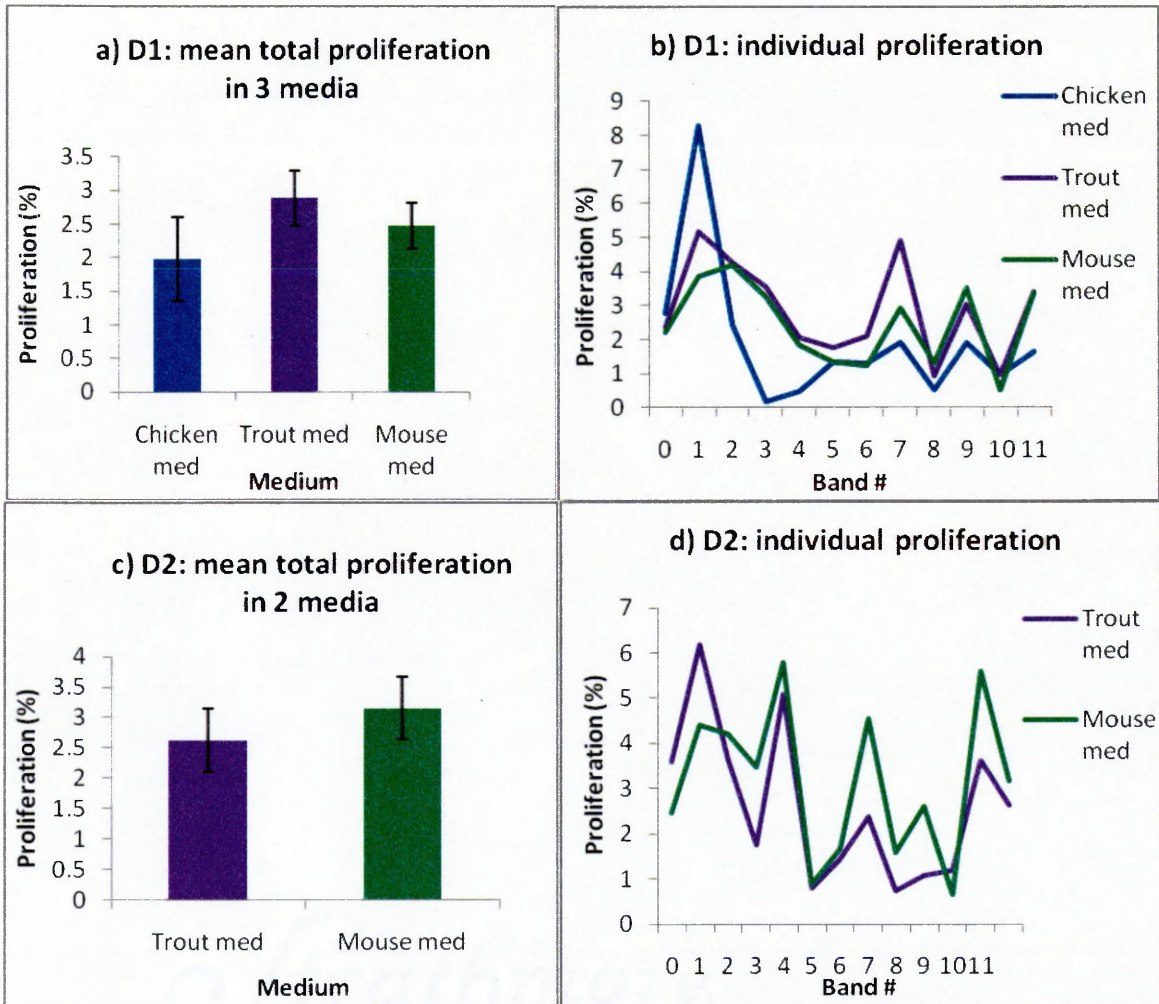
### *Flow cytometry gating:*

Total cell proliferation for starlings over three days was gated using one color flow cytometry histograms to ensure maximum consistency (Figure III.2.4a and b). Because anti-Pax5 antibody was not used on D1 of starling assay, total cell proliferation was measured over three days instead of B cell proliferation. However, LPS is a B cell specific mitogen, and only B cells would be expected to demonstrate significant levels of proliferation. Therefore total proliferation and B cell proliferation should be equivalent. This was supported by the data from D2 and D3. For example, as can be seen in Figure

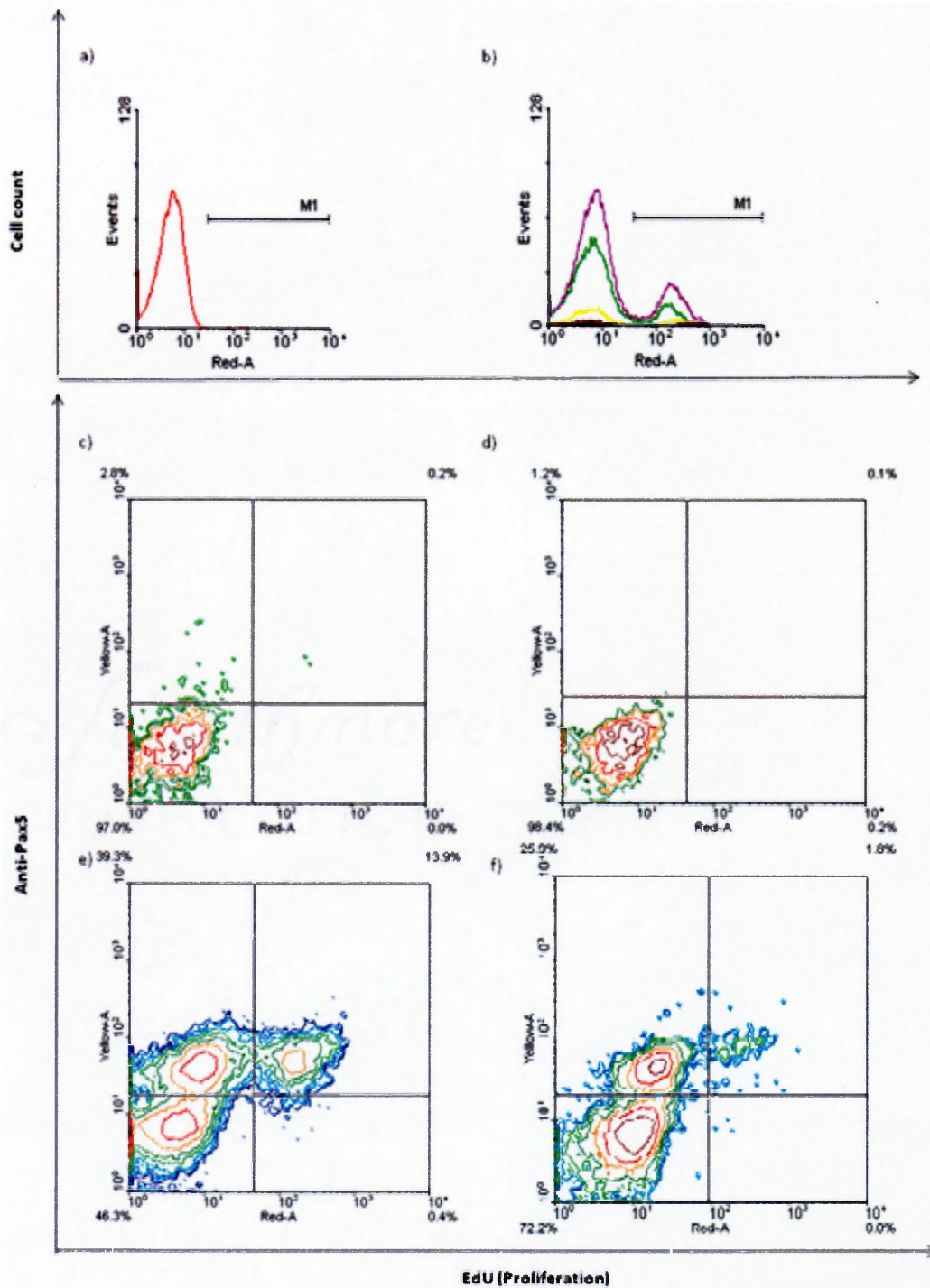
III.2.4e and f, all EdU+ cells were also Pax5+, meaning all proliferating cells were also B cells.

Data for frequency of resting B cell populations and ratios of proliferating to resting B cells in starlings and all zebra finch data were collected using two color flow cytometry and gated using quadrants to gate four populations (Figure III.2.4c-f).

