THESIS

THE BIOLOGICAL AND PHYSIOLOGICAL EFFECTS OF EXCESS COPPER IN JUVENILE MALLARDS (*Anas platyrhynchos)*: AN INVESTIGATION OF THE TOXICITY OF ACID MINE DRAINAGE IN WATERFOWL

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY STIVEN DANIEL FOSTER ENTITLED THE BIOLOGICAL AND PHYSIOLOGICAL EFFECTS OF COPPER IN JUVENILE MALLARDS (*Anus platyrhynchos*): AN INVESTIGATION OF THE TOXICTY OF ACID MINE DRAINAGE IN WATERFOWL BE ACCEPTED AS FULLFING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

Committee on Graduate Work

Advisor

Department Head/Director

ABSTRACT OF THESIS

THE BIOLOGICAL AND PHYSIOLOGICAL EFFECTS OF EXCESS COPPER IN JUVENILE MALLARDS (*Anus platyrhynchos*): AN INVESTIGATION OF THE TOXICITY OF ACID MINE DRAINAGE.

In the early 1990's, concentrations of copper in the Alamosa River were increased by the release of acid mine drainage from the Summitville Mine site. Concern about the potential impact to resident waterfowl led to an investigation of copper toxicity in juvenile mallards. The investigation described in this thesis included a small field survey and six laboratory studies. The field survey provided an indication of potential exposure concentrations and a relative measurement of copper exposures in mallards from the Alamosa River. The laboratory studies examined the biological and physiological effects of excess copper in juvenile mallards and the relationship between copper exposure and tissue copper accumulation.

Acute copper toxicity produced mortality in juvenile mallards that received a drinking water dose of 800 milligrams of copper per kilogram body weight per day (mg Cu/kg BW/d). Sublethal copper toxicity was quantified by decreased weight gain. Mallards experienced minor reductions in weight gain (10-20%) with exposures ranging from 70-210 mg Cu/kg BW/d. Weight gain was substantially decreased, by more than 50% compared with control birds, in mallards that received larger doses of copper (≥ 250 mg Cu/kg BW/d). Based on decreased weight gain, a dose of 20 mg Cu/kg BW/d was

determined to be a No Observed Adverse Effect Level (NOAEL) for copper in juvenile mallards.

Tissue copper concentrations were measured by flame atomic absorption spectrophotometry. Untreated mallards accumulated substantial concentrations of copper in their livers, up to 700 μg Cu/g on a dry weight basis. Hepatic copper increased significantly in mallards that received a dose greater than or equal to 160 mg Cu/kg BW/d.

Feather copper concentrations were significantly correlated with both dietary (R^2 = 0.99, $p < 0.001$) and drinking water exposure ($R^2 = 0.76$, $p < 0.001$). Analysis of feather tissue was determined to be the most sensitive method for evaluating copper exposure. Feather copper concentrations were significantly increased in mallards that received a dose greater than or equal to 70 mg Cu/kg BW/d.

Concentrations of copper in both liver and feather tissue reached their peak at a dose below the highest treatment level. Copper accumulation appears to be diminished in birds that received doses greater than or equal to 340 mg Cu/kg BW/d.

Drinking water pH was investigated for its ability to influence copper absorption. Copper accumulation in feather tissue was significantly reduced when exposure pH was decreased. In addition to influencing tissue copper accumulation, acidic water ($pH \leq 3.5$) produced signs of direct toxicity in juvenile mallards. Weight gain was significantly reduced in mallards exposed to drinking water at pH 3.5. Drinking water at pH 3.0 exceeded the LC_{50} for very young mallards (< 5 days old).

Evaluation of liver tissue from mallards that were collected on the Alamosa River indicated that these birds had increased exposure when compared to mallards from uncontaminated areas. Vegetation collected from the Alamosa River below the Wightman Fork contained a substantial concentration of copper, 463 mg Cu/kg. A diet that consisted solely of this copper-rich vegetation would provide mallards with approximately 88 mg Cu/kg BW/d. A similar dose of copper significantly reduced weight gain in laboratory mallards.

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Dedication

I would like to dedicate this thesis to the living memory of three men who inspired me to pursue an education in science: Stephen Reekie, Paul Max, and Paul Graves.

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Acronyms

A/G – Albumin/Globulin ratio

AST – Apartate Aminotranserase

BDL – Below Detection limit

BUN – Blood Urea Nitrogen

BW – Body Weight

CK – Creatine Kinase

CSU – Colorado State University

Cu – Copper

Cu -MT – Copper-bound Metallothionein

DW – Dry Weight

EPA – U.S. Environmental Protection Agency

FLAA – Flame Atomic Absorption Spectrophotometry

FWS – U. S. Fish and Wildlife Service

ICP-AA – Inductively Coupled Plasma Atomic Absorption spectrophotometry

HC – High Copper

HQ – Hazard Quotient

LD_x – Lethal Dose (for x% of the population)

LOAEL – Lowest Observed Adverse Effect Level

MT - Metallothionein

NBF – Neutral Buffered Formalin

NOAEL – No Observed Adverse Effect Level

NSL – No Significant Lesions

NWR – National Wildlife Refuge

PCV – Packed Cell Volume

QA/QC – Quality Assurance Quality Control

SD – Standard Deviation

SWPTL – Soil, Water, Plant, Testing Laboratory

T-MT – Total Metallothionein

USFWS – U.S. Fish and Wildlife Service

WBC – White Blood Cells

WW – Wet Weight

CHAPTER 1 INTRODUCTION

1.0 Purpose

Copper (Cu) is a biologically important mineral and the twenty-fifth most abundant metal in the Earth's crust (Sidhu, 1995, and Demayo, 1982). Mining and other industrial activities have increased environmental copper concentrations in certain areas, producing elevated exposures for resident wildlife populations. Adequate dietary intake of copper is important, but excess copper can result in adverse health effects (Sidhu, 1995). This thesis describes the biological and physiological effects of excess copper in juvenile mallard ducks (*Anas platyrhynchos*).

In 1986, the Summitville Mine Site in Southwestern Colorado began to release a substantial amount of copper into the Wightman Fork of the Alamosa River (King, 1995). The studies described in this thesis were conducted as part of a site investigation for the Summitville Mine Site, which was initiated by the U.S. Environmental Protection Agency (EPA) Region VIII and the Colorado Department of Public Health and Environment. These agencies wanted to evaluate the potential hazard of elevated copper concentrations in the Alamosa River to non-human terrestrial receptors. Waterfowl are among the classes of receptors that are potentially exposed to copper in this area.

1.1 Summitville Mine Site

From 1985 through 1992, the Summitville Mine produced gold from low-grade ore using cyanide heap-leach techniques. The mine's operator had ceased active mining and initiated environmental remediation when it declared bankruptcy in December 1992. In May 1994, EPA placed the site on the National Priorities List. Environmental problems at Summitville included leakage of cyanide-bearing solutions and acidic metal-rich drainage into the Wightman Fork of the Alamosa River (King, 1995). Early remediation efforts included the control of leaking adits, construction of a water treatment facility for cyanide removal, back-filling and capping the open mine pit (King, 1995). Despite the efforts to contain the contamination, a substantial amount of copper was released into the Alamosa River between 1992-1995 (King, 1995).

1.2 Waterfowl Habitat

The Alamosa River flows from the San Juan Mountains into the San Luis Valley (Figure 1.1). The valley is a major migratory pathway for a number of avian species, including some threatened and endangered species. Representative of both state and federal agencies felt that the rich avian resources in this area supported the need to investigate the potential risk to waterfowl from increased copper in the Alamosa River.

1.3 Study Design

The primary objective for this investigation was to obtain data that could be used to evaluate the risk to waterfowl from copper contamination in the Alamosa River below the

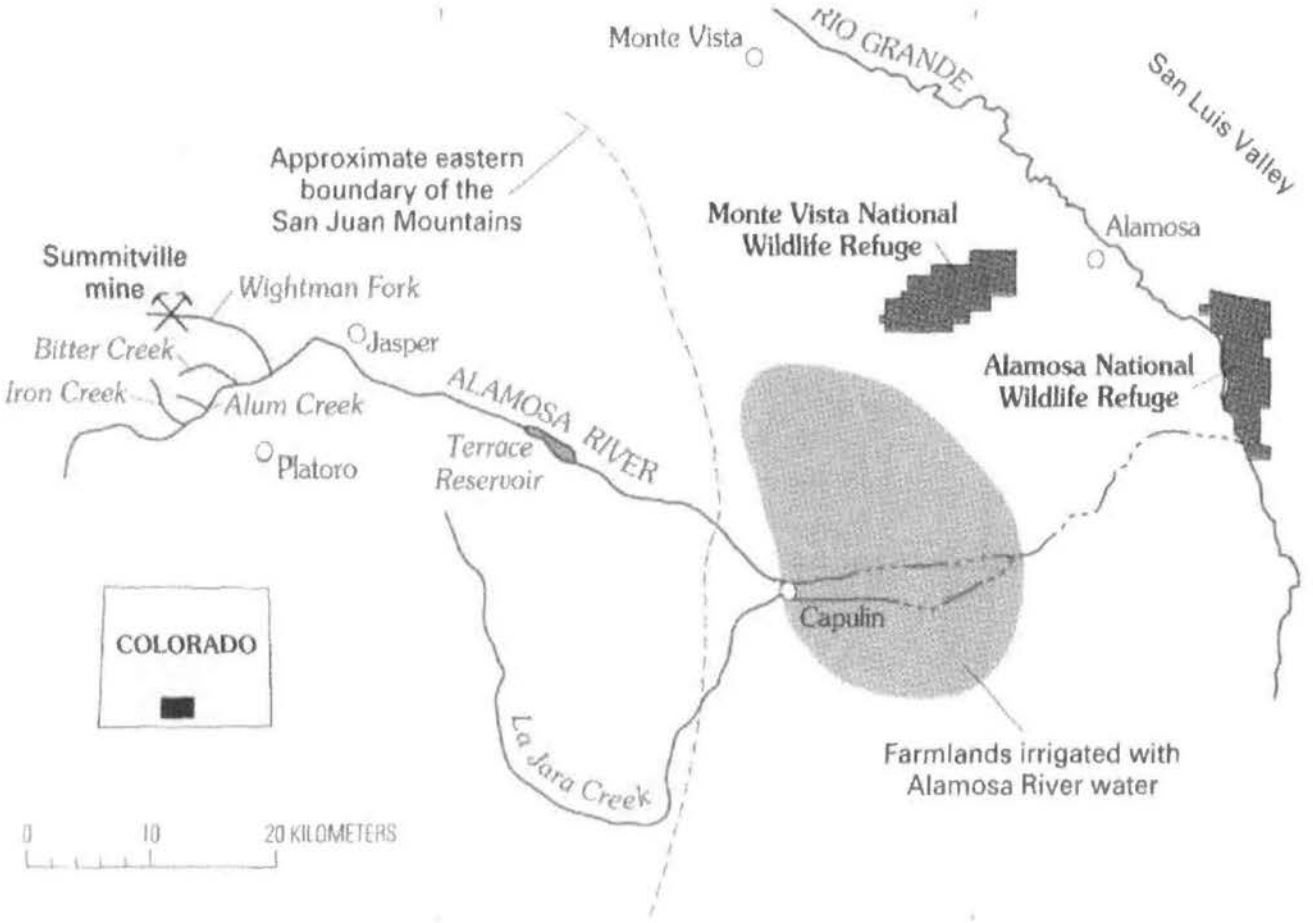


Figure 4.1 Map of the Alamosa River

Wightman Fork. To achieve this goal, a field survey was conducted to evaluate potential exposure conditions on the Alamosa River. The lack of duck samples available from the field precluded a conclusion on the impact of excess copper in mallards.

A series of laboratory studies were performed to define the toxicity of copper in juvenile mallards. Prior to this investigation there were very limited data on the toxicity of copper in mallards or any other species of waterfowl. Mallards were selected as a representative waterfowl species because there is breeding population of this species on the Alamosa River (Archuleta, 1995) and because their commercial availability facilitated laboratory investigations. This investigation focused on juvenile birds because they are potentially sensitive individuals and because pre-flight birds collected in the field have a definable exposure area and exposure period.

1.3.1 Field Survey

Early in the development of this project it was determined that it would not be feasible to conduct a series of controlled toxicity tests in the field. In the summer of 1995, juvenile mallards and environmental samples were collected from the Alamosa River for metals analysis. The objectives of field survey were to:

1) Collect site specific information about potential sources of copper exposure.
2) Compare the tissue copper concentration in juvenile mallards raised on the Alamosa River to birds from a reference area.

1.3.2 Laboratory Investigations

The lack of substantial mallard populations available for sampling on the Alamosa River raised the question of whether this could be due to direct copper toxicity to the birds. Because of the lack of adequate literature data on copper toxicity, a series of six toxicity studies were conducted with juvenile mallards in order to provide data to support the interpretation of the results of the field study. There were two objectives for the laboratory studies:

1) Determine safe and unsafe copper exposure in juvenile mallards.

2) Develop methods for assessing copper exposure in wild birds.

To achieve these goals, each of the laboratory studies was designed to collect a specific set of observations. The specific aims for the six studies were:

Study One - Determine a minimum concentration of dietary copper that would produce signs of toxicity.

Study Two - Compare the toxicity of dietary cupric acetate and cupric sulfate.

Study Three - Determine a concentration of copper in drinking water that would produce signs of toxicity and investigate the influence of pH on copper bioavailability.

Study Four - Determine a minimum concentration of copper in drinking water that would produce signs of toxicity, and to investigate the influence of pH on copper tissue accumulation.

Study Five – Evaluate the toxicity of copper and acidic pH water between previously observed levels of effect and no effect.

Study Six - Determine if prolonged exposure to low concentrations of copper in drinking water would produced signs of toxicity, or measurable increases in tissue copper concentrations.

CHAPTER 2 LITERATURE REVIEW

2.0 Biological Role of Copper

Copper is an essential nutrient required for a number of biological processes including: host defense mechanisms, red and white blood cell maturation, myocardial contraction, glucose and cholesterol metabolism (Olivares, 1996). Copper is also important for the synthesis of a number of enzymes including catalase, peroxidase, superoxide dismutase, and cytochrome oxidase (Goyer, 1996). Furthermore, copper is required for proper iron utilization and a deficiency of copper can result in anemia (Aiello, 1998). Copper supplementation is commonly used to increase production of several domestic species including swine and poultry (Cheeke, 1991). Recommended dietary copper levels vary among species: poultry 6 - 8 mg Cu/kg feed, horses and dairy cattle 10 mg Cu/kg feed, and swine 5 mg Cu/kg feed (Cheeke, 1991).

2.1 Copper Metabolism

2.1.1 Copper Absorption

In most mammalian species, the majority of copper absorption takes place in the duodenum and jejunum (NAS, 1980), but some copper appears to be absorbed from the stomach (Cousins, 1985). The mechanisms of copper absorption are not completely understood (Underwood, 1971). It is thought that copper is transported across the brush border surface of the small intestine bound to one or more absorbable ligands (Cousins,

1985). Copper absorption is influenced by a number of factors including dietary composition, the animal's age, the form of copper, the route of exposure, and the presence of other minerals in the diet (ATSDR, 1990). Dietary levels of zinc, molybdenum, and sulfate are reported to have a significant effect on copper absorption in mammals (NAS, 1980). Competition with intestinal binding ligands appears to be one the factors that limit copper absorption (Cousins, 1985).

2.1.2 Copper Binding Proteins

Copper metabolism consists mainly of its transfer to and from various organic ligands including the sulfhydryl, and imidazole groups on amino acids and proteins (ATSDR, 1990). Once copper is absorbed, cupric ions bind loosely to albumin (NAS, 1980). The albumin protein has a high affinity for binding copper on the *N* - terminus (Goode, 1988). Albumin facilitates the transport of copper to the liver where the majority of copper, approximately 90%, is thought to bind with the metalloprotein ceruloplasmin (Goode, 1988).

Ceruloplasmin is an acute phase protein whose synthesis is increased by acute inflammatory conditions such as bacterial or viral infections (Jain, 1993). There is conflicting evidence on the induction of ceruloplasmin with copper treatment (Cousins, 1985). In the liver, copper attaches tightly to the 6 or 7 copper binding sites on ceruloplasmin and is then released into general circulation (NAS, 1980). The function of this protein in copper metabolism is not completely characterized, but it has been

suggested that dysfunction in ceruloplasmin synthesis may be associated with increased susceptibility to copper toxicity (Chowrimootoo, 1996).

Metallothionein (MT) is a heat-stable metal binding protein. Synthesis of metallothionein is increased by exposure to a number of metals including mercury, cadmium, and zinc (Yamada, 1991). Metallothionein has a high affinity for binding copper, but copper is not considered a strong inducer of the metalloprotein (Eaton, 1980). However, because of its high affinity for copper, metallothionein is thought to play an important role in copper storage and distribution (Eaton, 1980).

2.1.3 Distribution

Sites of tissue copper accumulation vary among species, but the liver, kidneys, brain, and heart consistently contain the highest concentrations of copper (Underwood, 1971). Liver copper concentrations normally range, on a dry matter basis, from 15 to 30 mg Cu/kg for a wide variety of monogastric mammals and domestic fowl (Beck, 1956). The liver copper concentrations in ducks, cattle, and sheep are generally 10 times greater than most other species (NAS, 1980).

The preferential accumulation of copper by the liver, appears to involve more than just having first access to absorbed copper in the portal circulation because this trend is observed with both oral and parenteral administration (Ettinger, 1986). Unfortunately, little is known about the copper uptake mechanism in hepatocytes. The process appears to follow first-order kinetics and utilize a passive carrier-mediated mechanism (Ettinger, 1986, and Cousins, 1985).

Amino acids and albumin are the ligands which most likely present copper to the hepatocytes for transport across the plasma membrane (Cousins, 1985). Regardless of the mechanisms for copper uptake, in most species the liver is the primary storage organ and analysis of liver copper can often provide a reasonably reliable index of an animal's copper status (Underwood, 1971).

2.1.4 Excretion

The biliary system is the major excretory pathway for copper, but small quantities appear to be excreted directly into the intestine (NAS 1980). It has been suggested that ceruloplasmin may play an important role in regulating copper excretion (Chowrimootoo, 1996).

2.2 Copper Toxicity

Acute or chronic copper poisoning can occur when excess amounts of copper are ingested. Acute copper poisoning in non-avian species typically presents as severe gastroenteritis characterized by abdominal pain, diarrhea, anorexia, dehydration, and shock (Aiello, 1998). Copper appears to be corrosive to mucous membranes of the gastrointestinal tract (Nicholson, 1995). Chronic copper toxicosis is usually subclinical until the hepatic storage capacity is exceeded and then copper is released into the blood stream in massive amounts (Demayo, 1982). A hemolytic crisis, caused by the destruction of red blood cells, is commonly associated with the release of free copper from the liver (Demayo, 1982). There is a marked difference among species in their

ability to tolerate excess dietary copper levels. Dietary levels that are toxic to ruminants are well tolerated by non-ruminants (Underwood, 1971). Continued dietary exposure to 10 mg Cu/kg feed will produce toxicity in sheep (Cheeke, 1991). Patterns of hepatic copper storage vary among species; liver copper concentrations in sheep increase in proportion to their dietary intake, while rats maintain normal liver copper levels until a high dietary level is reached (Underwood, 1971).

Several disease conditions are associated with impaired copper regulation. A classic example is Wilson's disease, an inborn error of copper metabolism that affects humans. Wilson's disease is characterized by excessive accumulation of copper in the liver, kidney, brain, and cornea (Sarkar, 1983). The biochemical defect produced by this genetic disease is not known. However, it is has been suggested that an alteration in the copper excretion pathways may be the responsible for this condition (Chowrimootoo, 1996). A disease similar to Wilson's disease is found in the Bedlington Terrier (Aiello, 1998), suggesting that genetic predisposition to copper toxicity may exist in non-human species.

2.2.1 Copper Toxicity in Avian Species

Symptoms of copper toxicity in avian species include listlessness, ruffled feathers, drooping head, variable appetite, voiding of bluish-green seromucous urates, watery diarrhea, and weight loss (Pullar, 1940 and Aiello, 1998). Pathological lesions associated with copper toxicity have been found in the oral cavity, gizzard, proventriculus,

gastrointestinal tract, and liver (Jensen, 1991, Henderson, 1975, and Pullar, 1940). Reported lesions include burns or erosion in the lining of the gizzard, catarrhal gastroenteritis, and hemosiderin (Aiello, 1998, Goldberg, 1956).

Copper is widely used as a dietary supplement to increase weight gain in weight gain (Underwood, 1971). It has been reported that 10 - 100 mg Cu/kg feed can increase weight gain in chickens and turkeys (Christmas, 1981, and Waibel, 1963). Because of the potential economic benefit associated with increased weight gain, the poultry industry has investigated the affects of copper in several domestic avian species.

A 10 week dietary study conducted with chicks found that 570 mg Cu/kg in feed reduced weight gain by approximately 10% (Mehring, 1960). Turkey poults that received feed with 800 mg Cu/kg for 3 weeks experienced a 10% reduction in weight gain (Waibel, 1963). The survival rate in chicks was significantly reduced by greater than 40% after 10 weeks of exposure to 1,150 mg Cu/kg (Mehring, 1960).

The threshold of copper toxicity in avian species appears to be influenced by a number of factors including species, the form of copper, and dietary composition (Demayo, 1982). It has been reported that copper toxicity was significantly increased in turkey poults by changing their diet from a natural diet to a purified diet (Waibel, 1963). Conversely the addition of excess zinc to the diet has been demonstrated to decrease copper toxicity in turkey poults (Supplee, 1964).

2.2.2 Copper Toxicity in Waterfowl

Prior to this investigation there were limited data available on the toxicity of copper in waterfowl. An extensive literature search found one study on the toxicity of copper in ducks. Pullar reported that mallards which received gavage doses greater than or equal to 55 mg of copper carbonate per kg body weight per day (mg/kg BW/d) for 26 days experienced reduced weight gain and mortality (1940). This same author also found that a single gavage dose of 400 mg copper sulfate produced acute copper poisoning in adult mallards (Pullar, 1940). Pullar administered copper to several avian species, and found that ducks were more sensitive to copper treatment than either pigeons or chickens (1940). The dose of copper sulfate required to produce mortality in mallards was 400 mg per kg body weight (mg/kg BW) compared to 1500 mg for pigeons, and 3000 mg/kg BW for fowl (Pullar, 1940).

Several reports have documented copper treatment in ducks without adverse effects. Aylesbury ducklings provided with a diet that contained 100 mg/kg copper sulfate demonstrated increased growth and no signs of toxicity (King, 1975). Rowe and Prince offered adult mallards a choice between water with 100 mg/L copper sulfate or untreated drinking water and found that the birds preferred the copper - treated water; no health effects were reported (1983).

There are only a few recorded incidences of copper toxicity in waterfowl following environmental exposure. In one case, a group of 100 Canada geese were found dead following exposure to water that contained 600 mg/L copper sulfate (Henderson, 1974). These birds had extensive necrosis of the upper digestive tract and liver copper

concentration ranging from 56-97 mg/kg wet weight (WW); estimated as 204 - 353 mg/kg dry weight (DW) assuming a 72% moisture content.

Eight mute swans were found dead in a Japanese river that was thought to be contaminated with copper (no environmental data were provided). The mean liver copper concentration for these birds was 2150 ± 2280 mg Cu/kg DW (mean ± standard deviation) (Kobayashi, 1992). Pathological findings in these birds included focal necrosis of the liver and hepatic lipofuscin. Another study compared liver copper concentrations in mute swans collected from a contaminated lagoon in Denmark to birds from non-industrial areas (Clausen, 1978). Swans from the industrial area had a mean liver copper concentration of 1096 mg/kg (N = 32) and birds from the reference areas had a mean liver copper concentration of 391 mg/kg in (N = 15) one site and 418 mg/kg in another (N = 44).

2.3 Literature Values

2.3.1 Reported Concentrations of Copper in Waterfowl Tissues

Analysis of contaminant concentrations in tissues is a standard method for evaluating physiological uptake and biological accumulation. For many contaminants, including copper, the liver is the principle site of accumulation (Underwood, 1971). It has been reported that compared to other animals, ducks have relatively high levels of copper in their livers (Beck, 1956). Liver copper concentrations for turkeys and chickens typically range from 12.7 - 17.0 mg/kg on a dry weight (DW) basis (Beck, 1956). The reported

mean liver copper concentration for group of Muscovy ducks (N = 34) was 153 ± 21 mg/kg DW (Beck, 1956).

Table 2.1 summarizes the wide range of liver copper concentrations that have been reported for adult mallards. There is a clear difference between the liver copper concentrations for mallards that were raised in laboratories and those that were collected from the field. It seems likely that the increased hepatic copper concentrations in laboratory-raised ducks are the result of dietary differences. However, it should be noted that the reported copper concentration for the commercial feed used in the laboratory studies conducted by Di Giulio et al. was only 7 mg Cu/kg (1984a,b).

Table 2.1 Reported Liver Copper Concentrations in Adult Mallard Ducks

Source	N	Liver Cu (μg/g DW)	Reference
Field	15	35	Chupp (1964)
Field	157	52	Di Guilio et al. (1984a)
Field	8	61 ± 52 [a]	Archuleta (1992)
Unknown	34	153 ± 21	Beck (1956)
Laboratory controls	12	332 ± 84	Di Guilio et al. (1984a)
Laboratory controls	8	585 ± 133	Di Guilio et al. (1984b)

[a] Mean ± standard deviation

Metal concentrations in feathers have been used as a method for associating breeding populations with specific geographic areas (Ranta, 1978) and for evaluating metal contamination (Rose, 1982, Parker, 1985). Metal concentrations in feathers are thought to reflect dietary intake because metals are incorporated into feathers during growth (Parker, 1985).

Mallard feathers (N = 47) collected from an uncontaminated site in Ontario, Canada, had copper concentrations ranging from 0 - 23 μg Cu/g (Ranta, 1978).

2.3.2 Body Weight and Food Consumption

Body weights for mallards at various ages are available in the published literature, there is a general consensus that males weight more than females and that body weight at fledging is approximately 75% the weight of adult birds (EPA, 1993). Feed consumption is reported in a number of published studies, but water consumption is generally estimated with an allometric equation (EPA, 1993). Table 2.2 summarizes the data available in the literature for body weights and feed and water consumption values for mallard ducks.

Table 2.2 Reported Mallard Feed and Water Consumption Rates

Reference	Body weight (g)	Feed (g/d)	Feed (g/g BW/d)	Water (g/d)	Water (g/g BW/d)
Di Giulio (1984c)	1116 ± 36	105 ± 8	0.094	NA	NA
Di Giulio (1984c)	1201 ± 46	137 ± 13	0.114	NA	NA
EPA (1993)	1043 [a]	NA	NA	60	0.058
EPA (1993)	1225 [b]	NA	NA	67	0.055
Rowe (1983)	NA	NA	NA	157 ± 28	NA

Reported as mean ± standard deviation
[a] adult female, 740 at fledging (56 days).
[b] adult male, 817 at fledging (56 days).

2.3.3 Clinical Chemistry/Hematology Parameters

Clinical chemistry and hematology panels are standard diagnostic techniques. Changes in enzymes, ions, and cell types can provide an indication of an organism's physiological status. In many species, copper toxicity presents with signs of liver damage and hemolytic anemia (Aiello, 1998). Hepatocyte damage can be detected by elevated concentrations of glutamate dehydrogenase, sorbitol dehydrogenase and aspartate aminotransferase (AST) (Jain, 1993). Anemia can be diagnosed by reduced packed cell volume (PCV) and increased hemolysis (Jain, 1993). Table 2.3 summarizes some normal clinical chemistry values for mallards. Reference values were not obtained for several endpoints.

