

AN EVALUATION OF HEAT SHOCK PROTEIN EXPRESSION IN THE BLOOD CELLS
OF FREE-LIVING COMMON LOONS (*GAVIA IMMER*)

Ericka Leigh Griggs

Masters of Science in Integrated Biological Diversity
Western Connecticut State University

August 2022

Advisor: Michelle Y. Monette

Abstract

Common loons in the northeastern United States are subjected to a wide variety of environmental and anthropogenic stressors. Many of the biomarkers currently used to assess individual or population health of common loons are influenced by capture and handling, therefore there is a need to develop new biomarkers of physiological condition that can aid in the management of this species. Heat shock protein 70 (HSP70) is a stress protein found within the red blood cells of many vertebrates that has been used as a biomarker of general stress in avian species. The objective of our study was to evaluate the use of HSP70 as a potential intracellular biomarker of physiological condition of loons from across New England. We collected blood samples from adults and hatch-year chicks across Maine, New York, and Massachusetts during the 2021 breeding season, and measured indicators of stress at the circulatory (% hematocrit, plasma glucose, blood mercury), immune (heterophil to lymphocyte ratio), and cellular level (hemoglobin and HSP70 protein expression). Our results revealed that: (1) HSP70 is present in the red blood cells of common loons; (2) HSP70 expression does not vary significantly with age or sex; (3) HSP70 expression does not appear to be correlated to blood mercury levels, and (4) there is a trend that higher expression of HSP70 in blood cells may be associated with anemia in common loons. Together, the evidence we present suggests that HSP70 should be further investigated as a potential blood biomarker of physiological condition in loons when combined with other measurable factors of environmental and anthropogenic stress.

Table of Contents

Abstract.....2

Acknowledgments.....5

Introduction.....6

 Study Species.....6

 Current Biomarkers.....6

 Heat Shock Proteins (HSPs).....8

 Objective and Hypotheses.....9

Methodological Approach.....10

 Sample Collection.....10

 Blood Analysis.....11

 Heat Shock Protein (HSP70) Expression.....12

 Data Analysis and Statistics.....13

Results.....14

Discussion.....15

 Is HSP70 detectable in the red blood cells of common loons?.....15

 Are age or sex predictors of HSP70 protein abundance?.....16

 Are HSP70 levels correlated to blood Hg concentrations in common loons?.....17

 Are HSP70 levels correlated with other blood cell metrics?.....18

Summary of Major Findings.....21

Literature Cited.....22

Table and Figure Legends.....30

Tables and Figures.....	32
Table 1 & 2.....	32
Figure 1.....	33
Figure 2 & 3.....	34
Figure 4.....	35
Figure 5.....	36

Acknowledgments

To begin with, I would like to express my sincere gratitude and appreciation to my thesis advisor Dr. Michelle Y. Monette for giving me the opportunity to work on this project, providing valuable guidance and feedback, and challenging me to grow as a researcher. Dr. Monette provided me with unlimited support and unconditional guidance throughout my master's program. Additionally, I would like to extend my gratitude to Dr. Theodora Pinou for her guidance and mentoring throughout my degree process.

Without the support and collaboration of many people, this research would not have been possible. Lucas Savoy and the Biodiversity Research Institute deserve a tremendous thank you for providing funding for this project, helping to collect samples, and providing guidance, support, and feedback throughout the past two years. As well, I want to thank Dr. Nina Schoch and the staff at Adirondack Center for Loon Conservation for the opportunity to be involved in this project and for assistance with sampling. Also, I would like to thank Dr. Randall Walikonis from the Department of Physiology and Neurobiology at the University of Connecticut, Storrs for the use of the Licor Odyssey Infrared Scanner. Lastly, thank you to the members of my committee, Dr. Mark Pokras, Dr. James Paruk, and Dr. Patrice Boily, who provided guidance and support throughout my degree.

I am grateful to the National Science Foundation for awarding me a 2021 Graduate Research Fellowship, which has enabled me to complete this research. As a final note, I would like to thank my family, friends, and fellow graduate students for their constant support and love.

Introduction

Study Species

The common loon, *Gavia immer* (Brunnich, 1764), is a long-lived, low fecundity waterbird that migrates from marine waters to lakes within the northeastern United States for reproduction from April - September (Paruk et al., 2021a). The common loon is considered an important ecological indicator of aquatic ecosystem health due to its exposure to, and bioaccumulation of, pollutants such as mercury (Hg) through their diets (Evers, 2004). While Hg is known to negatively impact reproduction in loons (Evers et al., 2008), it is only one of the factors contributing to the decline of populations in North America. A recent study on breeding loons found that climate change stress interacting with other anthropogenic (e.g., human disturbance, shoreline development, recreation) and environmental (e.g., lake pH, fish abundance, temperature) factors has led to declines in loon populations in Canada (Bianchini et al., 2020). Scientists and managers have utilized extensive monitoring, education, and conservation efforts to demonstrate that these factors can be used to predict reproduction success (Mitro et al., 2008; Evers et al., 2008; Burgess and Meyer, 2008; Bianchini et al., 2020). However, it is still unclear how these factors are affecting other aspects of loon physiology.

Current Biomarkers

As a result of the difficulty of capture, blood is the most easy and abundant tissue to collect from live, wild loons. This limits biomarkers of stress to those that are strictly derived from within blood samples. In a free-living population, pinpointing a single stress factor is nearly impossible, so instead we have decided to target biomarkers that are general indicators of overall condition, like oxygen-carrying capacity, immune function, and hormonal changes. In wild

vertebrates, the most common measurements of blood oxygen-carrying capacity are hemoglobin and % hematocrit (Minias, 2020). Typically, birds in better health maintain higher levels of hemoglobin and/or hematocrit as this helps to increase aerobic performance needed to meet the high metabolic demands of their tissues (Coles et al., 2009). A deficiency of red blood cells is known as anemia, and non-regenerative anemia is the most common type of anemia described in birds (Jones, 2015). It has been suggested that non-regenerative anemia is an indirect stress response, and is usually a consequence of nutritional stress, chronic disease, or exposure to toxic substances (Johnstone et al., 2017). It is difficult to correlate a direct stressor with a decline in hemoglobin and/or % hematocrit in free-living birds, therefore both blood parameters are often used together as generalized indicators of anemia in loons (Haefele et al., 2006; Paruk et al., 2014). However, short-term stress from capture has been shown to influence both of these biomarkers (Gormally and Romero, 202), therefore the reference ranges of both metrics in common loons are caveated as stress induced.

Currently, there is no way to index immune function in birds from within the red blood cell (Johnstone et al., 2017). However, the H/L ratio, which is the ratio of heterophils to lymphocytes, is a well-known biomarker of avian stress derived from white blood cell counts (Maxwell, 1993). H/L ratios have been demonstrated to have predictive powers as stress indicators, with high ratios being generally indicative of high glucocorticoid levels (Davis et al., 2008). A reference range of white blood cell counts has been previously published in common loons for both adults and hatch-years (Haefele et al., 2006). However, to our knowledge the relationship between H/L ratios and stress hormones such as glucocorticoids has not been examined in common loons.

Circulating levels of glucocorticoid hormones is a common biomarker in free-living vertebrates, and corticosterone (CORT) is a widely measured stress hormone in birds (Romero, 2004). However, CORT is seldom used as a stress biomarker in common loons since it is known that capture and handling can influence short-term stress reactions in vertebrates (Johnston et al., 2017). As a potential alternative to measuring stress hormones, measurements of intracellular stress proteins from within the erythrocyte or red blood cell have been suggested due to their reduced fluctuation with capture stress (Johnstone et al., 2017). One such group of proteins that does not appear to be affected by capture stress in wild vertebrates are the heat shock proteins (HSPs) (O'Dell et al., 2014; Finger Jr. et al., 2021).