Samples that lacked sufficient cell numbers to form a clear population of lymphocytes in a dotplot were not included in analyses. Therefore N = 64 for analysis of zebra finch B cell proliferation.



**Figure III.2.3.** D1 proliferation in 3 types of media: a) mean proliferation and b) proliferation per individual bird sample, identified by band #. D2 proliferation in 2 types of media: c) mean proliferation and d) proliferation per sample identified by band #.



**Figure III.2.4.** One color flow cytometry histograms to measure total proliferation in starlings: a) control and b) overlay of D3 samples. Two color flow cytometry to gate B cell populations: c) starling with c555 and no EdU; d) zebra finch with c555 and no EdU; e) starling with anti-Pax5 conjugated to Alexa 555 and EdU; f) zebra finch with Ed-1 conjugated to Alexa 555 and EdU.

### Section 3. B lymphocyte proliferation data

#### *Starlings: total proliferation, resting B cells and ratios of proliferating to resting B cells*

In order to measure total LPS-stimulated cell proliferation, the 12 samples were collected and fixed on D1, D2, and D3 (Figure III.3.1). EdU was added 8 hours prior to fixation on D1 and D2 and 24 hours before fixation on D3. Because of this inconsistency, D3 is included on a separate graph. Mean total proliferation on D1 and D2 indicate a trend towards decreased cell proliferation in starlings dosed with 0.4 ppm MeHg versus 0.1 ppm ((D1:  $F_{1,10} = 0.832$ ,  $p = 0.383$ ; D2:  $F_{1,10} = 0.546$ ,  $p = 0.477$ ; Figure III.3.1a). Mean proliferation after 8 hours of EdU incorporation on D1 was 3.16% in the 0.1ppm dosing group and 2.32% in the 0.4ppm dosing group, while mean proliferation on D2 was 3.54% in the 0.1ppm samples and 2.77% in the 0.4ppm samples. Finally, mean proliferation after 24 hours of EdU incorporation on D3 was 17.08% in the 0.1ppm group and 18.73ppm in the 0.4ppm group ( $F_{1,9} = 1.494$ ,  $p = 0.253$ ). On D3, sample size decreased to 9 due poor sample quality.

Total LPS-stimulated proliferation in starlings was also evaluated for a relationship with tissue-Hg PC 1 (Figure III.3.1b, c, and d). Total D1 proliferation (Figure III.3.1b) had a weak negative linear relationship with tissue-Hg PC 1, best fitting the line: total proliferation =  $-0.649 * \text{Hg PC 1} + 2.472$  (95% confidence intervals:  $a = [-2.14; -0.202]$ ,  $b = [1.47; 3.52]$ ;  $r^2 = 0.0047$ ,  $t = -0.218$ ,  $p = 0.832$ ,  $N = 12$ ). Total D2 proliferation (Figure III.3.1c) similarly had a weak negative linear relationship with Hg PC 1, best fitting the line: total proliferation =  $-0.953 * \text{Hg PC 1} + 3.16$  (95% confidence intervals:  $a = [-3.13; 0.449]$ ,  $b = [1.95; 4.67]$ ;  $r^2 = 0.031$ ,  $t = -0.564$ ,  $p = 0.589$ ,  $N = 12$ ). Total D3

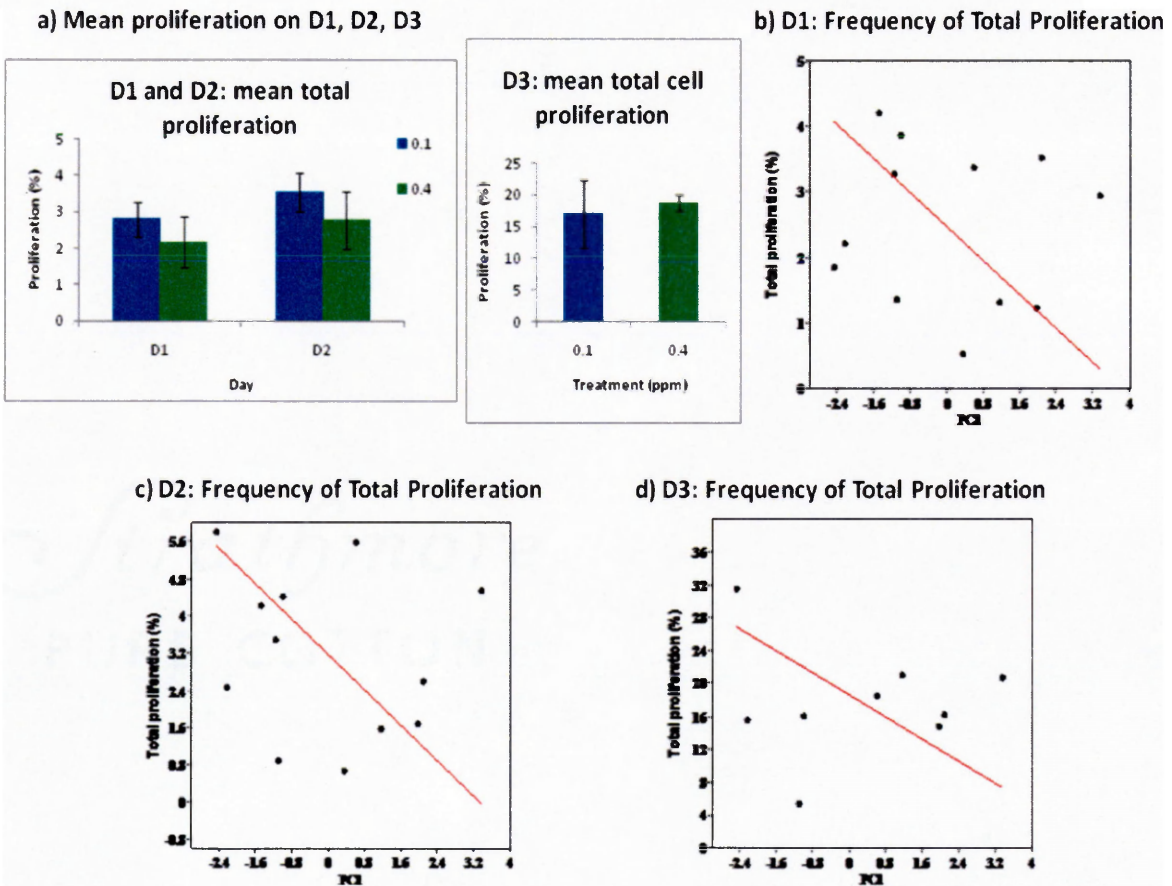
proliferation (Figure III.3.1d) showed the same general (lack of) pattern with Hg PC 1, best fitting the line: total proliferation =  $-3.36 * \text{Hg PC 1} + 18.6$  (95% confidence intervals:  $a = [-11.26; -0.918]$ ,  $b = [13.52; 26.42]$ ;  $r^2 = 0.075$ ,  $t = -0.198$ ,  $p = 0.861$ ,  $N = 9$ ).

Resting or non-proliferating B cells were measured on D2 and D3 (Figure III.3.2) in starlings. The mean frequency of resting B cells appeared to increase slightly at the higher (0.4ppm) level of mercury on D2 and D3 (D2:  $F_{1,10} = 0.128$ ,  $p = 0.728$ ; D3:  $F_{1,10} = 5.376$ ,  $p = 0.43$ ; Figure III.3.2a and b). To investigate this relationship further, frequency of resting B cells in each sample was plotted against Hg PC1. Again, a trend for positive relationships between Hg PC1 and frequency of resting B cells was found. On D2 (Figure III.3.2c), the relationship was weak (95% confidence intervals:  $a = [4.13; 21.74]$ ,  $b = [33.75; 51.84]$ ,  $r^2 = 0.029$ ,  $t = 0.542$ ,  $p = 0.594$ ,  $N = 12$ ), best fitting the line: % resting B cell frequency =  $6.34 * \text{Hg PC1} + 41$ . However, on D3 (Figure III.3.2d) this positive relationship was stronger even given the smaller sample size (95% confidence intervals:  $a = [2.97; 16.03]$ ,  $b = [34.76; 50.51]$ ,  $r^2 = 0.337$ ,  $t = 1.89$ ,  $p = 0.092$ ,  $N = 9$ ), best fitting the line: % resting B cell frequency =  $5.9406 * \text{Hg PC1} + 41.8$ .

