## IMPROVED CLINICOPATHOLOGIC DIAGNOSIS OF ACUTE LIVER DISEASE IN PSITTACIFORMES

by

#### LISA MICHELLE HOLTHAUS

(Under the Direction of Susan M. Williams)

#### ABSTRACT

Liver disease is a common malady that afflicts companion birds. Often, non-invasive tests, including history, fecal examination, clinical pathology, and diagnostic imaging can be nonspecific for liver disease. There is a lack of specific information regarding testing for avian liver disease. Thus, the purpose of this study was to evaluate the utility of hepatic testing in companion birds. Thirty Indian ring-necked parakeets (*Psittacula krameri manillensis*) were subjected to acute liver disease (traumatic liver injury or aflatoxicosis) to evaluate for changes in hepatic function tests reported to be of use in birds. The hepatic function tests were correlated with hepatic histopathology. The only significant changes in the variables measured and attributable to liver disease were sorbitol dehydrogenase (SDH) and glutamate dehydrogenase (GLDH). Based upon the two models of acute liver injury, overall findings suggest hepatic function testing is insensitive in detecting mild liver disease.

INDEX WORDS: Avian liver disease, Traumatic liver injury, Aflatoxicosis, Clinical pathology, Clinical enzymology, Hepatic function tests, Endoscopy, Hepatic histopathology

# IMPROVED CLINICOPATHOLOGIC DIAGNOSIS OF ACUTE LIVER DISEASE IN PSITTACIFORMES

by

## LISA MICHELLE HOLTHAUS

A.S., Macon State College, 2000

B.S.A., The University of Georgia, 2002

D.V.M., The University of Georgia, 2008

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment

of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

© 2009

Lisa Michelle Holthaus

All Rights Reserved

# IMPROVED CLINICOPATHOLOGIC DIAGNOSIS OF ACUTE LIVER DISEASE IN

## PSITTACIFORMES

by

## LISA MICHELLE HOLTHAUS

Major Professor:

Susan M. Williams

Committee:

Frederic S. Almy Stephen J. Hernandez-Divers

Electronic Version Approved:

Maureen Grasso Dean of the Graduate School The University of Georgia May 2009

## DEDICATION

To my parents, Bill and Mary Ellen Holthaus, for their unconditional love and support. To my birds, past and present, Maggie, Miggie, Heidi, Polly, Archie, Jack, and especially Cricket, for their love, devotion, and inspiration.

#### ACKNOWLEDGEMENTS

Susan M. Williams, DVM, PhD, DACPV

Stephen J. Hernandez-Divers, BVetMed, MRCVS, DZooMed, DACZM

Frederic S. Almy, DVM, MS, DACVP

Kenneth S. Latimer, DVM, PhD, DACVP

G. Heather Wilson Baron, DVM, DABVP

Michael McBride, DVM

Roger D. Wyatt, PhD

Shay Bush, MT (ASCP)

Jien Chen, PhD

Yijie Xue

Branson W. Ritchie, DVM, MS, PhD, DABVP, ECAMS

Christopher R. Gregory, DVM, PhD

Perry J. Bain, DVM, PhD, DACVP

The past and present clinical pathology staff and residents

Amanda R. Crawford

## TABLE OF CONTENTS

Page					
ACKNOWLEDGEMENTSv					
LIST OF TABLES					
LIST OF FIGURESix					
CHAPTER	CHAPT				
1 INTRODUCTION	1				
Purpose of the Study1					
2 AVIAN LIVER ANATOMY AND DISEASE	2				
Anatomy2					
Function5					
Disease5					
3 TRAUMATIC LIVER IINJURY	3				
Materials and Methods22					
Results					
Discussion					
Sources and Manufacturers					
4 AFLATOXICOSIS	4				
Materials and Methods54					
Results					
Discussion61					

	Sources and Manufacturers	63
5	CONCLUSION	78
REFERE	NCES	80

## LIST OF TABLES

Table 2.1: Clinical signs associated with avian liver disease    18
Table 2.2: The sensitivity and specificity of certain plasma chemistry variables in liver and
muscle disease based on experimental studies in pigeons
Table 3.1: Gender determination via endoscopic examination
Table 3.2: Systemic abnormalities via endoscopic examination in crush group
Table 3.3: Systemic abnormalities via endoscopic examination in biopsy sample population3
Table 3.4: Biochemical analysis at time 0 for fasted Indian ring-necked parakeets (n=30)30
Table 3.5: Biochemical analysis of crush treatment group taken over time (0-120 hours) for
Indian ring-necked parakeets
Table 3.6: Trend of each variable in crush treatment group over time (0-120 hours) for Indian
ring-necked parakeets
Table 4.1: Systemic abnormalities via endoscopic examination in aflatoxin group pre- and post-
treatment
Table 4.2: Gender determination via endoscopic examination    64
Table 4.3: Weight in grams of birds pre- and post- aflatoxin B1 treatment
Table 4.4: Biochemical analysis of aflatoxin treatment group taken over time (0-10 days) for
Indian ring-necked parakeets65
Table 4.5: Trend of each variable in aflatoxin treatment group over time (0-10 days) for Indian
ring-necked parakeets6

## LIST OF FIGURES

Figure 3.1: Left flank approach for endoscopic evaluation of a bird with surgical preparation 39
Figure 3.2: Representative views obtained during an endoscopic examination of the reproductive
tract
Figure 3.3: Representative views obtained during an endoscopic liver biopsy procedure40
Figure 3.4: Estimate of ln(ALP) for crush subgroups A and B over time (0-120 hours)41
Figure 3.5: Estimate of ln(ALT) for crush subgroups A and B over time (0-120 hours)42
Figure 3.6: Estimate of ln(AST) for crush subgroups A and B over time (0-120 hours)43
Figure 3.7: Estimate of ln(CK) for crush subgroups A and B over time (0-120 hours)44
Figure 3.8: Estimate of ln(LDH) for crush subgroups A and B over time (0-120 hours)45
Figure 3.9: Estimate of ln(CHOL) for crush subgroups A and B over time (0-120 hours)46
Figure 3.10: Estimate of ln(BA) for crush subgroups A and B over time (0-120 hours)47
Figure 3.11: Estimate of GGT for crush subgroups A and B over time (0-120 hours)
Figure 3.12: Estimate of GLDH for crush subgroups A and B over time (0-120 hours)49
Figure 3.13: Estimate of SDH for crush subgroups A and B over time (0-120 hours)50
Figure 4.1: Estimate of ln(ALP) for aflatoxin subgroups A and B over time (0-10 days)68
Figure 4.2: Estimate of ln(ALT) for aflatoxin subgroups A and B over time (0-10 days)69
Figure 4.3: Estimate of ln(AST) for aflatoxin subgroups A and B over time (0-10 days)70
Figure 4.4: Estimate of ln(CK) for aflatoxin subgroups A and B over time (0-10 days)71
Figure 4.5: Estimate of ln(LDH) for aflatoxin subgroups A and B over time (0-10 days)72

Figure 4.6: Estimate of ln(CHOL) for aflatoxin subgroups A and B over time (0-10 days)73
Figure 4.7: Estimate of ln(BA) for aflatoxin subgroups A and B over time (0-10 days)74
Figure 4.8: Estimate of GGT for aflatoxin subgroups A and B over time (0-10 days)75
Figure 4.9: Estimate of GLDH for aflatoxin subgroups A and B over time (0-10 days)76
Figure 4.10: Estimate of SDH for aflatoxin subgroups A and B over time (0-10 days)77

## CHAPTER 1

#### INTRODUCTION

The need for improved methods of liver disease testing has manifested as the popularity of pet birds has increased. Veterinary professionals are limited to nonspecific testing and liver biopsies for the evaluation of hepatic disease because, currently, noninvasive diagnostic procedures are inadequate. Presently biochemical testing for hepatic disease in animals is most advanced in cats and dogs.<sup>1(p230)</sup> Due to the many anatomical and physiological differences between mammals and birds, standard blood chemistry values cannot be interpreted equally among species.