Heat Shock Proteins (HSPs)

Certain environmental stressors can cause cellular proteins to unfold or misfold in response to cellular damage, which subsequently triggers a specific cellular response called the heat shock response (HSR) (Sørensen et al., 2003). The HSR is a mechanism for the cell to restore homeostasis, and its activation signals multiple pathways including the upregulation of molecular chaperone proteins called HSPs (Herring and Gawlik, 2007). It is generally understood that the role of HSPs in vertebrates is one of a house-keeping chaperone protein, and it has been demonstrated that a variety of different environmental stressors, including thermal and nonthermal stress can increase the expression of the inducible HSPs (Feder and Hoffman, 1999). The highly conserved HSP70 is one of the most well-studied classes of HSPs and is known to function early in the process of protein folding and stabilization during the cellular stress response (Tavaria et al., 1996). HSPs are found in all vertebrates and can produce proteins encoded by the HSP70 gene family in response to elevated temperatures (Lindquist and Craig, 1988). Although inducers of expression vary from organism to organism, in nearly all cells other

stressors such as anoxia, and certain heavy metals can also induce expression of HSP70 (Lindquist and Craig, 1988).

In avian species, the total specific activity of HSPs within a tissue is thought tmay help to maintain homeostasis during times of increased stress (Tomás et al., 2004). HSPs in birds have been correlated to stressors such as temperature (Hill et al., 2013), parasite load (Merino et al. 1998), predation risk (Thomson et al., 2010), sibling competition (Martinez-Padilla et al. 2004), pollution (Herring et al., 2014; Eeva et al., 2014), and nutritional stress (Moreno et al., 2002). HSPs have also been correlated to measurable physiological changes such as plasma antioxidant capacity (Moreno et al., 2013), H/L ratios (Moreno et al., 2002), and body condition (Martinez-de la Puente et al., 2007). These studies demonstrate that HSPs are either upregulated or downregulated depending on the type of stressor, making it unclear how HSPs function within avian red blood cells during stress. Thus, HSPs can be seen as mediators of the cellular stress response and may be better used as complementary to traditional immune and hormonal biomarkers when investigating impacts of anthropogenic and environmental stressors in birds (Ibanez-Alamo et al., 2020).

Objective and Hypothesis

The New England (NE) common loon population consists of several reproducing sub-populations throughout Maine, New Hampshire, Vermont, Massachusetts, Connecticut, and New York (Larison et al. 2021). Even though the NE population is considered stable and increasing, in several of these states, loons are classified as either threatened or a species of concern (Paruk et al., 2021a). With the help of intensive management and monitoring programs, the NE population continues a southward expansion into regions where the species has been extinct since the late 1800s (Paruk et al., 2014). Specifically, the 2020-2025 loon chick translocation

program on lakes in western and southeastern MA is reintroducing loons to more densely populated and industrialized areas in which they have not reproduced in over 100 years (Kneeland et al., 2020; Bent, 1919). Thus, future management of the population surrounding the translocation program in MA have raised concerns for the potential reproductive future of these loons since the majority of these birds were released to lakes with fish that have detectable Hg concentrations (Rose et al., 1999).

The main objective of this study was to evaluate the use of avian HSPs as a potential biomarker of physiological stress in loons across NE. Specifically, the following research questions were addressed: (1): Is HSP70 detectable in the red blood cells of common loons? If so, are age or sex, predictors of HSP70 protein abundance? (2): Are HSP70 levels correlated to blood Hg concentrations in common loons? (3) Are HSP70 levels correlated with other blood cell metrics? We hypothesized that circulating levels of HSP70 will provide a useful intracellular biomarker of stress in common loons and will add to existing tools to assess physiological condition in this species.

Methodological Approach

Sample Collection

Samples from adult and hatch-year loons were collected in three different geographic regions of the New England population (Massachusetts n=10, New York n=15, and Maine n=54) from June-September, 2021. Capture and sampling were achieved using an established night lighting technique (described in Evers, 2001). Territorial pairs were chosen opportunistically based on accessibility and availability of capture crews. All sampling was conducted in collaboration with the Biodiversity Research Institute (Portland, ME), which obtained the

necessary permits and animal handling protocols for capture and sample collection from this species (Protocol Number: 050621-6). Upon capture of both adults and hatch-years, weight, bill and tarsus morphometrics, and whole blood was collected, and each individual bird was banded for re-sighting. Blood was drawn from the metatarsal vein with a 21-gauge butterfly needle and stored on cold packs. All individuals were banded with an aluminum band numbered by the U.S. Geological Survey along with custom-formed plastic color bands (described in Evers, 2001). Sex in adults was determined by weight, vocalization, or previous band combinations. Sex was not determined for hatch-years. The sampling protocol was designed to maximize the number of known metrics that could be measured from the blood collected. Due to the difficulty of capturing free-living loons, sampling was done opportunistically and resulted in an inconsistent number of samples per each stress metric measured.

Blood was collected from each bird with the objective of getting at least 1 green top blood collection tube containing sodium heparin as an anticoagulant, and at least 2 heparinized capillary tubes. One collected green top blood collection tube was centrifuged for 15 minutes at a minimum speed of 12,000 rpm. Plasma was then separated from red blood cells, pipetted into a separate cryovial, and stored at -80°C until further analysis. Red blood cells were placed in a 1.5 mL cryovial, and immediately frozen in liquid nitrogen or dry ice before being placed in a -80°C freezer.

Blood Analysis

Hematocrit was measured in the field by several different field crews using a microcapillary centrifuge. Blood was centrifuged at 12,000 rpm until separation was achieved, and packed cell volume or % hematocrit was determined using a micro-capillary reader.

Samples were analyzed for total Hg content using a direct Hg analyzer (Nippon MA-3000; Biodiversity Research Institute, Portland, ME), as previously described (Adams et al., 2020). Blood smears were prepared and stored at room temperature before being sent to the Veterinary Diagnostic Lab at the University of Miami for the determination of differential white blood cell counts. Differential white blood cell counts determined the number of heterophils, monocytes, eosinophils, and basophils in each smear. H/L ratios were then determined by dividing the number of heterophils by the number of lymphocytes.

Frozen red blood cells were thawed, and 100 - 200 μ l of each sample was transferred to a 1.5 mL cryovial, homogenized with a hand-held homogenizer for 30 seconds, and centrifuged for 15 minutes at 12,000 rpm at 4° C. Hemoglobin was then quantified in each sample using a hemoglobin colorimetric detection kit (Invitrogen™, ThermoFisher Scientific, Waltham, MA). Plasma samples were thawed at room temperature and centrifuged at 12,000 rpm for 5 minutes at 4° C. Plasma glucose levels were quantified using a colorimetric glucose assay (Glucose HK reagent, Sigma #G3293).

Heat Shock Protein (HSP70) Expression

The expression of intracellular HSP70 was examined using SDS-PAGE followed by Western Immunoblotting in blood cell samples as previously described in birds (Tomás et al., 2004). To begin, 50 μ l of red blood cells was added to 200 μ l of homogenization buffer containing EDTA-free Protease Inhibitors (cOmplete™, Sigma Aldrich). Samples were then sonicated, centrifuged for 10 minutes at 12,000 rpm at 4° C, and the supernatant was removed. Total protein concentration (μ g/ μ l) of supernatant was quantified using the Bio-Rad Protein Assay (Bio-Rad Laboratories). Supernatants were then combined with 4x Laemmli Sample Buffer (4X LB) containing 10% β -mercaptoethanol before being loaded into Mini-PROTEAN

TGX Stain-Free Precast Gels (Bio-Rad Laboratories). Each gel was loaded with 660 µg total protein. Gels were run at 200 V for approximately 40 minutes and then transferred to a 0.2 µm PVDF membrane (Bio-Rad Laboratories) using a Trans-Blot Turbo Transfer System (Bio-Rad Laboratories). For immunoblotting, membranes were first blocked in 5 ml of 1% Bovine Serum Albumin (BSA) for 1 hour in phosphate-buffered saline containing 1% Tween (PBS-T) at room temperature, and then rinsed three times for 5 minutes in PBS-T. Membranes were incubated overnight at 4°C with a HSP70 monoclonal mouse antibody (Lk2, Sigma Aldrich) at a ratio of 1:2500 in PBS-T with 1% BSA. Membranes were then incubated for 2 hours at room temperature with an 800CW goat anti-mouse IG secondary antibody (LICOR Biosciences) at a ratio of 1:5000 in PBS-T with 1% BSA. Membranes were then rinsed three times for 5 minutes in PBS-T. All membranes were scanned on an Odyssey InfraRed Scanner (LICOR Biosciences), and protein expression of HSP70 was quantified using Image Studio Lite Western Blot Analysis Software version 3.1 (LICOR Biosciences). HSP70 band intensity was expressed relative to an internal control present on each gel.