The relationship between mercury level and frequencies of proliferating to resting cells was analyzed using an RMA regression of Hg PC1 and the ratio of proliferating to resting B cells, as with the earlier comparisons. Anti-Pax5 antibody was not used on D1. Therefore only D2 and D3 are included in analyses of ratios of proliferating to resting B cells. Mean values on both D2 and D3 indicate slightly decreased ratios of B cell proliferation in the 0.4ppm mercury dosing group compared to

the 0.1ppm mercury group (D2:  $F_{1,10} = 0.276$ ,  $p = 0.611$ ; D3:  $F_{1,10} = 0.218$ ,  $p = 0.651$ ; Figure III.3.3a and b). When evaluated by individual mercury levels rather than treatment dose, both D2 and D3 (Figure 3.3b and c) indicate a weak negative relationship between ratio of proliferating to resting B cells and Hg PC 1 (on D2: 95% confidence intervals:  $a = [-0.088; -0.009]$ ,  $b = [0.037; 0.117]$ ;  $r^2 = 0.050$ ,  $t = -0.722$ ,  $p = 0.493$  and on D3: 95% confidence intervals:  $a = [-0.277; -0.035]$ ,  $b = [0.302; 0.626]$ ;  $r^2 = 0.198$ ,  $t = -1.32$ ,  $p = 0.231$ ). The lines that best described these data were: ratio =  $-0.027 * \text{Hg PC 1} + 0.074$  on D2 and ratio =  $-0.105 * \text{Hg PC 1} + 0.44$  on D3.

In summary, as Hg PC1 values increased in starlings, total proliferation on days 1, 2, and 3 and ratio of proliferating to resting B cells on days 2 and 3 tended to decrease. By contrast, frequency of resting B cells tended to increase with greater mercury accumulation on days 2 and 3. Given the low sample sizes, and thus low statistical power to detect significant differences, the consistency of the trends in the data across the days and various measures of resting and proliferating cells indicates a biologically interesting phenomenon.



**Figure III.3.1.** Total proliferation in starlings over 3 days: a) mean proliferation at low mercury dose (0.1ppm) and high mercury dose (0.4ppm) over day 1, 2, and 3; b) proliferation on day 1 versus Hg PC1; c) proliferation on day 2 vs. Hg PC1; and d) proliferation on day 3 versus Hg PC1.



*Zebra finches: B cell proliferation, resting B cells and ratios of proliferating to resting B cells*

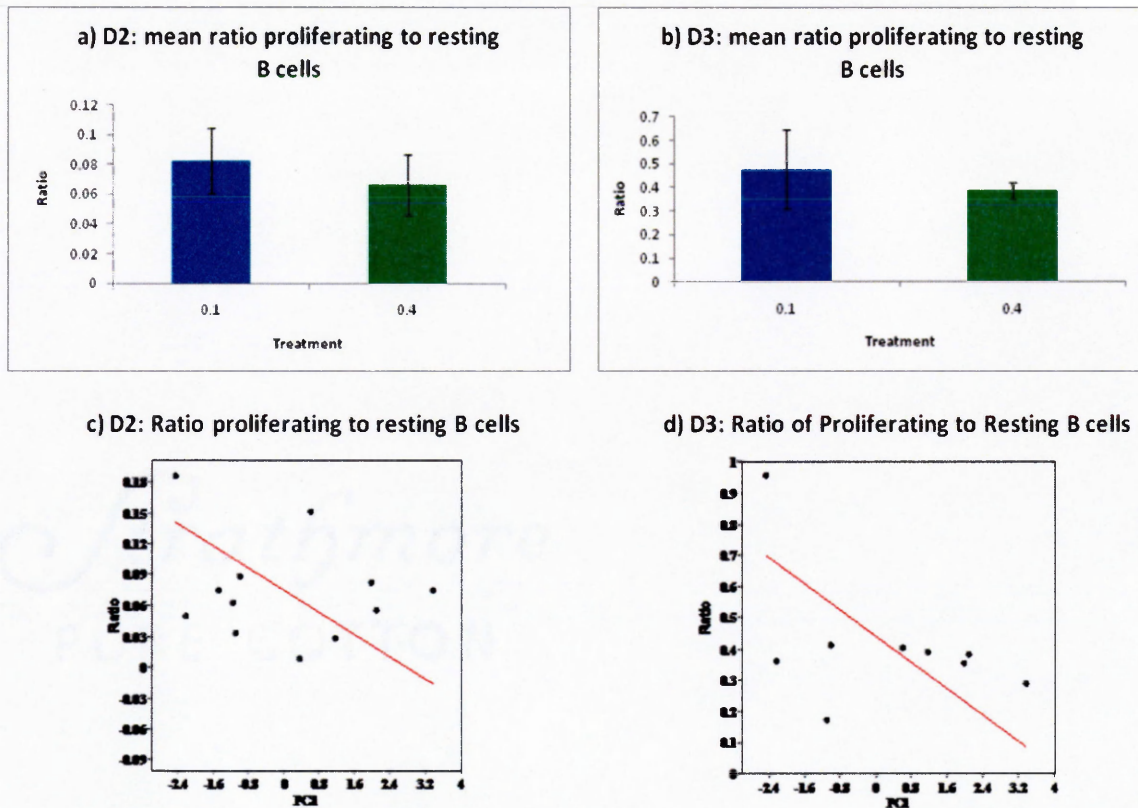
Due to low splenic cell counts, LPS-induced proliferation in zebra finch primary cultures was only measured on D1. Mean B cell proliferation decreased from the control group to 0.5ppm to 1.0ppm ( $F_{1,62} = 3.99$ ,  $p = 0.05$ ; Figure III.3.4a), and analysis of B cell proliferation in individual zebra finch samples vs. Hg PC1 (Figure III.3.4b) was consistent with the same relationship (95% confidence intervals:  $a = [-3.55; -0.851]$ ,  $b = [2.184; 3.89]$ ,  $r^2 = 0.0531$ ,  $t = -1.87$ ,  $p = 0.071$ ). These data were best described by the line: % B cell proliferation =  $-1.27 * \text{Hg PC 1} + 3.075$ . Both mean ( $F_{1,62} = 1.692$ ,  $p = 0.198$ ; Figure III.3.4c) and individual (Figure III.3.4d) frequencies of resting B cells demonstrated a weak positive relationship with mercury dose and Hg PC1 (95% confidence intervals:  $a = [5.11; 20.34]$ ,  $b = [34.1; 43.1]$ ,  $r^2 = 0.026$ ,  $t = 1.30$ ,  $p = 0.199$ ), best fitting the line: % resting B cells =  $6.691 * \text{Hg PC 1} + 38.714$ .

As with the starlings I examined the relationship between mercury level and relative abundance of proliferating B cells by calculating a ratio of proliferating to resting B cells (Figure III.3.5). The mean ratio of proliferating to resting B cells tended to decrease across dosing groups ( $F_{1,62} = 6.158$ ,  $p = 0.16$ ; Figure III.3.5a), and the ratio decreased notably with increasing Hg PC1 (Figure III.3.5b: 95% confidence intervals:  $a = [-0.071; -0.031]$ ,  $b = [0.063; 0.126]$ ,  $r^2 = 0.087$ ,  $t = -2.44$ ,  $p = 0.017$ ), best fitting the line: ratio =  $-0.05 * \text{Hg PC 1} + 0.1$ .

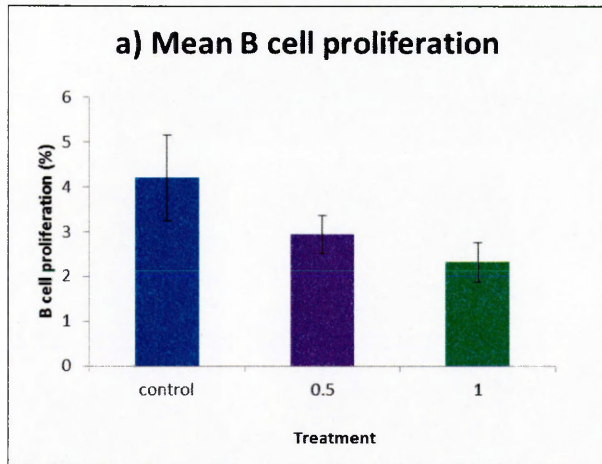
Overall, the same trends were observed in zebra finches and starlings; increased Hg PC1 was correlated with a decrease in B cell proliferation and ratio of proliferating to

resting B cells whereas frequency of resting B cells increased. The larger sample sizes rendered more of the zebra finch analyses statistically robust; however the consistency in the direction of the patterns across these two species lend themselves to indicating a similar biological process—mercury suppresses B cell proliferation in both of these songbirds.