#### **Purpose of the Study**

The purpose of this study was to establish appropriate reference intervals for hepatic enzyme activities in a selected parrot species (Psittaciformes), to evaluate changes in plasma hepatic biochemical parameters in acute avian liver disease caused by trauma and aflatoxicosis, and to compare biochemical changes with histologic lesions of the hepatic parenchyma. The biochemical tests of interest included alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), creatinine kinase (CK), lactate dehydrogenase (LDH), bile acids (BAs), cholesterol (CHOL), gamma-glutamyl transferase (GGT), glutamate dehydrogenase (GLDH), and sorbitol dehydrogenase (SDH). The overall aim was to provide a more accurate, noninvasive method to access liver disease in birds.

## CHAPTER 2

## AVIAN LIVER ANATOMY AND DISEASE

The liver is the largest and heaviest organ in the avian body; however, its size is relative to the species of bird, the bird's diet, and/or the bird's age.<sup>2</sup> Piscivorous and insectivorous species generally have the largest livers relative to the size of their body.<sup>2</sup> The effects of age on liver size have been examined in chickens (*Gallus*),<sup>3</sup> turkeys (*Meleagris*),<sup>4</sup> quails (*Coturnix*),<sup>5</sup> and geese (*Anser*).<sup>6</sup> In poults, for example, the relative weight of the liver peaks 4 days post-hatch in females and 6 days post-hatch in males and begins to slowly decline thereafter.<sup>4</sup> The color of the liver varies with age. In precocial hatchlings, the liver is bright yellow due to absorption of pigments and lipid from the yolk sac.<sup>2,7-9</sup> The liver's color begins to transition to red-brown 8-14 days in *Gallus* and 5-10 days in waterfowl (*Anas*) after hatching.<sup>2,8,10,11</sup> This darker color is also seen in altricial species at hatching.<sup>10</sup>

#### Anatomy

The liver is divided into right and left lobes by cranial and caudal longitudinal incisures. The lobes fuse cranially at midline by an interlobar part.<sup>2, 10, 12</sup> At least one liver lobe is subdivided in many avian species. In domestic fowl (*Gallus*) and turkeys (*Meleagris*), the left lobe is subdivided into caudodorsal and caudoventral parts.<sup>2, 9, 13</sup> Most birds from the families Caprimulgidae, Apodidae, and Trochilidae and the order Passeriformes have a subdivided right liver lobe.<sup>2</sup> The relative proportions of the two liver lobes vary both intra- and interspecifically.<sup>2</sup> The right liver lobe is most commonly larger in many avian species; however, the lobes can be of equal size.<sup>2, 10, 12-14</sup>

The liver lies in the cranial quadrant of the coelomic cavity with its cranioventral portions surrounding the cardiac apex. This finding is markedly different from mammals where the heart is completely surrounded by lung and the thoracoabdominal cavity is separated by a diaphragm.<sup>13</sup> The ventral border of a normal liver rarely extends beyond the caudal border of the sternum, with the exception of some large birds.<sup>8, 10</sup> The parietal surface of the liver, which is smooth and convex, is deeply marked by the direct relationship of the heart on its cranioventral surface. In contrast, the liver's visceral surface is concave and quite irregular. This irregularity is due to the proximity of adjacent organs and can vary between species. In most avian species, the proventriculus, ventriculus, and spleen consistently form impressions on either the right and/or left liver lobes.<sup>2, 10, 13</sup> In addition, other impressions have been documented but vary between species. The jejunum in Anas and Anser and the ascending and descending limbs of the duodenum as well as the umbilical vein and right testis in Gallus cause depressions in the right liver lobe.<sup>2</sup> The most notable interspecies difference is the absences or presence of a gallbladder. Some species of birds, including most psittacines, as well as horses, rats, and elephants lack a gallbladder.<sup>12, 14</sup> However, when present in birds, the gallbladder forms a pit-like depression on the visceral surface of the right liver lobe.<sup>2, 8, 10, 14</sup>

The avian liver, as in mammals, receives blood supply from the hepatic arteries and hepatic portal veins and is drained by hepatic veins.<sup>2, 10, 12, 15</sup> The hepatic arteries (right and left) arise from branches of bifurcation of the celiac artery and supply arterial blood to the liver.<sup>2, 10, 12, 15</sup> The interlobar notch of the liver, the gallbladder, and the proximal ends of the extrahepatic bile ducts receive blood from the right hepatic artery prior to it entering on the medial surface of

the left liver lobe.<sup>2</sup> Providing approximately 75% of the liver's blood supply, the hepatic portal veins (right and left) transport venous blood drained from the proventriculus, ventriculus, duodenum, pancreas, intestines, and cloaca.<sup>2, 10, 12</sup> The region of liver in which the hepatic portal veins supplies blood differs interspecifically.<sup>2</sup> The right hepatic portal vein is the larger of the two and supplies the right liver lobe, the interlobar tissue, and often a portion of the left liver lobe.<sup>2</sup> The left hepatic portal vein is restricted to providing blood to the left liver lobe; however, an anastomosis frequently occurs between the right and left hepatic portal veins.<sup>2</sup> The intrahepatic arteries divide in company with branches of the portal vein and tributaries of the bile duct.<sup>2</sup> The liver is drained majorly by the right and left hepatic veins and minorly by one or more middle hepatic veins. The caudal vena cava receives the blood from the right and left hepatic vein(s) at the interlobar notch of the liver.<sup>2</sup>

The microscopic structure of the avian liver closely resembles the liver of other vertebrates.<sup>2</sup> Each hepatic lobe is separated into indistinct lobules which consist of sinusoids and sheets of hepatocytes radiating around a central vein. The number of hepatocytes within the sheet varies between species. In the Red-necked Grebe (*Podiceps grisegena*), Prairie Chicken (*Tympanuchus cupido*), some passerine species, and mammals, the hepatocytes are one cell layer; however, in *Gallus*, the Wood Duck (*Aix sponsa*), and American Coot (*Fulica americana*) the hepatocytes are two cells muralium.<sup>2, 16</sup> The sinusoids receive blood from terminal portal venules and arterioles and are lined by endothelium and phagocytic Kupffer cells. Between the endothelium and hepatocytes is a space (space of Disse) that contains plasma. Microvilli of hepatocytes extend into the space of Disse allowing direct exchange of substances from the blood. Within the sheet of hepatocytes are bile canaliculi that collect bile from the hepatocytes.

Portal areas occur at the periphery of the lobules and consist of hepatic artery, portal vein, bile ducts, and lymphatic vessel. The bile canaliculi drain the lobules via the intrahepatic bile ductules. These ductules empty into the interlobular ducts of the portal areas which join together to form the lobar ducts; the lobar ducts unite at the hilum to form the right and left hepatic ducts. The two bile ducts (unique to birds) join to form the common hepatoenteric duct which opens into the duodenum.<sup>2</sup> A branch of the right hepatic duct either forms the right hepatoenteric duct (emptying into the duodenum) in most birds without a gallbladder or the hepatocystic duct (entering the gallbladder) in birds with a gallbladder. The right hepatoenteric duct is absent in the ostrich even though it lacks a gallbladder.<sup>2</sup>

## Function

The liver is an indispensable component of homeostasis.<sup>17</sup> The major embryonic function of the liver is the synthesis of red blood cells. Some hepatic hematopoietic activity remains active shortly after hatching, can occur at varying stages of health, and is a common finding in birds with chronic blood loss.<sup>10, 17</sup> After birth, the liver has an essential role in nutrient metabolism, protein synthesis, detoxification of xenobiotics, bile acid synthesis, storage of vitamins and minerals, ammonia detoxification, immunoregulation, and biliary excretion of biliverdin.<sup>18</sup>