Data Analysis and Statistics

All statistical analyses were performed with Sigma Plot (version 13.0). We used general linear models to examine the effects of sex and age on each of the following variables: weight, hemoglobin, hematocrit, H/L ratio, plasma glucose, blood Hg, and HSP70 protein expression. An objective of the study was to examine whether expression levels of HSP70 were correlated to commonly measured physiological variables in loons. In order to examine the *HSP70 x variable* relationship, we first performed a Pearson's product-moment correlation coefficient. We then executed multiple independent *HSP70 x variable* linear regression models to further examine the relationships between these indicators based on age and sex. Prior to all analyses, variables were

either log transformed or ranked to meet the assumptions of normality and homogeneity of variance, when necessary. Since multiple comparisons were involved, Bonferroni corrections were applied to correct significance values.

Results

We collected blood samples from a total of 79 common loons across ME, NY, and MA, including adult males (N=27), adult females (N=23) and hatch-years (N=29). Descriptive statistics are reported for all measured variables (weight, hemoglobin, hematocrit, H/L ratios, plasma glucose and blood Hg) in Table 1. Due to the small sample size from each state, geographic location was not a factor that was evaluated in relation to the measured variables.

We first examined the effect of age and sex on the following variables: hemoglobin, hematocrit, H/L ratio, plasma glucose and blood Hg. When comparing adults and hatch-years, we found significant differences for all of the variables examined ($P \leq 0.004$), except H/L ratios ($P = 0.179$, data not shown) (Fig. 1). Adults had significantly higher hemoglobin (Fig. 1A), % hematocrit (Fig. 1B), and blood Hg levels (Fig. 1D) than hatch-years ($P \leq 0.004$). However, hatch-years had significantly higher plasma glucose concentrations than adults (Fig. 1C, $P < 0.001$). There was no significant effect of sex on any of the measured variables ($P \geq 0.144$), with the exception of blood Hg where males had significantly higher levels as compared to females ($P = 0.011$) (Fig. 2A). Adult male blood Hg levels ranged from 0.7 - 3.8 ppm with a mean (\pm SD) of 1.6 ± 0.7 ppm (n=26), while females blood Hg levels ranged from 0.4 – 3.0 ppm with a mean (\pm SD) of 1.2 ± 0.6 ppm (n=21) (Table 1). Further, linear regression revealed that weight was significantly correlated to blood Hg concentration in common loons ($P = < 0.001$, $R^2 = 0.604$) (Fig. 2B).

HSP70 protein expression was quantified in the blood cells of loons using Western Immunoblotting (as shown in Fig. 3). For representative samples, a single band at ~70 kDa was detected. Neither age nor sex had a significant effect on HSP70 expression levels ($P \geq 0.206$) (Fig. 4). In adult and hatch-years, a Pearson's product-moment correlation coefficient was performed on the variables HSP70, hemoglobin, hematocrit, H/L ratio, plasma glucose, and blood Hg to determine the *HSP70 x variable* interaction (Table 2). Linear regression models determined hemoglobin, H/L ratio, plasma glucose, and blood Hg were not significantly correlated with HSP70 expression ($P \geq 0.28$) (Fig. 5). We found that % hematocrit had a trend toward a positive correlation to HSP70 expression in the common loons in our sample set ($P = 0.02$, $R^2 = 0.17$) (Fig. 5B).

Discussion

Common loons are an ecological species of importance that can only be regularly monitored for health during the breeding season. At present, only a few physiological biomarkers are available for assessing the physiological condition of free-living common loons. The objective of this study was to examine an intracellular biomarker in order to contribute to the on-going challenge of measuring stress in a species that is difficult to capture. Our results reveal several important findings: (1) HSP70 is present in the red blood cells of common loons; (2) age and sex are not predictors of variation in HSP70 expression levels; (3) HSP70 levels do not appear to be correlated to blood Hg concentrations, (4) there is a trend that elevated expression of HSP70 in blood cells may be associated with anemia in common loons.

Is HSP70 detectable in the red blood cells of common loons?

We were able to detect HSP70 in the red blood cells of both adult and hatch-year common loons. By using the method of blood collection and processing presented in Tomás et al., 2004, we were able to quantify HSP70 expression between different individuals within the NE population, indicating that this technique represents a reliable way to measure HSP70 in field studies of common loons. Although it is known that HSP70 can be detected in the red blood cells of other vertebrates, to our knowledge this is the first study to specifically investigate HSPs in the blood cells of common loons. As HSP70 was present in both adult and hatch-year blood cells, we suggest that this protein may play a significant role in blood cell dynamics, even though its function is not fully understood. As HSP expression has been linked to different environmental stressors (Feder and Hoffman, 1999), it may provide a new intracellular biomarker in loons when certain stress factors are known.

Are age or sex predictors of HSP70 protein abundance?

Our results suggest that age and sex are not predictors of HSP70 expression levels in loons. There have been few studies investigating the relationship between HSP expression with age and development, with the most prominent in *Drosophila* showing that even under controlled laboratory conditions, HSP70 expression exhibited a lot of variation, since individuals of the same species, at the same age, and same heat-shock displayed different levels of expression (Feder et al., 1996). Similarly, a study in rainbow trout (*Oncorhynchus mykiss*) was able to demonstrate significant increases in HSP70 in juveniles fed a metal-contaminated diet but not in the adults that were fed the same diet (Williams et al., 1996), suggesting a developmental difference in the HSP response. Studies such as these demonstrate the difficulty of not only comparing expression levels between adults and chicks, but also comparing HSP70 in

individuals of the same age. It should be noted that HSP70 expression levels in hatch-year chicks has previously been positively correlated with growth (Moreno et al., 2002), however due to the opportunistic nature of our study, we were not able to explore this idea further in hatch-year chicks.

Our results suggest that sex was not a significant predictor of HSP70 expression levels within our population. This is contradictory to a study in barn swallows (*Hirundo rustica*) that suggested males had higher HSP levels than females (Merino et al. 2002). The authors of this study suggested that this sex difference was related to differences associated with immune function changes related to parasitism (Merino et al. 2002). Similarly, a study in Antarctic penguins found that chinstrap penguins (*Pygoscelis antarctica*) showed significant differences in HSP60 levels between sexes, with females having higher levels; while gentoo penguins (*Pygoscelis papua*), on the other hand, showed opposite differences for both HSP70 and HSP60 levels (Barbosa et al., 2007). Together, these studies indicate that the effect of sex on HSP70 levels may differ across species. While adults were all in the NE population, we did not know the age of the adults in our study, nor did we know any life-history traits. HSP70 levels do not differ significantly between sexes in our generalized sample of adults of the same populations, indicating there is no sex difference in HSP70 levels when no direct stress source is present.

Are HSP70 levels correlated to blood Hg concentrations in common loons?