Table 2.3 Reported Clinical Chemistry Values for Mallard Ducks

Parameter	Normal Values
Packed cell volume (PCV) [a]	33.9 - 48.0
Heterophils[a]	2.6 - 13.3
Eosinophils [a]	0.25 - 1.44
Lymphocytes[a]	1.97 - 10.42
Monocytes[a]	0.04 - 0.57
Basophils[a]	0.06 - 0.71
Albumin[b]	1.5 - 1.7
Total protein[b]	3.56 - 4.61
Glucose[b]	185 – 215
Aspartate aminotransferase (AST)[c]	15.8 -16.2
Calcium[b]	9.4 - 9.8
Magnesium[b]	1.8
Phosphorus[b]	2.9 - 3.0
Creatinine[d]	0.25 - 0.28
Uric acid[c]	4.0 - 4.5[c]

Sources: Driver, 1981, and Fairbrother, 1990
[a] Units * 10^3 /ul
[b] Units mg/dl
[c] IU/L
[d] Units g/dl

CHAPTER 3 METHODS

3.0 Field Survey

In June 1995, a brief field survey was conducted on the Alamosa River by personnel from the U.S. Fish and Wildlife Service (USFWS), U.S. EPA Region VIII, and Colorado State University's (CSU) Department of Environmental Health. Mallards were collected from the Terrace Reservoir and wetland areas on the Alamosa River, upstream from the reservoir and below the Wightman Fork (Figure 2.1). USFWS biologists used tin-bismuth shot to collect the juvenile birds. An EPA veterinary toxicologist preformed full necropsies as soon as the mallards could be retrieved and weighed. The maximum amount of time that elapsed between collection and necropsy was approximately 10 minutes. Liver and kidney samples collected for metals analysis were placed in 15 ml polypropylene tubes and stored on ice. Liver and kidney samples collected for histopathological analysis were stored in 10% neutral buffered formalin (NBF). Soil, water, sediment, and vegetation samples were collected from each location where a bird obtained. When it was possible, macroinvertebrate samples were also collected.

The Alamosa National Wildlife Refuge (NWR) was selected as reference site for this study. Several attempts were made to collect similar aged birds from this site. Unfortunately, juvenile mallards in the area had already fledged and no birds were collected from the Alamosa NWR. Fortunately, metal tissue data collected by Andrew

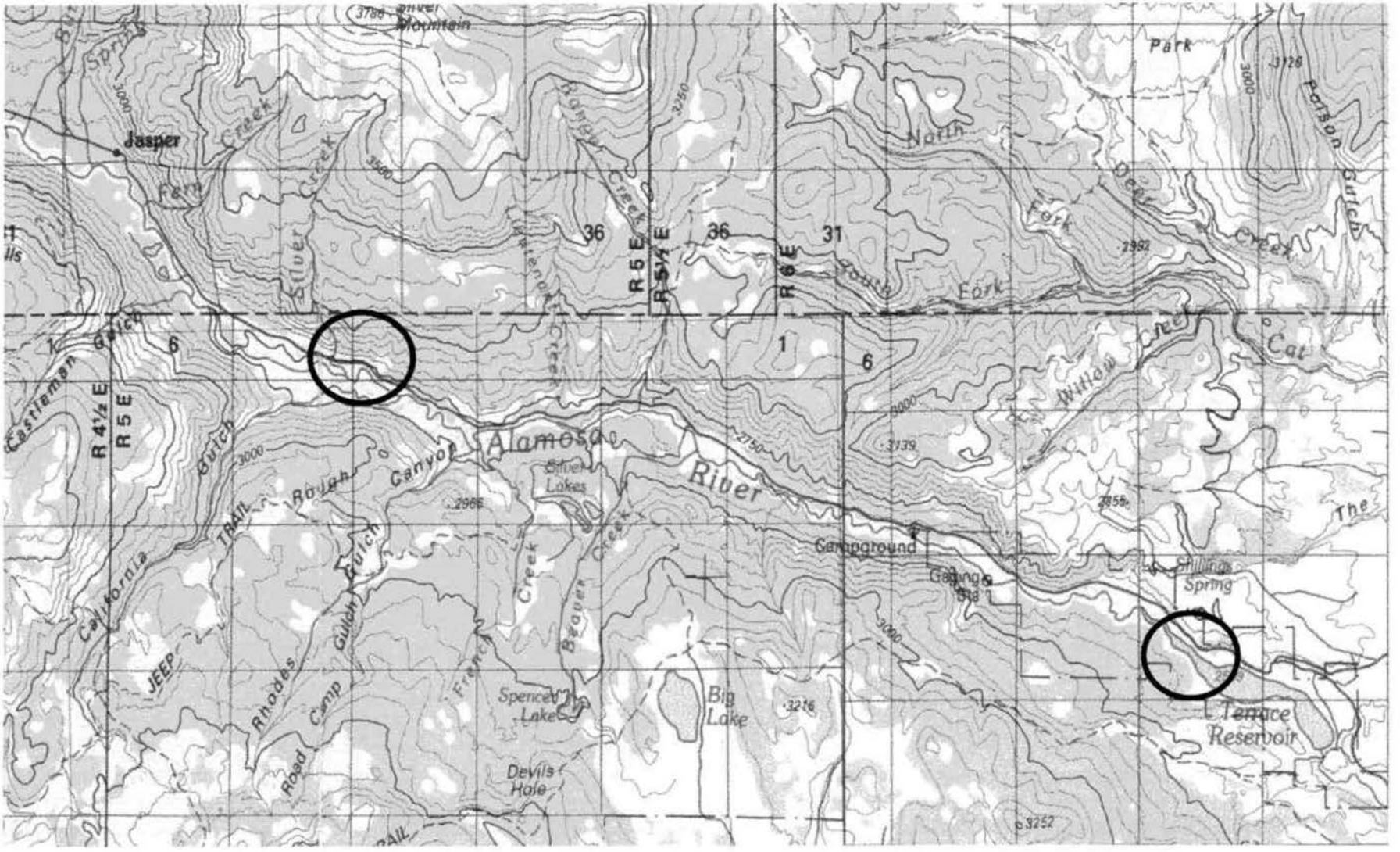


Figure 3.1 Sampling Sites

Circles identify sampling locations

Archuleta (USFWS) from the Monte Vista NWR during 1989-1990 were available as a source of reference site values (1992).

Environmental Science and Engineering, Inc. (ESE) performed the metals analyses of the duck tissues and the macroinvertebrate samples. Colorado State University's Soil, Water and Plant Testing Laboratory (SWPTL) performed the metals analysis of the water, sediment, and vegetation samples. Samples submitted to ESE and CSU SWPTL were digested in concentrated nitric acid and analyzed by inductive coupled plasma atomic absorption spectrophotometry (ICP-AA) for the concentrations of Al, Cd, Cu, Fe, Mo, and Zn using EPA method 7210.

3.1 Laboratory Studies

Six laboratory studies were conducted at CSU between the fall of 1995 and the spring of 1997 to evaluate the toxicity of copper in juvenile mallards. Because the six studies were conducted successively the results from the preceding study were used to influence the design of the subsequent studies.

3.1.1 Animal Care

Day old mallards were purchased from Whistling Wings, Inc. (Hanover, IL). The ducklings were sent by over-night delivery to the Laboratory Animal Resources Center at CSU. Upon arrival the two day old ducklings were examined and randomly placed into poultry brooder units. Randomization was achieved by removing birds from the shipment box and distributing them one at a time into separate incubation units. In this

manner the size of each unidentified group was increased at the same rate. Once all of the birds were allocated to an incubation unit, each of the brooder units was assigned to an experimental treatment by random drawing.

For the duration of the study, the mallards were kept in brooder units under controlled temperature and lighting at a CSU Laboratory Animal Resources facility. Mallards were raised according to a protocol approved by the CSU Animal Care and Use Committee. Assistance with animal care was provided by Laboratory Animal Resource staff (Study One), Jennifer Sadler and Suzanne Worker (Studies Two, Three, Four, Five and Six).

Room temperature was maintained between 22-28 °C and each brooder unit had a small heating source that allowed the birds a temperature gradient within the brooder. Ambient light was provided by overhead fluorescent lights on an automatic timer set to a photoperiod of 16 hours of light and 8 of hours dark. The length of the photoperiod was selected to match the natural light during late May and June, when juvenile mallards would be present on the Alamosa River.

The mallards were given free access to feed and water at all times. Water was provided in stainless steel troughs and feed was provided in galvanized steel troughs. The same lot of commercial game bird starter feed purchased from Ranch - Way Feeds (Ft. Collins, CO) was used in all six studies. Without added copper this feed contained 25 - 27 mg/kg of copper (N = 6), 28% protein (w/w), and 2.5% fat (w/w). Feed samples were collected in 50 ml polypropylene tubes and submitted to the CSU Soil, Water and Plant Testing Laboratory for analysis. Prior to analysis the samples were baked at 60-70 °C for 24 hours, ground, weighed (at room temperature), and digested with nitric acid and

heat. The feed samples were analyzed by ICP-AA according to EPA methodology 7210. The manufacturer reported the percentage of fat and protein in the diet.

Drinking water samples were collected in 5 ml polyethylene containers and acidified with 10% nitric acid. Analysis of drinking water was performed at the CSU Department of Environmental Health's Analytical Toxicology Laboratory using flame atomic absorption spectrophotometry (FLAA). Details of the FLAA methodologies used in this investigation and quality assurance / quality control (QA/QC) are presented in Section 3.1.6.

All mallards were given an acclimation period prior to initiation of the experimental treatments; the typical acclimation period was 24 hours. However, when then number of birds in a study exceeded the number of post-mortem evaluations that could be performed in a single day, the acclimation periods for some treatments groups were extended.

The mallards were housed and treated in groups of 5-9 duckling. Individual birds were identified with color coded and numerically labeled plastic leg bands. A specific color and number sequence was assigned to each group. Leg bands were placed on the individual birds during the first week of treatment. Prior to this time the ducklings were too small and the leg bands would fall off. During the course of treatment the leg bands had to be changed to accommodate the rapid growth of the mallards.

Copper treatments were administered in feed for Studies One and Two and in drinking water for Studies Three, Four, Five and Six. Treatment protocols are described in Section 3.3. During the exposure period, weight gain, feed and water consumption was measured at regular intervals. The body weight of each duck was measured at the beginning of the

study and twice weekly until the end of the study. The birds were weighed on a digital balance that was accurate to the nearest 0.01 g. The balance was designed to weigh live animals and corrected for movement by reporting an average reading for a 10 second period.

Feed consumption was measured in all six studies, and water consumption was measured in all of the drinking water studies. Feed and water consumption was measured by treatment groups rather than individuals. When new quantities of feed or water were added, the weight of the new feed/water, and the weight of the residual feed/water were recorded. Feed and water weights were obtained with a spring scale accurate to the nearest 1 g. In Studies Four, Five, and Six, when additional quantities of water were added and the previous portions were removed, drinking water pH was measured with a portable pH meter. The pH meter was accurate to the nearest 0.1 pH unit and was calibrated daily.

Daily feed and water consumption was calculated for each treatment group by subtracting the residual feed and water weight from the weight of the quantity added on the previous day. The consumption data were used to estimate daily dose, which was expressed as gram per gram body weight per day (g/g BW/d). The feed and water consumption data were converted to dose units by dividing the mass of feed or water consumed on a given date by the aggregate weight of the group on that day, and this quantity was then multiplied by the measured concentration of copper in the medium.

3.1.2 Chemicals

All of the reagents used in this investigation were obtained from a commercial vendor. Copper treatments were prepared from reagent grade copper (II) sulfate * 5 H_2O (powder), copper (II) acetate monohydrate (crystalline), or copper (II) carbonate basic (powder) obtained from Fisher Scientific (Springfield, NJ). Analytical chemistry was performed with analytical grade nitric acid (Fisher Scientific).

3.1.3 Experimental Treatments

3.1.3.1 Study One

The specific aim for Study One was to determine a minimum concentration of dietary copper that would produce toxicity in juvenile mallards. The study included three treatment groups and a control group. Copper, as cupric sulfate, was added to feed in concentrations of 200, 400, and 800 mg Cu/kg feed. The highest exposure concentration, 800 mg/kg, was selected because this concentration of dietary copper had been reported to produce adverse effects in turkey poults (Waibel, 1963). The control group was provided with untreated feed and all treatment groups were provided with deionized water. Each treatment group consisted of 6-8 ducklings. Mallards were given a 24 hour acclimation period prior to the initiation of experimental treatments. Copper treatments were administered from the third day post-hatch until the thirty-eighth day post-hatch.

. The length of the study was determined by body weight. When the mean body weight for the control group was equivalent to fledging birds, 750 - 817 g (EPA, 1993), the study was terminated.

The copper enriched diets were prepared by incorporating powdered cupric sulfate into a commercial feed with a stainless steel mixer. Copper was a weighed in plastic weigh boat on a top-loading digital balance accurate to the nearest 0.01g. The feed was stored in plastic bags and kept in large plastic containers with tight-fitting lids. Samples from each feed batch were collected in 15 ml polypropylene tubes for metals analysis.

3.1.3.2 Study Two

The specific aim for this study was to compare the toxicity of dietary cupric acetate and cupric sulfate. The study was designed to provide the two treatment groups with equal concentrations of copper as either cupric sulfate or cupric acetate. Unfortunately, an error in the feed preparation resulted in a copper concentration for the copper sulfate diet that was approximately half the concentration of the copper acetate diet. The control group was given untreated feed and all groups were provided with deionized water. Each group contained 8 ducklings. All of the birds were given a 24 hour acclimation period. Copper treatments were administered from the third day post-hatch until the thirty-eighth day post-hatch.

Copper was incorporated into the feed by the addition of concentrated solutions of either cupric sulfate or cupric acetate. The copper solutions were mixed into the feed

using a stainless steel mixer. The treated feed was placed in large polyethylene tubs, covered with nylon mesh, and dried at room temperature for 48 hours. After drying, the feed was stored in plastic lined containers with tight fitting lids. Feed samples from each batch were collected in 15 ml polypropylene tubes for metals analysis.

3.1.3.3 Study Three

The specific aims for Study Three were to determine a concentration of copper in drinking water that would produce signs of toxicity and to investigate the influence of pH on copper bioavailability. Cupric sulfate was added to deionized water at concentrations of 600 and 1200 mg Cu/L. Water pH was adjusted to 3.0 or 4.7 with sulfuric acid and/or sodium hydroxide. All treatment groups were provided with untreated feed and the control groups were provided with pH adjusted deionized water. The treatment groups were given either a 24, 48 or 72 hour acclimation period (Table 3.1). Copper treatments were administered on the third, fourth, and fifth day post-hatch and terminated on the eighth day post-hatch. This study was designed for 34 day duration, but was terminated after 96 hours due to signs of overt toxicity.

Table 3.1 Treatment Groups in Study Three

Copper treatment (mg/L Cu)	pH	N	Acclimation period (hr)
Control	3.0	9	48
600	3.0	8	48
Control	4.7	9	72
600	4.7	8	24
1200	4.7	7	24

Treatment solutions were prepared with deionized water in 18 L quantities. Copper sulfate was weighed in a plastic weight boat on a digital balance and transferred to an acid - rinsed polyethylene container. The copper solutions were mixed with a magnetic stir bar for at least 20 minutes. Acids and bases were added with Pasteur pipettes to achieve the desired pH.

3.1.3.4 Study Four

The specific aims for Study Four were to determine a minimum concentration of copper in drinking water that would produce signs of toxicity and to investigate the influence of pH on copper tissue accumulation. The study included nine treatment groups and three control groups (Table 3.2). The acclimation periods for each of the treatment groups are summarized in Table 3.3. Copper treatments were administered from the third and fourth day post-hatch until the seventeenth and eighteenth day post-hatch. A 14 day exposure period was used because the results from the previous investigations indicated that treatment effects could be evaluated within this time period.

Table 3.2 Treatment Groups in Study Four

	pH 4.5	**N**	**pH 5.5**	**N**	**pH 6.5**	**N**
Copper in	Control	6	Control	5	control	6
Drinking	2	6	2	6		
Water	10	6	10	6		
(mg/L)	50	7	50	7	50	6
	250	6	250	6		

Table 3.3 Acclimation Periods in Study Four

	pH 4.5	Acclimation period (hr)	pH 5.5	Acclimation period (hr)	pH 6.5	Acclimation period (hr)
Copper	Control	48	Control	48	control	72
in	2	24	2	24		
Drinking	10	24	10	24		
Water	50	24	50	24	50	72
(mg/L)	250	48	250	48		

Copper was administered to mallards in a soft reconstituted water which was prepared with deionized water and the addition of 30 mg/L $CaSO_4 * H_2O$, 30 mg/L $MgSO_4$, and 2 mg/L KCl. Hardness of the reconstituted water was calculated to be between 40-48 mg/L of $CaCO_3$ and sulfate levels were calculated to be between 80 - 85 mg/L for all of the treatment solutions. The sulfate and hardness levels were selected to approximate the conditions found in the Alamosa River in May and June of 1995 and 1996 (Ortiz, 1996).

Reconstituted water was prepared as a stock solution in large quantities (~ 70 L) in a 55 gal container. Each treatment solution was then prepared from the stock solution in smaller quantity (18 L) by altering the pH and/or the copper concentration of the stock solution. Solution pH was adjusted with sulfuric acid and sodium hydroxide. All solutions were prepared in acid - rinsed polyethylene containers. Each solution was mixed for at least 10 minutes with an electric stir plate and a magnetic stir bar.

The 250 mg Cu/L treatment was prepared by the addition of cupric sulfate and cupric carbonate. This approach was used for two reasons: 1) to maintain the sulfate levels within the defined range, and 2) because copper sulfate is insoluble at this concentration. After the addition of copper carbonate, the solution was acidified to pH 2.0 by titration of

sulfuric acid to liberate some of the carbonate as carbon dioxide. The acidified solution was mixed for 10 minutes before the pH was re-adjusted with sodium hydroxide.

3.1.3.5 Study Five

The specific aim for Study Five was to evaluate the toxicity of copper and acidic pH water between previously observed levels of effect and no effect. Copper was administered in reconstituted water at either 117 or 184 mg Cu/L. The copper concentrations for this study were selected because they were equally spaced between the 50 and 250 mg Cu/L. In the preceding study, minimal effects were observed in mallards treated with 50 mg Cu/L and substantial health effects were observed in birds treated with 250 mg Cu/L. Likewise, drinking water pH values of 3.5 and 4.0 were selected because no adverse effects were observed in previous study at pH 4.5 and significant effects were observed at pH 3.0.

The soft reconstituted drinking water was prepared as described in Section 3.3.4. The 117 mg Cu/L treatment was tested at pH 3.5, 4.0 and 4.5. The 184 mg Cu/L treatment was only tested at pH 4.5 (Table 3.3). The pH 3.5 and 4.0 control groups were provided with soft reconstituted water without added copper. Each group contained 7 birds and the exposure period was 14 days. Table 3.5 summarizes the acclimation periods for this study. Copper treatments were administered from the third, fourth, and fifth day post-hatch until the seventeenth, eighteenth, and nineteenth day post-hatch.

Table 3.4 Treatment Groups in Study Five

	pH 3.5	N	pH 4.0	N	pH 4.5	N
Copper	Control	7	control	7		
in drinking water	117	7	117	7	117	8
(mg/L)					184	7

Table 3.5 Acclimation Periods in Study Five

	pH 3.5	Acclimation period (hr)	pH 4.0	Acclimation period (hr)	pH 4.5	Acclimation period (hr)
Copper in	control	72	control	24		
Drinking	117	72	117	24	117	48
Water					184	48
(mg/L)						

3.1.3.6 Study Six

The specific aim of Study Six was to determine if prolonged exposure to low concentrations of copper in drinking water would produced signs of toxicity or measurable increases in tissue copper concentrations. In wild populations, the fledging period represents the maximum length of exposure for juvenile mallards. The length of exposure in this study was 34 days because it was determined in an earlier study that mallards raised in the laboratory for 34 days had approximately the same body weight as fledging birds.

Copper was administered in soft reconstituted drinking water at either 2 or 50 mg/L, a third treatment group was provided with a "river matrix." The river matrix contained 1.3 mg Al/L, 2.0 mg Cu/L, 1.8 mg Fe/L, 0.6 mg Mn/L, and 0.3 mg Zn/L. The control group was provided with soft reconstituted water without added copper. Drinking water pH for

all treatments was 4.5 and each group contained 8 birds. All treatment groups were given a 24 hour acclimation period.

Reconstituted water was prepared as previously described (section 3.1.3.4). The river matrix solution was prepared by the addition of: aluminum sulfate, ferric sulfate, magnesium sulfate, zinc sulfate and nickel (II) chloride to reconstituted water. The concentrations of all metals, except copper, used in the river matrix were selected by reviewing US Geological Survey water quality data for the Alamosa River during the months of May and June 1995-6 (Ortiz, 1996). Water pH was adjusted with sulfuric acid and sodium hydroxide.

3.1.4 Necropsy and Tissue Collection

At the end of each study, the mallards were euthanized by exsanguination under anesthesia. Anesthesia was induced with isoflurane, which was delivered from a small animal anesthesia machine equipped with a re-breathing circuit and an isoflurane vaporizer. The anesthesia mask was modified with a latex glove, which contained a narrow slit to create a tight seal around the bird's head. When a mallard was no longer responsive to external stimulus it was removed from the mask and transferred to a sink where the jugular vein was punctured with a sharp No. 10 scalpel blade.

Three blood samples, plasma/serum chemistry, hematology, and residue were collected in that order. Hematology samples were collected by draining blood off the tip of a scalpel blade into pre-labeled 5 ml tubes containing EDTA. Hematology samples were

immediately capped, inverted and then placed in a Styrofoam rack on top of wet ice. Plasma chemistry samples were collected by draining blood in pre-labeled 7 ml tubes containing heparin. The plasma chemistry samples were immediately capped, inverted and spun in a clinical centrifuge for 5 minutes. The plasma was transferred with a Pasteur pipette to a pre-labeled 1.5 µl tube and placed on wet ice. In Study One, serum was collected instead of plasma. Serum samples were collected in pre-labeled 7 ml tubes, allowed to clot and spun in centrifuge for several minutes. The serum was removed by pipette and placed in a pre-labeled 1.5 µl tube. Blood residue samples were collected in 7 ml tubes and stored at 4 °C. The amount of blood recovered from each bird varied, but the minimum volume was approximately 1 ml. On a few occasions there were complications in the exsanguination and blood samples could not be collected.

The spinal column was severed once all of the blood samples were collected. The body was then immersed in a weak detergent solution and the breast feathers were removed. The bird was transferred to the necropsy table where the peritoneal cavity was opened using a mid-line incision. Bile was collected with a 1 cc (ml) syringe and transferred to a pre-labeled 1.5 ml tube. The amount of bile recovered from each bird varied and on several occasions was not collected, but for birds that were sampled a minimum of 0.1 ml was obtained. Bile samples were collected for possible future analysis and the data are not included in this thesis.

The entire liver was dissected and placed in a plastic weight boat. Whole liver weight was recorded and two liver samples were collected for histopathological analysis. One section was taken from the tip of the left lobe and the other from the point of gall bladder

attachment. Both sections were placed in 10% NBF. The remaining portion of the liver was then washed in a 0.9% NaCl solution and divided into two samples: one for residue analysis, and one for biochemical analysis. The samples were weighed and placed into pre-labeled 7 ml polypropylene tubes. Samples for biochemical analysis were placed in a cooler on dry ice until they could be stored at -80 °C. The residue samples were place in a cooler on wet ice until they could be stored at 4 °C.

Following removal of the liver, the next priority was to collect the kidneys. First the spleen and thyroid were removed and then the intestinal tract was deflected to allow incisions along both sides of the kidney. The entire kidney was carefully dissected and placed in a weight boat. The kidney was weighed and a portion tissue from the area of gonadal attachment was removed and placed in formalin. The remainder of the kidney was washed in saline and divided into biochemical and residue samples, which were weighed and placed in pre-labeled 7 ml polypropylene tubes. The samples for biochemical analysis were placed in a cooler on dry ice until they could be stored at -80 °C. Residue samples were place in a cooler on wet ice until they could be stored at 4 °C.

The heart was removed and a lateral incision was made to examine the interior of the atria and ventricles. Observations of lesions, if present, were recorded. The next tissues to be removed were the intestinal tract, proventriculus, ventriculus, crop, and esophagus. Scissors were used to open the digestive tract from the esophagus to the cloaca. These tissues were examined for lesions and portions from each section were collected. A portion of the pancreas, duodenum, and the ileocecal junction were excised and placed in formalin.

The head was removed from the body, the cranial cavity opened with a small pair of rongeurs and the brain carefully removed. The oral cavity was examined for gross lesions. The tongue and soft palate were removed with a pair of small scissors. An eye was collected with the lachrymal gland attached.

A piece of skin with feather follicles attached was taken from the region where the mid-line incision of the peritoneal cavity was made. A group of primary feathers from the wing tip was removed, placed in a zip-lock bag with an identifying label, and stored at 4 ^{o}C for residue analysis. The left tibia tarsus was removed and cracked to collect a portion of bone marrow.

During necropsy a checklist was used to record the bird's identification (leg band number and color) and any lesions or abnormal tissue morphology. This check list delineated all of the tissues that were harvested and was used to verify that each of these were, in fact, taken from the bird. All of the samples were stored in 10% NBF unless otherwise noted. Only three tissues were submitted for histopathological analysis: liver, kidney and ventriculus. The remaining tissues were collected for possible future analysis.

Assistance with post-mortem examinations was provided by: Donna Carver, D.V.M. Ph.D., Dave Close, D.V.M., Patricia Newell, Cheryl Perkins, Christina Sigurdson, D.V.M., Sean Strom, and several students from the CSU Veterinary Medicine class of 2000.

3.1.5 Tissue Copper Analysis

Flame atomic absorption spectrophotometry (FLAA) was used to measure the copper levels in various tissues. These analyses were performed in the CSU Department of Environmental Health's Analytical Toxicology Laboratory. Assistance with metals analyses was provided by Patricia Newell, Cheryl Perkins, and Sean Strom.

Prior to analysis, all of the tissue samples were digested with concentrated nitric acid (70%) and microwave heat. All of the glass- and plasticware used for metals analysis were pre-rinsed with 10% nitric acid (v/v).

The liver and kidney samples were weighed on a digital balance and then placed in a digestion vessel with 10 volumes (w/v) of nitric acid. Feather samples were weighed, washed in 0.1% Triton-X detergent solution, rinsed three times with deionized water, cut into small pieces, and then placed in a digestion vessel with 10 volumes of nitric acid. Coagulated whole blood samples were homogenized in 2 ml of deionized water with a Teflon pestle; approximately 1 ml of the blood suspension was placed in a digestion vessel with 10 volumes (v/v) nitric acid.

Tissues were left in the digestion vessels for at least two hours before they were placed in the rotating carousel of a CEM digestion microwave model MDS 205 (Matthews, NC). The process of microwave digestion consisted of heating the samples for a 3 minute period at 25% power, allowing the samples to cool to room temperature, reheating the samples for 2 minutes at 60% power, cooling, and finally reheating for 2 minutes at 60% power. The tissue digests were then transferred to glass volumetric cylinders and the digestion vessels were rinsed several times with deionized water. The water from each

rinse was added to the volumetric cylinder and the samples were diluted to 20 ml with deionized water. Copper concentrations in some samples exceed the upper limit of the analytical method and required further dilution. The final volume was recorded and the diluted sample was transferred to a 30 ml polypropylene bottle and stored at room temperature until analysis by FLAA.

Sample absorbency was measured at 324.7 nm with a copper-specific lamp in a Varian 2000 Spectrophotometer (Victoria, Australia). Lamp current was 4 mA and the slit width was 0.5 nM. The flame was generated with a mixture of air and acetylene. The air flow was 13.5 L/min and acetylene flow was 2.0 L/min.

Prior to analysis, a calibration curve was generated with a group of copper standards. The calibration curve consisted of 0.5, 1.0, 5.0, 10.0 and 50.0 mg Cu/kg copper standards prepared in 10% nitric acid. The accuracy of the calibration curve was periodically checked with copper standards prepared by the National Bureau of Standards.

Samples were introduced manually and absorbency was measured with three consecutive readings and reported as an average. The measurement time was one second. The spectrophotometer was equipped with Varian 2000 software, which correlated mean sample absorbency with the calibration curve to provide a measurement of copper concentration. The detection limit was dependent on the range of standards used to establish the calibration curve; for tissue analysis the detection limit was typically 0.1 mg Cu/kg.

Quality control and quality assurance (QA/QC) techniques were applied for all analyses. Each set of samples included at least two duplicate samples, three reagent

blanks and four spike samples. Sample spikes were prepared by the addition of a known quantity of copper standard to a duplicate sample prior to digestion. The coefficient of variation between duplicates ranged from 1.2 - 16.1%. Percent recoveries for spiked samples ranged from 83.1 - 110.1%.

All of the raw FLAA data were entered into an Excel® spreadsheet (Microsoft, Redmond WA). These data were used to calculate tissue copper concentration as follows:

1) The mean absorbency for the blanks was subtracted from the sample reading.
2) The final volume of the sample was multiplied by the corrected sample absorbency and divided by the initial sample weight.