#### Disease

The avian liver is frequently afflicted with disease either as the primary organ or secondarily to other diseases.<sup>17</sup> Unfortunately, physical examination findings associated with liver disease are vague and non-definitive, and therefore, may be mistakenly attributed to other disease processes. A variety of clinical signs may be observed (Table 2.1); however, none of

these signs are pathognomonic for hepatic disease.<sup>14, 17, 19</sup> Since the liver is such a large organ and has the ability to regenerate, focal insults to the liver may not cause significant disease and many birds will be asymptomatic.<sup>14, 17</sup>

Noninfectious causes of avian liver disease are metabolic disorders, iron storage disorders, circulatory disorders, hepatotoxins, neoplasia, and trauma.<sup>14</sup> Metabolic disorders, such as fatty liver and kidney syndrome or fatty liver hemorrhagic syndrome, are associated with the consumption of high-energy, multi-nutrient deficient diets and restricted exercise which may lead to hepatic lipidosis.<sup>14</sup> Iron storage disease presents as abnormal accumulation of iron in the liver and other organs. This condition may be acquired (hemosiderosis) or possibly genetic (hemochromatosis). Excessive iron accumulation can cause both hepatic and cardiac disease. Portal hypertension, a disorder of the circulatory system, may cause a distended liver due to extreme congestion. Severe anemia also may be associated with centrilobular necrosis of hepatocytes secondary to hypoxia. Hepatotoxins include plants, alkaloids, mycotoxins, chemicals, and other poisons. In some cases, degeneration and necrosis of hepatocytes, bile duct proliferation, or fibrosis are evident. Hepatic tumors, such as bile duct carcinoma, originate in the liver and may metastasize to other tissues and organs. Rupture of the liver also may occur as a result of trauma.

Infectious agents responsible for liver disease include bacteria, *Chlamydophila*, viruses, fungi, helminths, trematodes, and protozoa.<sup>14, 20</sup> Many bacterial species such as *Staphylococcus* sp., *Salmonella* sp., *Pseudomonas* sp., *Pasteurella* sp., *Mycobacterium* sp., and *Escherichia coli* can cause liver disease.<sup>14</sup> One of the most common causes of liver disease is chlamydiosis (caused by *Chlamydophila psittaci*). Radiographic findings include hepatomegaly and splenomegaly. Birds may contract viral infections as a primary disease or in association with

other diseases. Some examples of viral infections that cause liver disease in birds include herpesviruses (Pacheco's disease virus); polyomavirus; and adenovirus. *Aspergillus fumigatus, A. flavus*, and *A. niger* are common opportunist fungi that invade the respiratory tract of immunocompromised birds; however, in more severe infections, hematogenous dissemination into visceral organs can occur.<sup>20</sup> Parasites such as helminths (cestodes, nematodes, and trematodes) can infest the hepatic parenchyma as well as the bile ducts of certain avian species. Various protozoa such as *Histomonas meleagridis* and *Atoxoplasma screnii* also can cause liver disease in Galliformes and canaries, respectively.<sup>14</sup>

Due to the ambiguity of clinical signs, avian liver disease is difficult to identify; therefore, a combination of tests is often necessary for an accurate diagnosis. In any situation where liver disease is suspected, the ideal minimum database should include anamnesis, fecal examination, complete blood count with leukocyte differential, clinical enzymology, and radiographs.<sup>14, 21</sup> Even with the information provided by these tests, more extensive testing, such as endoscopy with liver biopsy/histopathology, is generally necessary for a definitive diagnosis.

A thorough history is important in documenting potential exposure to infectious agents. In addition, the type and origin of the bird's diet is important in determining potential exposure to mycotoxins (*e.g.*, peanuts and peanut byproducts) or other food contaminants. A fecal examination is an invaluable tool for clinicians to readily access changes in color, frequency, consistency, and amount of feces, urine, and/or urates. Green or yellow discoloration of urates or urine is a strong indicator of liver disease.<sup>11, 14, 22, 23</sup>

Hematological and biochemical analyses are important diagnostic tools in determining the presence, degree, and, in some cases, cause of hepatobiliary disease. A complete blood cell

count with a differential may reveal changes in the red or white blood cells. In some animals with hepatobiliary disease, regenerative or non-regenerative anemia may be observed. Regenerative anemia is most often associated with blood loss; however, the cause is usually gastrointestinal ulceration and rarely liver disease.<sup>24</sup> Nonregenerative anemia, typically normocytic and normochromic, is more common in hepatobiliary disease and can indicate an iron deficiency. In some animals with liver disease, morphologic changes of the red blood cells (poikilocytes) may be identified and are most commonly due to altered erythrocyte membrane lipid content.<sup>24</sup> Fluctuations in the white blood cell count (WBC) may suggest an infectious agent. Toxic or viral disorders are commonly associated with a leukopenia, where a leukocytosis is often seen with bacterial or fungal infections.<sup>19</sup> Plasma enzymes as well as liver function tests are often used for diagnosing avian liver diseases. However, these tests are neither sensitive or specific since the test values may be within the reference interval in birds with liver disease, or inexplicably above the reference interval in clinical healthy birds.<sup>22</sup>

Hepatic dysfunction or failure can often result in hepatomegaly and subsequently be identified by full body radiographs; however, microhepatia from hepatic degeneration has also been reported.<sup>19, 22</sup> Therefore, for comparison, baseline radiographs should be obtained on a routine physical examination of each bird prior to onset of disease. Other diagnostic tests are available such as ultrasonography and may provide information concerning the size and structure of the liver; however, its usefulness is dependent on patient size and conformation, the presences of air sacs, and operator experience.<sup>25</sup> Currently, the best tool for a definitive diagnosis of liver disease is endoscopic biopsy with histopathology.

Clinical enzymology, as well as liver function tests, is probably the most specific noninvasive diagnostic method to detect liver disease. Increases in serum or plasma hepatic

enzyme activities are due to leakage of enzymes from damaged hepatocytes or induction of enzyme activity by bile ductular epithelial cells. The magnitude of increase in plasma or serum enzyme activity depends on the severity of hepatocellular damage, number of damaged hepatocytes, molecular size of the enzyme, degree of cellular enzyme activity, biological halflives of enzymes in serum or plasma, and the gradient and rate of enzyme leakage from cells.<sup>14,</sup> <sup>18, 26, 27</sup> GLDH, AST, ALT, LDH, GGT, and ALP are common enzymes whose activities have been evaluated in an attempt to diagnose liver disease; however, their utility in birds remains unclear.

#### Hepatocellular Leakage Enzymes

Any damage to the hepatocellular membrane will alter its permeability and subsequently cause an outflow of cytosolic enzymes into the extracellular space and, eventually, the blood.<sup>18</sup> Hepatocellular leakage enzymes include ALT, AST, SDH, LDH, and GLDH. As mentioned previously, the magnitude of increase in enzyme activity may be influenced by many factors. Thus, clinical signs and enzymology do not always correlate. The sensitivity of these enzymes in detecting liver damage is much greater than currently available liver function tests (Table 2.2); however, their specificity for liver disease varies interspecifically.<sup>14, 18</sup>

Alanine aminotransferase (previously, glutamate pyruvate transaminase) is found mainly in the liver but also in the kidneys, skeletal muscle, heart, spleen, lungs, pancreas, intestines, brain, and erythrocytes;<sup>26, 28-30</sup> nevertheless, its distribution within these tissues varies between species.<sup>31</sup> In dogs, cats, rabbits, rats, and humans, the highest activity of this enzyme is found in hepatocytes; therefore, significant increases in alanine aminotransferase activity are considered to be specific for liver disease.<sup>17, 30</sup> The degree of increase in ALT activity is directly related to the proportion of injured hepatocytes;<sup>24</sup> yet, it does not signify whether the injury is reversible or irreversible. In contrast, ALT activity in birds, horses, ruminants, and pigs is typically low and nonspecific with regard to liver. Most increases of ALT activity in these species are probably associated with muscle damage.<sup>18, 29-31</sup> Even in some avian patients with severe liver disease, ALT activity has been within the reference interval.<sup>29</sup> Also, ALT activity in birds is reportedly dependent on age and the season.<sup>29</sup> Although ALT is considered liver specific in some species of animals, other causes of an increase in this enzyme should not be overlooked. To differentiate hepatocellular damage from muscle damage, serum creatinine kinase (CK) activity should be measured; CK is the most sensitive and specific enzyme associated with striated muscle injury. An increase in ALT levels without a corresponding increase in serum CK levels indicates hepatocellular damage. Hemolyzed and lipemic serum or plasma samples can artifactually increase ALT levels. Following tissue damage in dogs, serum/plasma ALT activity increases within the first 12 hours, peaks within 12-24 hours, and returns to within reference interval in 2-3 weeks. The biological half-life of ALT is approximately 60 hours in dogs and 16 hours in racing pigeons.<sup>18, 24, 26, 32</sup> In dogs with acute liver disease, a 50% decline in serum activity of ALT within 2-3 days is usually associated with a good prognosis.<sup>24</sup>