We found detectable levels of Hg in the blood of all common loons within our population. All detected levels were sub-lethal, and all except one male had concentrations above the adverse effect threshold (3.0ppm), which has been published as being associated with negative impacts on reproductive behavior (Ever et al., 2008). We determined that Hg levels in our loons differed significantly according to their weight, with adult males having higher levels

than females and hatch-years. This result is consistent with previous literature as prey size relates to loon size, so Hg levels have been found to be higher in loons with higher weights (Evers et al., 1997). However, our results did not show a relationship between HSP70 levels and circulating blood Hg in either adult or hatch-year birds. This differs from a study conducted on great egret (*Ardea alba*) chicks in the Everglades which found that HSP70 protein expression was downregulated when levels of Hg in red blood cells were high (Herring et al., 2014). Even though the Florida study showed that Hg concentration influenced HSP70 levels, the chicks in that study were younger than ours and had higher concentrations of Hg than we detected in our hatch-years. Due to the unknown cumulative exposure to Hg in each individual, some adults may have been more acutely exposed to Hg during the season than others. In turn, this would influence HSP expression as acute Hg exposure might trigger cellular mechanisms that a more long-term accumulation of Hg would not, hence affecting HSP70 expression levels. There is also a possibility that the loons in our study had too low a concentration of blood Hg to elicit a HSR response that would warrant the elevation of HSP70. Therefore, further studies are needed to determine whether acute, sub-lethal Hg below 3.0 ppm results in any other measurable long-term physiological response, and whether those responses are associated with changes in HSP70 levels.

Are HSP70 levels correlated with other blood cell metrics?

In addition to measuring blood Hg levels, we also measured several other blood metrics including hemoglobin, hematocrit, plasma glucose, and H/L ratios to examine the relationship of other well-known indicators of physiological stress in birds and HSP70 expression levels. We found no relationship between hemoglobin, plasma glucose, and H/L ratios with HSP70 expression levels. Based on our comparison of hemoglobin between adults and hatch-years, our

results are consistent with the literature, showing that adults have higher values as compared to hatch-years (Haefele et al., 2006). However, we measured hemoglobin using only red blood cells instead of whole blood, therefore, the values reported in this study are overall higher than previously published values for loons (Paruk et al., 2014). It has been suggested that HSP70 is part of a molecular chaperone system that supports functions including the promotion of red blood cell differentiation and survival (Mathangasinghe et al., 2021). Therefore, we were surprised to see that hemoglobin concentration was unrelated to HSP70 expression levels since hemoglobin is one of the most numerous proteins within the red blood cells.

It is not surprising that there was no significant correlation between plasma glucose and HSP70 levels because these two parameters may be measuring separate physiological responses, and because glucose levels can be heavily influenced by food intake, as well as acute handling and restraint stress (Remage-Healey and Romero, 2001). However, we are able to report that hatch-years had significantly higher glucose levels than adults which is consistent with a previous study of blood glucose levels in wild caught loons (Haefele et al., 2006). In adults, plasma glucose levels have been reported to be higher in female loons than males (Kneeland et al., 2021). However, our data did not support these findings. Although these differences are not completely understood, a lab study on captive budgerigars (*Melopsittacus undulata*) concluded that adults have lower glucose levels than juveniles due to differences in response to capture and handling (Bailey et al., 1999). There may be a similar phenomenon in the plasma glucose levels of loons, with glucose level variation being based on how the individual bird is physiologically responding to capture stress. Further, glucose variation between individuals, coupled with HSP70's response to different stressors, may make the link between the two variables extremely difficult to define.

We found no correlation between age or sex with H/L ratio, and ‘normal’ parameters for H/L ratios in common loons are unknown. Similar to previous studies in birds, we also failed to find a relationship between H/L ratios and HSP70 protein expression (Moreno et al., 2002; Ruiz-Raya et al. 2022). We suggest that this lack of relationship could be due to the fact that H/L ratios can be affected by many factors including those related to immune function (Davis et al., 2008), whereas HSP70 expression may be regulated as part of a generalized stress response (Sørensen et al., 2003). Due to individual differences in H/L ratios, an exact immune stressor, such as a disease or parasite load, coupled with immune system analytes, might be able to reveal more about HSP70 expression and H/L ratios than just measuring these two parameters alone.

It has been previously reported that adult loons with hematocrit < 40% are considered anemic (Paruk et al., 2014; Paruk et al., 2021b). The hematocrit of males in our population ranged from 42-58%, while females ranged from 32-55%. A meta-analysis of 36 bird studies showed no difference in hematocrit between the sexes (Whitaker and Pearson, 2007), and our data was consistent with this finding. It is known that due to increased erythropoiesis that adult birds generally have higher hematocrit than chicks, and that is reflected in our data (Whitaker and Pearson, 2007). A wide variety of causes of different natural factors that include age, energy expenditure, parasitism, nutrition, and genetics could be the reason for the % hematocrit difference in adult loons (Whitaker and Pearson, 2007). Due to this, % hematocrit is difficult to use as a general indicator of health since it is a secondary response to a greater stressor decreasing aerobic capacity. We believe further exploration is needed to define the cause of lower % hematocrit before conclusions can be made on the relationship between HSP70 expression levels differences in males, females, and chicks, however our study suggests that elevated expression of HSP70 in blood cells may be associated with anemia in common loons.

Summary of Major Findings

As common loons continue a southward expansion, it is important for managers to understand the physiological health of individuals in these populations. HSP70 is an intracellular chaperone protein that does not appear to be affected by age or sex when it comes to measuring abundance within the red blood cell. In all loons that were sampled, HSP70 was expressed universally, which suggests it is likely playing a significant role in the function of blood cells. Hematocrit is the most common general indicator of health in loons, and we observed a trend that indicated birds are increasing HSP70 protein expression in response to lower hematocrit levels. However, a key component that was lacking from our study was a health or body condition index that could be used as a visual assessment of the bird at the time of capture. This would have allowed the evaluation of fitness for each bird at the time of capture, and any noticeable stress factors would have added validity to the type of biomarkers we used to target stress responses within the cells. Further research is needed to establish some kind of overall health or body condition index for the species so stress biomarkers, such as HSP70, can be useful in informing management practices. Based on the evidence we have presented thus far, we suggest that HSP70 should be further investigated as a potential blood biomarker of physiological condition in loons when combined with other measurable factors of environmental and anthropogenic stress.

Literature Cited

- Adams, E. M., Williams, K. A., Olsen, B. J., and Evers, D. C. (2020). Mercury exposure in migrating songbirds: correlations with physical condition. *Ecotoxicology*, 29(8), 1240-1253.
- Bailey, T. A., Wernery, U., Howlett, J., Naldo, J., and Samour, J. H. (1999). Age-related plasma chemistry changes in houbara and kori bustards in the United Arab Emirates. *Journal of Wildlife Diseases*, 35(1), 31-37.
- Barbosa, A., Merino, S., Benzal, J., Martínez, J., and García-Fraile, S. (2007). Population variability in heat shock proteins among three Antarctic penguin species. *Polar Biology*, 30(10), 1239-1244.
- Bent, A. C. (1919). *The life histories of North American birds*. Smithsonian Institution Press, Washington, D.C.
- Bianchini, K., Tozer, D. C., Alvo, R., Bhavsar, S. P., and Mallory, M. L. (2020). Drivers of declines in common loon (*Gavia immer*) productivity in Ontario, Canada. *Science of the Total Environment*, 738, 139724.
- Burgess, N. M., and Meyer, M. W. (2008). Methylmercury exposure associated with reduced productivity in common loons. *Ecotoxicology*, 17(2), 83-91.
- Coles, B. H., Crosta, L., Cruz-Martinez, L. A., De Herdt, P., Dorrestein, G., Harcourt-Brown, N., Harris, D. J., Jones, A. K., Krautwald-Junghanns, M., Macwhirter, P., Olsen, G. H., Pasmans, F., Pees, M., Redig, P. T., Robinson, I., Routh, A., Sanderson, S., Timossi, L.,