In Studies Three, Four, and Five, the moisture content of the liver samples was measured by placing a known weight of liver in a pre-baked aluminum weight boat. The sample was weighed and then dried at 80 °C for 24 hours. The percent moisture was calculated by dividing the sample's dry weight by the wet weight, subtracting this number from 1, and multiplying by 100.

3.1.6 Metallothionein Analysis

Concentrations of hepatic metallothionein were measured in the liver samples collected for biochemical analyses. The samples collected for this assay were stored at -80 °C until analysis. Tissue samples were analyzed for both the copper bound fraction (Cu - MT) and the total amount of heat stable copper binding protein (T - MT).

All of the tubes and glassware used in the MT assay were pre-rinsed with 10% nitric acid. Approximately 1 g of tissue was homogenized in four volumes (w/v) homogenization buffer (50 mM Tris-HCl, pH 7.4) using a Teflon pestle. An aliquot of homogenized liver was analyzed for protein content. The remainder of the homogenate was centrifuged at 15,000 x g for 10 minutes. The supernatant was heated at 100 °C for 10 minutes. After heat treatment the sample was vortexed, cooled on ice, and centrifuged at 15,000 x g for 10 minutes. The supernatant fraction was diluted with an equal volume of homogenization buffer. Aliquots (200 µl) of the diluted heat-treated supernatant fraction were transferred to a 1.5 ml tube for copper saturation/chelex treatment (in triplicate for both T -MT and Cu - MT and a single sample for protein analysis).

Copper saturation/chelex treatment was performed by adding 10 µl of 1 mM copper acetate monohydrate to tubes for T - MT determination and 10 µl of 1 mM sodium chloride to tubes for Cu - MT determination. Tubes were shaken and then spun on a tube rotator for 10 minutes. After mixing, 200 µl of a 66% (v/v) suspension of chelex 100 ion-exchange resin in Tris-HCl was added to each tube to remove unbound copper and the tubes were rotated for 15 minutes. The chelex treated samples were centrifuged and the supernatant was acidified with 2.5 ml of 1% nitric acid (v/v), capped, vortexed and stored at room temperature for a minimum of 12 hours before analysis by FLAA. The metals analysis homogenate was digested by adding 350 µl of concentrated nitric acid (70% v/v) and heating at 100 °C under a fume hood for 10 minutes. Instrument conditions and quality assurance protocols for metals analysis are described in Section 3.1.5.

Total protein analysis on the homogenate and supernatant fractions was performed in 96 well plates using a modification of the Coomassie® Reagent kit colormetric assay. Briefly, either sample or standard (10 µl) and 250 µl of Coomassie® Reagent to was added to each well, the plate was mixed for 30 seconds on a platform shaker, and the absorbency of the protein-dye complex was measured in a plate reader at 600 nm. Samples were run in triplicate and every fifth sample was a duplicate. A standard curve was prepared with dilutions of bovine serum albumin. Results for FLAA were corrected for protein content and reported as µg Cu/ g protein.

3.1.7 Clinical Chemistry

Plasma or serum samples were submitted to the CSU Veterinary Teaching Hospital's Clinical Pathology Laboratory for an "Exotic Profile 2." Serum was analyzed in Study One and plasma samples were used in all of the later studies at the request of Clinical Pathology Laboratory. Clinical chemistry analysis was performed with a Hitachi 911 Auto Sampler (Tokyo, Japan). The assays included in the avian panel were glucose, blood urea nitrogen (BUN), uric acid, albumin (A), globulin (G), A/G ratio, creatine kinase (CK), aspartate aminotransferase (AST), calcium, phosphorus, sodium, potassium, chloride, bicarbonate, anion gap, lipemia, hemolysis, and icterus. A novel procedure for analysis of creatine. Creatine is found in the serum and plasma of birds and reptiles and may be an indicator of renal function. Metabolism of creatine leads to the production of

hydrogen peroxide. Plasma creatine concentrations were determined by a colormetric indication of hydrogen peroxide generation (Vapp, 1997).

3.1.8 Hematology

Whole blood samples were submitted to the CSU Veterinary Teaching Hospital's Clinical Pathology Laboratory. Hematology samples were analyzed for total white blood cell (WBC), heterophil, lymphocyte, monocyte, eosinophil, basophil, plasma protein, and packed cell volume (PCV). The Eosinophil Unopette Method was used to indirectly calculate the total WBC manually with a hemocytometer. The differential count of the percent of heterophil, lymphocyte, monocyte, eosinophil, basophil was determined on a smear of blood stained with a Wright's stain. Total solids was determined with a refractometer and packed cell volume was measured by centrifugation in a hematocrit tube.

3.1.9 Histopathology

The following tissues were collected at necropsy for histopathological evaluation: liver, kidney, gonads, adrenal gland, spleen, gall bladder, esophagus, proventriculus, ventriculus, small intestine, duodenum, ileocecal junction, pancreas, heart, bursa, skin, tongue, soft palette, eye, lachrymal gland, brain, and bone marrow. All tissues were preserved in 10% neutral buffered formalin. Only liver, kidney and portions of the digestive tract were submitted for evaluation by a pathologist because of the cost

associated with histopathological analysis. Slide cassettes were prepared and sent to the CSU Histopathology Laboratory to be embedded in paraffin, sectioned, mounted, and stained. Hematoxylin and eosin staining was used in all six studies. In Study One additional slides were prepared with, rhodonein, a copper specific stain. All slides were read by a Board-certified veterinary pathologist without knowledge of the treatment status of the specimen.

3.1.10 Statistical Analysis

All data were evaluated using the Student Edition of Minitab® for Windows® release 9 or Minitab® 10.0 for Windows® (Addison – Wesley Publishing, Reading MA). Analysis of variance (ANOVA) and Tukey's pair-wise comparisons were used to identify significant differences between groups ($\alpha = 0.05$, $p < 0.05$). Regression analysis for was used to determine the correlation between two variables. All other analyses were performed in Microsoft Excel® for Windows.

CHAPTER 4 RESULTS

4.0 Field Study

During the last week of June 1995, juvenile mallards and environmental samples were collected from the Terrace Reservoir and wetlands along the Alamosa River. The field sampling was scheduled to occur when juvenile mallards were expected to be near the end of their fledging period. Unfortunately, in the course of a week only six mallards were obtained on the Alamosa River and none were collected from the reference site. The six birds included one adult female and two juvenile mallards from the Terrace Reservoir and three juvenile mallards from wetland areas upstream from the reservoir (Figure 2.1).

Metals analysis of the environmental samples indicates that copper concentrations are not uniformly distributed throughout the Alamosa River Basin (Table 4.1). Samples collected from the Terrace Reservoir contained substantially greater copper concentrations than those collected in the wetlands. Sediment samples from the wetlands and the Terrace Reservoir contained similar concentrations of copper. The wetland sediments with elevated copper concentrations might actually be river sediments that were carried into the wetlands during periods of high flow.

Table 4.1 Copper Concentrations in Environmental Samples Collected during the Field Survey

Sample Type	Wetlands	Terrace Reservoir
Water (N = 2)	0.01 ± 0 [ab]	0.35 ± 0.01 [ab]
Water (filtered) (N = 2)	0.01 ± 0 [b]	0.30 ± 0.03 [b]
Sediment	118.00 [c]	234.00 [c]
Vegetation (unwashed)	11.10 [c]	463.00 [c]
Vegetation (washed)	6.73 [c]	421.00 [c]
Marcoinvertebrates	24.60 [c]	38.00 [c]

[a] Mean ± standard deviation
[b] Units mg Cu/L
[c] Composite sample, units µg Cu/g

Liver copper concentrations were lower in mallards collected from wetland areas when compared to birds collected in the Terrace Reservoir (Table 4.2). Compared to the mallards collected at the Monte Vista NWR, mallards from both the wetlands and the Terrace Reservoir had substantially higher liver copper concentrations (Table 4.2). Copper concentrations in all kidney samples were below the detection limit (24.6 µg/g WW).

Table 4.2 Liver Copper Concentrations of Mallards Collected in the Field Survey

Sample Origin	N	Liver Copper (µg Cu/g DW)	Liver Copper (µg Cu/g WW)	Range (µg Cu/g WW)
Wetlands (juveniles)	3	187.9 ± 70.6 [ab]	53.0 ± 19.9 [a]	36.3 - 75.1
Alamosa River (juveniles)	2	478.7 ± 90.4 [b]	135.0 ± 25.5	117.0 – 153.0
Alamosa River (adult)	1	929.1 [b]	262.0	262.0
Monte Vista NWR (adults) [c]	8	60.7 ± 51.9	17.1 ± 14.6 [d]	1.5 - 38.6 [d]

[a] Mean ± standard deviation
[b] These numbers are dry weight estimations, calculated with 71.8% moisture.
[c] Data from Archuleta, 1992.
[d] Wet weight values were calculated from the reported mean percent moisture (71.8%).

4.1 Study One

In Study One, mallards were fed either a copper treated diet or a control diet for 34 days beginning on the fourth day post-hatch. Three copper treatments: high, medium and low were tested. Measured concentrations of copper in the feed are reported in Table 4.3. Copper concentrations in the high copper treatment diet varied by almost 50%, indicating that the powered copper sulfate was not evenly distributed in the feed.

Table 4.3 Dietary Copper Concentrations in Study One

	Control	Low	Medium	High
Calculated	NA	200	400	800
Analyzed	25.5 ± 1.5[ab]	218.5 ± 58.7[ab]	420 ± 87.7[ab]	1024.0 ± 503.5[ab]

[a] Units are mg Cu/kg feed
[b] Mean ± standard deviation, N = 3

4.1.1 Weight Gain and Feed Consumption in Study One

Weight gain in the medium and high exposure groups was significantly reduced ($p < 0.05$) when compared to the control group (Table 4.4). Weight gain for the high treatment group was 15% lower than the control group mean. No significant differences in feed consumption were observed between treatment groups (Table 4.5).

Table 4.4 Weight Gain in Study One

Treatment	N	Starting Weight (g)	Weight Gain (g)	Range (g)
Control	7	59.9 ± 6.1	775.4 ± 57.1[a]	683.9 – 862.4
Low	6	62.8 ± 5.3	737.2 ± 70.6	641.9 – 857.4
Medium	7	58.1 ± 4.4	707.6 ± 64.5 [b]	633.0 – 845.9
High	8	59.4 ± 6.1	658.7 ± 78.7 [c]	551.2 – 758.5

[a] Mean ± standard deviation
[b] Significantly different from control (ANOVA, Tukey's test, $p < 0.05$)
[c] Significantly different from control (ANOVA, Tukey's test, $p < 0.01$)

Table 4.5 Feed Consumption in Study One

Treatment	Feed consumption (g/g BW/d)
Control	0.22 ± 0.09[a]
Low	0.26 ± 0.11
Medium	0.27 ± 0.13
High	0.24 ± 0.12

[a] Mean ± standard deviation from 6 observations

4.1.2 Tissue Copper Concentrations in Study One

There were no significant differences in liver copper concentrations between treatment groups (Table 4.6). However, feather copper concentrations increased significantly with copper exposure (Table 4.7). A 2- and 3-fold increase in feather copper concentrations was observed in the medium and high treatment groups (Table 4.7).

Table 4.6 Liver Copper Concentrations in Study One

Treatment	N	Liver Copper (μg Cu/g WW)	Range (μg Cu/g WW)
Control	7	155.7 ± 16.6 [a]	100.8 – 196.3
Low	6	160.2 ± 9.8	92.2 – 205.5
Medium	7	143.8 ± 35.1	63.1 – 234.3
High	8	183.6 ± 17.0	121.1 – 266.9

[a] Mean ± standard deviation

Table 4.7 Feather Copper Concentrations in Study One

Treatment	N	Feather Copper (μg Cu/g)	Range (μg Cu/g)
Control	7	22.7 ± 5.7[a]	15.6 – 32.2
Low	6	35.3 ± 22.1	9.6 – 64.4
Medium	7	48.7 ± 8.3[b]	41.2 – 62.0
High	8	66.8 ± 6.7[bc]	56.0 – 73.2

[a] Mean ± standard deviation
[b] Significantly different from the control (ANOVA and Tukey's test, $p < 0.001$)
[c] Significantly different from all other groups (ANOVA and Tukey's test, $p < 0.001$)

4.1.3 Clinical Chemistry and Hematology Results for Study One

No significant differences were seen in any of the measured serum chemistry or hematology values (Tables 4.8 and 4.9). There was a wide range in the reported AST values, and the mean AST values for all of the treatment groups were larger than the reported range, 15.8-16.2 IU/L (Table 2.3). The glucose results for the medium treatment group were below the reported normal range, 185-215 mg/dl (Table 2.3).

Table 4.8 Serum Chemistry Result for Study One

Treatment	Control [a] (N = 7)	Low [a] (N = 6)	Medium [a] (N = 7)	High [a] (N = 8)
Glucose [b]	186.0 ± 1.5	190.0 ± 9.0	167.7 ± 6.8	196.1 ± 23.0
Uric acid [b]	12.6 ± 3.4	8.2 ± 4.1	3.6 ± 0.6	8.9 ± 4.5
Total protein [c]	3.6 ± 0.3	3.8 ± 0.4	3.7 ± 0.2	3.8 ± 0.3
Albumin [c]	1.4 ± 0.6	1.7 ± 0.2	1.7 ± 0.1	1.7 ± 0.2
Globulin [c]	2.2 ± 0.6	2.0 ± 0.2	2.0 ± 0.1	2.0 ± 0.2
A/G	0.7 ± 0.3	0.9 ± 0.1	0.8 ± 0.1	0.8 ± 0.1
AST [d]	109.0 ± 85.5	38.5 ±12.1	42.0 ±10.3	82.6 ± 97.1
Ca [b]	10.6 ± 0.4	11.1 ± 0.4	11.0 ± 0.2	11.5 ± 1.5
Phosphorus [b]	8.6 ± 0.9	7.1 ± 0.8	7.2 ± 0.3	7.8 ± 1.2
Lipemia [b]	19.0 ± 10.2	13.3 ± 7.5	7.9 ± 4.8	17.9 ± 21.1
Hemolysis [b]	29.9 ± 23.1	13.5 ± 6.5	12.0 ± 6.6	13.4 ± 10.0
Icterus [b]	0.9 ± 0.4	0.8 ± 0.4	1.0 ± 0.0	0.8 ± 0.5

[a] Reported as mean ± standard deviation
[b] Units are mg/dl
[c] Units are g/dl
[d] Units are IU/L

Table 4.9 Hematology Results for Study One

Treatment	Control [a] (N = 7)	200 [a] (N = 6)	400 [a] (N = 7)	800 [a] (N = 8)
WBC [b]	8.9 ± 4.7	18.3 ± 7.9	9.5 ± 3.7	14.3 ± 6.1
Heterophils [b]	4.9 ± 3.2	8.5 ± 3.1	5.2 ± 2.8	7.3 ± 3.1
Lymphophils [b]	3.2 ± 1.5	8.5 ± 5.9	3.4 ± 1.7	6.2 ± 3.8
Monocytes [b]	0.2 ± 0.2	0.6 ± 0.7	0.8 ± 0.9	0.3 ± 0.2
Eosinophils [b]	0.2 ± 0.1	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.3
Basophils [b]	0.5 ± 0.3	0.8 ± 0.6	0.5 ± 0.5	0.4 ± 0.3
Plasma protein [c]	4.1 ± 0.5	4.2 ± 0.4	4.0 ± 0.3	4.3 ± 0.4
PCV (%)	38.8 ± 3.2	38.0 ± 1.9	35.3 ± 0.4	36.6 ± 3.4

[a] Reported in mg Cu/kg as mean ± standard deviation
[b] Units are * $10^3/\mu l$
[c] Units are g/dl

4.1.4 Histopathology Results for Study One

Moderate to severe pathological changes were observed in six of the eight liver samples from the control group (Table 4.10). Similar frequencies of liver lesions were

observed in the low and high copper treatment groups. Liver lesions included hepatocellular swelling and microcavitary degeneration. No difference between treatment and control groups was observed in slides that were stained with the copper - specific stain rhodonien. Kidney lesions included swelling and degeneration of the tubular epithelial cells. Nearly all of the ventriculus samples exhibited some degree of degeneration in the kaolin layer. It was the pathologist's opinion that the lesions of the ventriculus could be artifacts of stress (Spraker, 1996).

Table 4.10 Frequency of Tissue Lesions in Study One

Treatment	Liver	Kidney	Ventriculus
Control	6/8	3/8	2/8
Low	4/6	1/6	0/8
Medium	1/7	2/7	2/7
High	6/8	2/8	2/8

Note: only lesions that were classified as moderate to severe were included in this comparison

4.2 Study Two

In Study Two, mallards were provided with treated feed that contained either copper sulfate or copper acetate for 34 days beginning on the fourth day post-hatch. Measured concentrations of copper in the feed are reported in Table 4.11. The concentration of copper in the acetate diet was approximately twice that of the sulfate diet due to an error in feed preparation.

Table 4.11 Dietary Copper Concentrations (mg/kg feed) in Study Two

	Control	Acetate	Sulfate
Calculated		1119	637
Analyzed (N = 2)	26.9 ± 1.2[a]	1024.8 ± 121.0[a]	503.4 ± 57.7[a]

[a] Mean ± standard deviation

4.2.1 Weight Gain and Consumption Rates for Study Two

At the end of the 34 day exposure period weight gain in the copper sulfate treatment group was significantly reduced ($p < 0.05$) when compared to the control group (Table 4.12). There were no significant differences in feed or water consumption between control and treatment groups (Table 4.13).

Table 4.12 Weight Gain in Study Two

Treatment	N	Starting Weight (g)	Weight Gain (g)	Range (g)
Control	8	56.2 ± 5.2	897.7 ± 116.6[a]	704.9 – 1039.9
Acetate	8	46.8 ± 6.3	911.6 ± 100.2	773.6 – 1022.6
Sulfate	8	54.9 ± 5.5	812.6 ± 87.0[b]	676.8 – 977.1

[a] Mean ± standard deviation

[b] Significantly different from control (ANOVA, Tukey's test, $p < 0.05$)

Table 4.13 Consumption Rates in Study Two

Treatment	Feed (g/g BW/d)	Water (ml/g BW/d)
Control	0.15 ± 0.04[a]	0.95 ± 0.31[a]
Acetate	0.14 ± 0.04	0.99 ± 0.34
Sulfate	0.15 ± 0.04	0.99 ± 0.35

[a] Mean ± standard deviation of 34 observations

4.2.2 Liver Copper Concentrations in Study Two

Hepatic copper concentrations in both of the treatment groups were significantly increased ($p < 0.01$) when compared to the control group (Table 4.14). There was a 2-fold increase in liver copper concentrations for the acetate treatment group when compared to the sulfate treatment group.

Table 4.14 Liver Copper Concentrations in Study Two

Treatment	N	Liver Copper (μg Cu/g WW)	Range (μg Cu/g WW)
Control	8	95.1 ± 43.9[a]	38.0 - 152.3
Acetate	8	352.5 ± 130.6 [bc]	108.8 - 515.5
Sulfate	8	153.4 ± 61.2[b]	88.1 - 271.8

[a] Mean ± standard deviation
[b] Significantly different from control (ANOVA, Tukey's test, $p < 0.01$)
[c] Significantly different from sulfate treatment, (ANOVA, Tukey's test, $p < 0.001$)

4.2.3 Clinical Chemistry and Hematology Results for Study Two

The plasma chemistry results did not demonstrate any significant differences between treatment groups (Tables 4.15). Likewise, there were no significant differences in the hematology results (4.16). The AST result for all treatment groups were greater than reported normal range (Table 2.3).

Table 4.15 Plasma Chemistry Results for Study Two

Treatment	Control (N = 8)	Acetate (N = 8)	Sulfate (N = 8)
Glucose [b]	229.1 ± 29.8	223.6 ± 25.7	200.9 ± 27.3
BUN [b]	1.6 ± 0.5	1.0 ± 0.0	1.8 ± 0.5
Uric acid [b]	6.1 ± 2.3	3.6 ± 0.9	4.8 ± 2.3
Creatine [b]	1.5 ± 0.4	1.7 ± 0.5	1.6 ± 0.5
Total protein [c]	3.6 ± 0.1	3.7 ± 0.5	3.7 ± 0.2
Albumin [c]	1.6 ± 0.1	1.6 ± 0.2	1.6 ± 0.1
Globulin [c]	2.0 ± 0.1	2.1 ± 0.3	2.1 ± 0.1
A/G ratio	0.8 ± 0.04	0.8 ± 0.07	0.8 ± 0.05
AST [d]	32.4 ± 18.6	30.3 ± 23.8	34.1 ± 19.4
Creatine kinase [d]	2122.0 ± 1326.0	1875.0 ± 1231.0	2317.0 ± 1580.0
Calcium [b]	10.6 ± 0.4	10.9 ± 0.6	9.4 ± 4.3
Phosphorus [b]	7.9 ± 2.5	7.5 ± 0.9	7.9 ± 2.5
Sodium [c]	145.9 ± 3.9	144.9 ± 1.1	145.5 ± 2.0
Potassium [e]	7.5 ± 0.9	7.5 ± 0.9	7.9 ± 2.5
Chloride [e]	110.0 ± 3.4	110.0 ± 1.5	110.0 ± 3.7
Bicarbonate [e]	23.1 ± 2.5	22.4 ± 2.2	22.8 ± 4.5
Anion gap	15.4 ± 3.2	15.6 ± 2.6	18.6 ± 10.5
Lipemia [b]	7.1 ± 6.0	6.0 ± 2.9	13.4 ± 6.3
Hemolysis [b]	8.1 ± 5.9	12.3 ± 7.5	9.4 ± 11.2
Icterus [b]	0.9 ± 0.4	0.6 ± 0.5	0.3 ± 0.5

[a] Mean ± standard deviation
[b] Units are mg/dl
[c] Units are g/dl
[d] Units are IU/L
[e] Units are meq/L

Table 4.16 Hematology Results for Study Two

Treatment	Control (N =8)	Acetate (N =8)	Sulfate (N =8)
WBC [a]	16.1 ± 10.5	16.8 ± 3.0	24.3 ± 6.7
Heterophiles [a]	4.4 ± 1.9	5.6 ± 1.4	11.0 ± 3.4
Lymphocytes [a]	10.9 ± 8.8	10.0 ± 1.5	12.1 ± 6.7
Monocytes [a]	0.4 ± 0.3	0.4 ± 0.1	0.5 ± 0.3
Eosinophiles [a]	0.3 ± 0.2	0.2 ± 0.1	0.7 ± 0.3
Basophiles [a]	0.4 ± 0.4	0.4 ± 0.4	0.6 ± 0.2
Plasma protein [b]	3.9 ± 0.1	4.2 ± 0.6	4.0 ± 0.3
PCV (%)	37.4 ± 1.2	37.8 ± 2.9	37.9 ± 3.9

[a] Units * $10^3/\mu l$
[b] Units g/dl

4.3 Study Three

In Study Three, mallard ducklings were exposed to copper in deionized water on the third to fifth days post-hatch. Exposure concentrations are reported in Table 4.17. This study was designed for a 34 day exposure period but was terminated after 4 days due to high mortality in both treatment and control groups. The pH 4.7 control group was maintained for 34 days to monitor survival. Clinical chemistry and histopathology samples were not collected because the study was terminated prematurely and several of the birds were found dead rather than euthanized.

Table 4.17 Water Copper Concentrations (mg Cu/L) in Study Three

Treatment	pH	N	Measured concentration
Control	3.0	4	BDL[a]
600	3.0	4	605.4 ± 4.8[b]
Control	4.7	4	BDL
600	4.7	4	607.0 ± 8.2
1200	4.7	4	1195.2 ± 24.3

[a] Below detection limit (0.1 mg Cu/L)
[b] Mean ± standard deviation

4.3.1 Mortality in Study Three

The copper treatments used in this study produced unexpectedly high mortality rates (Table 4.18). Exposure to 600 mg Cu/L at pH 4.7 for 96 hours resulted in 63% mortality. After 24 hours there was 86% mortality in the 1200 mg Cu/L treatment group. An unexpected observation for this study was the decreased survival rate in birds that consumed drinking water at pH 3.0 without added copper. Ducklings exposed to

drinking water at pH 3.0 without added copper experienced 86% mortality between day 4 and 5, while the entire pH 4.7 control group survived until euthanasia at day 34. The combination of pH 3.0 and 600 mg Cu/L appeared to increase the extent of mortality, and reduced the time until first mortality.

Table 4.18 Mortality in Study Three

Treatment (mg Cu/L)	pH	Mortalities/ Treated Birds	Mortality (%)	Days of Exposure to 1st Mortality	Length of exposure period [a] (days)
Control	3.0	6/9	67	4	5
600		7/8	88	1	4
Control	4.7	0/9	0	NA	34
600		5/8	63	2	4
1200		6/7	86	1	1

[a] When mortality in a treatment group exceed 50% the remaining birds were euthanized.

4.3.2 Liver Copper Concentrations in Study Three

The mean liver copper concentrations for ducks that were exposed to 600 mg Cu/L at both pH 3.0 and 4.7 were significantly elevated ($p < 0.001$) when compared to the respective control group (Table 4.19). Mean liver copper concentration for the 1200 mg Cu/L treatment group was not significantly different ($p > 0.05$) when compared to the respective control group. However, this group was terminated after 24 hours due to the high mortality rate, it is possible that the short exposure period precluded the accumulation of copper. Exposure to 600 mg Cu/L at pH 4.7 for 96 hours produced a mean liver copper concentration that was approximately twice that of ducks, which had been exposed to 1024 mg Cu/kg in feed (as copper acetate) for 34 days (Table 4.14). The

liver copper concentration for the 600 mg Cu/L pH 4.7, but not the 600 mg Cu/L pH 3.0, treatment group was significantly greater than the 1200 mg Cu/L pH 4.7 treatment group.

Table 4.19 Liver Copper Concentrations in Study Three

Treatment (mg Cu/L)	**N**	**pH**	**Liver copper (μg /g WW)**	**Range (μg /g WW)**
Control	9	3.0	25.6 ± 7.7 [a]	15.8 - 37.3
600	8	3.0	290.8 ± 183.2 [b]	66.0 - 501.3
Control	9	4.7	75.6 ± 29.2 [cd]	51.5 - 138.5
600	8	4.7	364.1 ± 132.5 [be]	142.5 - 500.3
1200	7	4.7	134.7 ± 135.4	45.5 - 396.4

[a] Mean ± standard deviation
[b] Significantly different from the respective control (ANOVA, Tukey's test, $p < 0.001$)
[c] 34 day-old bird
[d] Significantly different from the pH 3.0 control (ANOVA, Tukey's test, $p < 0.01$)
[e] Significantly different from 1200 mg Cu/L treatment group (ANOVA, Tukey's test, $p < 0.01$)

4.4 Study Four

In Study Four, mallards were exposed to copper in soft reconstituted water for 14 days starting on the third and fourth days post-hatch. Measured water pH and copper concentrations are summarized in Table 4.20.

Table 4.20 Water pH and Copper Concentrations in Study Four

Nominal (mg Cu/L)	pH	pH range	Measured (mg Cu/L)	N
Control	4.5	4.2 - 4.7	BDL [a]	4
2	4.5	4.3 - 4.6	2.3 ± 0.5 [b]	4
10	4.5	4.2 - 4.8	9.7 ± 0.9	4
50	4.5	4.4 - 4.8	51.6 ± 3.7	4
250	4.5	4.4 - 5.0	252.4 ± 8.5	4
Control	5.5	5.4 - 5.7	BDL	4
2	5.5	5.2 - 5.5	1.9 ± 0.7	4
10	5.5	5.2 - 5.6	10.1 ± 1.0	4
50	5.5	5.4 - 5.7	48.7 ± 4.1	4
250	5.5	5.5 - 5.9	249.3 ± 10.2	4
Control	6.5	6.4 -6.7	BDL	4
50	6.5	6.5 -6.6	52.4 ± 4.5	4

[a] Below detection limit (0.1 mg Cu/L)
[b] Mean ± standard deviation
[c] Units mg Cu/L

4.4.1 Weight Gain and Consumption Rates in Study Four

Weight gain was significantly reduced ($p < 0.001$) in mallard ducklings that were exposed to 250 mg Cu/L copper at both pH 4.5 and 5.5 when compared to their respective control groups and all other treatment groups (Table 4.21). Mean weight gain for both 250 mg Cu/L treatment groups were less than 50% of the respective control group means. During the last week of treatment, the ducklings in 250 mg Cu/L treatment groups were noticeably weak and listless.