High aspartate aminotransferase (previously, glutamate oxalacetate transaminase) activity has been reported in the liver, gastric mucosa, adipose tissue, skeletal muscle, heart, brain, and kidneys.<sup>26, 28-31</sup> The distribution of AST activity within these tissues varies between avian species.<sup>29</sup> AST is present in both the cytoplasm and mitochondria of cells. In cases of mild hepatocellular damage; the majority of AST plasma activity is due to the cytosolic isoenzyme. With more severe hepatocellular injury, mitochondrial isoenzyme of AST is released.<sup>18, 28</sup> The serum biological half-life of AST in dogs and cat is 5-12 hours and 77 minutes, respectively.<sup>24</sup>

The half-life of AST in racing pigeons is 7.5 hours, but values for other avian species have not been determined.<sup>26, 33</sup> Since AST activity is found in many tissues, it is not considered liver specific and increases in this enzyme may be associated with liver or muscle damage.<sup>18, 24, 29</sup> Even though AST activity is not specific for liver disease in birds, dogs, or cats, some investigators consider it more sensitive than ALT in detecting hepatocellular damage.<sup>24, 31</sup> Plasma AST activities of at least 230 IU/L<sup>29</sup> in birds is considered increased, but due to low specificity, the increased activity may be associated with either liver or muscle damage. An AST > 800 IU/L is highly suggestive of severe hepatic injury, especially if other clinical signs of liver disease are present.<sup>31</sup> Age related fluctuations in AST have been identified in several avian species and should be considered when interpreting the results.<sup>29</sup> In dogs and cats, an increase in AST generally parallels an increase in ALT; however if serum AST values are greater than serum ALT, muscle damage<sup>24</sup> or cystic hyperplasia-pyometra complex<sup>34</sup> should be suspected.

In all animals including humans, sorbitol dehydrogenase (also known as iditol dehydrogenase) is predominately present in the cytoplasm and mitochondria of the liver with small amounts in the kidneys and seminal vesicles and is considered a specific indicator of hepatocellular damage.<sup>18, 35</sup> SDH is superior to ALT and AST in detecting liver disease in horses, cattle, sheep, and goats. In birds, SDH is also specific for liver disease, but, according to Thrall,<sup>31(p487)</sup> SDH has no diagnostic advantage over glutamate dehydrogenase (discussed below). The biological half-life of SDH is very short (< 12 hours); therefore, any increase in this enzyme usually indicates acute hepatocellular injury.<sup>18</sup> Hemolysis and lipemia have been shown to interfere with SDH analysis.<sup>35</sup>

The lactate dehydrogenase enzyme is found in many tissues such as the heart, liver, skeletal muscle, kidneys, bone and erythrocytes, and therefore, should not be regarded as liver

specific.<sup>18, 29, 31, 36</sup> Five LDH isoenzymes are present in serum/plasma and can be separated based on their electrophoretic mobility to determine the source of increased activity.<sup>29, 36</sup> Increased activity of LDH can be seen with liver, muscle damage, or hemolysis.<sup>18, 29</sup> According to Thrall,<sup>31(p486)</sup> LDH activity in normal birds has a very high upper reference limit of 1000 IU/L and is nonspecific for hepatocellular injury. This may limit its usefulness as a diagnostic test for liver disease, and as a result, the measurement of AST is preferred by Thrall.<sup>31</sup> However, Hochleithner<sup>29(p230)</sup> indicates that increases in LDH activity are usually observed in psittacines with hepatic diseases and, based on its short biological half-life (< 1 hour),<sup>26</sup> can provide more information on the duration of liver disease compared to AST (i.e. persistent increases in LDH indicate ongoing hepatocellular damage). Seasonal fluctuations and gender have been reported to affect the activity of LDH in birds, especially canary finches. Hemolyzed serum/plasma samples can artifactually increase LDH activity.<sup>31</sup> Thus, the value of LDH in assessing avian liver disease is potentially diminished.

Glutamate dehydrogenase is a mitochondrial enzyme found in numerous tissues. The liver contains the highest concentration of this enzyme in rodents, ovine, bovine, feline, and canine species.<sup>30</sup> In Galliformes (chickens, ducks, and turkeys) and Columbiformes (racing pigeons) the most significant GLDH activity is associated with the liver, kidneys, and brain; in Psittaformes (budgerigars), the kidneys contain the highest amount of GLDH.<sup>29</sup> Due to its mitochondrial location, increases in GLDH activity reflect leakage from severely damaged or necrotic cells. GLDH is a sensitive and specific marker of liver disease in animals. The biological half-life of GLDH in plasma is approximately 14 hours in cattle and < 1 hour in racing pigons.<sup>26, 30, 32</sup>

#### Inducible Hepatic Enzymes

Hepatobiliary injury can be assessed by the presence of inducible enzymes in the blood. Induced hepatic enzymes include alkaline phosphatase and gamma-glutamyl transferase. These enzymes are membrane-bound and only increase in serum/plasma concentration with increased synthesis, canalicular activity or injury, cholestasis, or solubilization of bile salts.<sup>18, 37</sup>

Alkaline phosphatase is found in hepatocytes, neutrophils (except birds<sup>38</sup>), prostate, placenta, kidneys, osteoblasts, and duodenum.<sup>17, 18, 29, 33, 39</sup> In pigeons, chickens, and turkeys, high ALP activity was found in the kidneys and duodenum, but at low levels in the liver.<sup>29</sup> Five clinically important ALP isoenzymes are present in serum/plasma and can be differentiated based on their electrophoretic mobility to determine the source of increased activity; the ALP isoenzymes include hepatic, bone, corticosteroid-induced (dog only), placenta, and intestinal forms.<sup>18</sup> The liver ALP isoenzyme (L-ALP) is specific for hepatic disease, especially intra- or extra-hepatic cholestasis, and may precede other biochemical abnormalities such as hyperbilirubinemia.<sup>18</sup> However, in feline cholestasis, L-ALP is less sensitive than GGT which usually has a more dramatic increase in measured activity. Feline hyperthyroidism commonly stimulates an increase in ALP activity associated with either the liver and/or bone isoenzyme. The serum biological half-life of L-ALP is approximately 72 hours in the dog and considerably shorter in the cat. The latter makes increases in ALP in the cat sensitive and specific for cholestatic liver disease. The sensitivity of ALT in diagnosing hepatobiliary disease in large animals is limited due to its wide reference interval. Even though the activity of ALP is low in the liver of birds, Hochleithner<sup>29(p229)</sup>suggests that an increase in this enzyme is most commonly seen with liver disease. On the other hand, Thrall<sup>31(487)</sup> states increases in ALP are useless for detecting hepatobiliary disease and instead are primarily associated with osteoblastic activity.<sup>23</sup>,

<sup>33</sup> Other causes of an increase ALP concentration include nutritional secondary hyperparathyroidism, enteritis, preovulation condition in hens, and seasonal variations.<sup>29,31</sup>

Gamma-glutamyl transferase activity is found in hepatocytes and mammary, renal tubular, and biliary epithelial cells.<sup>18</sup> Measurable activity has been found in the brain and kidneys of racing pigeons (Columba livia domestica), the kidneys and duodenum of budgerigars (Melopsittacus undulatus), and only the kidneys in African grey parrots (Psittacus erithacus).<sup>29,</sup> <sup>33</sup> This enzyme is predominately associated with the brush border of biliary and renal tubular epithelium. Serum GGT activity is usually the result of enzyme induction in hepatocytes or biliary epithelial cells.<sup>18, 29</sup> In humans, GGT is one of the most sensitive indicators of hepatobiliary disease and may be the only elevated parameter.<sup>40</sup> Also, it is commonly used as screening test for occult alcoholism.<sup>40</sup> The mechanisms of alcohol-related increases in GGT are induction by ethanol, leakage from damaged hepatocytes, synthesis secondary to cholestasis, and diffusion across hepatic cell membranes.<sup>41</sup> In most animals, with the exception of cats, GGT is less sensitive but more specific than ALP in detecting cholestasis. The usefulness of GGT activity for diagnosing hepatobiliary disease in birds seems to be unreliable since increased GGT activity may be associated with renal rather than liver disease.<sup>29</sup> The serum biological half-life of GGT in dogs and horses is approximately 72 hours.<sup>18, 32</sup> The half-life of GGT in birds has not been established.