- Tully, T. N., and Worell, A. B. (2009). *Handbook of Avian Medicine*. (Second Edition). Saunders-Elsevier, London.
- Davis, A. K., Maney, D. L., and Maerz, J. C. (2008). The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology*, 22(5), 760-772.
- Eeva, T., Rainio, M., Berglund, Å., Kanerva, M., Stauffer, J., Stöwe, M., and Ruuskanen, S. (2014). Experimental manipulation of dietary lead levels in great tit nestlings: limited effects on growth, physiology, and survival. *Ecotoxicology*, 23(5), 914-928.
- Evers, D. C. (2001). Common Loon population studies: Continental mercury patterns and breeding territory philopatry. University of Minnesota.
- Evers, D. C., Lane, O. P., Savoy, L., and Goodale, W. (2004) Assessing the impacts of methylmercury on piscivorous wildlife using a wildlife criterion value based on the common loon, 1998-2003. Unpubl. report BRI 2004-05 submitted to the Maine Department of Environment.
- Evers, D. C. (2004). Status assessment and conservation plan for the Common Loon (*Gavia immer*) in North America. US Fish and Wildlife Service.
- Evers, D. C., Savoy, L. J., DeSorbo, C. R., Yates, D. E., Hanson, W., Taylor, K. M., and Fair, J. (2008). Adverse effects from environmental mercury loads on breeding common loons. *Ecotoxicology*, 17(2), 69-81.
- Fair, J., Whitaker, S., and Pearson, B. (2007). Sources of variation in haematocrit in birds. *Ibis*, 149(3), 535-552.

- Feder, M. E., Cartano, N. V., Milos, L., Krebs, R. A., and Lindquist, S. L. (1996). Effect of engineering hsp70 copy number on hsp70 expression and tolerance of ecologically relevant heat shock in larvae and pupae of *Drosophila melanogaster*. *Journal of Experimental Biology*, 199, 1837–1844.
- Feder, M. E., and Hofmann, G. E. (1999). Heat-shock proteins, molecular chaperones, and the stress response: evolutionary and ecological physiology. *Annual Review of Physiology*, 61(1), 243-282.
- Finger Jr, J. W., Kelley, M. D., Zhang, Y., Hamilton, M. T., Elsey, R. M., Mendonca, M. T., and Kavazis, A. (2021). Short-term capture stress and its effects on corticosterone levels and heat shock proteins in captive American Alligators (*Alligator mississippiensis*). *Canadian Journal of Zoology*, 99(8), 665-671.
- Gormally, B. M., & Romero, L. M. (2020). What are you actually measuring? A review of techniques that integrate the stress response on distinct time-scales. *Functional Ecology*, 34(10), 2030-2044.
- Haeefele, H. J., Sidor, I., and Evers, D. C. (2006). Hematologic and physiologic reference ranges for free-ranging adult and young common loons (*Gavia immer*). *Journal of Avian Medicine and Surgery*, 20(3), 202-203.
- Herring, G., and Gawlik, D. E. (2007). The role of stress proteins in the study of allostatic overload in birds: use and applicability to current studies in avian ecology. *The Scientific World Journal*, 7, 1596-1602.
- Herring, G., Eagles-Smith, C. A., Gawlik, D. E., Beerens, J. M., and Ackerman, J. T. (2014). Physiological condition of juvenile wading birds in relation to multiple landscape

- stressors in the Florida everglades: effects of hydrology, prey availability, and mercury bioaccumulation. *PLoS One*, 9(9), e106447.
- Hill, G. E., Fu, X., Balenger, S., McGraw, K. J., Giraudeau, M., and Hood, W. R. (2013). Changes in concentrations of circulating heat-shock proteins in House Finches in response to different environmental stressors. *Journal of Field Ornithology*, 84(4), 416-424.
- Ibáñez-Álamo, J. D., Jimeno, B., Gil, D., Thomson, R. L., Aguirre, J. I., Díez-Fernández, A., and Figuerola, J. (2020). Physiological stress does not increase with urbanization in European blackbirds: Evidence from hormonal, immunological and cellular indicators. *Science of the Total Environment*, 721, 137332.
- Johnstone, C. P., Lill, A., and Reina, R. D. (2017). Use of erythrocyte indicators of health and condition in vertebrate ecophysiology: a review and appraisal. *Biological Reviews*, 92(1), 150-168.
- Jones, M. P. (2015). Avian hematology. *Clinics in laboratory medicine*, 35(3), 649-659.
- Kneeland, M., Berman, E., Grade, T., Cooley, J., Vogel, H., Schoch, N., and Pokras, M. (2020). Plasma biochemistry and protein electrophoresis reference intervals of the common loon (*Gavia immer*). *Journal of Zoo and Wildlife Medicine*, 51(3), 561-570.
- Larison, B., Lindsay, A. R., Bossu, C., Sorenson, M. D., Kaplan, J. D., Evers, D. C., and Ruegg, K. 2021. Leveraging genomics to understand threats to migratory birds. *Evolutionary applications*, 14(6), 1646-1658.

- Lindquist, S., and Craig, E. A. (1988). The heat-shock proteins. *Annual review of genetics*, 22(1), 631-677.
- Martínez-Padilla, J., Martínez, J., Dávila, J. A., Merino, S., Moreno, J., and Millán, J. (2004). Within-brood size differences, sex and parasites determine blood stress protein levels in Eurasian kestrel nestlings. *Functional Ecology*, 18(3), 426-434.
- Martínez-de la Puente, J., Merino, S., Moreno, J., Tomás, G., Morales, J., Lobato, E., and Martínez, J. (2007). Are eggshell spottiness and colour indicators of health and condition in blue tits *Cyanistes caeruleus*? *Journal of Avian Biology*, 38(3), 377-384.
- Maxwell, M. H. (1993). Avian blood leucocyte responses to stress. *World's Poultry Science Journal*, 49(1), 34-43.
- Mathangasinghe, Y., Fauvet, B., Jane, S. M., Goloubinoff, P., and Nillegoda, N. B. (2021). The Hsp70 chaperone system: distinct roles in erythrocyte formation and maintenance. *Haematologica*, 106(6), 1519.
- Merino, S., Martínez, J., Barbosa, A., Møller, A. P., De Lope, F., Pérez, J., and Rodríguez Caabeiro, F. (1998). Increase in a heat-shock protein from blood cells in response of nestling house martins (*Delichon urbica*) to parasitism: an experimental approach. *Oecologia*, 116(3), 343-347.
- Minias, P. (2020). Ecology and evolution of blood oxygen-carrying capacity in birds. *The American Naturalist*, 195(5), 788-801.

- Mitro, M. G., Evers, D. C., Meyer, M. W., & Piper, W. H. (2008). Common loon survival rates and mercury in New England and Wisconsin. *The Journal of Wildlife Management*, 72(3), 665-673.
- Moreno, J., Merino, S., Martínez, J., Sanz, J., and Arriero, E. (2002). Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Ecoscience*, 9(4), 434-439.
- Moreno, J., Morales, J., and Martínez, J. (2013). HSP70 level in blood is associated with eggshell blue-green colouration the pied flycatcher. *Avian Biology Research*, 6(4), 297-301.
- O'Dell, D. A., Carlo, M. A., Kimmitt, A., Bikowski, E., Morris, K. R., and Dolby, A. (2014). A comparison of techniques measuring stress in birds. *Virginia Journal of Science*, 65(3), 3.
- Paruk, J. D., Adams, E. M., Uher-Koch, H., Kovach, K. A., Long IV, D., Perkins, C., and Evers, D. C. (2016). Polycyclic aromatic hydrocarbons in blood related to lower body mass in common loons. *Science of the Total Environment*, 565, 360-368.
- Paruk, J. D., Evers, D. C., McIntyre, J. W., Barr, J. F., Mager, J., and Piper, W. H., 2021a. Common Loon (*Gavia immer*), version 2.0. In *Birds of the World* (P. G. Rodewald and B. K. Keeney, Editors). Cornell Lab of Ornithology, Ithaca, NY, USA. Retrieved from *Birds of the World*: <https://birdsoftheworld.org/bow/species/comloo/2.0>.
- Paruk, J.D., U-Koch, H., Byrd, A., Kovach, K., Dolley, A., Hernandez J., and Indri. N.S, 2021b. Evidence of Subclinical Inflammation relates to PAH exposure in Overwintering Common Loons (*Gavia immer*). *Waterbirds*, 44(3): 317-323.