Weight gain for all three of the 50 mg Cu/L treatment groups was reduced by more than 15% when compared to the respective control groups. Reduced weight gain (20%) was also observed in the 2 mg Cu/L pH 5.5 treatment group. There were no significant

differences ($p > 0.05$) in weight gain between the different pH groups, which received the same copper treatment.

Table 4.21 Weight Gain in Study Four

Treatment (mg Cu/L)	pH	N	Starting Weight (g)	Weight Gain (g)
Control	4.5	6	55.5 ± 8.4	256.5 ± 40.2[a]
2	4.5	6	60.7 ± 5.3	231.9 ± 27.5
10	4.5	6	60.1 ± 6.9	242.5 ± 29.6
50	4.5	7	58.4 ± 7.2	184.2 ± 26.4[b]
250	4.5	6	57.4 ± 6.6	94.7 ± 46.2[bc]
Control	5.5	5	61.7 ± 3.9	224.6 ± 16.7
2	5.5	6	49.2 ± 6.5	180.2 ± 28.8[b]
10	5.5	6	66.9 ± 7.5	258.9 ± 46.1
50	5.5	7	54.5 ± 5.9	183.4 ± 25.8[b]
250	5.5	6	60.3 ± 4.3	73.8 ± 39.8[bc]
Control	6.5	6	59.4 ± 6.3	237.3 ± 11.7
50	6.5	6	61.2 ± 5.6	198.2 ± 26.3

[a] Mean ± standard deviation

[b] Significantly different from the respective control (ANOVA and Tukey's test, $p < 0.001$)

[c] Significantly different from all other treatment groups with the same pH (ANOVA and Tukey's test, $p < 0.001$)

There were no significant differences among mean feed consumption rates for any of the treatment groups (Table 4.22). Likewise, there were no significant differences in water consumption between treatment groups.

Table 4.22 Feed and Water Consumption Rates for Study Four

Treatment (mg Cu/L)	pH	Feed (g/g BW/d)	Water (g/g BW/d)
Control	4.5	0.23 ± 0.07[a]	1.12 ± 0.22[a]
2	4.5	0.18 ± 0.07	1.06 ± 0.22
10	4.5	0.23 ± 0.07	1.16 ± 0.09
50	4.5	0.24 ± 0.06	1.14 ± 0.23
250	4.5	0.22 ± 0.11	1.67 ± 0.27
Control	5.5	0.24 ± 0.08	1.00 ± 0.10
2	5.5	0.28 ± 0.08	1.37 ± 0.36
10	5.5	0.24 ± 0.06	1.21 ± 0.06
50	5.5	0.25 ± 0.09	1.03 ± 0.25
250	5.5	0.18 ± 0.09	1.76 ± 0.41[b]
Control	6.5	0.22 ± 0.07	1.15 ± 0.13
50	6.5	0.24 ± 0.06	1.42 ± 0.41

[a] Mean ± standard deviation for 12 observations
[b] Significantly different from the control (ANOVA, Tukey's test, $p < 0.001$)

4.4.2 Tissue Copper Concentrations in Study Four

Mallards in the 250 mg Cu/L treatment groups at both pH 4.5 and 5.5 had elevated liver copper concentrations (Table 4.23). One individual in the 250 mg Cu/L treatment group (pH 5.5) had an exceptionally high concentration of copper, 5749 μg Cu/g DW. When the mean hepatic copper concentrations were calculated, the individual with the largest hepatic copper concentration was excluded because it appears to be an outlier (i.e., more than 4 standard deviations above the mean) (Kleinbaum, 1988). Mean liver copper concentrations for the 250 mg Cu/L treatment groups at both pH 4.5 and 5.5 were significantly increased ($p < 0.001$) when compared to the respective control groups.

Table 4.23 Liver Copper Concentrations in Study Four

Treatment (mg Cu/L)	pH	N	Liver Copper (μg Cu/g DW)	Range (μg Cu/g DW)
Control	4.5	6	199.8 ± 75.9[a]	85.2 - 286.5
2	4.5	6	295.5 ± 93.3	201.6 - 460.5
10	4.5	6	185.6 ± 82.9	89.3 - 318.5
50	4.5	7	197.3 ± 82.3	110.9 - 339.5
250	4.5	6	1546.7 ± 408.6[b]	1100.8 - 2129.1
Control	5.5	5	335.1 ± 134.7	231.5 - 555.1
2	5.5	6	216.1 ± 90.1	141.1 - 367.9
10	5.5	6	252.9 ± 56.4	202.0 - 347.0
50	5.5	7	174.4 ± 56.6	73.6 - 234.5
250	5.5	6	1524.1 ± 746.5[bc]	857.4 - 2510.1
Control	6.5	6	228.3 ± 62.7	120.6 - 275.3
50	6.5	6	137.6 ± 36.7	64.6 - 164.5

[a] Mean ± standard deviation

[b] Significantly different from the respective control group (ANOVA, Tukey's test, $p < 0.001$).

[c] An outlier, with 5748.9 μg Cu/g, was excluded from the mean. The mean ± standard deviation including the outlier is 2228.3 ± 1849.5.

There were no significant differences ($p > 0.05$) between the blood copper concentrations in the 250 mg Cu/L treatment groups and their respective control groups (Table 4.24). Kidney copper concentrations for both the 250 mg Cu/L treatment groups were significantly greater ($p < 0.05$) than the respective control group means (Table 4.25). The mallard with the exceptionally large hepatic copper concentration also had very high concentration of copper in its kidney. The kidney copper concentration for this individual was more than 4 standard deviations greater than the group mean so it was treated as an outlier and also omitted from the calculation of the mean.

Table 4.24 Blood Copper Concentrations in Study Four

Treatment (mg Cu/L)	pH	N	Blood Copper (μg Cu/g)	Range (μg Cu/g)
Control	4.5	4	1.4 ± 0.8[a]	0.9 - 2.5
250	4.5	5	1.8 ± 1.0	1.0 - 3.4
Control	5.5	5	1.4 ± 0.8	0.8 - 2.7
250	5.5	6	1.8 ± 0.6	1.1 - 2.8

[a] Mean ± standard deviation

Table 4.25 Kidney Copper Concentrations in Study Four

Treatment (mg Cu/L)	pH	N	Kidney Copper (μg Cu/g)	Range (μg Cu/g)
Control	4.5	6	4.1 ± 0.6[a]	3.0 - 4.7
2	4.5	6	4.2 ± 0.6	3.4 - 4.9
10	4.5	6	3.3 ± 0.6	2.7 - 4.1
50	4.5	7	3.9 ± 0.6	3.3 - 4.8
250	4.5	6	9.9 ± 5.6[b]	5.9 - 21.1
Control	5.5	5	3.6 ± 0.7	2.7 - 4.7
2	5.5	6	3.3 ± 0.5	2.7 - 3.8
10	5.5	6	5.3 ± 1.6	3.7 - 7.5
50	5.5	7	4.6 ± 2.2	3.3 - 9.2
250	5.5	6	13.4 ± 4.6[cd]	7.7 – 18.9
Control	6.5	6	3.3 ± 0.4	2.9 - 4.0
50	6.5	6	3.9 ± 0.7	3.0 - 5.1

[a] Mean ± standard deviation
[b] Significantly different from pH 4.5 control (ANOVA, Tukey's test, $p < 0.001$)
[c] The individual with the largest kidney copper concentration, 61.4 μg Cu/g was omitted from the mean. The mean ± standard deviation including the outlier was 21.4 ± 20.0.
[d] Significantly different from pH 5.5 control (ANOVA, Tukey's test, $p < 0.05$)

Feather copper concentrations in both the 250 mg Cu/L treatment groups were significantly greater ($p < 0.001$) than their respective control groups (Table 4.26). The mean feather copper concentration for the 250 mg Cu/L pH 5.5 treatment groups was

significantly greater ($p < 0.001$) than the mean for the 250 mg Cu/L pH 4.5 treatment group. The individual in the 250 mg Cu/L pH 5.5 treatment group with the exceptionally large liver and kidney copper concentrations also had the largest feather copper concentration, 231 μg Cu/g. However, the feather copper concentration in this individual was less than 2 standard deviation greater than the mean, so it was not treated as an outlier. The mean feather copper concentration for the 50 mg Cu/L pH 6.5 treatment group was significantly greater ($p < 0.001$) than the pH 6.5 control and all other 50 mg Cu/L treatment groups.

Table 4.26 Feather Copper Concentrations for Study Four

Treatment (mg Cu/L)	**pH**	**N**	**Feather Copper (μg Cu/g)**	**Range (μg Cu/g)**
Control	4.5	6	15.0 ± 6.8 [a]	6.7 - 22.1
2	4.5	6	8.8 ± 1.7	7.3 - 10.4
10	4.5	6	18.4 ± 4.9	14.3 - 25.1
50	4.5	7	21.5 ± 5.1	15.2 - 26.4
250	4.5	6	65.0 ± 20.5 [b]	37.6 - 93.1
Control	5.5	5	12.5 ± 3.7	7.2 - 16.1
2	5.5	6	13.5 ± 3.0	10.8 - 18.3
10	5.5	6	20.7 ± 10.3	11.2 - 27.1
50	5.5	7	26.5 ± 7.2	17.2 - 38.1
250	5.5	6	144.0 ± 64.6 [cd]	49.7 - 231.0
Control	6.5	6	13.1 ± 2.1	9.6 - 13.7
50	6.5	6	66.3 ± 27.1 [ef]	43.1 - 102.0

[a] Mean ± standard deviation
[b] Significantly different from pH 4.5 control (ANOVA, Tukey's test, $p < 0.001$)
[c] Significantly different from pH 5.5 control (ANOVA, Tukey's test, $p < 0.001$)
[d] Significantly different from 250 mg Cu/L pH 4.5 (ANOVA, Tukey's test $p < 0.001$)
[e] Significantly different from control, pH 6.5 (ANOVA, Tukey's test, $p < 0.001$)
[f] Significantly different from 50 mg Cu/L pH 4.5 and 5.5 (ANOVA, Tukey's test, $p < 0.001$)

4.4.3 Clinical Chemistry and Hematology Results for Study Four

Mean plasma concentrations of total protein, albumin, globulin, uric acid, and potassium in the 250 mg Cu/L pH 5.5 treatment group were significantly different ($p < 0.001$) from the respective control group (Table 4.28a). The elevated mean uric acid concentration in the 250 mg Cu/L treatment group (pH 5.5) was produced by one bird, the individual with exceptionally high liver and kidney copper concentrations. The plasma concentration of uric acid in the high copper bird was 17.2 mg/dl. When this individual was omitted from the average, the mean (4.3 ± 2.3 mg/dl) was not significantly increased ($p > 0.05$). The elevated mean potassium concentration was also associated with one bird. The bird with an elevated plasma potassium concentration (14.3 meq/L) had increased, but not exceptionally large concentrations of copper in its liver and kidney.

The mean lipemia values for the 50 mg Cu/L treatment groups at both pH 4.5 and 5.5 were significantly elevated ($p < 0.001$) when compared to the respective control groups, but the increased means in both cases was created by one individual. The presence of elevated plasma lipids can occur if the sample is improperly prepared (Vapp, 1997). No significant differences were seen among any of the hematology parameters (Tables 4.27b, 4.28b, and 4.29b).

Table 4.27a Clinical Chemistry Results from Study Four - I

Treatment	Control, pH 4.5	2, pH 4.5	10, pH 4.5	50, pH 4.5	250, pH 4.5
Glucose [a]	210 ± 13	230 ± 21	222 ± 14	216 ± 14	223 ± 10
BUN [a]	1.3 ± 0.5	1.5 ± 0.6	1.7 ± 0.8	2.1 ± 0.4	1.5 ± 0.6
Uric Acid [a]	3.8 ± 0.6	5.1 ± 1.5	5.1 ± 1.5	4.2 ± 1.1	3.0 ± 1.3
Total Protein [b]	3.2 ± 0.2	3.3 ± 0.3	8.2 ± 11.7	3.2 ± 0.3	3.0 ± 0.6
Albumin [b]	1.6 ± 0.1	1.6 ± 0.2	1.7 ± 0.1	1.6 ± 0.3	1.5 ± 0.2
Globulin [b]	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.6 ± 0.3	1.5 ± 0.2
A/G	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.3	1.1 ± 0.5
AST [c]	46 ± 25	139 ± 144	144 ± 196	123 ± 87	116 ± 167
CK [c*]	10.9 ± 4.1	7.6 ± 8.3	7.2 ± 9.5	6.7 ± 4.9	5.6 ± 7.2
Creatine [c]	1.0 ± 0.2	1.7 ± 1.0	2.5 ± 2.5	1.9 ± 0.9	1.5 ± 0.4
Ca [a]	9.9 ± 0.3	9.7 ± 0.5	10.1 ± 0.6	10.0 ± 0.4	9.4 ± 2.2
Phosphate [a]	8.2 ± 0.1	8.1 ± 0.7	8.3 ± 1.2	8.7 ± 1.5	7.8 ± 0.9
Na [d]	143 ± 1.4	144 ± 2.5	146 ± 1.3	147 ± 5.1	145 ± 3.6
K [d]	3.8 ± 0.2	4.4 ± 0.7	4.0 ± 0.5	4.3 ± 0.9	4.6 ± 2.5
Cl [d]	114 ± 1.4	114 ± 2.8	115 ± 2.6	116 ± 4.9	114 ± 2.6
Bicarbonate [d]	19.1 ± 1.7	17.1 ± 1.1	17.5 ± 1.5	17.6 ± 1.8	17.0 ± 1.3
Anion Gap	13.8 ± 2.5	17.7 ± 2.1	18.0 ± 2.1	17.4 ± 2.3	18.6 ± 1.8
Lipemia [a]	11.0 ± 5.8	10.3 ± 6.4	8.5 ± 9.8	***49.7 ± 78.8***	8.2 ± 5.3
Hemolysis [a]	16.8 ± 5.1	53.6 ± 44.3	35.8 ± 50.7	35.4 ± 40.9	67.3± 110.7
Icterus [a]	0.3 ± 0.5	0.8 ± 0.4	0.7 ± 0.5	0.6 ± 0.5	0.2 ± 0.4

Reported as mean ± standard deviation

Bold number are significantly different from control (ANOVA, $p<0.001$, $\alpha = 0.05$)

[a] Units are mg/dl

[b] Units are g/dl

[c] Units are IU/L (* CK units are * 1000)

[d] Units are meq/L

Table 4.27b Hematology Results for Study Four - I

Treatment	Control, pH 4.5	2, pH 4.5	10, pH 4.5	50, pH 4.5	250, pH 4.5
WBC [a]	18.0 ± 10.0	6.8 ± 2.9	6.7 ± 3.3	6.9 ± 1.4	11.2 ± 4.4
Heterophils [a]	5.3 ± 1.7	3.5 ± 1.8	3.6 ± 1.5	2.9 ± 0.6	5.7 ± 3.2
Lymphocytes [a]	11.8 ± 10.3	2.6 ± 1.4	3.3 ± 2.9	3.2 ± 2.0	5.3 ± 1.7
Monocyte [a]	0.4 ± 0.2	0.3 ± 0.3	0.6 ± 0.4	0.4 ± 0.2	0.6 ± 0.3
Eosinophil [a]	0.3 ± 0.2	0.1± 0.0	0.3 ± 0.0	0.3 ± 0.3	0.3 ± 0.0
Basophil [a]	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
Protein[b]	4.0 ± 0.3	4.3 ± 0.5	4.2 ± 0.1	4.1 ± 0.5	3.9 ± 0.4
PCV (%)	36.8 ±1.0	38.0 ± 2.8	37.5 ± 3.6	36.2 ± 1.5	34.0 ± 2.6

Reported as mean ± standard deviation

[a] Units * $10^3/\mu l$

[b] Units g/dl

Table 4.28a Clinical Chemistry Results for Study Four - II

Treatment	Control, pH 5.5	2, pH 5.5	10, pH 5.5	50, pH 5.5	250, pH 5.5
Glucose [a]	215 ± 15	231 ± 16	271 ± 40	204 ± 17	203 ± 34
BUN [a]	2.0 ± 0.0	1.2 ± 0.4	1.8 ± 1.0	2.0 ± 0.7	1.8 ± 1.2
Uric Acid [a]	5.1 ± 1.8	4.7 ± 1.4	5.7 ± 2.1	4.9 ± 1.4	***6.5 ± 5.6***
Total Protein[b]	3.1 ± 0.2	3.2 ± 0.2	3.4 ± 0.2	3.2 ± 0.3	***2.1 ± 0.7***
Albumin [b]	1.2 ± 0.7	1.6 ± 0.1	1.7 ± 0.2	1.5 ± 0.3	***0.9 ± 0.4***
Globulin [b]	1.9 ± 0.8	1.6 ± 0.1	1.7 ± 0.2	1.7 ± 0.3	***1.2 ± 0.4***
A/G	0.8 ± 0.4	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.2	0.8 ± 0.1
AST [c]	72 ± 42	68 ± 30	83 ± 87	84 ± 56	70 ± 24
CK [c*]	4.5 ± 3.2	4.0 ± 1.9	4.9 ± 5.1	3.2 ± 2.7	4.9 ± 3.3
Ca [a]	9.9 ± 0.2	10.1 ± 0.5	10.5 ± 0.9	10.2 ± 0.3	8.1 ± 4.1
Phosphate [a]	8.8 ± 0.8	7.4 ± 1.0	8.8 ± 2.8	8.3 ± 1.0	8.9 ± 2.5
Na [d]	144 ± 1.5	145 ± 1.9	145 ± 2.9	145 ± 1.7	144 ± 4.0
K [d]	4.4 ± 0.5	3.7 ± 0.2	5.1 ± 2.4	4.0 ± 0.7	***6.1 ± 4.3***
Cl [d]	115 ± 2.1	114 ± 2.6	114 ± 3.6	114 ± 3.1	115 ± 1.1
Bicarbonate [d]	17.7 ± 1.9	18.0 ± 1.6	16.6 ± 4.2	16.4 ± 1.7	16.6 ± 1.7
Anion Gap	15.6 ± 1.8	16.7 ± 1.5	19.5 ± 7.2	18.4 ± 1.5	13.7 ± 3.1
Lipemia [a]	14.2 ± 7.3	4.2 ± 2.6	7.0 ± 4.9	***51.0 ± 86.7***	5.5 ± 4.3
Hemolysis [a]	49.8 ± 44.4	25.2 ± 14.4	41.5 ± 37.7	25.4 ± 9.2	18.5 ± 13.0
Icterus [a]	0.6 ± 0.6	0.8 ± 0.4	1.0 ± 0.0	0.2 ± 0.5	0.2 ± 0.4

Reported as mean ± standard deviation

Bold number are significantly different from control (ANOVA, Tukey's test, $p < 0.001$, $\alpha = 0.05$)

[a] Units are mg/dl

[b] Units are g/dl

[c] Units are IU/L

[d] Units are meq/L

Table 4.28b Hematology Results for Study Four - II

Treatment	Control, pH 5.5	2, pH 5.5	10, pH 5.5	50, pH 5.5	250, pH 5.5
WBC [a]	6.2 ± 0.5	7.5 ± 1.8	12.7 ± 7.3	4.6 ± 1.7	11.0 ± 3.6
Heterophils [a]	3.9 ± 1.1	3.3 ± 1.0	4.2 ± 2.8	2.6 ± 1.0	5.8 ± 2.6
Lymphocytes [a]	1.9 ± 1.1	3.3 ± 1.6	7.2 ± 8.3	1.4 ± 0.7	4.5 ± 1.8
Monocyte [a]	0.3 ± 0.3	0.5 ± 0.3	0.6 ± 0.4	0.3 ± 0.2	0.6 ± 0.3
Eosinophil [a]	0.2 ± 0.1	0.5 ± 0.6	0.5 ± 0.4	0.5 ± 0.0	0.7 ± 0.0
Basophil [a]	0.2 ± 0.1	0.2 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	0.3 ± 0.2
Protein[b]	4.3 ± 0.6	4.1 ± 0.2	4.2 ± 0.2	3.9 ± 0.5	2.8 ± 0.8
PCV (%)	34.3 ± 2.8	37.4 ± 1.8	40.5 ± 3.6	29.0 ± 6.6	30.0 ± 6.9

Reported as mean ± standard deviation

[a] units * 10^3 /µl

[b] units g/dl

Table 4.29a Clinical Chemistry Results for Study Four - III

Treatment	Control, pH 6.5	50, pH 6.5
Glucose [a]	192 ± 16	235 ± 19
BUN [a]	2.0 ± 0.7	1.5 ± 0.8
Uric Acid [a]	4.4 ± 1.6	3.5 ± 1.0
Total Protein [b]	3.2 ± 0.2	3.1 ± 0.1
Albumin [b]	1.6 ± 0.2	1.5 ± 0.1
Globulin [b]	1.6 ± 0.1	1.5 ± 0.1
A/G	1.0 ± 0.1	1.0 ± 0.2
AST [c]	78 ± 38	63 ± 49
CK [c*]	4.1 ± 2.4	2.8 ± 1.5
Ca [a]	10.1 ± 0.5	9.8 ± 0.4
Phosphate [a]	8.7 ± 1.3	7.6 ± 0.8
Na [d]	144 ± 2.5	146 ± 2.6
K [d]	3.9 ± 0.4	3.6 ± 0.5
Cl [d]	113 ± 1.8	114 ± 3.1
Bicarbonate [d]	17.0 ± 3.0	18.1 ± 2.1
Anion Gap	17.8 ± 2.8	17.3 ± 1.6
Lipemia [a]	17.6 ± 22.9	5.5 ± 4.3
Hemolysis [a]	20.0 ± 18.6	18.8 ± 11.4
Icterus [a]	0.6 ± 0.6	0.7 ± 0.5

Reported as mean ± standard deviation
[a] Units are mg/dl
[b] Units are g/dl
[c] Units are IU/L
[d] Units are meq/L

Table 4.29b Hematology Results for Study Four - III

Treatment	Control, pH 6.5	50, pH 6.5
WBC [a]	12.0 ± 2.6	13.6 ± 4.8
Heterophils [a]	3.2 ± 1.3	3.3 ± 1.4
Lymphocytes [a]	8.0 ± 2.5	9.1 ± 3.8
Monocyte [a]	0.5 ± 0.4	0.3 ± 0.2
Eosinophil [a]	0.7 ± 0.0	0.8 ± 0.7
Basophil [a]	0.1 ± 0.0	0.1 ± 0.1
Plasma Protein[b]	4.0 ± 0.3	4.2 ± 0.4
PCV (%)	37.3 ± 1.2	37.5 ± 6.6

Reported as mean ± standard deviation
[a] units * 10^3 /µl
[b] units g/dl

4.4.4 Histopathology Results for Study Four

An increased frequency of liver lesions was observed in both the 10 and 250 mg Cu/L treatment groups, but not in the 50 mg Cu/L treatment group at pH 5.5 (Table 4.30). Abnormal pigmentation of hepatic macrophages was observed in three birds that were exposed to 250 mg Cu/L at both pH 4.5 and pH 5.5. One bird from each of the high dose groups presented with green-brown lipofuscin-type pigments in their sinusoidal macrophages and a second bird from the 250 mg Cu/L pH 5.5 treatment group had darker and larger brown hemosiderin-type granules. Lipofuscin is the presence of pigmented residues from lysosomal digestion and hemosiderin is an insoluble protein produced by phagocytic digestion of hematin (Trump, 1980). The mallard which appear to have hepatic hemosiderin was also the bird with exceptionally large concentrations of copper in its liver and kidney. This individual also displayed several abnormal plasma chemistry results including decreased glucose, increased BUN, increased uric acid, and decreased protein levels (total, albumin, and globulin).

Table 4.30 Frequency of Tissue Lesions in Study Four

Treatment	pH	Liver lesion	Kidney lesion	Ventriculus lesion
Control	4.5	1/6 [a]	1/6	NSL [b]
2	4.5	NSL	NSL	NSL
10	4.5	1/6	1/6	NSL
50	4.5	NSL	NSL	NSL
250	4.5	1/6	1/6	2/6
Control	5.5	NSL	1/5	NSL
2	5.5	NSL	2/6	NSL
10	5.5	3/6	NSL	NSL
50	5.5	NSL	NSL	NSL
250	5.5	4/6	NSL	1/6
Control	6.5	2/6	NSL	NSL
50	6.5	1/6	NSL	NSL

[a] Number of affected individuals / group's total size
[b] No Significant Lesions

4.4.5 Hepatic Metallothionein Concentrations in Study Four

Hepatic concentrations of metallothionein were evaluated for the pH 4.5 treatment groups. Only samples from the pH 4.5 treatment group were analyzed because of the time-intensive nature of the assay. Samples were analyzed for both the total and the copper bound fraction of metallothionein. Both fractions was significantly increased ($p < 0.001$) in the 250 mg Cu/L treatment group when compared to all other treatment groups (Table 4.31). The percentage of copper bound metallothionein was highest in the control group.

Table 4.31 Hepatic Metallothionein Concentrations in Study Four

Treatment (mg Cu/L)	pH	N	Cu metallothionein (μg Cu/g protein)	Total metallothionein (μg Cu/g protein)	Copper-bound (%)
0	4.5	6	2.25 ± 0.87 [a]	2.36 ± 0.37 [a]	95 [b]
2	4.5	7	2.15 ± 0.37	3.38 ± 0.46	64
10	4.5	6	2.57 ± 0.91	2.22 ± 0.62	72
50	4.5	7	2.04 ± 0.49	2.82 ± 0.63	72
250	4.5	6	3.48 ± 0.61 [c]	4.39 ± 0.83 [c]	79

[a] Mean ± standard deviation

[b] Calculated by the mean Cu - MT/ mean T - MT

[c] Significantly different from all other treatment groups (ANOVA, Tukey's test, $p < 0.001$)

4.5 Study Five

In Study Five, juvenile mallards were exposed to copper in soft reconstituted water for 14 days starting on the third and fourth days post-hatch. Measured water pH and copper concentrations are summarized in Table 4.32.

Table 4.32 Water pH and Copper Concentrations (mg Cu/L) in Study Five

Nominal (mg Cu/L)	pH	pH range	Measured (mg Cu/L)	N
Control	3.5	3.2 - 3.6	BDL[a]	4
117	3.5	3.3 - 3.6	115.4 ± 4.3 [b]	4
Control	4.0	3.9 - 4.2	BDL	4
117	4.0	4.0 - 4.4	116.2 ± 3.3	4
117	4.5	4.2 - 4.7	115.8 ± 3.9	4
184	4.5	4.4 - 4.8	189.0 ± 8.3	4

[a] Below detection limit (0.1 mg Cu/L)

[b] Mean ± standard deviation

4.5.1 Weight Gain and Consumption Rate in Study Five

Weight gain was significantly reduced ($p < 0.05$) in all of the copper treatment groups, and the pH 3.5 control group when compared to the pH 4.0 control group (Table 4.33). Weight gain in the pH 4.5 treatment groups was compared to the pH 4.0 control group because there was no pH 4.5 control group. The 117 pH 3.5 treatment group was compared to the 4.0 control group because the pH 3.5 control group demonstrated a significant reduction ($p < 0.001$) in weight gain. There was not a significant difference in weight gain between the pH 3.0 control and the 117 mg Cu/L pH 3.0 treatment group. There were no significant differences ($p > 0.05$) among group feed or water consumption rat

Table 4.33 Weight Gain in Study Five

Treatment (mg Cu/L)	pH	N	Starting Weight (g)	Weight gain (g)
Control	3.5	7	57.3 ± 4.3	174.7 ± 19.3 [ab]
117	3.5	7	66.1 ± 5.6	189.3 ± 35.2[b]
Control	4.0	7	64.7 ± 7.1	240.1 ± 34.1
117	4.0	7	61.7 ± 7.9	182.0 ± 28.1[b]
117	4.5	8	61.3 ± 4.5	196.2 ± 53.9[b]
184	4.5	7	58.3 ± 5.1	65.2 ± 37.0 [cd]

[a] Mean ± standard deviation
[b] Significantly different from control pH 4.0 (ANOVA, Tukey's test, $p < 0.05$)
[c] Significantly different from control pH 4.0 (ANOVA, Tukey's test, $p < 0.001$)
[d] Significantly different from 115 pH 4.5 (ANOVA, Tukey's test, $p < 0.001$)

Table 4.34 Feed and Water Consumption Rates for Study Five

Treatment (mg Cu/L)	pH	Feed (g/g BW/d)	Water (g/g BW/d)
Control	3.5	0.20 ± 0.12[a]	2.28 ± 0.97[a]
117	3.5	0.22 ± 0.06	1.72 ± 0.46
Control	4.0	0.16 ± 0.09	1.32 ± 0.46
117	4.0	0.21 ± 0.03	1.39 ± 0.63
117	4.5	0.19 ± 0.05	1.34 ± 0.42
184	4.5	0.15 ± 0.03	2.19 ± 1.03

[a] Mean ± standard deviation for 12 observations

4.5.2 Tissue Copper Concentrations in Study Five

All copper treatment groups had liver copper concentrations that were significantly greater than their respective control group ($p < 0.001$). One individual in the 184 mg Cu/L treatment group had an extremely elevated concentration of liver copper, 9280 μg Cu/g DW. When the mean hepatic copper concentrations were calculated, the individual with the largest hepatic copper concentration was excluded because it appears to be an outlier (i.e., more than 4 standard deviations above the mean) (Kleinbaum, 1988). The mean liver copper concentration for the 184 mg Cu/L treatment group at pH 4.5 was significantly greater ($p < 0.001$), almost 50% higher, than the mean for the 117 mg Cu/L treatment group at pH 4.5 (Table 4.35). Mallards in the 117 mg Cu/L treatment group at pH 4.5 had significantly greater ($p < 0.001$) liver copper concentrations when compared to the 117 mg Cu/L treatment groups at either pH 3.5 or 4.0, suggesting that hepatic copper accumulation is reduced as drinking water pH decreases.