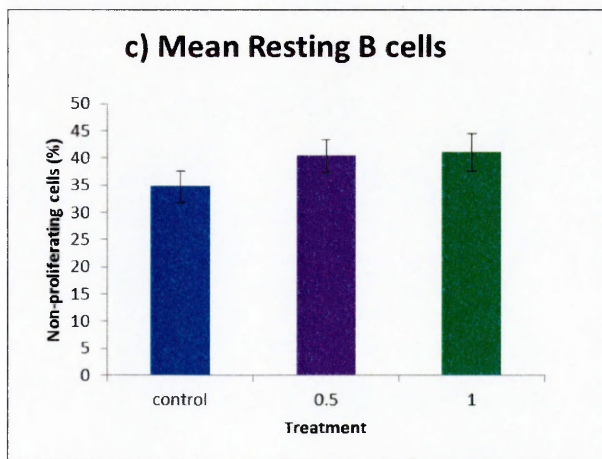
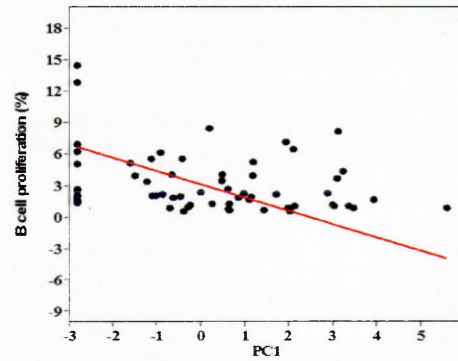
Nine reference samples from a separate population of zebra finches were also run to compare D1 vs. D2 proliferation in zebra finches. These preliminary data suggest that slightly higher levels of proliferation occur on D1 vs. D2 (Figure III.3.6).



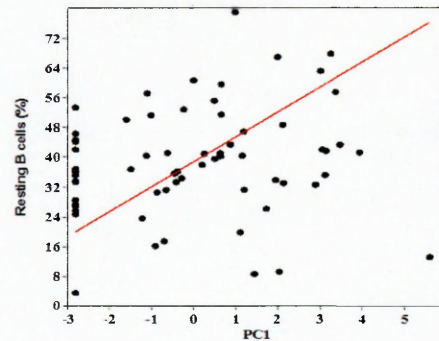
**Figure III.3.3.** Ratio of proliferating to resting (non-proliferating) B cells in starlings a) D2 means, b) D2 ratios versus Hg PC1; c) D3 means and d) D3 ratios versus Hg PC1.



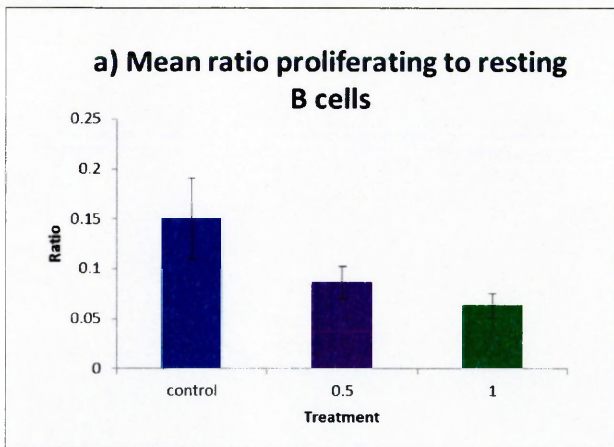
**b) % B cell proliferation vs. PC 1**



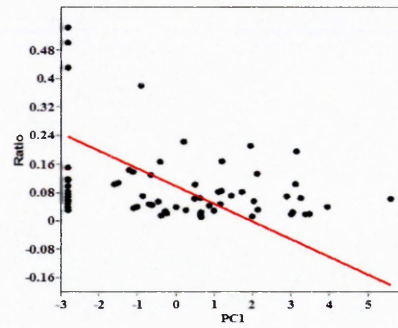
**d) % Resting B cells vs. PC 1**



**Figure III.3.4.** Frequency of different B cell populations in zebra finches: a) mean B cell proliferation in each dosing group; b) B cell proliferation per sample versus Hg PC1; c) mean resting (non-proliferating) B cells per dosing group and d) resting B cells per sample versus Hg PC1.

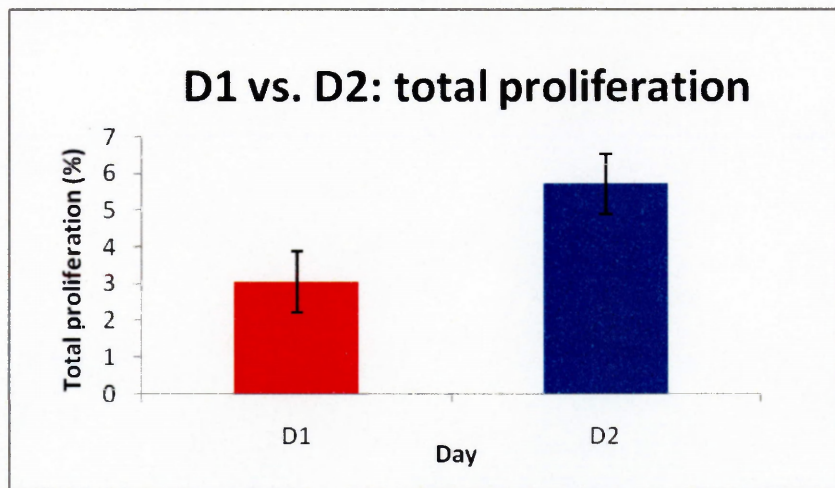


**b) Ratio proliferating to resting B cells**



**Figure III.3.5.** Ratio of proliferating to resting B cells in zebra finches: a) means and b) versus Hg PC1.

*Strathmore*  
PURE COTTON



**Figure III.3.6.** Zebra finch: D1 vs. D2 data for future experiments

*Strathmore*  
PURE COTTON

## Chapter IV.

### Discussion

This study sought to develop reliable assays of immunocompetence in songbirds and to use these assays to measure the effects of MeHg on starlings and zebra finches. A brief discussion of the macrophage phagocytosis assay for songbirds is included, but because no data were collected for this assay, the primary focus of this discussion will be on the methods and results for the B lymphocyte proliferation assay. In order to develop a B cell proliferation assay, a reliable B cell specific marker as well as a proliferation medium were required. Establishing songbird reagents allowed us to measure the effects of mercury on one important endpoint of the immune system, B lymphocyte proliferation. I also discuss several possible mechanisms by which mercury may have caused the observed results. This study is a starting point for future research on the effects of contaminants on songbird immunocompetence. However, in order to gain a more complete understanding of the impact of dietary mercury, the macrophage phagocytosis assay along with additional cellular assays of immunocompetence will need to be conducted.

#### **4.1 Macrophage phagocytosis method: future directions**

Although macrophage phagocytosis could not be measured here due to loss of cell viability, future studies should consider inclusion of this assay because it represents an important measure of immunocompetence (Fournier et al., 2000). Therefore, I propose several suggestions for future development and application of this assay. First,

although macrophage-like cells were usually observed in this study, yield and consistency may be improved through incubation of small liver sections with pronase to digest parenchymal liver tissue prior to filtration and subsequent culture (Alabraba et al., 2007). My most important finding however is that, while at least one study recommends the use of cryopreserved blood samples for immunological assays (Finkelstein et al, 2003), this method does not appear to be successful for liver cells. For this reason, the cryopreserved mercury-dosed samples collected here did not provide any data. Future studies should use fresh liver cells whenever possible, or use a modified method for cryofreezing.

There is much variability in the literature as to the optimal incubation period for adherent cells in culture, often depending on the method of isolation. These range from 2 hours (Alabraba et al., 2007) to 10 days (Kitani et al., 2010). Based on these studies and the fact that after 24 hours, cell viability greatly decreased, 20 hours was chosen for cell culturing. Still, future studies should test shorter time spans to potentially increase yield of macrophage-like cells. As the images from this study suggest, macrophage-like cells were at relatively low concentrations, especially in samples where trypan blue was not used to quench fluorescence (Figure III.1.1). Kupffer cells are highly adherent (Alabraba et al., 2007), and it may not be necessary to incubate cultures for more than a few hours.