- Remage-Healey, L., & Romero, L. M. (2001). Corticosterone and insulin interact to regulate glucose and triglyceride levels during stress in a bird. *American Journal of Physiology Regulatory, Integrative and Comparative Physiology*, 281(3), R994-R1003.
- Romero, L. M. (2004). Physiological stress in ecology: lessons from biomedical research. *Trends in Ecology and Evolution*, 19(5), 249-255.
- Rose, J., Hutcheson, M. S., West, C. R., Pancorbo, O., Hulme, K., Cooperman, A., and Screpetis, A. (1999). Fish mercury distribution in Massachusetts, USA lakes. *Environmental Toxicology and Chemistry: An International Journal*, 18(7), 1370-1379.
- Ruiz-Raya, F., Abaurrea, T., Vigo, R., & Soler, M. (2022). Physiological stress responses to nonmimetic model brood parasite eggs: Leukocyte profiles and heat-shock protein Hsp70 levels. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology*.
- Sørensen, J. G., Kristensen, T. N., & Loeschcke, V. (2003). The evolutionary and ecological role of heat shock proteins. *Ecology Letters*, 6(11), 1025-1037.
- Tavaria, M., Gabriele, T., Kola, I., and Anderson, R. L. (1996). A hitchhiker's guide to the human Hsp70 family. *Cell Stress & Chaperones*, 1(1), 23.
- Thomson, R. L., Tomás, G., Forsman, J. T., Broggi, J., and Mönkkönen, M. (2010). Predator proximity as a stressor in breeding flycatchers: mass loss, stress protein induction, and elevated provisioning. *Ecology*, 91(6), 1832-1840.
- Tomás, G., Martínez, J., and Merino, S. (2004). Collection and analysis of blood samples to detect stress proteins in wild birds. *Journal of Field Ornithology*, 75(3), 281-287.

Williams, J. H., Farag, A. M., Stansbury, M. A., Young, P. A., Bergman, H. L., and Petersen, N. S. (1996). Accumulation of hsp70 in juvenile and adult rainbow trout gill exposed to metal-contaminated water and/or diet. *Environmental Toxicology and Chemistry*, 15, 1324–1328.

Table and Figure Legends

Table 1. Descriptive statistics of weight and blood variables for adult and hatch-year common loons.

Table 2. Comparison values of Pearson product-moment correlation coefficient in adult and hatch-year common loons to assess the relationship between HSP70 expression and the variables hemoglobin, hematocrit, H/L ratio, plasma glucose, and blood Hg. Significance $p = \leq 0.01$.

Fig. 1. Blood analytes of adult and hatch year common loons. (A) hemoglobin, (B) hematocrit, (C) plasma glucose and (D) blood hg represented as middle 50% of the data (box), 25th and 95th percentiles (lower and upper hinges), median (line) and minimum and maximum (whiskers) for each age class. A significant difference ($p = \leq 0.01$) between adults and hatch year chicks was found for all blood analytes displayed.

Fig. 2. Comparisons of blood hg and weight in and hatch-year common loons. (A) adult male and female blood hg represented as middle 50% of the data (box), 25th and 95th percentiles (lower and upper hinges), median (line) and minimum and maximum (whiskers) for each age class. (B) Linear regression correlation representing blood hg compared to weight of common loons. A significant difference ($p = \leq 0.05$) between groups is displayed for blood hg.

Fig. 3. Representative Western blot showing HSP70 protein is present in the blood cells of adult and hatch-year common loons. a = adults, hy = hatch-year chicks. A single band at ~70 kDa was detected in all samples.

Fig. 4. Metrics of adult and hatch-year common loons stratified by age (A) and adult common loons stratified by sex (B). HSP70 relative density are represented as middle 50% of the data (box), 25th and 95th percentiles (lower and upper hinges), median (line) and minimum and maximum (whiskers) for each age class.

Fig. 5. Multiple linear regression correlations reporting the relationship between HSP70 and (A) hemoglobin, (B) hematocrit, (C) H/L ratio, (D) plasma glucose, and (E) blood hg. Significance defined as ($p \leq 0.01$).

Tables and Figures

Table 1.

Variable	<i>Adult Male</i>			<i>Adult Female</i>			<i>Hatch Year</i>		
	N	Mean ± SD	Range	N	Mean ± SD	Range	N	Mean ± SD	Range
Weight (g)	24	5914 ± 610	4400 - 7250	21	4711 ± 237	4400 - 5200	25	2886 ± 1097	600 - 4500
Hemoglobin (g/dL)	24	24.9 ± 5.4	12.7 - 32.3	21	23.4 ± 5.0	14.5 - 33.7	27	20.5 ± 5.0	12.1 - 30.2
Hematocrit (%)	12	50 ± 5	42 - 58	9	50 ± 7	32 - 55	9	30 ± 10	23 - 42
H/L Ratio	14	2.9 ± 3.6	0.43 - 12.3	14	2.4 ± 3.7	0.1 - 14.3	9	2.6 ± 1.1	0.96 - 4.3
Plasma Glucose (g/ml)	17	242.8 ± 62.8	148.3 - 369.9	13	254.0 ± 58.7	193.5 - 431.3	26	304.7 ± 66.8	180.4 - 432.5
Blood Hg (ppm)	26	1.6 ± 0.7	0.7 - 3.8	21	1.2 ± 0.6	0.4 - 3.0	27	0.2 ± 0.1	0.1 - 0.5

Table 2.

<i>HSP70 x variable</i>	<i>Adult</i>			<i>Hatch Year</i>		
Variable	N	R²	P	N	R²	P
Hemoglobin	45	0.08	0.58	26	0.27	0.18
Hematocrit	21	-0.18	0.44	8	-0.60	0.11
H/L Ratio	26	-0.01	0.97	8	-0.06	0.90
Plasma Glucose	29	-0.30	0.12	25	-0.10	0.63
Blood Hg	44	-0.14	0.38	25	-0.28	0.18

Figure 1.