Table 4.35 Liver Copper Concentrations in Study Five

Treatment (mg Cu/L)	pH	N	Liver Copper (μg Cu/g DW)	Range (μg Cu/g DW)
Control	3.5	7	188.5 ± 41.6[a]	136.2 - 255.8
117	3.5	7	918.1 ± 381.8[b]	311.0 - 1309.6
Control	4.0	7	357.6 ± 138.5	177.6 - 512.7
117	4.0	7	726.4 ± 282.0[c]	220.4 - 1020.1
117	4.5	8	1276.4 ± 568.6[c]	851.7 - 2569.3
184	4.5	7	2346.8 ± 965.1 [cde]	1183.0 – 4029.4

[a] Mean ± standard deviation
[b] Significantly different from pH 3.5 control (ANOVA, Tukey's test, $p < 0.001$)
[c] Significantly different from pH 4.0 control (ANOVA, Tukey's test, $p < 0.001$)
[d] Significantly different from 117 mg Cu/L pH 4.5 (ANOVA, Tukey's test, $p < 0.001$)
[e] An outlier, with 9280 μg Cu/g, was excluded from the mean. The mean ± standard deviation including the outlier is 3646.7 ± 2670.0.

The mean kidney copper concentration in the 184 mg Cu/L pH 4.5 treatment group was significantly greater ($p < 0.001$) than the mean for the pH 4.0 control group (Table 4.36). No kidney copper value was available for the individual that was considered an outlier because of its elevated liver copper concentration and consequently it is not included in the mean.

Table 4.36 Kidney Copper Concentrations in Study Five

Treatment (mg Cu/L)	pH	N	Kidney Copper (μg Cu/g)	Range (μg Cu/g)
Control	3.5	6	3.5 ± 0.4[a]	3.1 - 4.2
117	3.5	7	6.4 ± 3.0	4.2 - 12.2
Control	4.0	7	4.2 ± 0.6	3.5 - 5.0
117	4.0	7	5.0 ± 1.2	3.7 - 6.6
117	4.5	8	5.0 ± 1.1	3.9 - 6.7
184	4.5	5	10.4 ± 5.2[b]	4.2 - 16.5

[a] Mean ± standard deviation
[b] Significantly different from pH 4.0 control (ANOVA, Tukey's test, $p < 0.001$)

The mean feather copper concentration for the 184 mg Cu/L, pH 4.5, treatment group was significantly greater ($p < 0.001$) than all other treatment groups (Table 4.37). The individual in the 184 mg Cu/L treatment group with an exceptionally large hepatic copper concentration reported the lowest feather copper concentration in its treatment group, 53 μg Cu/g. Feather copper concentrations were significantly elevated in all of the 117 mg Cu/L treatment groups when compared to the pH 4.0 control group. The 117 mg Cu/L treatment group at pH 4.5 had significantly greater feather copper concentrations than either the 117 mg Cu/L at pH 3.5 or 4.0.

Table 4.37 Feather Copper Concentrations in Study Five

Treatment (mg Cu/L)	pH	N	Feather Copper (μg /g)	Range (μg /g)
Control	3.5	7	23.6 ± 19.5[a]	10.4 - 62.9
117	3.5	7	37.3 ± 14.7[b]	13.1 - 53.0
Control	4.0	7	7.7 ± 1.7	5.4 - 10.2
117	4.0	7	49.9 ± 12.0[c]	31.6 - 70.2
117	4.5	8	73.7 ± 22.6 [cd]	43.7 - 105.0
184	4.5	7	107.6 ± 40.6 [cd]	31.0 - 168.0

[a] Mean ± standard deviation
[b] Significantly different from control pH 3.5 (ANOVA, Tukey's test, $p < 0.001$)
[c] Significantly different from control pH 4.0 (ANOVA, Tukey's test, $p < 0.001$)
[d] Significantly different from 117 mg Cu/L pH 4.0 (ANOVA, Tukey's test, $p < 0.001$)

4.5.3 Clinical Chemistry Results for Study Five

Mallards treated with 117 mg Cu/L at pH 4.0 and pH 3.5 had mean bicarbonate and anion gap values that were significantly different ($p < 0.001$) than the pH 4.0 control

Table 4.38a Clinical Chemistry Results for Study Five - I

Parameter	Control, pH 3.5	117, pH 3.5
Glucose [a]	198.2 ± 9.3	216.0 ± 5.4
Bun [a]	1.3 ± 0.8	1.3 ± 0.5
Uric acid [a]	3.2 ± 1.0	4.4 ± 1.2
Total protein [b]	2.9 ± 0.2	3.2 ± 0.8
Albumin [b]	1.4 ± 0.2	1.5 ± 0.6
Globulin [b]	1.5 ± 0.1	1.6 ± 0.3
A/G	0.9 ± 0.1	0.9 ± 0.1
AST[c]	54.1 ± 49.2	29.2 ± 23.3
CK[c*]	4.2 ± 5.1	1.1 ± 0.4
Calcium [d]	10.0 ± 0.3	10.2 ± 0.8
P [d]	8.1 ± 1.0	7.2 ± 1.0
Na [d]	145.7 ± 4.2	142.7 ± 3.3
K [d]	4.2 ± 0.8	3.6 ± 0.3
Cl [d]	115.4 ± 1.9	112.3 ± 2.6
Bicarbonate [d]	17.7 ± 1.9	***20.1 ± 1.7***
Anion gap	16.9 ± 16.8	***13.8 ± 1.9***
Lipemia [a]	10.6 ± 10.9	2.7 ± 3.4
Hemolysis [a]	25.0 ± 22.3	16.7 ± 10.0
Icterus [a]	0.6 ± 0.5	0.8 ± 0.4
Creatine [b]	1.06 ± 0.6	2.22 ± 1.4

Reported as mean ± standard deviation

Bold number are significantly different from the respective control group (ANOVA, Tukey's test, $p<0.001$)

[a] Units are mg/dl

[b] Units are g/dl

[c] Units are IU/L (* CK units are * 1000)

[d] Units are meq/L

Table 4.38b Hematology Results for Study Five - I

Parameter	Control, pH 3.5	117, pH 3.5
WBC[a]	11.2 ± 6.1	17.0 ± 14.7
Heterophiles[a]	5.3 ± 4.1	8.5 ± 12.8
Lymphocyte[a]	5.2 ± 3.6	6.6 ± 3.8
Monocytes[a]	0.3 ± 0.1	1.3 ± 2.3
Eosinophils[a]	0.1 ± 0.1	0.5 ± 0.5
Basophiles[a]	0.4 ± 0.2	0.6 ± 0.6
Protein[b]	4.0 ± 0.7	4.1 ± 0.7
PCV (%)	34.3 ± 2.3	35.9 ± 2.1

Reported as mean ± standard deviation

[a] Units * $10^3/\mu l$

[b] Units g/dl

Table 4.39a Clinical Chemistry Results for Study Five - II

Parameter	Control, pH 4.0	117, pH 4.0
Glucose [a]	200.1 ± 26.3	204.0 ± 10.9
Bun [a]	2.0 ± 1.0	1.1 ± 0.4
Uric acid [a]	6.5 ± 3.4	4.4 ± 1.1
Total protein [b]	3.2 ± 0.3	3.1 ± 0.3
Albumin [b]	1.7 ± 0.2	1.6 ± 0.1
Globulin [b]	1.5 ± 0.2	1.6 ± 0.2
A/G	1.1 ± 0.19	1.0 ± 0.1
AST[c]	80.4 ± 68.5	41.0 ± 35.9
CK[c*]	3.1 ± 2.4	2.4 ± 2.2
Calcium [d]	10.4 ± 0.5	10.1 ± 0.3
P [d]	10.2 ± 3.5	7.4 ± 0.6
Na [d]	144.1 ± 3.9	142.7 ± 2.4
K [d]	4.4 ± 0.8	3.8 ± 0.1
Cl [d]	113.1 ± 3.4	113.0 ± 2.4
Bicarbonate [d]	14.8 ± 4.8	***20.2 ± 1.0***
Anion gap	21.3 ± 8.4	***13.3 ± 1.8***
Lipemia [a]	13.2 ± 16.7	5.1 ± 3.7
Hemolysis [a]	41.0 ± 44.3	22.8 ± 11.8
Icterus [a]	0.7 ± 0.5	0.4 ± 0.5
Creatine [d]	1.17 ± 0.4	1.65 ± 1.1

Reported as mean ± standard deviation

Bold number are significantly different from the respective control group (ANOVA, Tukey's test, $p<0.05$)

[a] Units are mg/dl

[b] Units are g/dl

[c] Units are IU/L (* CK units are * 1000)

[d] Units are meq/L

Table 4.39b Hematology Results for Study Five - II

Parameter	Control, pH 4.0	117, pH 4.0
WBC[a]	11.2 ± 4.7	7.4 ± 3.4
Heterophiles [a]	4.3 ± 1.3	3.8 ± 2.4
Lymphocytes [a]	6.1 ± 3.6	2.9 ± 1.4
Monocytes [a]	0.4 ± 0.2	0.3 ± 0.1
Eosinophils [a]	0.1 ± 0.0	0.3 ± 0.3
Basophiles [a]	0.4 ± 0.2	0.3 ± 0.3
Plasma Protein [b]	3.9 ± 0.4	4.0 ± 0.3
PCV (%)	37.3 ± 3.6	37.1 ± 1.8

Reported as mean ± standard deviation

[a] Units * $10^3/\mu l$

[b] Units g/dl

Table 4.40a Clinical Chemistry Results for Study Five - III

Parameter	117, pH 4.5	184, pH 4.5
Glucose [a]	218.0 ± 15.5	205.2 ± 13.6
Bun [a]	1.5 ± 1.1	2.2 ± 1.0
Uric acid [a]	5.2 ± 1.6	4.3 ± 2.0
Total protein [b]	3.0 ± 0.3	2.8 ± 0.2
Albumin [b]	1.6 ± 0.2	1.5 ± 0.2
Globulin [b]	1.4 ± 0.3	1.3 ± 0.2
A/G	1.1 ± 0.1	1.1 ± 0.3
AST[c]	44.4 ± 55.3	43.3 ± 27.3
CK[c*]	2.4 ± 3.1	2.6 ± 1.9
Calcium [a]	9.7 ± 0.7	9.3 ± 0.2
P [d]	7.1 ± 0.9	6.8 ± 0.7
Na [d]	143.0 ± 3.3	143.0 ± 1.8
K [d]	4.5 ± 1.1	3.9 ± 0.5
Cl [d]	144.0 ± 4.3	115.2 ± 1.9
Bicarbonate [d]	16.8 ± 2.2	17.1 ± 1.5
Anion gap	16.9 ± 2.6	14.5 ± 2.1
Lipemia [a]	12.4 ± 9.5	14.1 ± 2.6
Hemolysis [a]	45.3 ± 51	22.5 ± 18.8
Icterus [a]	0.6 ± 0.5	0.0 ± 0
Creatine [b]	2.33 ± 1.3	1.67 ± 0.7

Reported as mean ± standard deviation

Bold number are significantly different from the respective control group (ANOVA, Tukey's test, $p<0.05$)

[a] Units are mg/dl

[b] Units are g/dl

[c] Units are IU/L (* CK units are * 1000)

[d] Units are meq/L

Table 4.40b Hematology Results for Study Five - III

Parameter	117, pH 4.5	184, pH 4.5
WBC[a]	8.7 ± 3.3	8.3 ± 5.6
Heterophiles [a]	3.0 ± 1.9	6.0 ± 4.5
Lymphocytes [a]	5.0 ± 1.8	1.8 ± 1.4
Monocytes [a]	0.5 ± 0.4	0.3 ± 0.3
Eosinophils [a]	0.4 ± 0.3	0.3 ± 0.3
Basophiles [a]	0.1 ± 0.1	0.1 ± 0.1
Plasma Protein [b]	4.1 ± 0.4	3.8 ± 0.4
PCV (%)	34.0 ± 7.0	32.4 ± 7.3

Reported as mean ± standard deviation

[a] Units * $10^3/\mu l$

[b] Units g/dl

group (Tables 4.38a, 4.39a). There were no significant differences ($p > 0.05$) among any of the measured hematology parameters (Tables 4.38b, 4.39b, and 4.40b).

4.5.4 Histopathology Results for Study Five

Microscopic evaluation of tissues from kidney, liver, and ventriculus demonstrated no trend in either the prevalence or types of lesion among treatment groups. No visible lesions were observed in any of the kidney samples. All of the ventriculus samples, from both treatment and control groups, had some degree of kaolin degeneration (Table 4.41). A section of intestinal tissue was submitted for the mallard with an exceptionally high liver copper concentration because it had an unusual appearance. The pathologist observed nodules of necrotic fat spherules, thin connective tissue walls, but no inflammation (Nordin, 1997).

Table 4.41 Frequency of Tissue Lesions in Study Five

Treatment (mg Cu/L)	**pH**	**Liver lesions frequency**	**Ventriculus lesions frequency**
Control	3.5	2/7[a]	5/7[a]
117	3.5	3/7	7/7
Control	4.0	0/7	4/7
117	4.0	0/7	5/7
117	4.5	1/8	6/8
184	4.5	1/7	4/7

[a] Number of affected individuals / total group size

4.5.5 Hepatic Concentrations of Metallothionein in Study Five

Liver tissue was analyzed for concentrations of both the total and the copper bound metallothionein. No significant differences were seen between treatment groups for either fraction (Table 4.42). Copper treatment groups contained the largest percentage of copper bound protein.

Table 4.42 Concentrations of Hepatic Metallothionein in Study Five

Treatment (mg Cu/L)	**pH**	**Cu metallothionein (μg Cu/g protein)**	**Total metallothionein (μg Cu/g protein)**	**Copper bound (%)**
Control	3.5	0.96 ± 0.39[a]	1.55 ± 0.55[a]	62[b]
117	3.5	1.45 ± 1.19	1.72 ± 0.13	84
Control	4.0	1.20 ± 0.73	1.93 ± 0.88	62
117	4.0	2.21 ± 1.33	2.52 ± 1.65	88
117	4.5	1.15 ± 0.88	1.43 ± 0.97	80
184	4.5	1.65 ± 1.55	1.84 ± 1.80	90

[a] Mean ± standard deviation
[b] Calculated as the mean Cu - MT/ mean T- MT

4.6 Study Six

Juvenile mallards were exposed to copper in soft reconstituted water for 34 days beginning on the third day post-hatch. The nominal and measured exposure concentrations are reported in Table 4.43.

Table 4.43 Copper Concentrations in Study Six Treatment Solutions

Calculated (mg Cu/L)	Control (N = 4)	2 (N = 4)	River Matrix (N = 4)	50 (N = 4)
Analyzed (mg Cu/L)	BDL[a]	2.2 ± 0.8 [b]	1.9 ± 1.0	47.0 ± 4.4

[a] Below detection limit (0.1 mg Cu/L)
[b] Mean ± standard deviation,

4.6.1 Weight Gain and Consumption Rates in Study Six

Weight gain for the 50 mg Cu/L treatment group was significantly reduced ($p < 0.001$), by more than 10%, when compared to the control group (Table 4.44). There were no significant differences ($p > 0.05$) among feed or water consumption rates for any of the treatment groups (Table 4.45).

Table 4.44 Weight Gain in Study Six

Treatment (mg Cu/L)	**N**	**Starting Weight (g)**	**Weight gain (g)**
Control	8	64.8 ± 5.8	778.6 ± 60.3[a]
2	8	63.2 ± 4.5	746.3 ± 81.6
River matrix (2)	8	59.9 ± 4.5	830.2 ± 108.6
50	8	63.6 ± 6.2	683.0 ± 91.2[b]

[a] Mean ± standard deviation
[b] Significantly different from control (ANOVA, Tukey's test, $p < 0.001$)

Table 4.45 Feed and Water Consumption Rates in Study Six

Treatment (mg Cu/L)	**N**	**Feed (g/g BW/d)**	**Water (ml/g BW/d)**
Control	8	0.42 ± 0.05[a]	1.21 ± 0.51[a]
2	8	0.38 ± 0.02	1.06 ± 0.28
River matrix (2)	8	0.41 ± 0.09	1.15 ± 0.33
50	8	0.40 ± 0.02	1.44 ± 0.26

[a] Mean ± standard deviation

4.6.2 Tissue Copper Concentrations in Study Six

There were no significant differences among the liver or kidney copper concentrations for any of the treatment groups (Tables 4.46 and 4.47). Mean feather copper concentration for the 50 mg Cu/L treatment group was significantly greater ($p < 0.05$) than the control and 2 mg Cu/L treatment group (Table 4.48).

Table 4.46 Liver Copper Concentrations in Study Six

Treatment (mg Cu/L)	**N**	**Liver copper (μg Cu/g DW)**	**Range (μg Cu/g DW)**
Control	8	456.7 ± 76.1[a]	274.1 - 510.9
2	8	429.4 ± 174.5	236.7 - 777.5
River matrix (2)	8	472.4 ± 111.8	360.7 - 660.7
50	8	444.8 ± 201.7	192.2 - 786.3

[a] Mean ± standard deviation

Table 4.47 Kidney Copper Concentrations in Study Six

Treatment (mg Cu/L)	**N**	**Kidney Copper (μg Cu/g)**	**Range (μg Cu/g)**
Control	7	5.2 ± 0.4	4.8 - 5.7
2	8	5.5 ± 1.0	4.1 - 7.6
River matrix (2)	8	5.3 ± 0.6	4.4 - 6.4
50	8	6.4 ± 1.3	4.2 - 8.4

[a] Mean ± standard deviation

Table 4.48 Feather Copper Concentrations for Study Six

Treatment	**N**	**Feather Copper (μg Cu/g)**	**Range (μg Cu/g)**
Control	8	10.6 ± 5.0[a]	2.1 - 17.1
2	8	9.9 ± 2.3	5.6 - 14.1
River matrix (2)	8	14.6 ± 6.0	8.8 - 24.5
50	7	22.8 ± 7.0[b]	16.5 - 36.8

[a] Mean ± standard deviation

[b] Significantly different from the control and 2 mg Cu/L treatment group (ANOVA, Tukey's test, $p < 0.005$)

4.6.3. *Clinical chemistry Results for Study Six*

No significant differences were observed among the measured plasma chemistry results (Tables 4.49). Likewise, there were no significant differences in the measured hematology parameters between treatment groups (Table 4.50).

Table 4.49 Clinical Chemistry Results for Study Six

Treatment (mg Cu/L)	**Control (N = 8)**	**2 (N = 8)**	**River (N = 8)**	**50 (N = 8)**
Glucose[a]	199.9 ± 9.3	204.1 ± 22.6	208.2 ± 27.6	208.2 ± 13.9
Bun[a]	1.6 ± 0.7	1.1 ± 0.4	1.3 ± 0.5	1.3 ± 0.5
Uric acid[a]	3.9 ± 1.2	4.2 ± 1.6	4.2 ± 1.1	4.4 ± 1.3
Total protein[b]	3.6 ± 0.3	3.4 ± 0.3	3.4 ± 0.4	3.5 ± 0.3
Albumin[b]	1.8 ± 0.2	1.7 ± 0.1	1.7 ± 0.2	1.7 ± 0.2
Globulin[b]	1.8 ± 0.2	1.7 ± 0.2	1.7 ± 0.2	1.8 ± 0.2
A/G	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	0.9 ± 0.1
AST[c]	55.4 ± 82.6	21.3 ± 7.4	32.8 ± 15.2	35.5 ± 26.1
CK[c]	3.2 ± 4.0	1.4 ± 0.4	2.1 ± 1.3	2.5 ± 1.9
Calcium[a]	11.0 ± 0.5	11.0 ± 0.5	10.7 ± 0.6	10.8 ± 0.5
P[a]	8.0 ± 2.0	7.8 ± 1.8	8.2 ± 0.7	7.9 ± 0.8
Na[d]	144.8 ± 2.7	146.1 ± 1.4	145.0 ± 2.3	143.3 ± 3.3
K[d]	4.7 ± 2.4	4.3 ± 1.3	4.6 ± 1.1	4.2 ± 1.6
Cl[d]	111.6 ± 1.4	112.3 ± 1.9	111.4 ± 1.2	111.5 ± 2.2
Bicarbonate[d]	17.7 ± 3.4	18.6 ± 2.8	17.0 ± 2.6	18.2 ± 1.4
Anion gap	21.1 ± 5.6	19.8 ± 3.2	21.1 ± 3.3	18.0 ± 0.9
Lipemia[a]	8.0 ± 5.8	13.4 ± 10.1	20.5 ± 11.5	6.2 ± 5.0
Hemolysis[a]	31.9 ± 20.4	14.4 ± 9.7	36.0 ± 39.5	30.0 ± 25.8
Icterus[a]	0.9 ± 0.4	0.9 ± 0.4	0.9 ± 0.4	1.0 ± 0.0

Reported as mean ± standard deviation
[a] Units are mg/dl
[b] Units are g/dl
[c] Units are IU/L (*CK units are * 1000)
[d] Units are meq/L

Table 4.50 Hematology Results for Study Six

Treatment (mg Cu/L)	Control (N = 8)	2 (N = 8)	River (N = 8)	50 (N = 8)
WBC	16.4 ± 6.5 [a]	11.3 ± 4.8 [a]	14.7 ± 6.0 [a]	14.5 ± 5.8 [a]
Heterophiles	6.8 ± 2.1	4.2 ± 1.8	5.9 ± 1.9	5.3 ± 4.3
Lymphocyte	8.2 ± 5.5	5.8 ± 3.3	7.8 ± 4.7	7.8 ± 2.2
Monocytes	0.6 ± 0.2	0.5 ± 0.3	0.4 ± 0.8	0.7 ± 0.3
Eosinophils	0.4 ± 0.4	0.4 ± 0.2	0.3 ± 0.2	0.5 ± 0.2
Basophiles	0.6 ± 0.2	0.5 ± 0.4	0.9 ± 0.4	0.7 ± 0.4
Plasma protein	4.4 ± 0.5	4.0 ± 0.3	4.4 ± 0.4	4.1 ± 0.4
PCV (%)	36.1 ± 4.5	38.3 ± 3.4	34.1 ± 3.5	36.2 ± 3.5

[a] Mean ± standard deviation, all units * $10^3/\mu l$ except plasma protein (g/dl)

4.6.4 Histopathology Results for Study Six

An increased prevalence of liver lesions was seen in the river matrix and 50 mg Cu/L treatment groups (Table 4.51). Lesions included hepatocyte vacuolization, and pericholangitis. Periductulitis was observed in one bird from the river matrix treatment group. Erosion of the kaolin layer associated was noted in nearly all of the ventriculus samples regardless of the treatment.

Table 4.51 Frequency of Tissue Lesions for Study Six

Treatment (mg Cu/L)	Liver lesion Frequency	Kidney lesion Frequency	Ventriculus lesion Frequency
Control	1/8[a]	NSL[b]	8/8
2	1/8	NSL	8/8
River	4/8	1/8	5/8
50	3/8	NSL	7/8

[a] Number of affected individuals / total group size
[b] No significant lesions

4.7 Data Analysis

In the preceding sections of this chapter the results from each of the individual studies are presented separately. In this section, selected results are compared to emphasize patterns and trends in the data.

4.7.1 Exposure Concentrations and Dose Calculations

A fundamental principle of toxicology is the dose-response relationship, which correlates biological or physiological effects to known quantities of a test substance. Essential to this association is the ability to measure the amount of substance consumed by (or administered to) a test subject. There are two basic approaches to quantifying exposure: clinical studies generally measure treatments by dose, which is expressed as milligrams of the test substance per kilograms body weight per day (mg/BW kg/d), whereas field investigations or other ecologically based studies tend to estimate exposure as a function of consumption rate and media concentration. In this section, the measured consumption rates and exposure concentrations from the laboratory studies are used to calculate an estimated daily dose for each treatment group.

4.7.1.1 Feed Consumption

Feed consumption rates for the control groups from the 14 day studies ranged from 0.18 - 0.33 g/g BW/d with a mean and standard deviation of 0.22 ± 0.01. Feed consumption rates for the control groups from the 34 day studies ranged from 0.07 – 0.32

g/g BW/d with a mean and standard deviation of 0.17 ± 0.6. Feed consumption rates between the 14 day and 34 day studies were not significantly different ($p > 0.05$). The feed consumption rate calculated from 52 observations of control groups in both the 14 day and 34 day studies was 0.18 ± 0.06 g/g BW/d. Exposure to a range of copper concentrations did not appear to influence consumption rates, even in treatment groups in which weight gain was reduced by more than 50% (Tables 4.22 and 4.34). Likewise, feed consumption was not reduced in mallards that demonstrated reduced weight gain when provided with drinking water at pH 3.5 (Table 4.34). The mean feed consumption rate for all treatment groups, based on 166 observations was 0.20 ± 0.05 g/g BW/d.

4.7.1.2 Water Consumption

Drinking water consumption rates for the control groups in both the 14 and 34 day studies ranged from 0.7 - 2.8 ml/g BW/d with a mean ± standard deviation of 1.1 ± 0.1 ml/g BW/d (N = 36). Elevated but not significantly different mean water consumption rates were observed in the pH 3.0 and 3.5 control groups; mean and standard deviation of 2.0 ± 0.5 (N = 3) and 2.3 ± 1.0 (N = 3) respectively. The mean water consumption rate for all treatment groups was 1.3 ± 0.1 ml/g BW/d (N = 103).

4.7.1.3 Estimated Copper Dose

Copper doses were calculated for each treatment group by multiplying the measured concentrations of copper in the treatment media by the mean consumption rate (Table 4.52). Because of the uncertainty associated with the dose calculation all of the values were rounded to two significant digits. The estimated dose from untreated feed was 5 mg Cu/kg BW/d (i.e., all control groups), based on the measured concentration of copper.