The target used for phagocytosis should also be carefully considered. While some studies employ the use of synthetic microbeads (Li et al., 2006), which may be easier to manipulate and are available in a large range of sizes, the biological relevance

of the target must also be considered. Ultimately, fluorescence conjugated *E. coli* BioParticles were the best target for phagocytosis for my purposes. In addition, while adherent cells were able to engulf large numbers of BioParticles (Figure III.1.1b), it would also be informative to opsonize BioParticles with antibody to better mimic *in vivo* phagocytosis. Because opsonization also increases uptake however, duration of incubation with BioParticles may require shortening.

Finally, there are multiple ways in which phagocytosed particles can be counted. These range from simple light microscopy (Finkelstein et al., 2007) and microplate fluorescence readers (Chok et al., 1992) to transmission electron microscopy (TEM) (Olwell et al., 2006) and flow cytometry (Holloway et al., 2003, Hart et al., 2008). These techniques vary greatly in their accuracy and efficiency. Basic microscopy does not allow for distinction between ingested and adherent cells. One possible solution is through the addition of trypan blue, which can be used to quench extracellular fluorescence. This is more commonly seen however, in studies using microplate readers that measure total fluorescence as a proxy for phagocytosis levels when cell number is controlled (Chok et al., 1992). This does not allow for the number of cells engulfing various numbers of particles to be assessed, a measure which has previously been found to be affected by mercury (Finkelstein et al., 2007). Flow cytometry accommodates this but also requires detachment of adherent Kupffer cells from culture plates, which is reportedly very difficult (Alabraba et al., 2002). Still, flow cytometry is a good option for future study. However, for the current study, my aim was to develop two distinct

methodologies of measuring immune response. Therefore, my plan was to collect data through confocal microscopy.

Figure III.1.1b demonstrates that trypan blue may not fully quench extracellular fluorescence. Therefore, I recommend taking z-stacks or cellular cross-sections in order to create a three dimensional image of cells using confocal microscopy. Taken together, trypan blue quenching and confocal microscopy will more reliably indicate which particles are engulfed. Previous studies have included between 100-300 total macrophage-like cells per sample (Finkelstein et al., 2007; Sun et al., 2008) and have counted the number of cells engulfing 1-2, 3-4, 5-10, >10 particles (Finkelstein et al., 2007) as well as the phagocytic index or average number of particles engulfed per cell (Sun et al., 2008). Although this is not the most efficient method, these measures are likely the most accurate way to assess phagocytosis.

Despite the fact that many studies have been conducted on the effects of mercury on phagocytosis (Christensen et al., 1993; Fournier et al., 2000; Holloway et al., 2003; Finkelstein et al., 2007; Sun et al., 2008), these studies have been somewhat inconclusive. Some studies suggest that mercury suppresses phagocytosis (Christensen et al., 1993; Finkelstein et al., 2007) while others suggest that there is no effect (Holloway et al., 2003). This is most likely the result of methodological differences between the studies. Efficiency of phagocytosis may be affected by the concentration of substrate (Finkelstein et al., 2007) as opposed to overall ability to phagocytose. In addition, once particles are ingested, mercury may affect their transport and processing.

Mercury is known to affect microtubule polymerization (Miura et al., 1984) and tubulin stains are readily available. Therefore future studies should consider visualizing particle processing and ingestion alongside studies of phagocytosis. Some pathogens such as *Listeria monocytogenes*, a deadly human pathogen, have evolved mechanisms of escaping the phagosomal pathway (Birmingham et al., 2008). If mercury suppresses functioning of this pathway, it could increase the number of pathogens that are able to evade immune defenses, thus rendering an organism more susceptible to pathogens.

#### **4.2 B cell proliferation assay development**

##### *B cell marker*

Measuring B cell proliferation in songbirds presents a challenge due to the paucity of B cell-specific markers for songbirds. Research in avian immunology has focused almost exclusively on galliformes, particularly chickens, due to their economic importance. It is not surprising then, that the majority of the anti-bird IgM and IgG antibodies that exist have been raised against chickens. The relatively few immunological studies conducted in other avian orders often use anti-chicken antibodies with the assumption that the anti-chicken antibodies will cross-react with the appropriate cell or protein type from their species of interest. However, this is not always the case as exemplified by my results from testing one anti-chicken IgM antibody for cross-reactivity with starling and zebra finch spleen cells. Western blotting was unsuccessful in detecting cross-reactivity of a fluorescence conjugated anti-chicken IgM ( $\mu$  heavy chain) antibody (Rockland) with passerine immunoglobulin, and it appeared from using flow cytometry that this antibody does not have a high affinity for any

component within the passerine spleen cell population (data not shown). Therefore this antibody could not be used as a marker to measure B cell proliferation in this study.

This is perhaps not surprising because, while there is considerable homology in the immunoglobulin heavy chain gene within galliformes, anseriformes, such as ducks, only have 51-54% homology with chicken heavy chain constant regions (Choi et al., 2010). Studies comparing the Ig gene in passeriformes and galliformes have yet to be conducted, but it is likely they would exhibit still less homology. Still, it is possible that a different anti-chicken antibody could be employed successfully, as at least one anti-chicken antibody has been shown through Western blots and ELISA to cross-react with serum from passerine species (Martínez et al., 2003). Furthermore, while the antibody tested here was against the  $\mu$  heavy chain, it is possible that an antibody against the chicken light chain ( $\lambda$ ) might be more successful since antibodies against light chains may have stronger cross-reactivity (Cray and Villar, 2008).

To my knowledge, the only antibody that has been developed specifically for use in a passerine species is a rabbit anti-red-winged blackbird IgG antibody, which was used in an ELISA to measure mitogen-induced antibody production (Hasselquist et al., 1999). This antibody has not been validated for its specificity to red-winged blackbird antibody using immunological techniques such as a Western blot (Martínez et al., 2003). For this reason, I ran a Western blot using this antibody against both starling and zebra finch spleen cellular fraction and blood serum. The anti-red-winged blackbird antibody exhibited a certain amount of nonspecific binding but consistently bound to proteins of

about 13, 25 and 60kDa (Figure III.2.2a). However, most known avian  $\mu$  heavy chains have a large range from about 55-86kDa and light chains between 21 and 30kDa (Martínez et al., 2003; Lundqvist et al., 2006; Cray and Villar, 2008). The avian antibody with the lowest molecular weight known, a truncated version of IgY, which currently is only known to exist in anseriformes, has a heavy chain with a molecular weight of 37-42kDa (Bando and Higgins, 1996). Based on these observations, the specificity of the anti-red-winged blackbird antibody remains unclear.

Another common approach taken in immunological studies in organisms, for which markers are lacking, is to use a well-conserved transcription factor, specific to the cell type of interest (Zwollo et al., 2008). One such marker for B cells is the 53kDa protein, Pax5, which contains a paired box or DNA binding domain that is well conserved in human, mice, and zebrafish (Zwollo et al., 2005) but has yet to be tested in any avian species. An alignment of the Pax5 sequence in mice, zebra fish, chicken and the predicted sequence in zebra finch, suggests that the paired box domain is also well conserved in both species of bird (Figure III.2.1a). Thus Pax5 was a strong candidate for use as a B cell specific marker. An anti-Pax5 antibody described previously (Zwollo et al., 2005) was tested using Western blot analysis with starling and zebra finch spleen cellular extract and blood serum. The expected 53kDa band appeared in lanes containing starling and zebra finch cellular extract but not in the lanes containing serum (Figure III.2.2b). This showed that the anti-Pax5 antibody successfully cross-reacted with Pax5 in zebra finch and starling cells and did not non-specifically bind with proteins in the serum. Therefore, Pax5 could be used as a B cell marker for this study.