#### Liver Function Tests

Liver function tests represent a broad range of normal functions performed by the liver. Approximately 70-80% of hepatocytes must be damaged before signs of dysfunction are evident on a biochemical profile.<sup>17, 18, 27</sup> Products produced or metabolized by the liver serve as hepatic function tests and include albumin,  $\alpha$ - and  $\beta$ -globulins, clotting factors, bilirubin, bile acids, ammonia, cholesterol, triglycerides, uric acid, and glucose; only a few of these tests will be discussed.

Cholesterol is a precursor of steroid hormones and bile acids and is an essential component of mammalian cell membranes.<sup>29</sup> It can be derived from the diet and/or synthesized by a pathway which occurs in most cells in the body but particularly in the cells of the liver and intestines.<sup>24, 29, 42, 43</sup> Lipoproteins (chylomicrons from the liver and very-low-density lipoproteins from the intestines) transport cholesterol in the blood. Changes in plasma cholesterol concentration can be associated with a number of conditions. Increased hepatic synthesis and/or decreased biliary excretion of cholesterol commonly results in hypercholesterolemia in dogs and cats. Hypercholesterolemia is often seen with extrahepatic biliary obstruction, hepatic fibrosis, bile duct hyperplasia, and fatty liver disease in birds;<sup>11, 29, 43</sup> other causes include hypothyroidism, xanthomatosis, high-fat diets, cystic ovarian disease, reproductive activity, and postprandial hyperlipidemia.<sup>29, 43, 44</sup> Higher cholesterol concentrations are seen in carnivorous birds as well as male budgerigars.<sup>29</sup> Chronic end-stage liver disease, aflatoxicosis, reduced fat diets, *E. coli* endotoxemia, spirochetosis, and maldigestion or malabsorption may cause decreased plasma cholesterol levels. Starvation in birds reportedly may cause either hypocholesterolemia<sup>31</sup> or hypercholesterolemia.<sup>29</sup> Hypocholesterolemia occurs in approximately 2/3 of dogs and cats with portosystemic vascular anomalies as well as chronic end-stage liver disease due to a loss of functional hepatic mass. The diagnostic value of serum/plasma cholesterol concentrations is considered poor in dogs and birds with hepatic disease.<sup>18, 29</sup>

Bile acids are lipid emulsifiers synthesized exclusively from cholesterol in the liver and are the main constituents of bile. The primary bile acids in birds are cholic acid, allocholic acid,

and chenodeoxycholic acid.<sup>12, 29, 31, 45</sup> They are excreted into bile and undergo enterohepatic circulation. Even though some birds lack a gallbladder, BAs are continuously secreted.<sup>33</sup> Bile acid determination is useful to assess liver function in humans and domestic animals, including birds because all major hepatic functions (extraction, conjugation, and secretion) are involved in this process.<sup>33, 45-47</sup> Since BAs are sensitive and specific indicators of liver disease, they are also helpful in distinguishing between liver and muscle damage.<sup>17</sup> Pre- and post- prandial BA samples are recommended in order to distinguish between a postprandial increase or increase due to hepatobiliary disease;<sup>33, 43</sup> however, preprandial sampling can be difficult in birds with a crop. In addition,  $Harr^{43}$  and Hochleithner *et al*<sup>19</sup> report that fasting is not necessary in birds without a gallbladder. This statement is in direct contrast to a study conducted by Lumeij<sup>48</sup> where postprandial BAs were evaluated and compared in birds with (Mallards) and without (racing pigeons) gallbladders. The results indicated that postprandial sampling affected the concentration of BAs in both species of birds; however, the degree of increase was less significant than in birds with hepatobiliary disease. Therefore, a 12-hour fast is recommended in most birds but sample timing depends on the species of bird, their diet (24-hour fast in carnivorous birds), and their health status.<sup>31, 43, 49</sup> Normal BA concentrations are higher in birds than mammals; Amazon parrots naturally have higher concentrations of BA than other psittacines.<sup>31,43</sup> An increase in BA concentrations is suggestive of abnormal hepatic uptake, storage, or perfusion;<sup>17, 31</sup> values > 100  $\mu$ mol/L are suspicious for hepatobiliary disease in birds.<sup>33</sup> Falsely increased BA concentrations occur with moderately to severely lipemic and hemolyzed specimens.<sup>17, 31, 50</sup> A decreased BA concentration may occur with intestinal malabsorption, chronic hepatitis, hepatic fibrosis, and atrophy of the liver.<sup>11, 29, 43, 50</sup>

Bilirubin is the primary bile pigment found in the serum of domestic animals and is the yellow byproduct of heme catabolism. In dogs and cats, increased concentrations of serum bilirubin often indicate hepatic or hemolytic disease and results in yellow discoloration of the skin and/or conjunctiva; however, it usefulness in detecting hepatobiliary disease in birds is poor.<sup>31</sup> Birds, with the exception of some *Gallus*,<sup>43</sup> and reptiles lack the enzymes (biliverdin reductase<sup>12, 18, 33, 43</sup> and glucuronyl transferase<sup>12</sup>) necessary to convert biliverdin to bilirubin; therefore, they do not produce bilirubin. However, intestinal bacteria may produce biliverdin reductase and subsequently, bilirubin at low levels.<sup>19, 31, 43</sup> Increased concentrations of biliverdin are often seen with liver disease. Clinical signs are usually associated with discolored urine, urates, and/or plasma and rarely icterus.<sup>17, 19, 31, 33</sup> Even though increased concentrations of biliverdin availability of commercial assays is limited.<sup>31</sup> Thus, the utility of biliverdin measurement in the detection of avian liver disease is low.

## Table 2.1 Clinical signs associated with avian liver disease.

Nonspecific signs

- Anorexia (or reduced appetite)
- Depression
- Dyspnea
- Lethargy
- Weight loss
- Weakness
- Diarrhea
- Polyuria
- Polydipsia
- Vomiting
- Poor feather quality (abnormal molt)

More specific signs

- Green or yellow urates
- Hepatomegaly (occasionally visible in smaller Passeriformes)
- Ascites
- Coagulopathies
- Melena
- Hematochezia
- Overgrown and/or bruised beak and nails
- Malcolored feathers
- Feather picking
- Pruritus
- Icterus (rare)
- Sudden death

Variable	Liver Disease		Muscle Disease	
variable	Specificity	Sensitivity	Specificity	Sensitivity
<b>BA</b> <sup>1</sup>	+++	+++	-	-
GGT <sup>2</sup>	+++	+	-	-
AST <sup>3</sup>	-	+++	-	+++
$ALT^4$	-	++	-	+++
ALP <sup>5</sup>	-	-	-	-
GLDH <sup>6</sup>	+++	$+^{a}$	-	-
CK <sup>7</sup>	-	-	+++	+++
LDH <sup>8</sup>	-	+	-	+