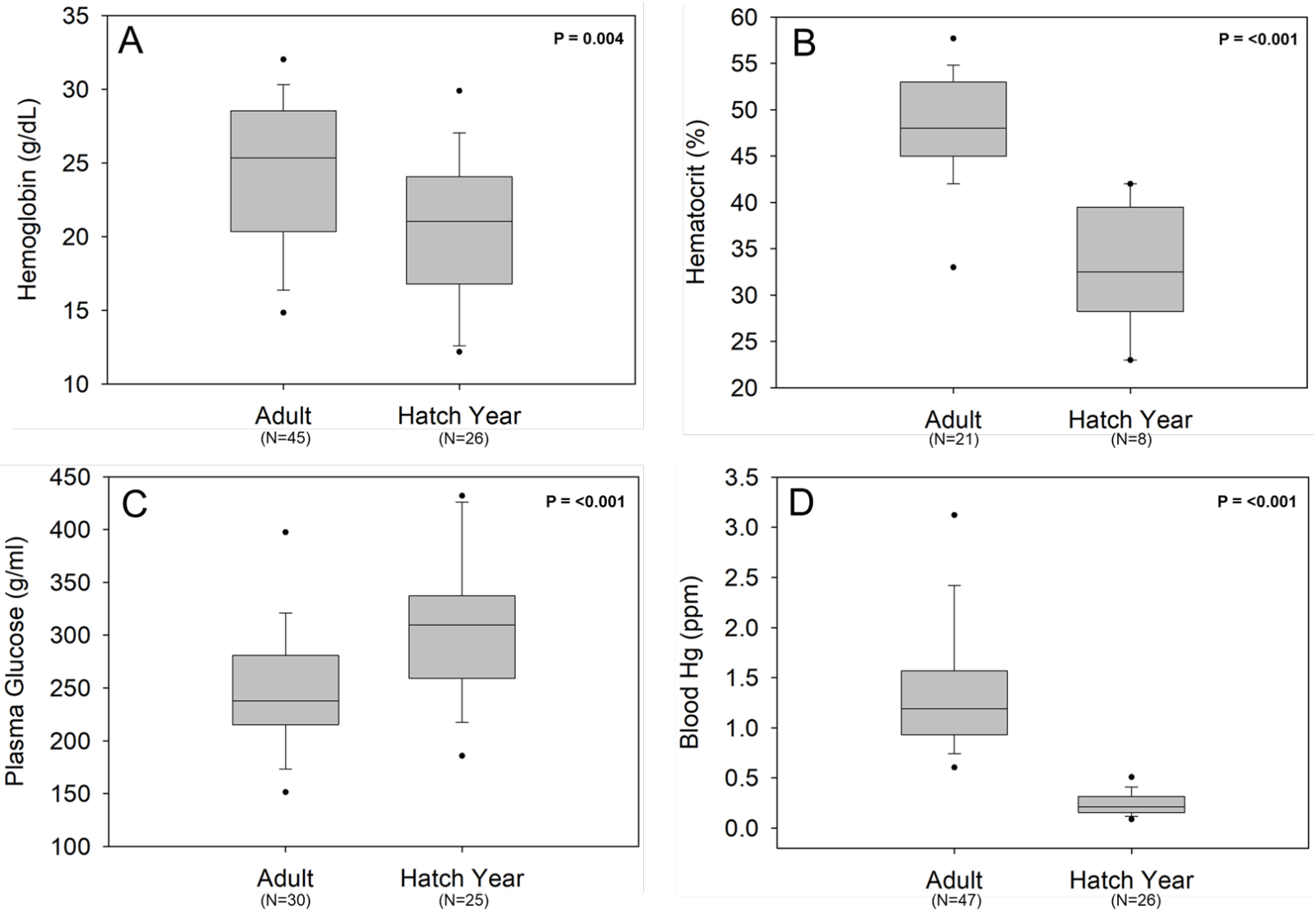


Figure 2.

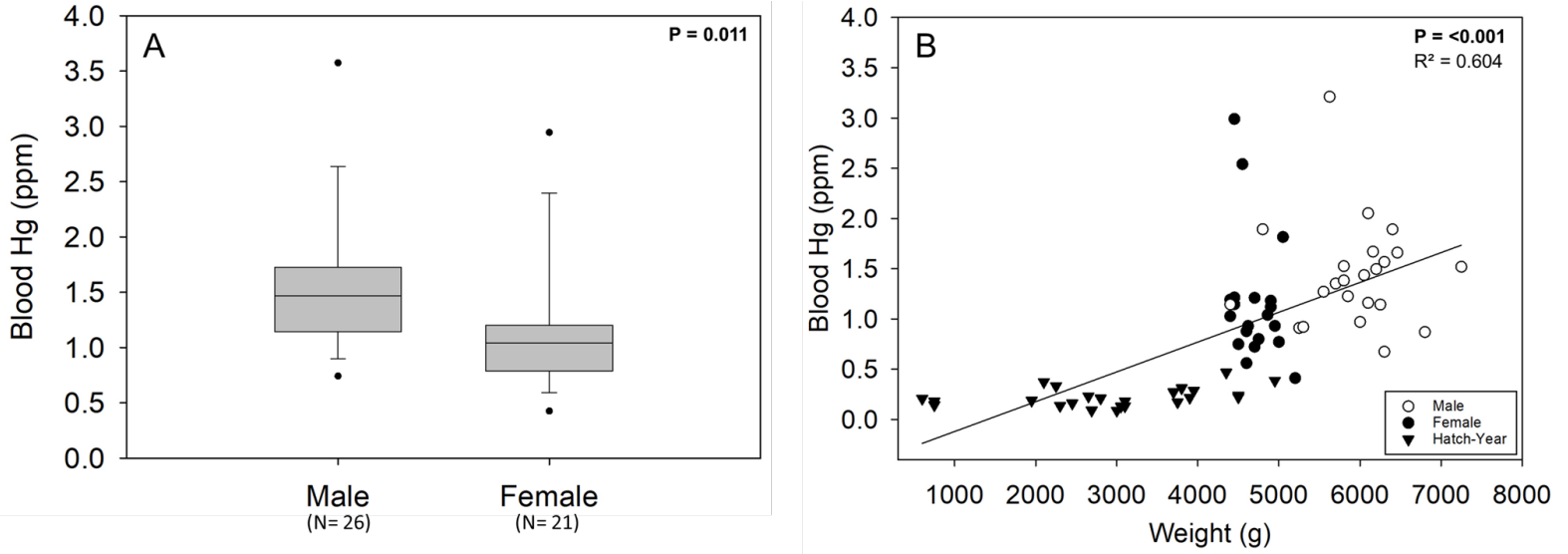


Figure 3

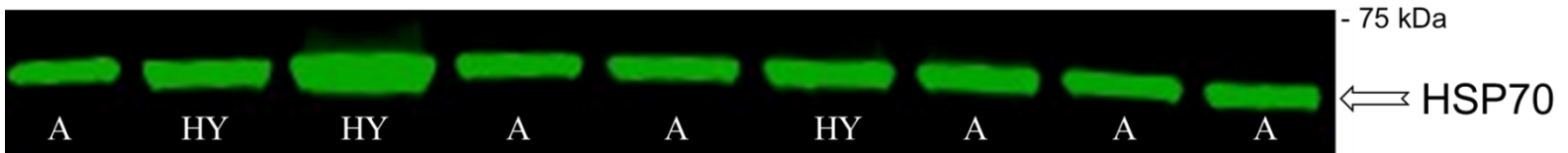
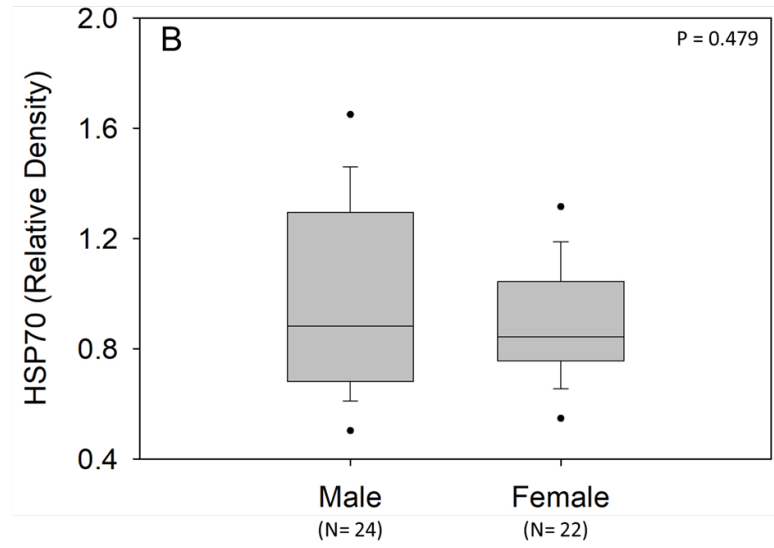
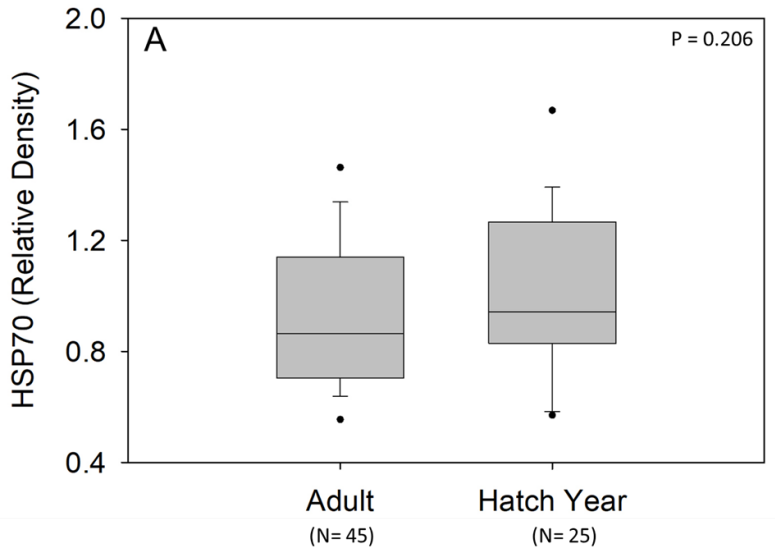