Preliminary flow cytometry trials further suggested that anti-Pax5 antibody could be used in conjunction with the proliferation marker EdU, to characterize proliferating and non-proliferating B cell populations (Figure III.2.4c-f). Pax5 is expressed at high levels in cells appearing early in the B cell lineage such as precursor and mature naïve B cells. However, as B cells are activated and begin to differentiate, Pax5 is slowly downregulated and is absent in plasma cells. Thus, when Pax5 and EdU are combined in two-color flow cytometry, several B cell populations may be defined. The Pax5-/EdU- population represents the population of both all non-B, non-proliferating cells, and the plasma cells in the spleen. The Pax5+/EdU- population includes resting B cells (which express high levels of Pax5). The Pax5+/EdU+ population represents the proliferating plasmablasts, which still express Pax5. Pax5-/EdU+ cells are any proliferating non-B cells, including proliferating T cells, none of which were observed at notable frequencies (Figure III.2.4e and f). Non-B cells did not proliferate in measurable quantities because a B cell specific mitogen (LPS) was used to stimulate proliferation (Keller et al., 2006). Plasma cells were not observed because it likely takes longer than 24 hours, the maximum time length in which cells were incubated with EdU, for avian spleen cells to differentiate into plasma cells based on studies in other organisms (Zwollo et al., 2008; Shaffer et al., 2004). A measure of the antibody-secreting plasma cell population in zebra finches would be a useful addition to this study however, as this cell type is a critical component of the humoral immune system's parasite-fighting function.

### *Proliferation medium*

Because passerine spleen cells are not commonly cultured, it was necessary to test the base medium and supplemental nutrients that would best support proliferation. Higher levels of proliferation would likely increase statistical power for detecting any differences between mercury-dosed and control treatment groups. The media tested included two that have been used previously in primary culture of spleen cells from trout and mice (Yui and Kaattari, 1987) and a chicken medium that is commonly used to culture DT40 cells. All three media were tested on starling cells on day 1, and trout and mouse medium, which both contain an RPMI base, were found to support higher levels of proliferation than the chicken medium (Figure III.2.3a and b).

The base components of all media tested were the same or very similar. All three contained either DMEM (chicken) or RPMI (trout and mouse) medium, a serum of either FBS (chicken and mouse) or FCS (trout), and BME. The chicken and trout media contained additional components, such as sodium bicarbonate and tryptose phosphate broth in the chicken media, or nucleosides and glutamine in the trout medium. Both the trout and mouse medium contained antibiotics while the chicken medium did not, suggesting that growth of bacteria may have played a role in the lower level of proliferation in the chicken medium. Because trout and mouse medium supported comparable levels of proliferation, it is likely that the additional factors present in the trout medium were not necessary for cultures of avian cells.

In order to ensure that the observed levels of proliferation were not simply an artifact of the medium, a Spearman's rank analysis was conducted. This analysis

illustrated that proliferation in all three media was positively correlated (Figure III.2.3c and d), demonstrating that the proliferation frequencies were biologically meaningful. Furthermore, because proliferation levels were greatest in the trout medium on day 1 and mouse medium on day 2, I concluded that either medium would support sufficient levels of proliferation to run a meaningful analysis. Mouse medium was chosen both because it is simpler than trout medium and because it is most commonly used for vertebrate *in vitro* B cell cultures.

#### **4.3 B cell proliferation data: starlings**

None of the apparent relationships observed between proliferation and mercury treatment or Hg PC1 were statistically significant in starlings. However, with a total sample size of only 12, this is perhaps not too surprising. The fact that the direction of these trends were consistent across all three days of proliferation, and were consistent with the zebra finch proliferation data, suggests that they may be biologically relevant and as such, merit further discussion and investigation.

Frequency of total proliferation was measured over three days in starlings, and a trend toward a decrease in mean proliferation was observed in the 0.4ppm mercury-dosed group versus the 0.1ppm group on days 1 and 2 (Figure III.3.1a). The 0.4ppm group demonstrated a decrease of about 25% from the 0.1ppm group on day 1 and 20% on day 2. When this trend was examined more closely by comparing total proliferation to individual Hg PC1 values, a weak negative relationship emerged on all three days (Figure III.3.1b, c and d). Again, although this trend was not statistically significant, it suggests that starlings that accumulate higher levels of mercury in their organs will likely

experience immunosuppression. I hypothesize that this decrease is biologically relevant, as 20-25% inhibition of lymphocyte proliferation would lead to a weakened ability of an organism to mount an appropriate immune response. The most likely direct result would be that fewer plasma cells would be formed, with a subsequent drop in serum levels of secreted Ig and reduced immune protection.

The negative relationship between total proliferation and greater mercury accumulation suggests two possible mechanisms for the effects of mercury. Mercury accumulation in B cells may inhibit proliferation directly or alternatively, it may lead to increased apoptosis, which would result in an apparent decrease in the frequency of proliferating cells. Because the frequency of resting B cells on day 2 and day 3 was greater in individuals with higher mercury, accumulation of mercury may induce apoptosis, but only in proliferating cells (Figure III.3.2). Note that because anti-Pax5 was not used on day 1, different populations of B cells, including resting B cells, could not be defined and measured. If resting cells that accumulated mercury were targeted for apoptosis, individuals with higher mercury accumulation would, in contrast, be expected to also have lower frequencies of resting B cells. Thus, apoptosis in proliferating cells is one possible explanation for the decreased proliferation and the relative increase in resting B cells in mercury-dosed groups. Without the use of an apoptosis marker, it is impossible to distinguish between an increase in apoptosis and direct inhibition of proliferation, and the two explanations may not be mutually exclusive. These mechanisms will be discussed in more detail below.

On day 3, the difference in mean frequency of total proliferation between 0.1 and 0.4 ppm treatment groups disappeared (Figure III.3.1a), in other words, both low and high MeHg groups proliferated at the same rate. This suggests that the primary effect of MeHg may be to delay the induction of proliferation, something that would be detectable only early in the response, such as in day 1 or day 2, in agreement with my results. This is further supported by the frequencies of resting B cells in the two mercury-dosed groups. Individuals with *higher mercury accumulation* may also have an *increased frequency of resting B cells*, because mercury slows down the ability of these B cells to proliferate. This same trend was observed when ratios of proliferating to non-proliferating B cells were calculated. The proportion of proliferating cells decreased at higher mercury levels (Figure III.3.3). Based on these data, I propose the following model: mercury accumulation (in B or T lymphocytes or antigen presenting cells) leads to delayed onset of proliferation, which subsequently leads to reduced humoral immune function. However, to demonstrate this conclusively, a study with larger sample size or higher mercury levels would be necessary.

#### **4.4 B cell proliferation data: zebra finches**

The trends observed in the 12 starling samples were evident and more pronounced, when a similar study was conducted in a much larger sample size of zebra finches with higher mercury exposure. Mean B cell proliferation was lower in the two mercury treatment groups than the non-dosed, reference population (Figure III.3.4a), and B cell proliferation also decreased in individual zebra finches with higher Hg accumulation (Figure III.3.4b). Again, the frequency of resting B cells was slightly

elevated in the mercury treatment groups versus the control (Figure III.3.4c), suggesting that mercury either induces apoptosis or inhibits or delays proliferation. These trends were stronger in zebra finches than in starlings but not statistically significant. However, the ratio of proliferating to non-proliferating B cells did significantly decrease as Hg PC1 increased (Figure III.3.5b), supporting the hypothesis that mercury accumulation in the blood and organs suppresses or delays the ability of B cells to proliferate in response to antigens.

#### **4.5 Field based studies**

Although previous tests of the avian immune response after mercury exposure are difficult to interpret, my results are consistent with earlier findings reported from field studies. The PHA skin response purports to measure T cell proliferation, and as such, is difficult to compare to this study on B cell proliferation. A more relevant field test to compare to the current study is the sheep red blood cell (SRBC) assay, which measures one aspect of B cell function, namely, antibody secretion. It is important to note however, that injection of SRBC leads to B cell activation that is T cell dependent as opposed to the LPS used in my study, which activates B cells in a T cell independent manner. This means that interpretations of this test should also take into account possible modulations in T cell behavior.

Studies employing the SRBC assay found a nonsignificant trend towards declining antibody production in mercury-exposed versus reference adult songbirds (Hawley et al., 2009) or a significant decrease in antibody production in juvenile waterbirds (Kenow et al., 2007). Again, these results are consistent with the results from my current study

but are difficult to interpret and do not elucidate any type of mechanism. If, as I have proposed, sublethal levels of mercury do not inhibit, but instead delay, B cell proliferation and function, a decrease in antibody production would not be observed if data were collected only at the time point of maximal antibody production. Data collected at multiple time points would help determine whether antibody secretion is slowed or completely inhibited.