Table 2.2 The sensitivity and specificity of certain plasma chemistry variables in liver and muscle disease based on experimental studies in pigeons.<sup>14, 33</sup>

- low + few ++ moderate +++ high

<sup>a</sup>Only with hepatocellular necrosis

 $^{1}BA = Bile Acid$ 

 ${}^{2}GGT = Gamma-Glutamyl Transferase$  ${}^{3}AST = Aspartate Aminotransferase$  ${}^{4}ALT = Alanine Aminotransferase$ 

 ${}^{5}ALP = Alkaline Phosphatase$ 

<sup>6</sup>GLDH = Glutamate Dehydrogenase

 $^{7}CK = Creatinine Kinase$ 

<sup>8</sup>LDH = Lactate Dehydrogenase

## CHAPTER 3

#### TRAUMATIC LIVER INJURY

The liver is particularly prone to injury due to its size, relative stiff architecture, and fixed abdominal position.<sup>51</sup> Traumatic liver injuries are often a result of blunt or penetrating abdominal trauma involving several organs; therefore, isolated liver injuries are uncommon.<sup>52</sup> In dogs and cats, the most common causes of trauma to the liver are falls, motor vehicle accidents, and blunt abdominal injuries.<sup>52, 53</sup> However, an underlying hepatic disorder, most common in birds and cats, can increase the sensitivity of the liver to trauma and, subsequently, result in rupture of the liver.<sup>14, 53</sup>

Traumatic injury to the liver can vary in severity and has an associated range of morbidity and mortality.<sup>54</sup> Significant blunt abdominal trauma may result in short or long term complications. In humans, less than 5% of blunt liver injuries result in hepato-vascular or biliary fistula, delayed hemorrhage, abscess, and hepatic cyst formation.<sup>54</sup> In most incidences, adults and children with blunt liver injuries only require conservative treatment.<sup>54</sup> However in dogs and cats, blunt abdominal trauma can be life-threatening due to severe hemorrhage. Commonly, these animals present with depressed mentation, pale mucous membranes, delayed capillary refill time, tachycardia, and poor peripheral pulse quality. Though, in some cases, survival is possible if the hemorrhage is confined to one hepatic peritoneal cavity (birds only) or to a subcapsular hematoma.<sup>14, 52</sup>

Injuries to the liver can be challenging to diagnosis because results of the initial abdominal examination can be unreliable.<sup>55, 56</sup> However, increases in certain biochemical tests (ALT, AST, LDH, and ALP) have been demonstrated to be associated with blunt liver injury in dogs, cats, and humans.<sup>52, 55, 57-60</sup> Increased activity of ALT, AST, and LDH is seen with acute hepatocellular damage and is more dramatic with blunt trauma to the liver, reflecting greater hepatocellular damage.<sup>58</sup> Furthermore, ALT and AST have been shown to correlate with the degree of hepatic injury.<sup>56, 57, 59, 60</sup> The serum levels of ALT, AST, and LDH peak within the first 24 hours after hepatic injury and decrease rapidly 4 days post-injury.<sup>55, 58</sup> Hennes *et al*<sup>55</sup> reported a series of 87 pediatric patients with blunt abdominal trauma and found that significantly increased serum hepatic enzyme activities (AST > 450 IU/L and ALT > 250 IU/L) accurately identified children with hepatic injury and reliably correlated with the computed tomography results; therefore, it was concluded that AST > 450 IU/L and ALT > 250 IU/L will accurately diagnose hepatic injury. Ritchie *et al*<sup>54</sup> performed statistical analysis on Hennes *et al*<sup>55</sup> data and data from four other retrospective studies in order to calculate the sensitivity and specificity of AST and ALT as predictors for blunt liver trauma in people; the mean sensitivity was 94.2% (range, 78-100%) and the mean specificity was 88.6% (range, 77-100%). In dogs, cats, and humans, serum ALP has been shown to gradually increase over time following trauma to the liver; <sup>52, 60</sup> however, in a study of experimental liver trauma in rabbits, <sup>56</sup> ALP activity was not increased except in cases of extensive liver damage.

Traumatic liver injury and its effects on biochemical parameters has been poorly studied in birds. Lierz *et al*<sup>61</sup> examined the effects of liver biopsy on blood chemistry in 19 wild raptors. Following liver damage, the activities of GLDH and AST increased dramatically, peaked on day 3, and returned toward baseline. The effects on ALT and ALP were less significant over the 11 day study; ALT had little change and ALP gradually increased from baseline. Therefore, the specific aim of this part of the study was to evaluate changes in plasma hepatic biochemical parameters in acute avian liver disease caused by trauma and to compare biochemical changes with histologic lesions of the hepatic parenchyma.

#### **Materials and Methods**

The study protocol was approved by the University of Georgia's Institutional Animal Care and Use Committee (IACUC No. A2003-10199-0). Thirty flighted, juvenile to young adult Indian ring-necked parakeets (*Psittacula krameri manillensis*) of unknown sex (later determined) were purchased from licensed breeders in Georgia and Florida. The 30 birds were housed in 6 cages (4 cages of 5 birds, 1 cage of 4 birds, and 1 cage of 6 birds) and maintained in a room with an ambient temperature of approximately 73°F at the University of Georgia College of Veterinary Medicine's Animal Resources. They were exposed to cycles of 12 hours of light followed by 12 hours of dark. Their diet consisted of commercial parrot seeds with occasional fruit and millet seeds; water was available *ad libitum*. On arrival, all 30 birds were physically examined by licensed veterinarians and were found to be clinically healthy. The birds acclimatized to the research facility for approximately two months prior to the start of the study.

All 30 birds were to be subjected to phlebotomy with biochemical analysis and endoscopic liver biopsies. Ten of the thirty parakeets were randomly assigned to a group known as the crush group. These birds were to receive addition liver injury via biopsy forceps. The remaining twenty birds that were biopsied but not crushed were called the biopsy group. Postoperation phlebotomy was performed in all 30 birds at various time periods.

On the day of surgery, food was withheld from all parakeets for approximately 12 hours before induction of anesthesia. The birds received Ketoprofen (2 mg/kg, IM) 15-20 minutes prior to induction of anesthesia. Based on the American Society of Anesthesiologists score, each bird was considered Class 1.<sup>62</sup> Anesthesia was induced with 3% to 5% isoflurane<sup>a</sup> in oxygen by use of an over-the-head mask that was connected to a Bickford PC-2 Non-Rebreathing System.<sup>b</sup> After induction, each bird was maintained at a surgical plane of anesthesia via the mask during the time necessary for phlebotomy and surgical preparation. Between 200-400 µl of blood was drawn from the right jugular vein (occasionally the left jugular, left brachial and/or right brachial veins) of all 30 parakeets with a 3-mL syringe and 25-gauge needle as described by Campbell<sup>14(p177)</sup> and placed into a lithium heparin Microtainer®<sup>c</sup> tube. Blood samples were stored on ice during the surgical procedures; plasma was harvested and biochemistry analysis was performed on an automated analyzer<sup>d</sup> at the end of the procedure. The birds were then intubated by use of an uncuffed endotracheal tube (internal diameter, 2 mm) with a Murphy eye.<sup>e</sup> During the surgical procedure, ventilation was assisted by the use of a pressure-limited mechanical ventilator;<sup>f</sup> adjustments were made to maintain an end-tidal CO<sub>2</sub> reading between 35 to 45 mmHg. A surgical depth of anesthesia was maintained in each bird by use of 1% to 3% isoflurane in 100% oxygen (flow rate: 1 L/min). The depth of anesthesia was monitored by evaluating reflexes, end-tidal capnography,<sup>g</sup> cardiac Doppler ultrasonography,<sup>h</sup> and esophageal temperature measurement.<sup>i</sup> The risk of developing hypothermia was minimized by placing water circulating heating pads under each bird and maintaining the surgical room at an ambient temperature of approximately 75°F.