Figure 4



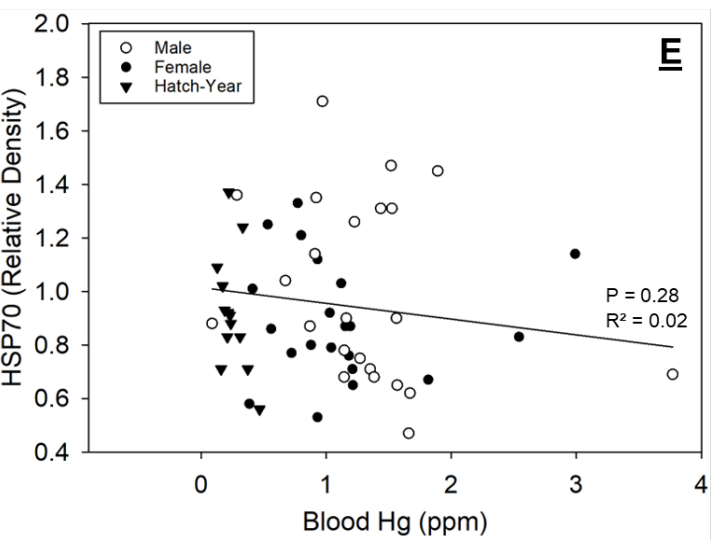
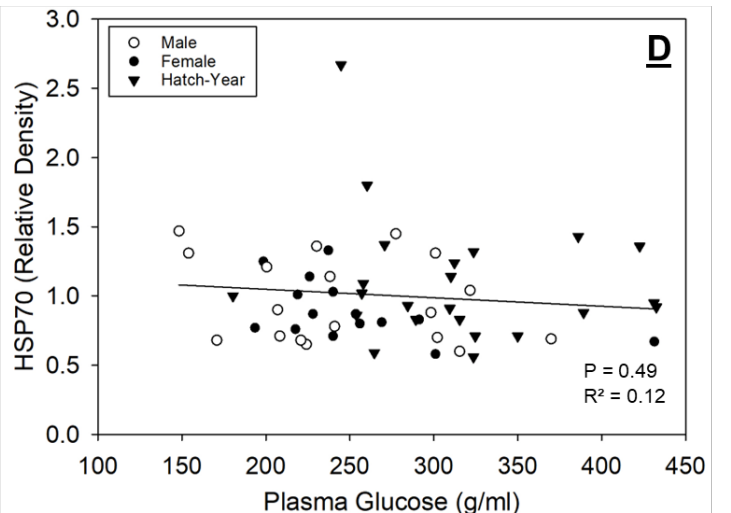
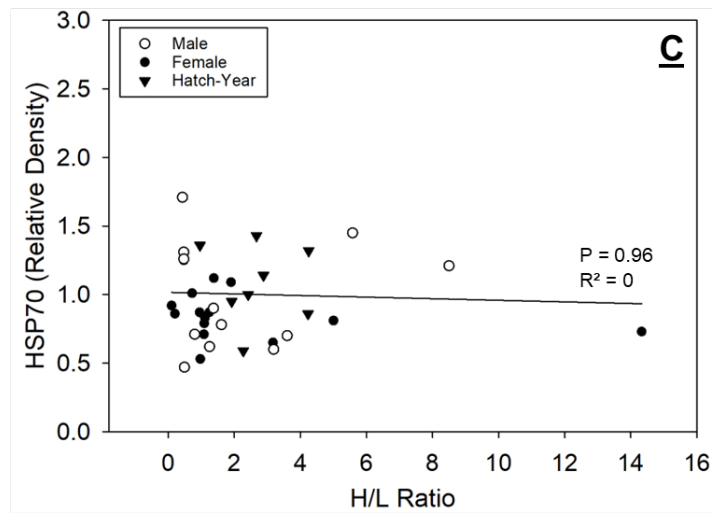
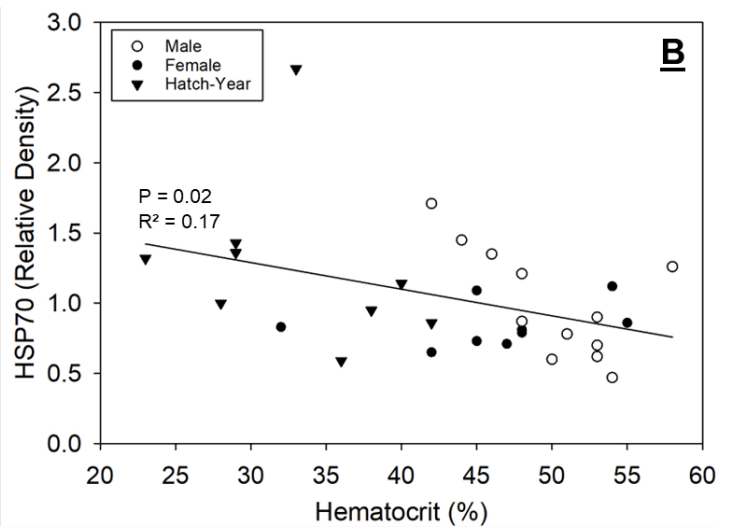
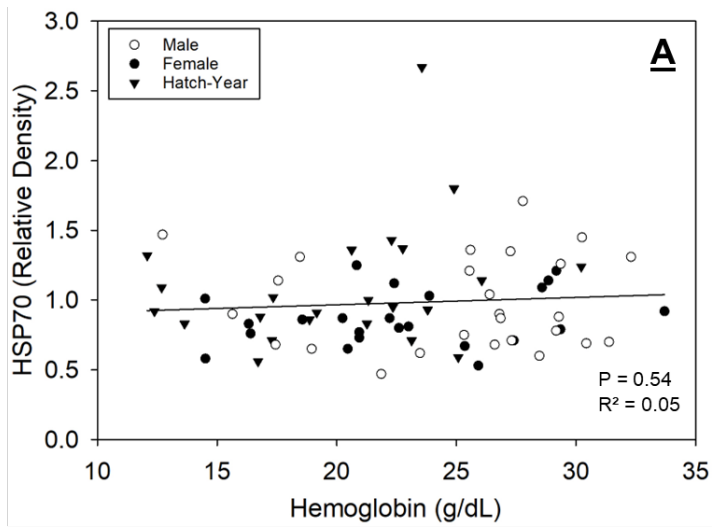


Figure 5