#### **4.6 Possible mechanisms**

While there are many proposed mechanisms for the effects of mercury on the immune response, very little consensus has been reached. However, it is well known that mercury readily binds sulfhydryl groups including tubulin, thus blocking microtubule formation (Miura et al., 1984; Brown et al., 1988). This could lead to the decreased proliferation observed in this study because microtubule polymerization is the central process involved in mitosis and cell proliferation. Correct microtubule formation and alignment is the critical mitotic checkpoint. If a cell cannot pass this checkpoint, it will be suspended in mitosis until microtubule alignment is corrected or apoptosis will eventually be induced. These cells will have incorporated EdU and will also be Pax5+ and would appear as plasmablasts in two-color flow cytometry. If mercury inhibits proliferation by suspending cells at the metaphase checkpoint, I would have expected to see higher levels of proliferating cells in the mercury-exposed populations, which was not the case. However, if apoptosis is induced in cells suspended at this checkpoint, then this could lead to the observed decrease in the Pax+/EdU+ population in mercury-exposed samples.

There is strong evidence to suggest that mercury induces apoptosis in immune cells, but the mechanism and susceptible cell types are unclear. For example, one study found that mercury exposure led to induction of apoptosis in resting T cells (Shenker et al., 1997). Interestingly, mitogen-activated T cells did not undergo apoptosis in response to mercury, which might be due to the ability of activated cells to produce glutathione and metallothionein. These proteins bind and sequester mercury, potentially lessening its physiological effects (Boening, 1999). However, induction of apoptosis in resting but not activated lymphocytes runs counter to the trend observed here of increased resting B cells and decreased proliferating cells in mercury-exposed samples.

A later study on the mechanism of apoptosis induction by mercury found that the concentration of mercury added to murine spleen cells in culture may affect the mechanism and timing of apoptosis (Pheng et al. 2000). The study concluded that the addition of very low levels of MeHg might trigger an apoptotic pathway that is distinct from that initiated in cells exposed to relatively high, but sublethal, levels of mercury. This would explain why studies that used low levels of MeHg observed decreased apoptosis in activated T lymphocytes (Shenker et al., 1997), as opposed to the oft-reported decrease in the population of mitogen-stimulated proliferating lymphocytes (Brown et al., 1984; Day et al., 2007; Das et al., 2008), accompanied by the inhibition of microtubule polymerization (Brown et al., 1984). Whereas low levels of mercury may induce apoptosis in resting T cells, at higher levels of mercury, mitotic T cells are more sensitive to apoptosis, likely due to the disassembly of their microtubules (Pheng et al.,

2000). Although apoptosis and microtubule polymerization were not measured directly in my study, the induction of cell death during mitosis upon higher mercury exposure is one reasonable explanation for the diminished population of proliferating B cells.

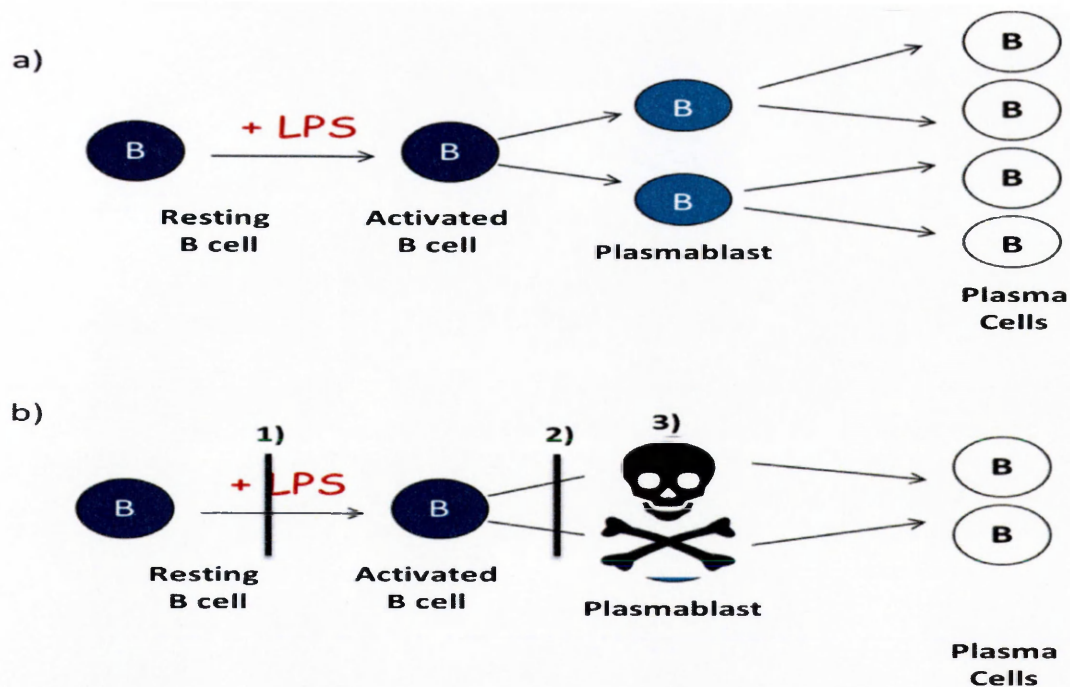
Microtubules are also critical for many other aspects of basic cell functioning, including vesicular transport and cell structure. If, instead of inducing apoptosis, microtubule disassembly leads to inhibition of these basic cellular processes, it would be likely that entry into mitosis would be inhibited or slowed. This presents an alternative explanation for the increase in resting B cells and the decrease in proliferating plasmablasts in mercury-exposed samples.

Another possible explanation for the decrease in B cell proliferation is the inhibition or dysregulation of signal transduction pathways necessary for lymphocyte proliferation and differentiation. For example, one study found that inorganic mercury led to diminished activity of the elements downstream of T cell receptor signal transduction, notably Ras, the small GTPase required for activating the mitogen-activated protein kinase (MAPK) pathway (Ziemba et al., 2006). This pathway relies on a signal cascade, which both amplifies signal and affects multiple endpoints of cellular function such as growth, proliferation and gene expression. Thus, even sublethal levels of mercury could have serious effects on numerous aspects of T cell function.

A separate line of evidence demonstrates that while the functions of T cells that are activated through the normal T cell receptor pathway may be suppressed, unstimulated T cells may be directly activated by inorganic mercury, leading to

upregulation of the MAPK pathway. Thus, mercury causes dysregulation of the MAPK pathway in T cells by decreasing sensitivity to pathogens while constitutively activating the downstream MAPK pathway even in the absence of bound ligand (Haase et al., 2011). This has important implications for an organism's health because even though the mechanism is likely different, constitutive activation of the MAPK pathway has been implicated in a large percentage of cancers. This pathway was not tested directly here, but if MeHg modulates the MAPK pathway, which is an important regulator of cell proliferation, this could lead to the results observed here.

It is important to note that whereas I isolated spleen cells from songbirds that were exposed to dietary mercury throughout their adult lifetimes, the majority of previous studies that explore potential mechanisms added mercury to peripheral blood lymphocyte (PBL) cultures *in vitro* over the span of several days (Shenker et al., 1997; Pheng et al., 2000; Ziemba et al., 2006; Haase et al., 2011). Several studies that tested the effects of naturally-occurring and *in vitro* mercury exposure on T and B lymphocytes found a dose-dependent decrease in lymphocyte proliferation in both cases implying that both types of mercury exposure have the same effect (Day et al., 2007; Das et al., 2008). However, these studies did not explore the potential mechanisms in detail.



**Figure IV.1.1.** a) Normal B cell proliferation and b) model for potential effect of MeHg on B cell proliferation: MeHg may inhibit signal transduction pathways involved in LPS activation (1), it may delay proliferation (2), or it may induce apoptosis in plasmablasts (3), all resulting in formation of fewer plasma cells.

The difference in species of mercury and type of exposure makes it difficult to interpret many of the previous studies in the context of the current study. Although my results are consistent with the apoptosis studies that employed higher levels of mercury, it is impossible to translate the mercury levels added to PBL cultures *in vitro* to the results of my long-term dosing study. In addition, most of the studies on signal transduction have employed inorganic mercury, which may have very different effects on the immune response than MeHg. I also tested the effect of mercury on B lymphocyte proliferation whereas the majority of previous studies on apoptosis as well as signal transduction have focused on T lymphocytes. Due to their function, these two cell types are likely differentially affected by mercury. However, both types of lymphocytes rely heavily on the well-conserved pathways of apoptosis and the MAPK

signaling in order to mount an appropriate immune response. Given the observed decrease in B cell proliferation and relative increase in the frequency of resting B cells in mercury-exposed samples, it seems reasonable to propose that that long-term MeHg exposure also increases apoptosis in proliferating cells, while inhibiting mitogen-induced activation of the MAPK pathway in songbirds.