Each parakeet was positioned in right lateral recumbency with the wings secured dorsally and the left leg secured cranially; the surgical site was prepped by plucking the surrounding

feathers and cleansed by alternating with povidone iodine and alcohol (Figure 3.1). A small skin incision was made on the left flank with a No. 15 scalpel blade and the underlying tissue was bluntly dissected with small straight mosquito hemostats until the left caudal air sac was penetrated. A rigid 30°-viewing telescope<sup>j</sup> (2.7 mm X 18mm) housed within a 14.5-F (4.8 mm) operating sheath<sup>j</sup> was introduced through the incision at the junction of the caudal edge of the eighth rib and the flexor cruris medialis muscle.<sup>63</sup> The telescope was connected to an endoscopic video system<sup>1</sup> (xenon light source, camera, and monitor). Endoscopically-guided 1.7 mm scissors<sup>j</sup> were used to gently dissect through the coalescent membranes of the left caudal thoracic air sac and the hepatic peritoneal cavity (HPC). The endoscope was positioned at different angles and depths, as described by Taylor,<sup>63(p331)</sup> in order to evaluate the health of various coelomic structures including the gender of each bird (Table 3.1; Figure 3.2); the organ abnormalities are listed in Tables 3.2 and 3.3. After which, two liver biopsies were collected from the lateral border of each liver by using 1.7 mm biopsy forceps<sup>j</sup> through the instrument channel of the operating sheath (Figure 3.3). Each tissue specimen was gently transferred from the forceps to a biopsy cassette by a moistened cotton-tip applicator and placed in containers with neutral-buffered 10% formalin. Samples were routinely processed, embedded in paraffin blocks, and thin sections placed on slides and stained for microscopic examination. After the biopsies were completed, the birds assigned to the crush group received additional liver injury by positioning 1.6 mm retrieval forceps at 3 random sites and clamping down. Following the surgical procedures, the endoscopic instruments were removed, and the skin and muscle incisions were closed by use of a single, simple interrupted 4-0 Monocryl<sup>TM</sup> suture.<sup>k</sup> Subcutaneous fluids (8 ml of 2.5% dextrose) were administered in the right lateral flank prior to anesthetic recovery on 100% oxygen for approximately five minutes. All surgical instruments

were sterilized by immersion in 2.4% alkaline glutaraldehyde solution<sup>1</sup> for 15 minutes and rinsed with sterile water between each surgical procedure. After recovery from anesthesia, the birds were returned to their enclosures and monitored for any change in behavior, food intake, or wound dehiscence; no abnormalities were noted.

Twelve hours post-operatively, the 10 birds in the crush group were divided into subgroups A and B with 5 birds in each group and the 20 birds in the biopsy group were also divided into subgroups A and B with 10 birds in each group; phlebotomy was performed in both crush and biopsy subgroup A. Twenty-four hours following the surgical procedures, phlebotomy was performed in both crush and biopsy subgroup B. Serial phlebotomy was performed in crush subgroups A and B at alternating 12 hour intervals for a total of 120 hours. Between 200-400 µl of blood was drawn from the right jugular vein (occasionally the left jugular, left brachial and/or right brachial veins) of each bird. Plasma was harvested from the lithium heparin tubes after each time point and analyzed on an automated analyzer<sup>d</sup> within 2 hours of phlebotomy. The 10 biochemical analytes assayed were ALP, ALT, AST, CK, LDH, CHOL, BA, GGT, GLDH, and SDH. Throughout the study, the birds were housed in the same manner as described above. The health of the birds was observed and recorded; only minor abnormalities were noted such as poor feather quality (including broken blood feathers) and hematomas (most consistent with venipuncture).

#### Statistical Analysis

Since the distribution of the data was highly skewed, an appropriate transformation (the natural logarithm denoted by ln) on the response variables was needed to normalize the error terms and stabilize the variance. To determine which variables needed to be transformed, a
comparison was performed for each variable's Q-Q plot of the original scaled variable to that of the transformed variable to identify which scale had a distribution closer to normal. Transformation appeared to be appropriate for ALP, ALT, AST, CK, LDH, BA and CHOL but not for GGT, GLDH, and SDH. Therefore, changes were analyzed in ln(ALP), ln(ALT), ln(AST), ln(CK), ln(LDH), ln(BA), ln(CHOL), GGT, GLDH and SDH before and after liver damage in the crush study.

A Student's *t* test was used to compare effect of gender on measured variables for all 30 birds. To examine the effect of liver biopsy on the 10 variables after 12 hours and 24 hours, a Student's *t* test was performed for the 10 birds in the biopsy group measured at the  $12^{th}$  hour and for the 10 birds in the biopsy group measured at the  $24^{th}$  hour. A Student's *t* test was also used to compare the 10 birds in the crush group to the 20 birds in the biopsy group at the  $12^{th}$  hour and  $24^{th}$  hour. All 10 variables of crush subgroup A and B were separately compared to the baseline data (t=0) by the use of repeated measure ANOVA. Values of p < 0.05 were considered significant.

# Results

Bird handling, anesthesia, surgical procedures, and phlebotomy were all completed and well tolerated with minor complications. One bird in the crush group developed bradycardia which was treated with 0.05 mL of atropine IV. Mild hemorrhage was observed with some of the liver biopsies; however, no medical intervention was necessary.

In the baseline data (t=0) of all 30 birds, the sample quantity was insufficient to determine CHOL in 15 birds, BAs in 7 birds, GLDH in 3 birds, and SDH in 1 bird. 95% confidence intervals (CIs) were established for the means of the 10 variables at baseline (Table

3.4). One bird had a very large SDH value of 66.6 U/L and was identified as an outlier. The distribution of SDH was close to being normal if this outlier was excluded; therefore, the 95% CI for SDH was obtained without this outlier. Gender was compared and determined not to be a significant factor for any of the 10 variables. Because of the ambiguity between immature and inactive reproductive organs, age was not statistically examined.

In the biopsy sample population, the post-operative effect of liver biopsy on the 10 variables at 12 hours and 24 hours were examined for significance. Log-scaled ALP, AST, CK, LDH, CHOL, and original-scaled SDH were significant at the 12<sup>th</sup> hour and log-scaled ALT, AST, CK, LDH, and CHOL were significant at the 24<sup>th</sup> hour. Log-scaled ALP and CHOL decreased significantly and log-scaled ALT, AST, CK, LDH, and original-scaled SDH increased significantly after 12 and 24 hours. In particularly, log-scaled ALP significantly decreased (p-value = 0.0001) by approximately 21 % after 12 hours compared to time 0.

When comparing the biopsy sample population and the crush group, log-scaled LDH increased significantly by approximately 215 % (p = 0.0003) after 12 hours for birds in the crush group only; log-scaled ALT increased significantly after 24 hours. No other variables were statistically significant. Only one bird in the crush group had baseline CHOL measurement; therefore, a comparative analysis was not performed on this variable.

The post-operative data in the crush subgroups A and B were compared to the corresponding baseline data. A separate analysis was performed for the two crush subgroups over time (0-120 hours) because the two groups were not similar to each other with respect to most of the 10 variables; consequently, the means for the 12<sup>th</sup>, 36<sup>th</sup>, 60<sup>th</sup>, 84<sup>th</sup>, and 108<sup>th</sup> hours

were not compared to the means for 24<sup>th</sup>, 48<sup>th</sup>, 72<sup>nd</sup>, 96<sup>th</sup>, and 120<sup>th</sup> hours. The results are listed in Table 3.5.

To examine the trend of each variable over the experimental period of the crush study, the least squares means of the time points were plotted. The plots for crush subgroups A and B were very similar for ln(CK) and ln(LDH), different but with similar patterns for ln(ALP), ln(AST), ln(CHOL), GLDH, and SDH, and quite different for other variables (Table 3.6 and Figures 3.4-3.13). The data at t=24 for subgroup A contained an erroneous BA value of 0.5  $\mu$ mol/L which substantially lowered the mean BA for this group. If this outlier is excluded, the mean BA for subgroup A at 24 hours most likely follows a linear pattern.