Table 4.52 Estimated Copper Dose

Study	Treatment/ Nominal	pH	Feed Copper [a]	Feed Dose [bc]	Water Copper [d]	Water Dose [be]	Total Dose [b]
1	Low		219	40	< 1	< 1	40
	Medium		420	80	< 1	< 1	90
	High		1027	210	< 1	< 1	210
2	Acetate		1025	210	< 1	< 1	210
	Sulfate		503	100	< 1	< 1	100
3	600[d]	3.0	26	5	605	790	800
	600	4.7	26	5	607	790	800
	1200	4.7		5	1195	1550	1600
4	2	4.5	26	5	2	5	10
	10	4.5	26	5	10	20	20
	50	4.5	26	5	52	70	70
	250	4.5	26	5	252	330	340
	2	5.5	26	5	2	5	10
	10	5.5	26	5	10	10	20
	50	5.5	26	5	49	60	70
	250	5.5	26	5	250	325	330
	50	6.5	26	5	52	73	80
5	117	3.5	26	5	115	150	160
	117	4.0	26	5	116	151	160
	117	4.5	26	5	116	154	160
	184	4.5	26	5	189	246	250
6	2	4.5	26	5	2	3	10
	River	4.5	26	5	2	3	10
	50	4.5	26	5	47	61	70

[a] Units are mg Cu/kg
[b] Units are mg Cu/kg BW/d
[c] Feed copper concentration multiplied by 0.2 g/kg BW
[d] Units are mg Cu/L
[e] Water copper concentration multiplied by 1.3 ml/kg BW

4.7.2 Weight Gain

Weight gain is one of the most common methods for evaluating treatment toxicity. Reduced weight gain was calculated as the percent reduction in weight gain between the treatment and control groups using the following equation:

Percent reduction in weight gain =

[1 - (weight gain treatment group/weight gain control group)] x 100

The weight gain results from Studies One and Two are combined in Figure 4.1. Weight gain was significantly correlated with copper dose ($p < 0.001$, $R^2 = 0.99$). The association is described by the following equation:

$$y = -0.0578x - 3.1907$$

Weight gain was significantly decreased ($p < 0.001$) in mallards with dietary exposure to 90 mg Cu/kg BW/d or greater when compared to the respective control groups (Figure 4.1). Weight gain was decreased by 8 to 15% in mallards that received between 90 and 210 mg Cu/kg BW/d. One notable exception to this trend of decreasing weight gain with increasing dietary copper exposure was the copper acetate treatment group, which received a dose of 210 µg Cu/g BW/d without a reduction in weight gain (Figure 4.1). The same dose of dietary copper as copper sulfate, 210 µg Cu/g BW/d, reduced mean weight gain by 15% suggesting that copper sulfate and copper acetate may have distinct toxic potencies.

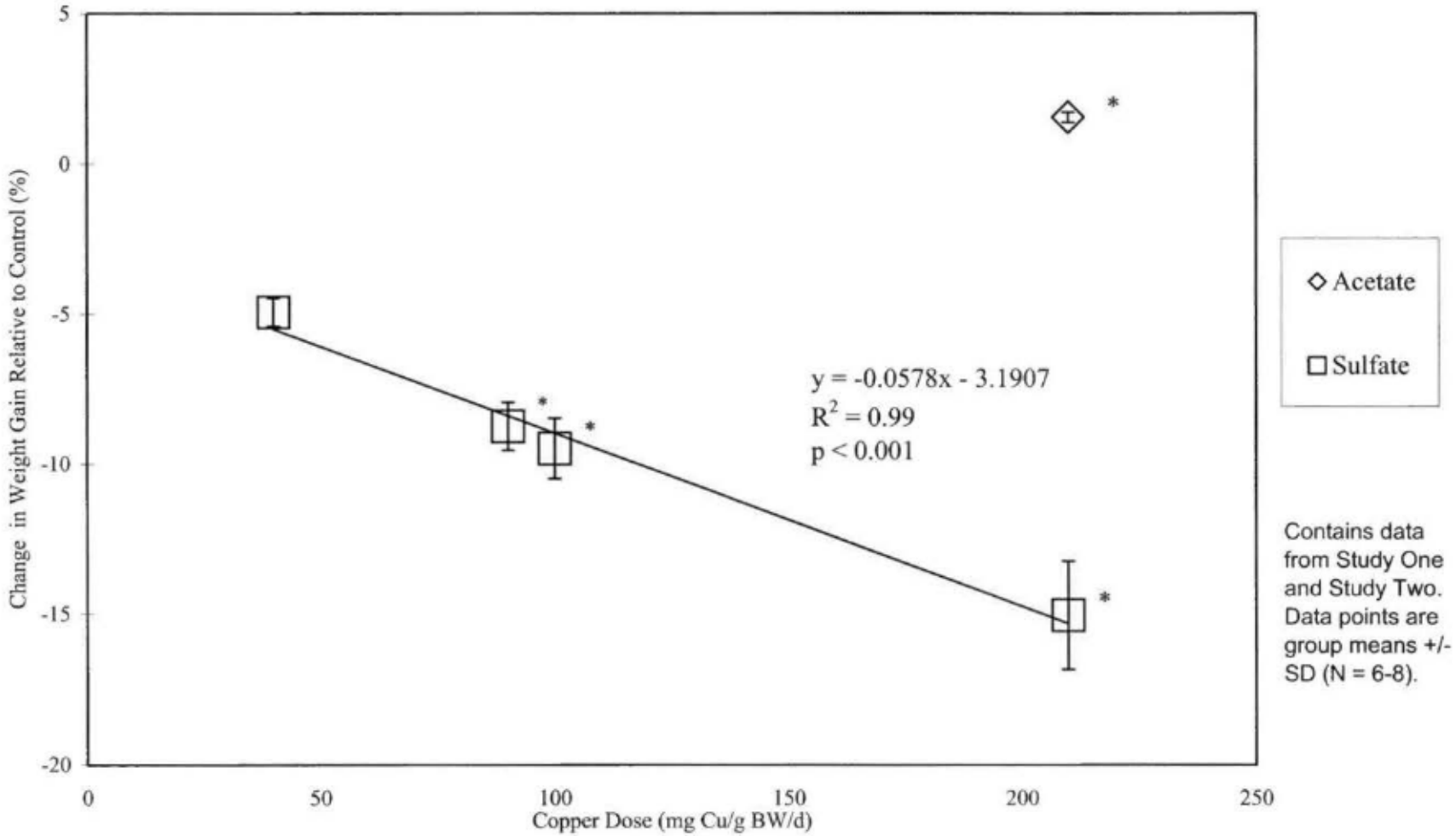


Figure 4.1 Reduced Weight Gain with Dietary Exposure to Copper for 34 Days

* - Significantly different from respective controls (ANOVA, Tukey's test, $p < 0.001$)

The weight gain results from Studies Four and Five are combined in Figure 4.2. Significantly decreased weight gain was observed in mallards with drinking water exposures between 60 and 160 mg Cu/kg BW/d when compared to the respective control groups. Weight gain in mallards that received between 60 and 160 mg Cu/kg BW/d was moderately decreased (10 to 20%). In contrast, weight gain was reduced by more than 50% in mallards that were exposed to doses of copper greater than or equal to 250 mg Cu/kg BW/d. The considerable difference in weight gain reduction between mallards that received 160 mg Cu/kg BW/d and those that received 250 mg/kg BW/d suggests that there may be an effect threshold at an exposure level above 160 mg Cu/kg BW/d.

The weight gain for mallards in both the drinking water and dietary exposures at 10 days post-hatch (Studies One, Two, Four and Five and Six) are combined in Figure 4.3. Study Three was excluded from this comparison because the short duration of the study. There was no apparent difference in weight gain between the drinking water and dietary treatment groups that received comparable doses of copper.

4.7.3 Tissue Copper

One of the main objectives for the laboratory investigation was to determine a suitable method for evaluating copper exposure in juvenile mallards. Copper concentrations in several tissues were evaluated for their potential as biological indicators, or biomarkers, of copper exposure.

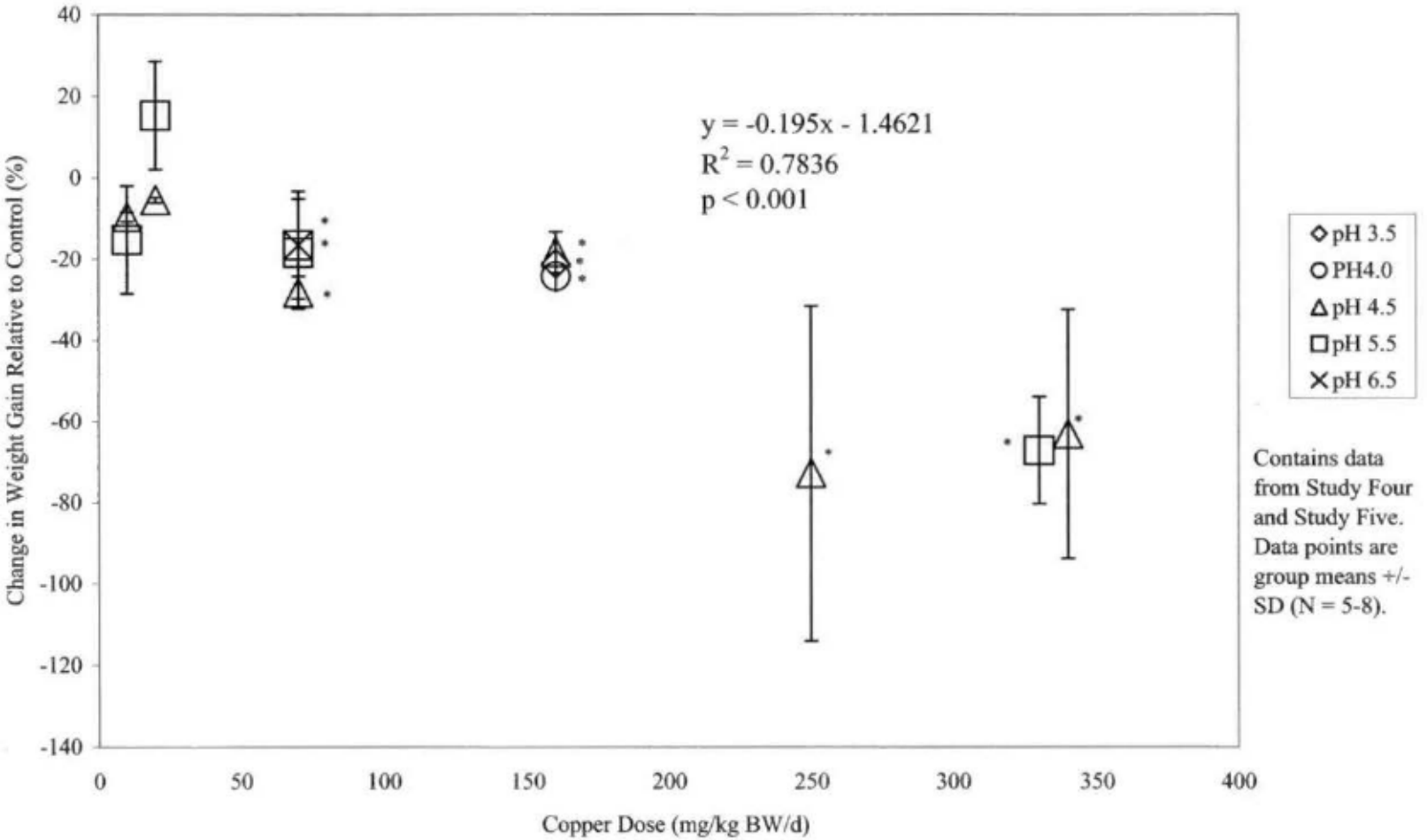


Figure 4.2 Reduced Weight Gain with Drinking Water Exposure to Copper for 14 Days

* - Significantly different for the respective control (ANOVA, Tukey's test, $p < 0.05$)

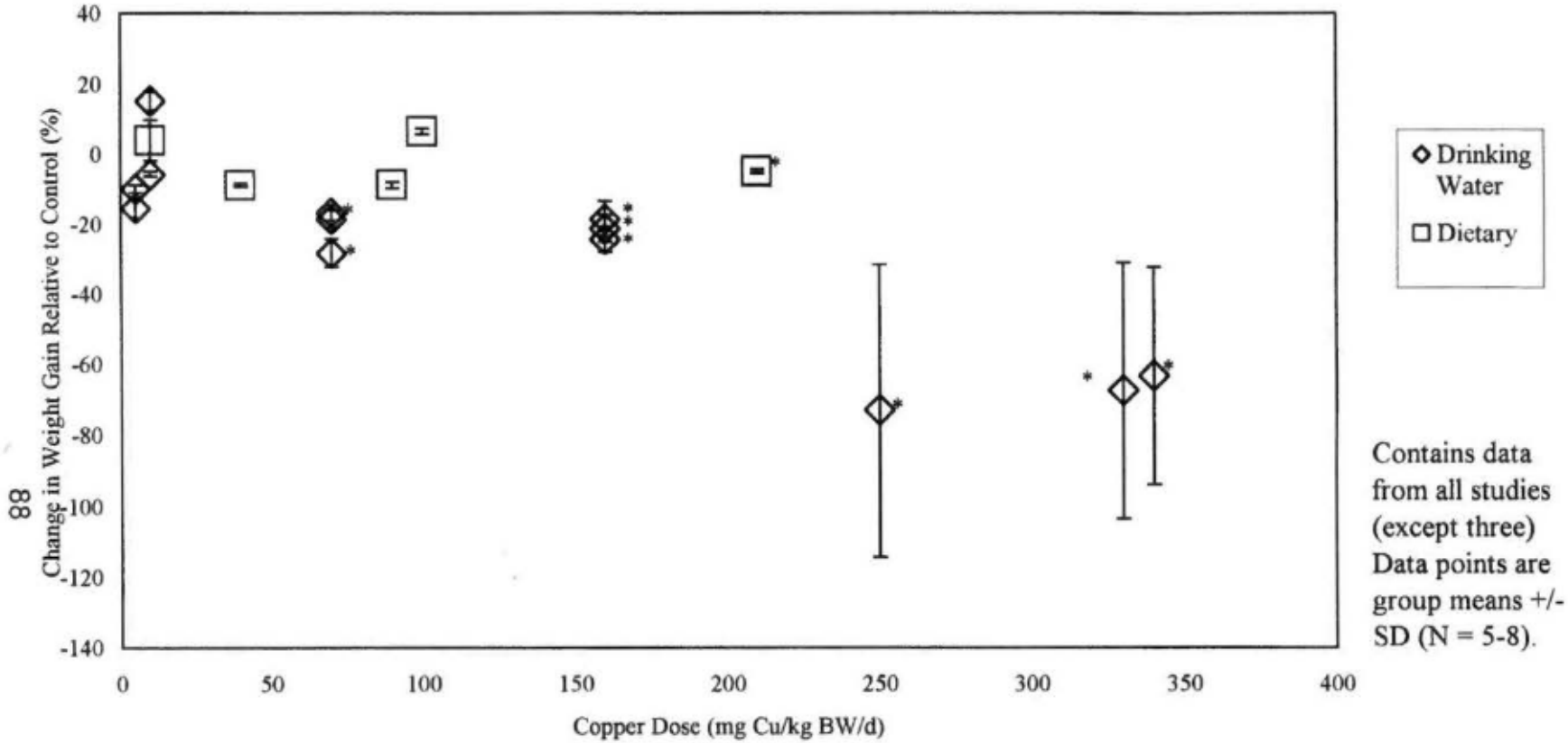


Figure 4.3 Comparison of Body Weight Between 34 Day Dietary and 14 Day Drinking Water Studies

* -Significantly different from respective control group (ANOVA, Tukey's test, $p < 0.001$)

4.7.3.1 Liver Copper

Liver copper concentrations in untreated birds from the 34 day dietary studies ranged from 138 to 713 μg/g DW. Liver copper results from Studies One and Two are combined in Figure 4.4. The copper acetate treatment group was the only dietary treatment group with significantly ($p < 0.05$) elevated hepatic copper concentrations (Figure 4.4). Liver copper concentrations in the dietary copper acetate treatment group were significantly greater ($p < 0.05$) when compared to the dietary copper sulfate group which received the same dose of copper (Table 4.53) suggesting that copper acetate may have an increased absorption rate.

Table 4.53 Liver Copper Concentrations Following Exposure to Dietary Copper Sulfate and Copper Acetate

Treatment	**Estimated Copper Dose (mg Cu/kg BW/d)**	**Liver Copper (μg Cu/g DW)**
Acetate (Study 2)	210	1282 ± 475 [ab]
Sulfate (Study 1)	210	668 ± 197 [c]

[a] Mean ± standard deviation
[b] Estimated dry weight based on 72.5% moisture
[c] Significantly different from the acetate treatment groups, ANOVA and Tukey's test, $p < 0.05$

Liver copper concentrations in untreated birds from the 14 day drinking water studies ranged from 85 - 555 μg/g DW. Liver copper results from Studies Four and Five are combined in Figure 4.5. Hepatic copper concentrations were significantly increased in mallards that received a dose greater than or equal to 160 mg Cu/kg BW/d (Figure 4.5). Hepatic copper concentrations appeared to reach a peak at dose of 250 mg Cu/kg BW/d;

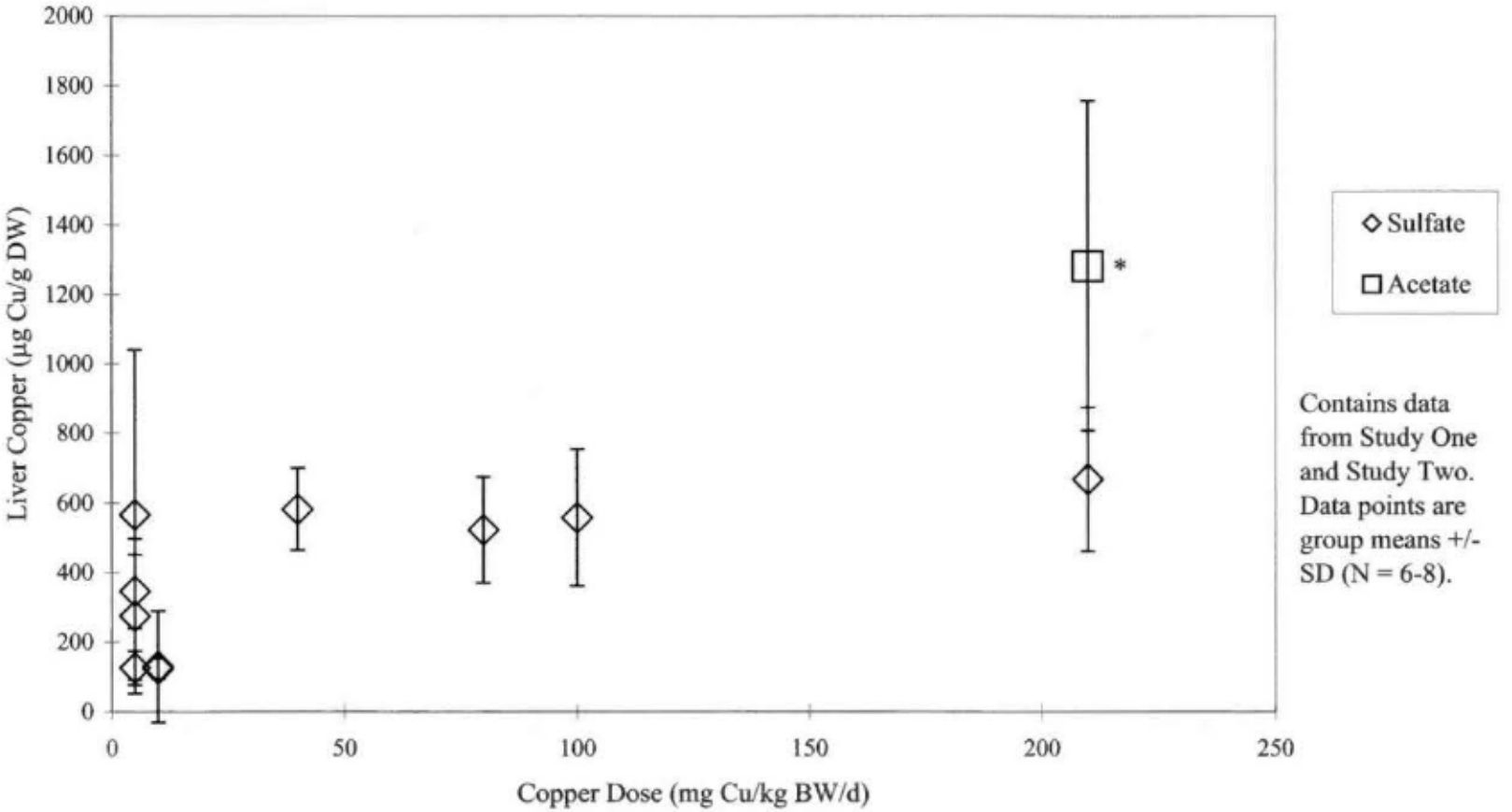


Figure 4.4 Liver Copper Accumulation with Dietary Exposure for 34 Days

* - Significantly different from control (ANOVA, Tukey's test, p >0.001)

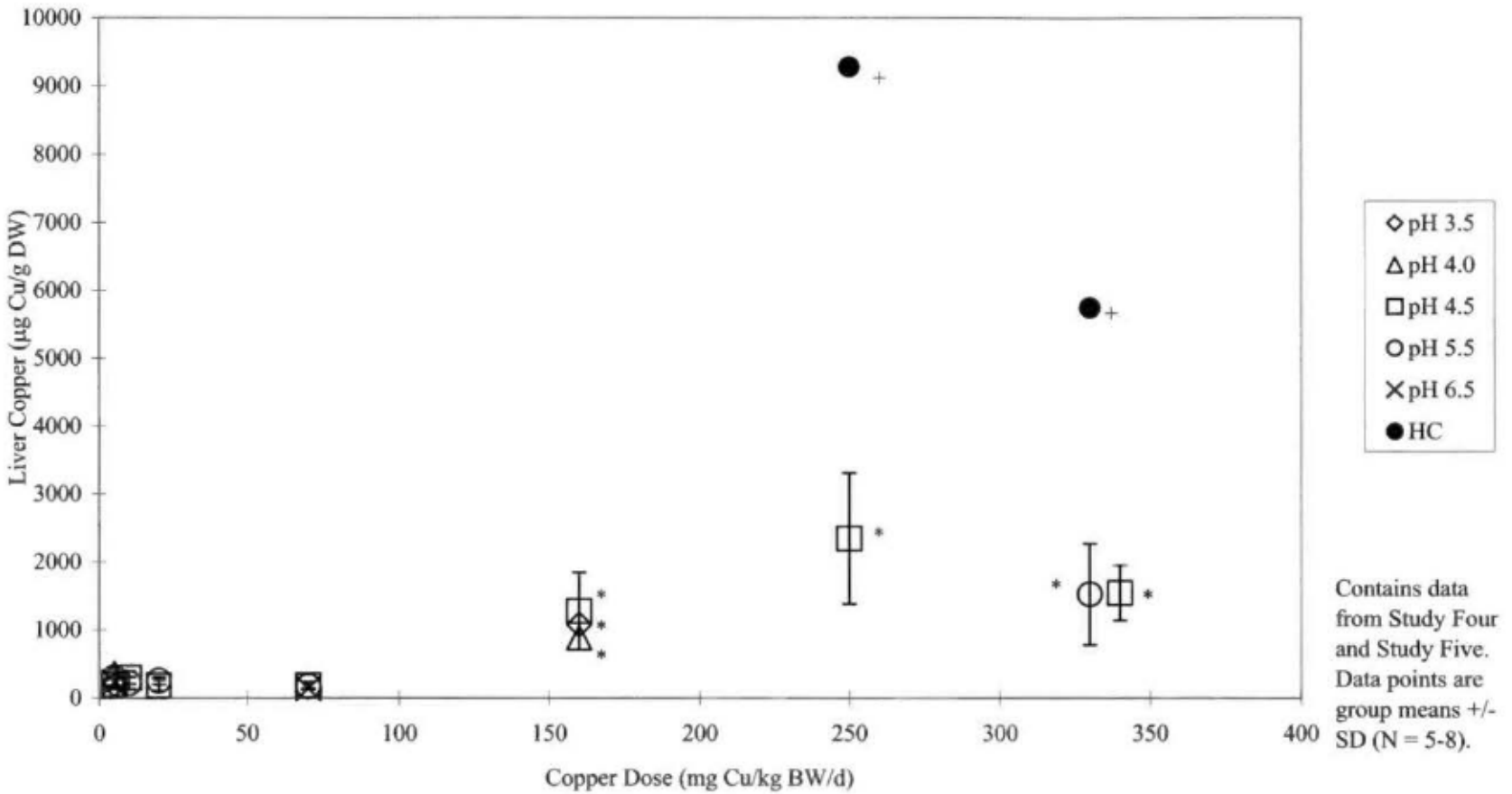


Figure 4.5 Liver Copper Accumulation with Drinking Water Exposure for 14 Days

* - Significantly different from respective control (ANOVA, Tukey's test, $p < 0.05$)
+ -Individual birds with high copper accumulation (HC)

mallards that were exposed to larger doses of copper had lower liver copper concentrations.

Two of the birds that received large doses of copper, 250 and 330 mg Cu/kg BW, accumulated substantial concentrations of copper in their livers (identified as High Copper or HC in Figure 4.5). The liver copper concentrations in these two individuals were three to four times greater than other birds that received the same dose of copper. Because the hepatic copper concentrations in these birds were more than four standard deviations above the mean they were treated as outliers, removed from the data set and the mean was recalculated (Tables 4.23 and 4.35).

4.7.3.2 Kidney Copper

Kidney copper concentrations in untreated birds ranged from 2.74 - 4.96 μg Cu/g DW in the 14 day studies and 4.82 - 5.71 μg Cu/g DW in a 34 day study. Renal copper concentrations were significantly increased ($p < 0.001$) in mallards that received 250 mg Cu/kg BW/d or greater (Figure 4.6). One of the birds with exceptionally large hepatic copper concentrations (Figure 4.5) also had a substantially increased kidney copper concentration. Because the kidney copper concentration for this bird was more then four standard deviations greater than the mean it was omitted from the mean. Unfortunately, no kidney copper data was obtained for the second mallard with extremely large hepatic copper concentration because the entire kidney sample was accidentally fixed in 10% NBF during necropsy.