#### **4.7 Implications**

While the mechanism remains unknown, the immunomodulation observed here is likely also observed in the wild as the MeHg levels used in this study are comparable to those in the wild. Starlings exposed to 0.4ppm MeHg accumulated blood-Hg levels (~2 ppm) comparable to mid-range levels of mercury exposure at a site with a legacy of an industrial mercury point source (Cristol et al., 2010) whereas 0.1ppm resulted in levels (~0.5 ppm) comparable to birds at sites with only atmospheric mercury inputs. No formal control was used, however, making it difficult to fully interpret the effects of the low dose of mercury on B cell proliferation in starlings. The zebra finch high dose (1.0ppm) resulted in zebra finches accumulating levels (~12 ppm) of mercury comparable to the highest levels of mercury ever observed in the field (Cristol et al., 2010) whereas the intermediate dose (0.5ppm) resulted in levels (~5 ppm) comparable to many songbirds at a site with a mercury point source (Cristol et al., 2010). Thus the effects observed in this study are applicable to songbirds at contaminated sites in the wild.

Our results suggest that the mercury levels present at polluted sites cause immunosuppression in songbirds. Both starlings and zebra finches that accumulated

higher mercury levels appeared to experience a decrease in both lymphocyte proliferation and the ratio of proliferating to resting B cells. Although most of these trends were not statistically significant, it is more important to consider whether these decreases in proliferation are biologically significant as opposed to representing normal variation within a healthy population (Adamo et al., 2004).

The starling sample size was smaller, thus making it unlikely that statistically significant differences in mercury dosed versus reference populations would be detected. However, a mean of 20-25% decreased cell proliferation on days 1 and 2 in the higher mercury treatment group is still remarkable and could indicate immunosuppression. Furthermore, this decrease appeared to be weakly correlated with an increase in individual mercury accumulation on all 3 days. The fact that these results were consistent across 3 days of proliferation in starlings, as well as occurring in 2 different songbird species, suggests that this trend may be robust and biologically significant. Regardless of mechanism, if mercury inhibits or slows B cell proliferation in an individual, that songbird will be more susceptible to pathogens, which may also lead to greater predation risk. Even if the observed decreases in B cell proliferation are within the range for normal functioning, if a songbird is immunocompromised by additional stressors or exposed to a high level of parasites, this could severely lessen an individual's chance of survival.

Furthermore, immunosuppression may also have a severe impact on fitness. If mercury suppresses immune function, a greater amount of energy will be required to

mount an effective immune response at a cost to other endpoints such as reproduction. Studies have shown that increased exposure to parasites leads to decreased reproductive output in adults (Fitze et al., 2004) and slowed growth in fledglings (O'Brien and Dawson, 2007). This is likely due to increased allocation of energy to survival and immune response. Decreased reproductive success represents a direct fitness cost to the individual, which could in turn affect the stability of songbird populations as a whole. Many songbird populations have experienced significant declines in the past several decades due to both natural and anthropogenic causes including climate change and industrial development (Friesen et al., 1995; Both et al., 2006). These factors can lead to increased predation and rapid spread of disease, problems that would be exacerbated by mercury-exposed individuals with suppressed immune response. Therefore, the cleanup of contaminated sites is a crucial aspect of an effective songbird conservation effort.

#### **4.8 Limitations and future directions**

This study aimed to develop the first lab-based assays of songbird immune response to mercury. While I was successful in developing one assay, this study had several limitations, generating multiple possibilities for future study. First, because, after processing, zebra finch spleens generally contained less than the optimal minimum number of cells for culturing ( $2 \times 10^6$  cells), all data were collected and fixed on day 1. Therefore it is unknown how well mercury-exposed B cells would proliferate at different time points. This would be particularly interesting as it would test the hypothesis that MeHg delays proliferation but does not lead to irreversible inhibition. A brief test of B

cell proliferation in 9 reference zebra finch spleen samples on day 1 versus day 2 suggested that B cell proliferation increases on day 2 (Figure III.3.6). Thus, if possible, future tests should measure proliferation on day 1 and 2, when greater individual differences in proliferation may be observed.

Another possible reason that statistically significant differences between treatment groups were not observed in most cases may be the ease of life in the aviary. The cages for both zebra finches and starlings were relatively small, so that birds did not have to fly far to gain access to the food and water that were provided *ad libitum*. It is likely that these birds were not exposed to a significant amount of stress or challenges to the immune system. Therefore the effects of mercury on immune response may not be fully comparable to the effects in the wild.

While this study is an important starting point in understanding the effects of mercury on songbirds on a cellular level, more information is needed to fully understand the significance of these effects. Acute mercury exposure induces apoptosis in T lymphocytes (Shenker et al., 1997; Pheng et al., 2000), and one possible explanation for my results is that it also induces apoptosis in proliferating B cells. Early in my study I began testing the commonly used apoptosis marker, Annexin V, but these studies were not pursued for lack of time and the limited number of available cells. Future studies in songbirds should consider including an apoptosis marker alongside measures of lymphocyte proliferation in order to provide a more complete picture of the cellular effects of mercury.

When assaying immunocompetence in any species, it is important to measure multiple aspects of the immune response. Taken alone, decreased B cell proliferation in response to mercury is difficult to interpret. Another aspect of adaptive or even innate immunity may simply be compensating for this weakness (Adamo et al., 2004). Conversely, if mercury inhibits the ability of organs such as the liver to remove toxins from the blood stream, lymphocytes may be exposed to higher levels of other toxins, which then inhibit their function. Thus, the effects of mercury on the immune system may be indirect. For both of these reasons, liver macrophage phagocytosis will be an important assay to complete as well.

Although it is beyond the scope of the current study, and new markers may need to be developed, there are multiple additional endpoints that should be tested. Here, T cell independent (LPS) activation of B cells was measured. The benefit of this approach is that the ability of B cells to proliferate was measured in isolation from T cell function. In addition, since the majority of B cells are activated by LPS, it is easier to compare samples. For this same reason however, mitogen activated B cell proliferation may not be the best method for discerning finer differences in individual immunity. Future studies should consider using specific antigen such as TNP-KLH (Trinitrophenyl hapten-keyhole limpet hemocyanin), which would activate a smaller subsection of B cells to proliferate.

If T cell dependent B cell proliferation is measured, then it would be necessary to also measure T cell proliferation and function. This would allow for conclusions of

whether B cell proliferation itself was affected in addition to or instead of the function of T cells. Moreover, the majority of previous studies have focused on the effects and mechanisms of mercury in T cells. Assays of T cell proliferation and function in songbirds would therefore better allow for comparison to previous studies. One important aspect of T cell function that could be measured is cytokine production. The current lack of antibodies directed against songbird cytokines presents a challenge, but cytokine production is important for stimulation and activation of B cells in the T cell dependent pathway of B cell activation. In addition, although this would not directly test the effect of mercury on the MAPK pathway, which is important in proliferation and cytokine production, it might help inform the direction of future study in this area.

## Conclusion

This study represents the first step in determining the effects of sublethal levels of mercury on the songbird immune response. I have developed a macrophage phagocytosis assay that can be used in future songbird studies to measure this critical aspect of the innate immune system. I have also established a flow cytometric assay that can be used in future studies of songbird B cells, and I measured the effects of mercury on mitogen-induced B cell proliferation. Both starlings and zebra finches that accumulated higher levels of mercury tended to have decreased B cell proliferation accompanied by a decrease in the ratio of proliferating to resting B cells. In relation to a decreased frequency of proliferating B cells, these individuals also experienced an increase in resting B cells. Based on previous study on the effects of mercury, suppressed B cell proliferation in songbirds may be due to a mechanism involving apoptosis or signal transduction. However, it is impossible to propose a mechanism based on this study. Studies of both T and B cell proliferation and function as well as apoptosis and potentially signal transduction are needed to help provide a more complete understanding of the effects of mercury.

Based on the results observed here, I propose that sublethal levels of MeHg delays B cell proliferation, thus making it more difficult for songbirds to mount a rapid adaptive immune response. This would lead to a greater susceptibility to parasites, which could pose a major threat to songbirds in the wild. It is important to understand whether mercury levels representative of those found in the wild have an effect on the

immune system, so that it is possible to better educate environmental agencies, polluters, and the general public about the dangers of mercury contamination.

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