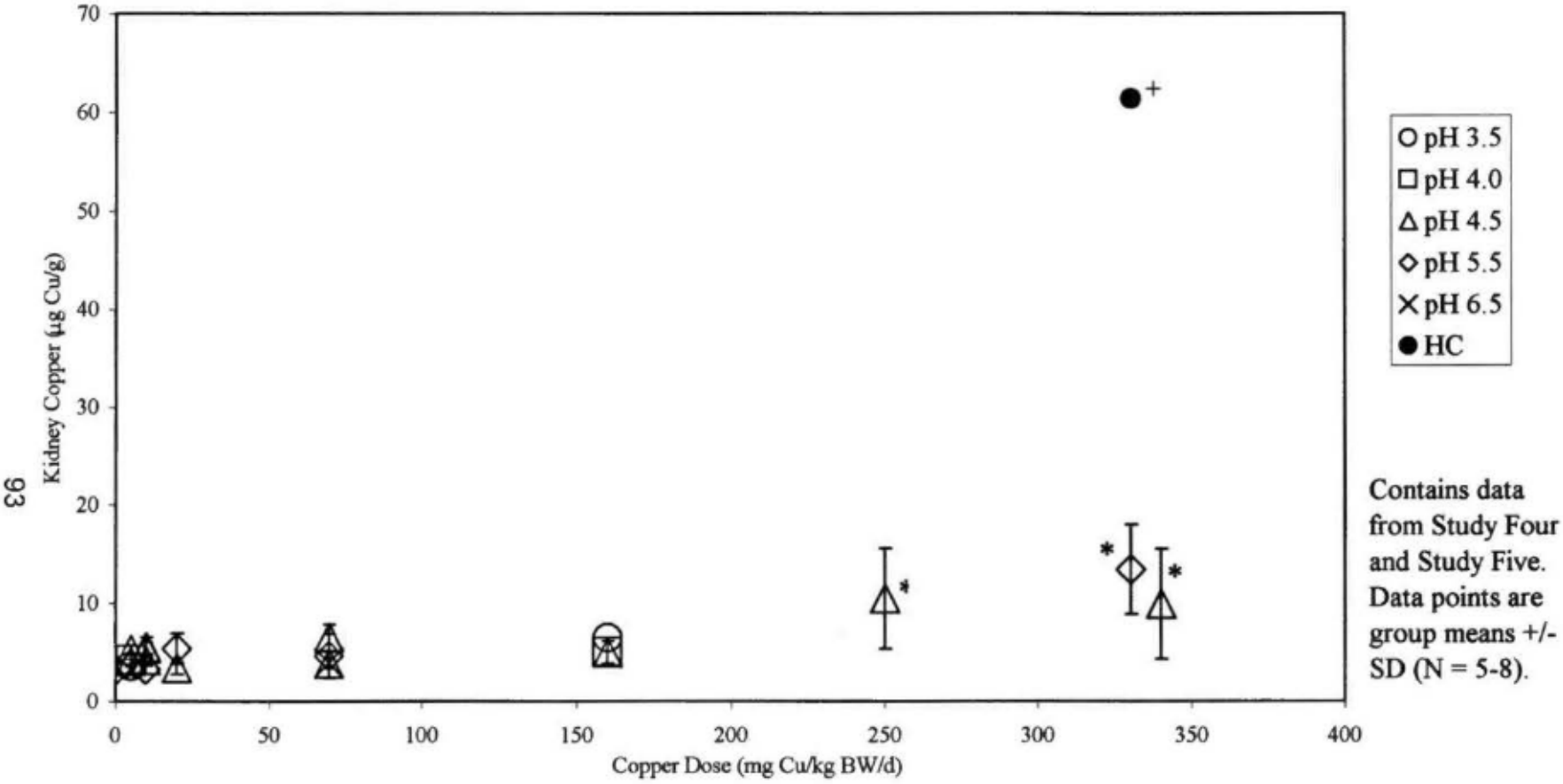


Figure 4.6 Kidney Copper Accumulation with Drinking Water Exposure for 14 Days

* - Significantly different from respective control group (ANOVA, Tukey's test, $p < 0.05$)
+ - Individual bird with High Copper (HC)

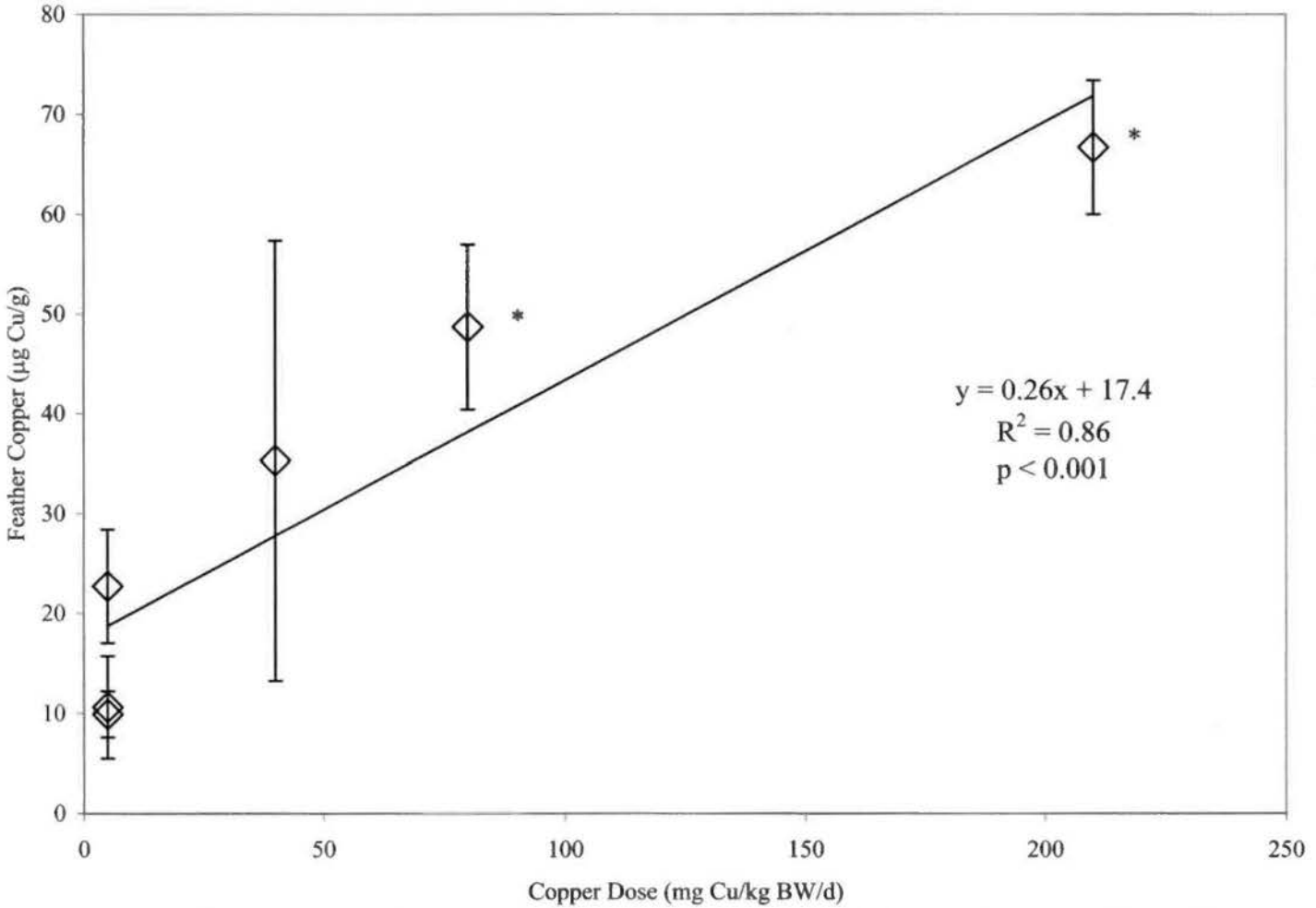
4.7.3.3 Feather Copper

Feather copper concentrations in untreated birds ranged from 2.1 - 32.2 μg Cu/g in the 34 day studies. Feather copper results from Studies One and Two are combined in Figure 4.7. Significantly increased feather copper concentrations were observed in mallards that received a dietary dose of 90 mg Cu/kg BW/d or greater. Feather copper concentrations were significantly correlated ($R^2 = 0.99$, $p < 0.001$) with dietary copper treatment. The association between dietary treatment and feather copper accumulation is described with the equation:

$$\textit{Feather Copper Concentration} = 0.28\ (\textit{Dose of Dietary Copper}) + 14.71$$

Feather copper concentration in untreated birds ranged from 5.4 - 22.1 μg Cu/g in the 14 day drinking water studies. Feather copper results from Studies Four and Five are combined in Figure 4.8. Significantly increased feather copper concentrations were observed in mallards that received a drinking water dose of 160 μg Cu/kg BW/d or greater. Feather copper concentrations in the pH 4.5 treatment groups appeared to reach a peak at dose of 250 mg Cu/kg BW/d; mallards that were exposed to larger doses of copper had lower feather copper concentrations. Feather copper concentrations were significantly correlated ($R^2 = 0.76$, $p < 0.001$) with drinking water exposure. The association between drinking water treatment and feather copper accumulation is described with the equation:

$$\textit{Feather Copper} = 0.41\ (\textit{Dose of Copper in Drinking Water}) + 13.27$$



Contains data from Study One and Study Two. Data points are group means +/- SD (N = 6-8).

Figure 4.7 Feather Copper Accumulation with Dietary Exposure for 34 Days

* - Significantly different from control (ANOVA, Tukey's test, $p < 0.05$)

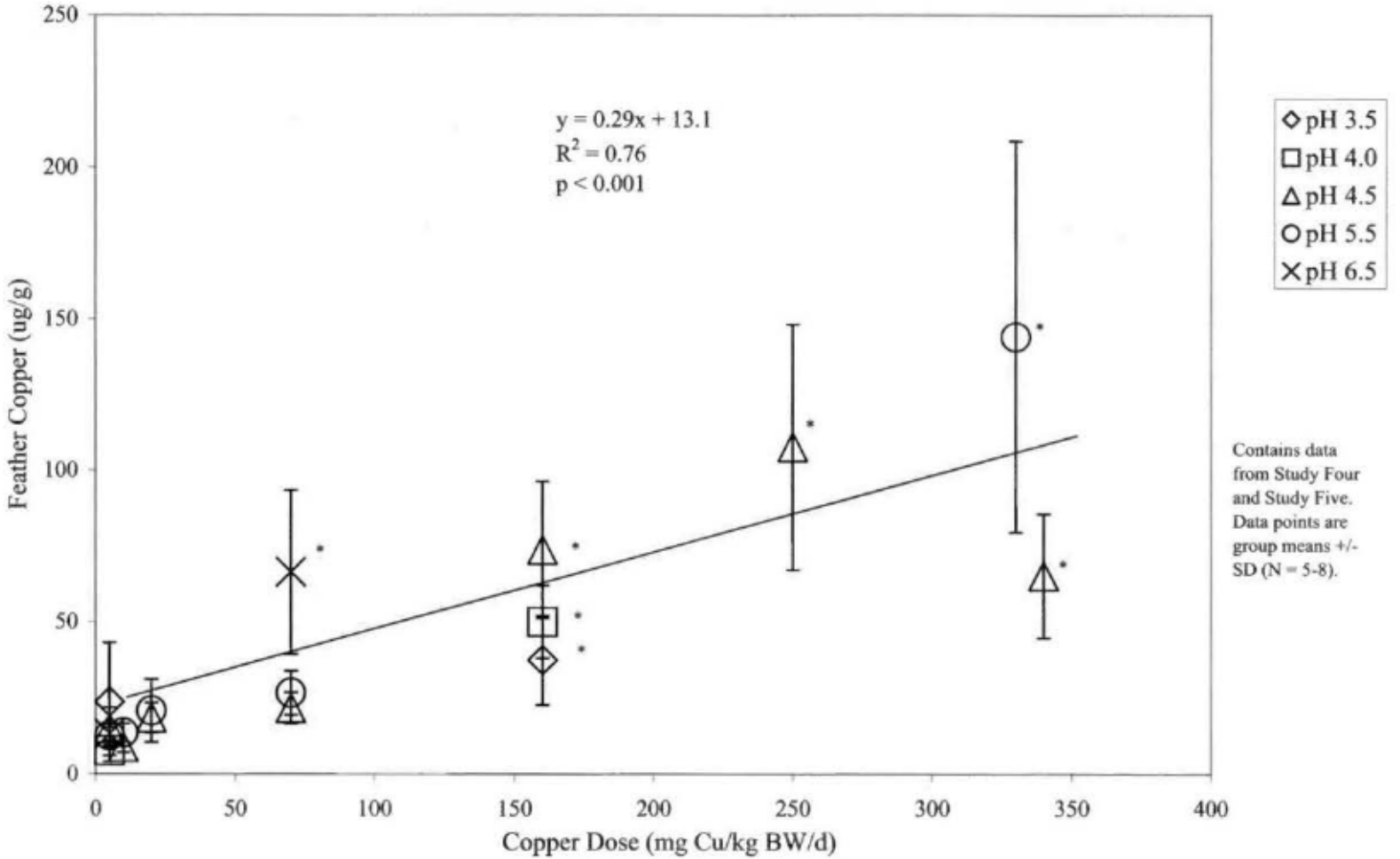


Figure 4.8 Feather Copper Accumulation with Drinking Water Exposure for 14 Days

* - Significantly different from respective control (ANOVA, Tukey's test, p <0.05)

Drinking water pH was negatively associated with feather copper accumulation (Figure 4.9). Feather copper concentration were significantly increased in the 50, 117, and 250 mg Cu/L treatment groups when compared to a respective control group and there were significant differences between the various pH exposure at all three treatment levels. The significant difference between feather copper concentrations for treatment groups that received the same copper concentrations at different drinking water pH suggest that feather copper accumulation is reduced when the metal is present in acidic water.

4.7.3.4 Association Between Feather Copper and Liver Copper Concentrations

Both liver and feather copper concentrations were significantly elevated in mallards that were exposed to large doses of copper in their drinking water for 14 days (Figures 4.5 and 4.8). Figure 4.10 depicts the association between the natural log (ln) liver and ln feather copper concentrations for individual birds. There is a significant correlation ($p < 0.001$, $R^2 = 0.34$) between ln feather and ln liver copper concentrations. The following equation describes the association between liver copper and feather copper:

$$ln\, y = 0.51\, ln\, x + 0.27$$

Copper measurements in feather samples could represent a non-lethal approach for assessment of copper exposure and the associated adverse effects.

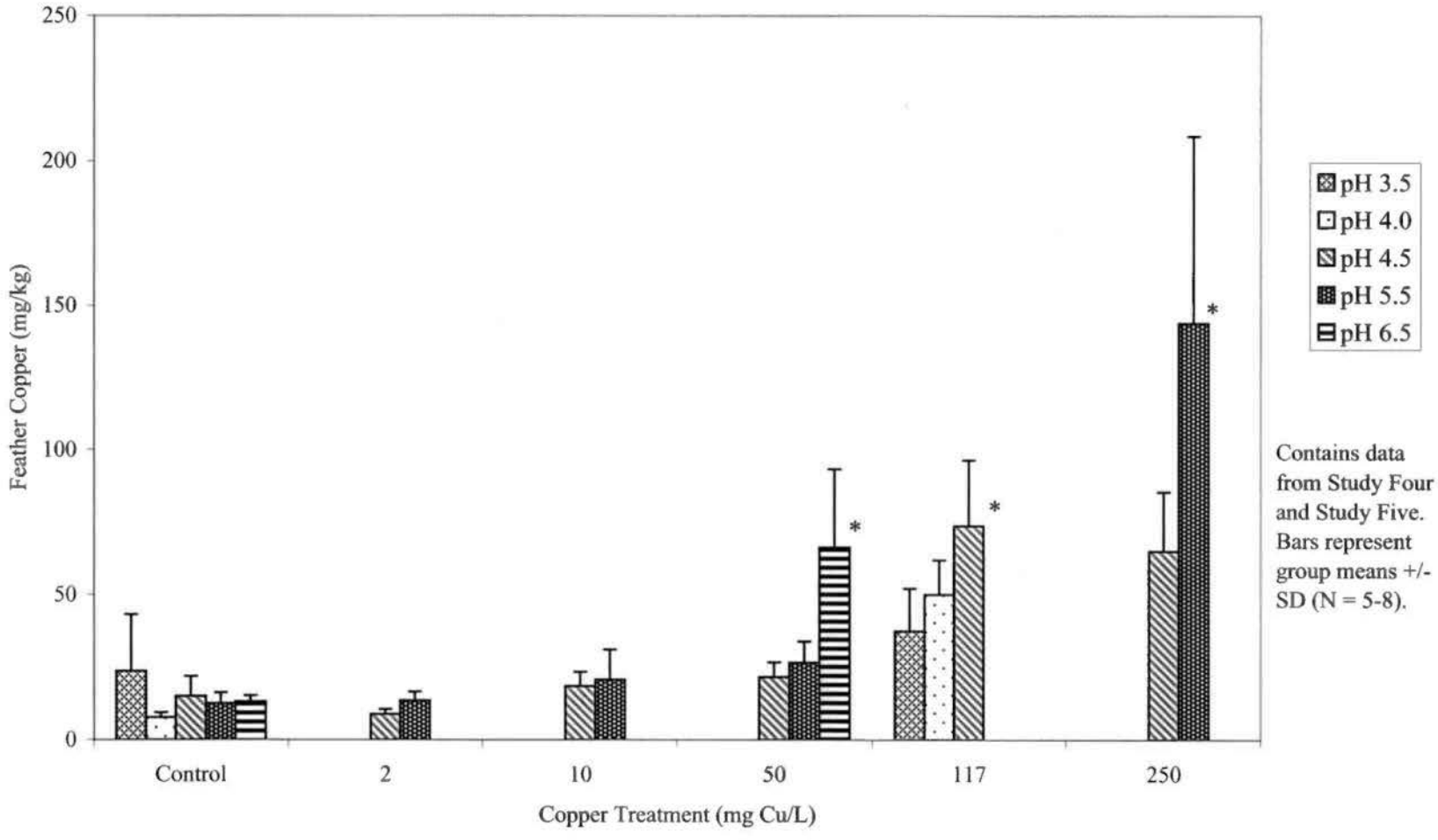


Figure 4.9 The Influence of Drinking Water pH on Feather Copper Accumulation

* - Significantly different from matched treatment group (ANOVA, Tukey's test, $p < 0.05$)

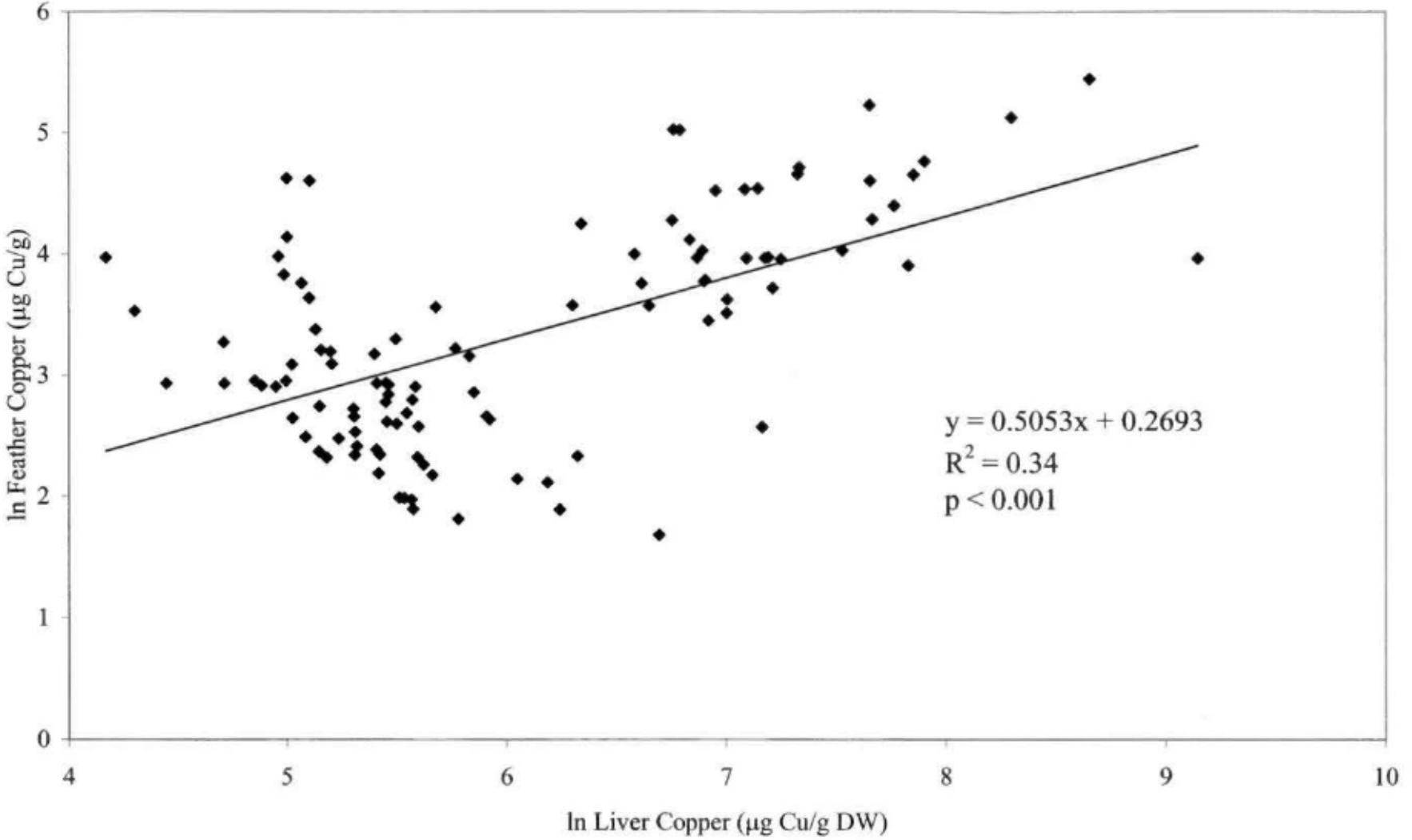


Figure 4.10 Liver Copper and Feather Copper Accumulation in individuals from the 14 Day Drinking Water Studies

4.7.4 Tissue Copper Concentrations and Treatment Effects

Tissue copper concentrations were significantly increased in mallards that were exposed to elevated concentrations of copper (Figures 4.5, 4.6, 4.7, and 4.8). Likewise, weight gain was significantly reduced in mallards that received large dose of copper (Figures 4.1, and 4.2). This section examines the association between the biological measurement of exposure and the biological measurements of treatment toxicity.

Figure 4.11 demonstrates that there is a significant ($p < 0.001$) correlation ($R^2 = 0.53$) between weight gain and liver copper concentrations for individual birds. The relationship between weight gain and liver copper accumulation is described with the following equation:

$$y = -0.0729x+263.1$$

The individuals that were considered as high copper accumulators were excluded from this regression analysis.

Figure 4.12 demonstrated that there is also a significant association between weight gain and feather copper concentration ($p < 0.001$). The following equation describes the relationship between weight gain and feather copper accumulation:

$$y = -0.6791x + 262.86$$

The correlation coefficient ($R^2 = 0.21$) for feather copper and weight gain is substantially lower than the correlation coefficient for liver copper and weight gain ($R^2 = 0.53$). Because liver copper concentrations appears to be a good indicator of reduced weight gain, liver copper concentrations were used to investigation potential associations with other markers of treatment toxicity.

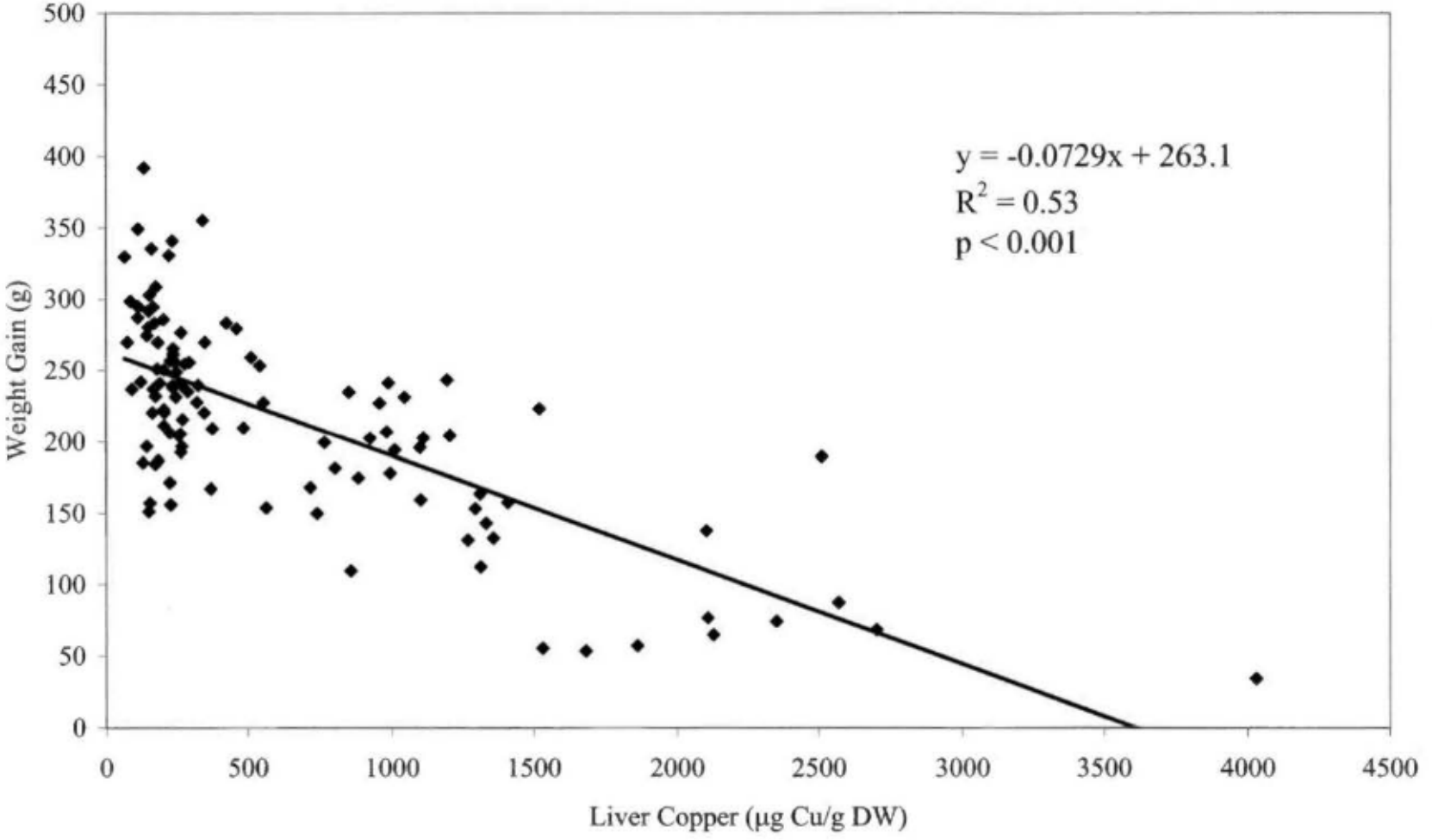


Figure 4.11 Weight Gain and Liver Copper Accumulation in Individual Birds from the 14 Day Drinking Water Studies

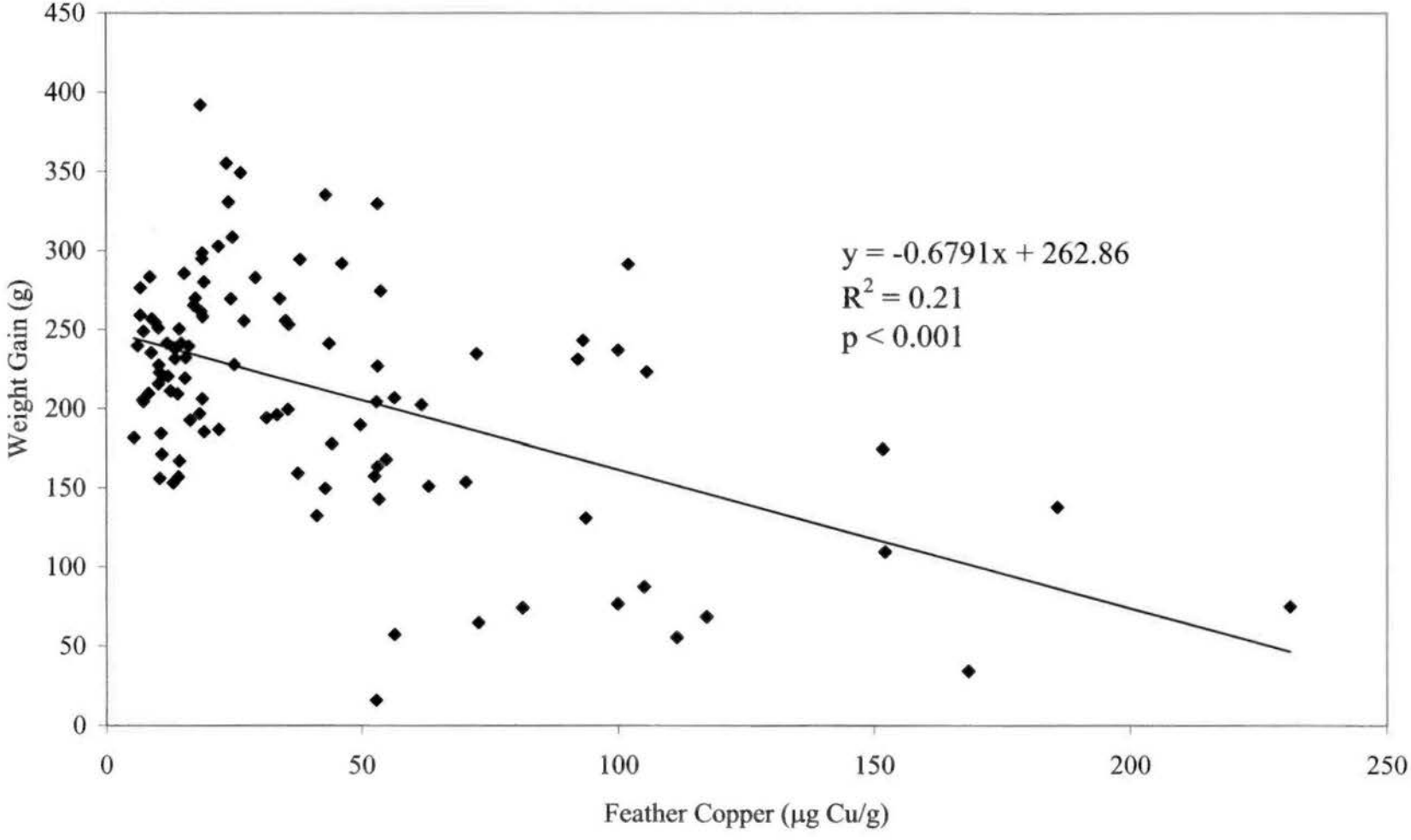


Figure 4.12 Weight Gain and Feather Copper Accumulation in Individual Birds from the 14 Day Drinking Water Studies

4.7.4.1 Clinical Chemistry / Hematology and Liver Copper

Regression analysis was used to evaluate the association between clinical chemistry/ hematology parameters and liver copper concentrations for individual birds. The individuals with extremely elevated liver copper concentrations (Figure 4.5) were included in these analyses in spite of the fact that their ability to accumulate copper appears to be atypical, because they may provide valuable information about the adverse effects associated with biological copper accumulation. Several parameters appear to be significantly associated with elevated liver copper concentrations (Table 4.54). Packed cell volume (PCV) and total protein concentration had the greatest correlation with liver copper concentrations, 0.33 and 0.31 receptively.

Table 4.54 Associations Between Liver Copper Concentration and Clinical Chemistry Parameters

Parameter	Equation	R^2	p - Value
PCV	y = -0.0021x +37.42	0.33	< 0.001
Total Protein	y = -0.0003x + 3.27	0.31	<0.001
Globulin	y = -0.0002x + 1.66	0.22	<0.001
Plasma Protein	y = -0.0002x + 4.16	0.21	<0.001
Uric Acid	y = -0.001x + 4.22	0.17	<0.001
Albumin	y = -0.001x + 1.61	0.16	< 0.05
BUN	y = -0.0003x + 1.46	0.09	< 0.05
Glucose	y = -0.007x + 221	0.06	< 0.05
Anion Gap	y = -0.0007x + 17.6	0.04	< 0.05

CHAPTER 5 DISCUSSION

5.0 Overview

The studies described in this thesis were conducted as part of a site-specific investigation of the Summitville Mine Site. The release of a copper-rich acid mine drainage from the Summitville Mine raised concerns about the potential impact to waterfowl living on the Alamosa River. A review of the published literature revealed that there was a paucity of data on the effects of excess copper in waterfowl. A field survey was conducted to determine exposure pathways for resident waterfowl and investigate the ecological impact elevated copper concentrations. However, the relatively small size of the waterfowl population on the Alamosa River and the lack of information on the effects of copper in waterfowl precluded any conclusions regarding the impact of copper on birds in the field.

A laboratory investigation was developed to collect information which could be used to support an ecological risk assessment for juvenile waterfowl raised on the Alamosa River. There were two goals for the laboratory investigation:

1) Determine the level of copper exposure that is toxic to juvenile mallards.

2) Establish methods for evaluating copper exposure in juvenile mallards.

5.1 Treatment Effects

One of the central objectives for this investigation was to characterize the effects of copper toxicity in juvenile mallards. Treatment toxicity was evaluated with plasma/serum chemistry panels, hematology panels, tissue histopathology, and weight gain.

5.1.1 Clinical Chemistry and Hematology

Clinical chemistry and Hematology panels have been successfully used in a number of copper toxicity studies to document a variety of adverse health effects. Examples of findings from copper toxicity investigations include: reduced PCV as a marker for copper - induced anemia in chickens (Goldberg, 1956), and the measurement of elevated liver enzymes in plasma or serum samples in a number of species (Jain, 1993).

The 250 mg Cu/L pH 5.5 treatment group demonstrated the most significant changes in their clinical chemistry parameters (Table 4.28a). Changes in these birds included: elevated uric acid, increased potassium, decreased total protein, decreased albumin, and decreased globulin. Regression analysis of clinical chemistry/hematology results and liver copper concentrations found significant correlations with nine parameters including PCV, protein concentrations, and uric acid (Table 4.54).

Decreased PCV is an important indicator of anemia (Rich, 1995). An investigation of copper-induced anemia in chickens found that anemia developed after three to four weeks of copper treatment and mortality occurred one to two weeks later (Goldberg, 1956). The finding that several mallards with elevated liver copper concentrations had a PCV below

30% suggests that these birds may have been experiencing the first signs of copper-induced anemia.

Decreased plasma concentrations of albumin can occur when hepatic protein synthesis is impaired, but decreased levels of globulin are an indication of protein-losing enteropathy (Sherding, 1994). Catarrhal gastroenteritis has been reported as a sign of copper toxicity in poultry (Aiello, 1998). Copper is reportedly corrosive to the mucous membrane of the gastrointestinal tract (Nicholson, 1995), and disruption of the mucosal barrier is one of the leading causes of protein-losing enteropathy (Sherding, 1994). Decreased concentrations of albumin, globulin and total proteins were observed in mallards with elevated liver copper concentrations, suggesting that the gastrointestinal tract was damaged in these birds. Unfortunately, histopathological evaluation was not performed on intestinal tissue samples from every treatment groups. However, histopathological evaluation of intestinal tissue from a bird with an elevated liver copper concentration provided evidence of intestinal tissue damage (Section 5.1.2).

Elevated plasma concentrations of uric acid are an indication of renal dysfunction (Aiello, 1998). Increased concentrations of plasma potassium can occur with cell damage, metabolic acidosis, or acute renal failure (Aiello, 1998). The mallards with elevated plasma levels of uric acid and potassium also exhibited elevated kidney copper concentrations (Table 4.25) suggesting a possible association between elevated concentrations of copper in the kidneys and renal dysfunction.

Mallards that were exposed to 117 mg Cu/L at both pH 3.5 and 4.0 had significantly elevated plasma bicarbonate and decreased anion gap values (Table 4.38a). Anion gap is

calculated as the ratio of sodium and potassium to chloride and bicarbonate (Aiello, 1998). Plasma bicarbonate concentrations are increased when carbon dioxide levels are decreased (Aiello, 1998). The decreased anion gap and increased plasma concentrations of bicarbonate are indicative of compensatory alkalosis, a condition that can be triggered as a compensatory mechanism for acidosis (Aiello, 1998).

In mammals, ingestion of excess copper commonly produces hepatotoxicity (Demayo, 1982). No evidence of hepatocyte damage was observed in mallards that demonstrated other signs of copper toxicity (i.e., reduced weight gain). Furthermore, no increases in the frequency of liver lesions or plasma concentrations of AST were seen in mallards that were exposed to the largest non-lethal concentration of copper in drinking water that was tested, 250 mg Cu/L.

The plasma concentrations of AST in both treatment and control groups were consistently higher than the reported normal value for mallards (Table 2.3). Plasma concentrations of AST can be increased by both hepatic or muscle injury. Increased AST due to skeletal muscle injury is associated with increased CK (Johnson, 1994). Plasma concentrations of AST and CK were highly correlated ($R^2 = 0.86$), indicating that the elevated concentrations of AST may have been due to muscle damage, perhaps a consequence of housing conditions, rather than hepatocyte damage.

5.1.2 Histopathology

Histopathology is a standard technique for evaluating the toxic effects of experimental treatments. The prevalence and type of lesions found in histology samples can help determine target tissues and provide insight about a compound's mechanism of toxicity..

An increased frequency of lesions was observed in several treatment groups. Liver samples from the high copper treatment group in Study One exhibited microcavitary degeneration and other mild hepatocellular changes (Table 4.10). However, similar lesions were also observed in the control group. The prevalence of hepatic lesions in both treatment and control birds suggests that these changes may have been associated with the processes of rearing and handling, and are not associated with treatment.

Abnormal macrophage pigmentation was observed in 3 of 12 birds (25%) that received doses of 330 or 340 mg Cu/kg BW/d (Table 4.30). These treatment groups received the highest non-lethal water exposure tested (250 mg Cu/L) (Table 4.52). The pigmented cells were identified as lipofuscin- and hemosiderin-type pigmentation. Lipofuscin or "aging pigments" are typically found in older animals and are produced by the accumulation non-biological material and other cellular debris in the digestive vacuoles of phagocytes (Trump, 1980). Sublethal injury to the liver following elevated metal exposure could result in the formation of autophagic vacuoles, producing lipofuscin-type pigments in juvenile birds. Hemosiderosis is a condition associated with increased levels of insoluble iron or other metals in the bloodstream (Trump, 1980). An increased frequency of "iron-containing lipofuscin" was previously reported in chickens

with copper-induced anemia (Goldberg, 1956), and in a group of swans collect from a copper contaminated river (Kobayashi, 1992).

The bird with the hemosiderin-type granules had extremely elevated tissue copper concentrations (liver, kidney, and feather) and extremely reduced weight gain. This bird also had several abnormal plasma chemistry results including decreased glucose, increased BUN, increased uric acid, and decreased protein levels (total, albumin, and globulin). The other birds with abnormal hepatic pigmentation had significantly elevated tissue copper concentrations, and reduced weight gain, but to a lesser extent than other birds which did not exhibit hepatic pigmentation. If the hepatic pigments were produced by copper treatment, it is not clear why it would be present in some birds that received large doses of copper but not in others that received the same treatment. Even if the pigments are associated with copper treatment, these tissue changes are not considered as evidence of extensive tissue damage (Nordin, 1997).

A sample of intestinal tissue from one of the mallards that accumulated exceptionally large tissue copper concentrations contained deteriorated connective tissue and necrotic fat spherules (Section 4.5.4). It has previously been reported that copper is corrosive to the gastrointestinal tract (Nicholson, 1995) and the finding of deteriorated intestinal connective tissue supports this observation. Unfortunately, samples of intestinal tissue for all of the treatment were not submitted for histopathological analysis.

In Study Six, an increased frequency of hepatocyte vacuolization was observed in birds from both the river matrix (4/8), and the 50 mg Cu/L treatment group (3/8) (Table 4.51).

However, there was no clear association between these observations and copper treatment.

5.1.3 Weight Gain

Exposure to elevated copper concentrations in feed and drinking water significantly reduced weight gain in 12 treatment groups (Figures 4.1 and 4.2). In every case, depressed weight gain occurred without a significant reduction in feed consumption ($p > 0.05$) (Tables 4.5, 4.13, 4.22, 4.34, and 4.45). Weight gain was moderately reduced with exposures between 70 to 210 mg Cu/kg BW/d. Mallards that received a dose of 250 mg Cu/kg BW/d or greater had extremely reduced weight gain. The dramatic decrease in weight gain between mallards that received 210 and 250 mg Cu/kg BW/d suggests that there is an effect threshold between these two doses. Exposures below the threshold appear to produce mild to moderate effects, whereas exposures above the threshold substantially reduced weight gain.

Pullar reported that adult mallards which were exposed to large doses of copper experience a significant loss in body weight (1940). Decreased weight gain as a result of excess copper ingestion suggests that copper interferes with some essential metabolic process. Mallards that were exposed to 340 mg Cu/kg BW/d (250 mg Cu/L at pH 4.5) presented signs of protein losing enteropathy (Table 4.28a), indicating that excess copper can adversely effect gastrointestinal tissue. Tissue copper accumulation was also reduced in mallards that received the largest doses of copper (Figure 4.5) suggesting that copper

absorption is decreased when administered in large doses. Large doses of copper may interfere with the physiological and biochemical processes of gastrointestinal absorption; decreased absorption of feed nutrients would decrease weight gain.

Weight gain was significantly decreased in mallards that received copper sulfate treatment when compared birds that received copper acetate treatment (Table 4.53). It is possible that the observed reduction in weight gain for the dietary studies may be associated with increased sulfate concentrations. However, in the drinking water studies reduced weight gain was observed between copper treatment groups with equivalent sulfate concentrations (~ 80 - 85 mg/L). If decreased weight gain is associated with reduce feed absorption resulting from injury of the gastrointestinal tissue, one possible explanation for the absence of reduced weight gain in mallards that were exposed to copper acetate is that copper acetate is less caustic to gastrointestinal tissue than copper sulfate.

As a parameter for measuring treatment effects, reduced weight gain demonstrated several important qualities including consistency between studies, a clear dose-response relationship, and coherence with existing knowledge. However, reduced weight gain does have some limitations as an indicator of treatment: reduced weight gain is not a biological effect that is specific to copper treatment (i.e., it is affected by other factors), and the exact physiological mechanisms responsible for the effect are unknown.

5.2 Exposure Assessment

One the goals of the investigation was to establish a method for evaluating copper exposure in juvenile mallards. The concentrations of liver copper, kidney copper, blood copper, feather copper, and hepatic metallothionein were evaluated for their ability to assess copper exposure in juvenile mallards.

5.2.1 Liver Copper

Liver residue is commonly viewed as a reliable index of exposure for a wide variety of contaminants, including copper (Underwood, 1971). The pattern of hepatic copper accumulation varies among species; liver copper concentration in sheep increase proportion to their dietary intake, while rats maintain normal liver copper levels until a high dietary level is reached (Underwood, 1971). Mallards with minimal copper intake appear to sequester substantial concentration of copper in their livers. Prior to this investigation it was reported that liver copper concentration in mallards and other ducks are typically greater than most avian species (Beck, 1956, and Beck 1962).

Mallards raised on untreated commercial feed for 34 days accumulated substantial concentrations of copper in their livers, up to 700 μg Cu/g DW (Figure 4.4). Commercial feeds, like the one used in this study, typically contain copper concentrations greater than the recommended dietary level to accelerate weight gain (Cheeke, 1991). The basal diet contained approximately 25 mg Cu/kg DW (Table 4.3). The recommended dietary level for fowl is 9 mg Cu/kg (Cheeke, 1991). A comparison of the previously reported liver copper data suggests that ducks which are raised in the laboratory on commercial feed

have substantially greater hepatic copper concentration when compared to mallards that have been collected in the field (Table 2.2). When compared to the diet of wild mallards, commercially prepared diets are likely to be relatively rich in copper. Copper concentrations in the vegetation samples collected from uncontaminated wetland areas along the Alamosa River (Table 4.1) contained less than half the concentration of copper in untreated feed.

Liver copper concentrations in mallards that received as much as 100 mg Cu/kg BW/d were indistinguishable from the liver copper concentrations of untreated birds. The absence of elevated hepatic copper concentrations in mallards that received a 4-fold increase in exposure suggests that mallards are able to regulate their liver copper levels until a threshold concentration is exceeded. Liver copper concentrations were significantly increased in mallards that were exposed to 160 mg Cu/kg BW/d or greater (Figure 4.5). Hepatic copper concentrations appeared to reach a peak in mallards that received 250 mg Cu/kg BW/d; lower liver copper concentrations were seen in mallards that received larger doses of copper (Figure 4.5). Extremely large doses of copper ($\geq$ 330 mg Cu/kg BW/d) may disrupt or saturate the physiological and/or biochemical processes of copper absorption.

There was a significant difference in hepatic copper accumulation between the acetate and sulfate treatments which received the same dose of dietary copper (Table 4.53). The variation in hepatic copper accumulation between these two forms of copper suggests that the biological uptake or bioavailability of copper is dependent on the counter-ion. It has previously been reported that several other forms of copper (copper carbonate, copper

oxalate) have increased copper absorption in mammals when compared to copper sulfate (Cousins, 1985). The absence of decreased weight gain in the copper acetate treatment group (Table 4.12), but increased liver copper concentrations suggest that copper acetate is more bioavailable but less toxic than copper sulfate.

Several birds that received high doses of copper accumulated exceptionally large concentrations of copper in their livers (≥ 5749 μg/g DW) (Figure 4.5). These birds may have consumed larger quantities of water, or they may have had a predisposition to copper accumulation. Ceruloplasmin is an α-globulin that is believed to play an important role in regulating tissue copper levels. Excess tissue copper accumulation in Wilson's disease has been associated with abnormal expression of ceruloplasmin (Chowrimootoo, 1996). The mallards that accumulated extremely large concentrations of copper in their livers may have had an impaired capacity to synthesize ceruloplasmin or some other copper regulating proteins.

5.2.2 Kidney Copper

Compared to liver tissue relatively low levels of copper were found in the kidneys. Renal copper concentrations were less 8 μg Cu/g WW for a wide range of copper exposures. Significantly increased kidney copper concentrations were only observed in mallards that received large doses of copper (≥ 250 mg Cu/kg BW) (Figure 4.6). One of the mallards with the exceptionally large hepatic copper concentrations also exhibited the highest kidney copper concentrations. No kidney copper results were obtained for the

other mallard that was considered a high copper accumulator based on liver copper data. The relatively low levels of copper that were present in kidneys that suggests that is not a primary site of copper deposition.

5.2.3 Blood Copper

No increases in blood copper concentrations were observed in mallards that received the largest doses copper (340 μg Cu/kg BW/d) (Table 4.24). Even one of the mallards that was considered to be high copper accumulators because of its elevated liver and kidney copper concentrations, did not have increased levels of copper in its blood (Table 4.24. In order to minimize analytical costs, and because there were no significant differences in the blood copper concentrations between the control groups and the 250 mg Cu/L treatment groups at pH 4.5 and 5.5, blood copper concentrations were only measured in Study Four.

Increased concentrations of copper in the blood occurs in mammals when the capacity for hepatic storage is exceeded (Demayo, 1984). The absence of increased copper concentrations in the blood of mallards that received as much as 340 mg Cu/kg BW/d suggests that this level of exposure is below the dose required to exceed the hepatic storage capacity of juvenile mallards.

5.2.4 Feather Copper

Feather copper concentrations were significantly increased at a relatively low dose (≥ 70 mg Cu/kg BW), when compared to other tissues (Figures 4.7 and 4.8). Because feather copper was significantly increased at lower doses than were required to increase liver copper concentrations it appears that feather copper accumulation is not subject to the same level of physiological regulation as internal tissues and organs. It has previously been suggested that metal levels in avian plumage reflect dietary assimilation (Rose, 1981).

Feather copper concentrations exposures were significantly correlated with both dietary exposure ($R^2 = 0.86$) and drinking water exposures ($R^2 = 0.76$) (Figures 4.7 and 4.8). The slopes of the dose response curves for the dietary and drinking water exposures were similar for both dietary and drinking water exposures, 0.28 and 0.29 respectively (Section 4.7.3.3). Feather copper accumulation appears to be a suitable method for assessing copper exposure from multiple environmental sources.

Feather copper concentrations in the highest dose group, 340 mg Cu/g BW, were lower than other treatment groups that received smaller doses of copper (Figure 4.8). As discussed earlier, a similar pattern was observed in liver copper concentrations. If copper accumulation was reduced in liver tissue, but not feather tissue it would suggest that large doses of copper interfere with hepatocyte uptake. The hypothesis that ingestion of large concentrations copper interfere with intestinal absorption is supported by the finding that both feather and liver copper concentration are reduced when exposures exceeded a plateau concentration (~ 340 mg Cu/kg BW/d).

One of the mallards with an unusually large liver copper concentration, 5749 μg Cu/g DW, also had the largest feather copper concentration 231 μg Cu/g. However, the mallard with the largest liver copper concentration, 9280 μg Cu/g DW, had a relatively low concentration of copper in its feather tissue, 53 μg Cu/g. The pre-disposition for hyper-copper accumulation may be associated with the absence of a biological copper ligand that regulates copper distribution. If the protein that normally removes copper from the liver is missing, or in limited quantity, accumulation in other depots, like feather, may be increased.

5.2.5 Hepatic Metallothionein

Concentrations of hepatic metallothionein were analyzed in the pH 4.5 treatment groups from Study Four and all of the treatment groups in Study Five. The copper bound fraction, and total metallothionein levels were significantly increased ($p < 0.001$) in mallards that received 340 mg Cu/kg BW/d (Table 4.42). The concentrations of copper binding protein were not significantly decreased or increased in a mallard that was considered to be a high copper accumulator (MT data were only available for one of two the high copper accumulators because only the pH 4.5 treatment groups from Study Four were analyzed). Regression analysis of individual liver copper concentrations and hepatic metallothionein concentrations support the conclusion that the liver copper accumulation is not associated with hepatic metallothionein expression. The absence of a clear dose-response relationship between copper exposure and metallothionein

concentrations in mallards that received large concentrations of copper, as much as 250 mg Cu/kg BW/d (Table 4.42), suggests that metallothionein synthesis in mallards is not significantly induced by copper treatment at or below this level of exposure.

5.3 Biological Effects of Low pH Drinking Water

Initially, drinking water pH was investigated for its potential to influence copper bioavailability. However, the decreased survival of birds that consumed drinking water at pH 3.0 without added copper demonstrated that acidic water can be directly toxic to recently hatched mallard ducklings (Table 4.18). In a subsequent study, the toxicity of acidic water was confirmed by the reduced weight gain in mallards that consumed drinking water at pH 3.5 without added copper (Table 4.33).

Drinking water with a pH of 3.0 may have produced mortality in young birds because their developing systems were not capable of maintaining an acid-base homeostasis. It is not clear why mallards would be sensitive to pHs that are easily tolerated by other organisms. Mammals can consume solutions with a pH of 2.0 without any adverse effects (Lewis, 1987). The observation that acidic water is toxic to juvenile mallards was one the most interesting aspects of this investigation. However, because evaluating the toxicity of acidic water was not one of the main objectives for the investigation this relationship may not have been adequately studied. Further investigations should monitor blood gas and blood pH in waterfowl following exposure to acidic water.

The influence of pH on copper bioavailability was indirectly assessed by measuring tissue copper accumulation in treatment groups that were provided the same concentrations of copper at different pH levels. It was anticipated that acidic pH water would increase tissue copper accumulation in juvenile mallards, but feather copper concentrations were reduced rather then increased in treatment groups that received highly acidic drinking water (Figure 4.9). It is not clear why tissue copper accumulation was reduced in mallards that received low pH copper exposures, but it appears that copper absorption is reduced by decreased pH. Decreased copper absorption at low pH suggests that ligand-bound copper, rather than free copper, is the predominate mechanism for copper uptake (Cousins, 1985).

5.4 Estimated Copper Doses

Copper doses were calculated from measured exposure concentrations and mean consumption rates (Table 4.52). Mean consumption rates were used to calculate dose because feed and water consumption was not measured for individual birds. The mean consumption rates were calculated from all treatment to reduce the substantial but not statistically significant variation between treatment groups and between studies.

The mean feed consumption for this investigation, 0.2 g/g BW/d, was approximately double the previously reported values of feed consumption (Table 2.2). The elevated consumption rates in this investigation may be the result of dietary or environmental factors associated with the laboratory setting such as temperature or population density.

The drinking water consumption rate for this investigation, 1.3 ml/g BW/d, was considerably larger than the published estimate of water consumption for mallards (0.06 g/g BW/d); water consumption in the mallard was predicted with an allometric model that estimates consumption as a factor of body size and may not properly reflect the consumption of young birds (EPA, 1983). The model for predicting water consumption is a generic model for all avian species and may not accurately reflect intake for juvenile mallards. Rowe reported a mean water consumption rate of 157 ± 28 g/d in untreated adult mallards (1983), which for a 750 g bird would be approximately 0.21 ml/g BW/d. The drinking water consumption rate for this investigation maybe greater than the previously reported value because factors associated with the animal care such as ambient temperature, population density, and diet, or it may reflect differences between juvenile and adult birds.

5.5 Evaluation of Toxicity

The toxicity of a compound is defined by its dose-response curve, which is described with toxicity reference values. There are several common toxicity reference values that are derived from the dose-response curve: the No observed Adverse Effect Level (NOAEL), the Lowest Observed Adverse Effect Level (LOAEL), and Lethal Dose for some percentage of the population (LD_x).

Weight gain was the endpoint that was used to define treatment toxicity. The largest dose of dietary copper that did not produce a significant reduction in weight gain, 40

mg/kg BW/d is designated as a NOAEL (Table 5.1). The smallest dose to produce a significant reduction in weight gain, 90 mg/kg BW/d, is considered a LOAEL. A lethal dose of dietary copper sulfate was not established but is in excess of 200 mg Cu/kg BW/d.

The largest dose of copper in drinking water that did not produce a significant reduction in weight gain, 20 mg/kg BW/d, is designated as a NOAEL (Table 5.1). The smallest dose to produce a significant reduction in weight gain, 60 mg/kg BW/d, is considered a LOAEL. A dose of 800 mg/kg BW/d was lethal to more than 50% of the population after 96 hours of exposure. An actual LD_x value was not calculated because of the limited number of observations, but the LD_{50} would fall within the range of 340 – 800 mg Cu/kg BW/d.

The lowest pH that did not produce a significant reduction in weight gain, 4.0, is designated as a NOAEL (Table 5.1). The highest pH to produce a significant reduction in weight gain, 3.5, is considered a LOAEL. Exposure to pH 3.0 was lethal to more than 50% of the population after 96 hours of exposure.

Table 5.1 Toxicity Benchmarks

Benchmark	**Dietary Dose (mg/kg BW/d)**	**Drinking Water Dose (mg/kg BW/d)**	**pH**
NOAEL	40	20	4.0
LOAEL	80	60	3.5
$LD_{>50}$		800	3.0

5.6 Evaluation of Risk to Waterfowl on the Alamosa River

The purpose of this investigation was to generate data that could be used to assess the potential impact of environmental copper exposure in waterfowl and collect data that could be used to support an ecological risk assessment for the Summitville Mine Site. A complete risk assessment for mallards on the Alamosa River is beyond the scope of this thesis; however, an interpretation of the laboratory study results with respect to field data is certainly warranted.

Environmental samples collected during the field survey provide an indication of potential exposure concentrations along the Alamosa River. Elevated copper concentrations were found in water, vegetation, and sediment samples from the Terrace Reservoir (Table 4.1). The data from the field survey suggest that the wetland areas, which are believed to be the breeding ground for resident waterfowl, may not be hydrologically connected to the river. Prior to this study it was assumed that the wetlands were fed by Alamosa River water, and would contain elevated copper concentrations. The substantially lower copper concentration in the wetland samples suggests that these areas may be fed by uncontaminated spring water.

The largest concentrations of copper in environmental media collected from the Alamosa River were found in unwashed vegetation samples. Vegetation typically compromises only about 30% of the diet of adults mallards (Eldridge, 1990) and juvenile mallards feed almost entirely on invertebrates for the first month of life (Ringelman,

1992). Following standard EPA guidance for a baseline risk calculation (EPA, 1989), it was assumed 100% of the target receptor's diet is composed of the contaminated media. The expected daily intake of copper from unwashed vegetation is 88 mg/kg BW, which would produce a hazard quotient (HQ) greater than two (Table 5.2). Copper concentrations in the surface water from the Alamosa River were much lower than the copper concentrations in the vegetation samples. The estimated daily dose from drinking Alamosa River water contaminated at the level observed in the field study (Table 4.41) is 0.45 mg/kg BW, which predicts a HQ of 0.02 (Table 5.2). A HQ that exceeds one is generally considered to be an indication of an unacceptable risk to a population (EPA, 1989).

The increased risk to juvenile mallards from consumption of copper-rich vegetation is based on the assumption that the copper in the vegetation has the same bioavailability as copper sulfate added to a commercially prepared feed. However, the results of this investigation indicate that copper bioavailability varies with the form of copper (Table 4.53). It is not know if the bioavailability of copper in vegetation would be increased or decreased when compared to copper sulfate added to commercial feed.

Table 5.2 Estimated Risk to Juvenile Mallards on the Alamosa River

Exposure Source	**Estimated Daily Intake**	**NOAEL (mg Cu/kg BW/d)**	**HQ [a]**
Vegetation	88 mg/kg BW	40	2.2
Surface Water	0.45 ml/kg BW	20	0.02

[a] HQ = daily intake / NOAEL

A dose of 88 mg Cu/kg BW/d was estimated for the consumption of copper - rich vegetation. Mallards that received a similar dose of dietary copper in the laboratory studies, 90 mg Cu/kg BW, experienced a 10% reduction in weight gain when compared a control group (Figure 5.2). It seems reasonable to assume that juvenile mallards consuming vegetation from the Terrace Reservoir would experience a similar decrease in weight gain; however, it not known what if, any effect this would have on the population. To accurately characterize the impact of copper to waterfowl living along the Alamosa River would require a study of reproductive success, which was beyond the scope of this project.

Liver and kidney samples were collected from mallards during the field survey to provide a relative assessment of copper exposures for ducks living on the Alamosa River. Juvenile mallards collected from contaminated areas of the Alamosa River had substantially greater hepatic copper concentrations when compared to birds collected in the wetland areas or the Monte Vista NWR (Table 4.2). The increased hepatic copper concentrations in the Alamosa River birds indicate that they are exposed to larger concentrations of copper. The elevated hepatic copper concentration in mallards from the Terrace Reservoir is consistent with increased copper concentrations in the environmental samples collected from that area. However, the liver copper concentrations in the Alamosa River birds were comparable to the levels found in the control groups from the laboratory studies. The estimated dose of copper from the consumption of Alamosa River vegetation, 88 mg Cu/kg BW/d, is considerably less than the level of exposure that

was found to produce elevated liver copper concentrations (≥160 mg Cu/kg BW/d) (Figure 4.5). Increased feather copper concentrations are expected from exposure to copper at the level which is predicted for mallards that consume aquatic vegetation from the Alamosa River, 88 mg Cu/kg BW/d (Figure 4.8). Unfortunately, feather tissue was not collected during the field survey. Ideally, the field survey should have been conducted after the laboratory studies had identified the most appropriate method for evaluating copper exposure. In summary, mallards on the Alamosa River appear to have increased copper exposure when compared to birds from uncontaminated areas, but the magnitude of exposure can not be established from the available information.

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