Biopsy procedures were successfully performed in all cases, with minimal hemorrhage. The liver biopsy specimens from each parakeet were routinely stained with hematoxylin and eosin (HE) stain (60 sections total). Sections were stained with acid-fast (AF) stain (30 sections total) and Perls' Prussian blue (PPB) stain (26 sections total) when deemed necessary. All sections were examined by light microscopy and scored with either ND (not determined), 0 (normal, 0-5%), 1+ (mild, 5-25%), 2+ (low moderate, 26-50%), 3+ (high moderate, 51-74%), or 4+ (severe,  $\geq$  75%) based on the severity of histological changes. In the HE sections for all 30 birds, crush artifact affected 0-5% of liver sections from 6 birds, 6-25% of liver sections in 13 birds, 26-50% of liver sections in 5 birds, 51-74% of liver sections in 5 birds, and  $\geq$  75% of liver sections in 1 bird. In most cases, the crush artifact was confined to the periphery of the section leaving a sufficient amount of undamaged tissue for evaluation. The microscopic changes observed in all 30 birds included hepatocellular anisocytosis (ND in 26 birds, 0 in 1 bird, 1+ in 3 birds) and anisokaryosis (ND in 1 bird, 0 in 4 birds, 1+ in 18 birds, 2+ in 6 birds, 3+ in 1 bird), hepatic lipidosis (0 in 24 birds, 1+ in 4 birds, 2+ in 1 bird, 4+ in 1 bird), hepatocellular necrosis (0 in 26 birds, 1+ scattered necrosis in 1 bird and focal necrosis in 3 birds), heterophilic hepatitis with variable degrees of lymphocytic or histiocytic infiltrates (0 in 6 birds, 1+ in 20 birds, 2+ in 4 birds), glycogen-laden hepatocytes (0 in 3 birds, 1+ in 14 birds, 2+ in 4 birds, 3+ in 7 birds, 4+ in 2 birds), and iron-laden hepatocytes (0 in 13 birds, 1+ in 7 birds, 2+ in 8 birds, 3+ in 2 birds). In the HE sections for the crush group, crush artifact affected 0-5% of liver sections from 3 birds, 6-25% of liver sections in 6 birds, and 26-50% of liver sections in one bird. The histological changes in the crush group included hepatocellular anisocytosis (ND in 9 birds, 0 in 1 bird) and anisokaryosis (0 in 2 birds, 1+ in 7 birds, 2+ in 1 bird), hepatic lipidosis (0 in 7 birds, 1+ in 1 bird), hepatocellular necrosis (0 in 9 birds, 1+ focal necrosis in 1 bird), heterophilic hepatitis with variable degrees of lymphocytic infiltrates (0 in 1 bird, 1+ in 8 birds, 2+ in 1 bird), glycogen-laden hepatocytes (0 in 4 birds, 1+ in 1 bird, 2+ in 2 birds, 3+ in 5 birds, 4+ in 1 bird), and iron-laden hepatocytes (0 in 4 birds, 1+ in 4 birds, 2+ in 1 bird, 3+ in 5 birds, 4+ in 1 bird), and iron-laden hepatocytes (0 in 4 birds, 1+ in 4 birds, 2+ in 1 bird, 3+ in 1 bird). Neither hepatic fibrosis nor bile duct hyperplasia was observed in any of the liver specimens. The AF stained sections were negative for acid-fast bacteria.

# Discussion

To the author's knowledge, this was the first study conducted in birds that examined the effects of traumatic liver injury via endoscopy on certain biochemical parameters. Liver injury was ensued by an endoscopic biopsy  $\pm$  crush of hepatic parenchyma. Microscopic examination of the liver biopsies indicated normal variance within clinically healthy birds. Biochemical analysis of all 30 birds was performed prior to any liver damage; a 95% confidence interval for all 10 variables was established. In this study, gender was found not to have an effect on any of the variables.

Biochemical analysis of the biopsy sample population revealed a highly significant decrease in ALP at the 12<sup>th</sup> hour; however, the reason for the hypophosphatasemia at this time point is unknown. Interestingly, ALP was not significantly different at 24 hours after surgery. The mean CHOL was decreased at the 12<sup>th</sup> and 24<sup>th</sup> hours without a change in BA. Since BA is considered sensitive and specific for liver disease,<sup>31, 33</sup> a decrease in cholesterol is mostly likely secondary to a non-hepatic reason such as decreased dietary intake.<sup>29</sup> Other significant biochemical abnormalities included increased mean activities of ALT (t=24), AST (t=12, 24), LDH (t=12, 24), SDH (t=12) and CK (t=12, 24). The increase in SDH 12 hours after liver damage with a rapid return towards baseline supports its liver specificity and short elimination half-life.<sup>18</sup> The increased activities of AST, LDH, and CK at 12 and 24 hours after surgery are most consistent with muscle damage associated with surgical manipulation and handling.

Comparing the biochemical analysis of the biopsy sample population vs. the crush group, a significant increase in the means of LDH at the 12<sup>th</sup> hour and ALT at the 24<sup>th</sup> hour was observed in the crush group only. Neither one of these biomarkers is liver specific and muscle injury is the primary consideration for the increased activity of these two enzymes.<sup>18, 29-31</sup> In both groups, mean CK was significantly increased and further supports muscle damage as the cause of increased LDH and ALT.

The crush group's data was examined and compared to the baseline values. All variables were significant at least at one time point during the study.

With one exception, ALP activities were consistently decreased after surgery in the crush group. Although the specific mechanism of the decreased ALP activity in the birds of this group is uncertain, a significant decrease in ALP activity has been reported with isoflurane

administration in humans.<sup>64</sup> However, a few other reports in humans<sup>65, 66</sup> as well as in dogs,<sup>67</sup> cats,<sup>68</sup> goats,<sup>69</sup> rabbits,<sup>70</sup> and American kestrels (*Falco sparverius*)<sup>71</sup> have not shown a significant change in ALP activity associated with isoflurane anesthesia. The decrease in ALP activity in people due to anesthesia is speculated to be the result of decreased induction from non-hepatic organs.<sup>64</sup> Since the liver was deliberately injured during surgery, ALP activity may have also decreased with the loss of hepatocytes, but this effect, if it did occur, is probably minimal given the number of hepatocytes affected.

The activities of AST, ALT, and LDH were significantly increased in the crush group; however, there was always a concurrent increase in CK activity. In birds, these enzymes (AST, ALT, and LDH) have a broad tissue distribution with the highest concentration of AST and ALT in the skeletal muscle, liver, and kidneys and LDH in the skeletal muscle, liver, kidneys, and brain; therefore, these enzymes can be increased with non-hepatic disease.<sup>17, 33, 72</sup> CK is specific and sensitivity for muscle damage in birds and thus, should be examined simultaneously with the other enzymes. The increased activities of AST, ALT, and LDH in the crush group were most likely the result of muscle damage rather than hepatic disease since there was concurrent increased activity of CK without significant change in the other variables measured.

Twenty-four hours post-operatively, a significant decrease in mean CHOL was noted in the crush group; however, the results were skewed due to the lack of one sample at this time point (n=4). The amount of sample was insufficient from multiple birds at numerous time points (t=0, 48, 84, 96, 108, and 120) and resulted in unreliable data with low statistical power. Even when considering the effects of low sample numbers, CHOL concentration did not appear to change substantially throughout the study and its concentration was likely unaffected by liver injury.

Bile acid concentrations were not significantly increased at any time after endoscopy in the crush group. The reference range of bile acids for subgroup B at 48 hours was based on one transformed value and therefore, was highly skewed due to the lack of sufficient samples. The lack of significant change in BA concentration was most likely a result of insufficient damage to functional hepatocytes. In other words, the crush injury applied was not extensive enough to cause a > 70% reduction in functional hepatocytes necessary for observation of a significant increase in bile acid concentration.<sup>17, 18, 27</sup> Instead, the mild changes in BA concentration from t=0 (fasted sample) to t=120 were most likely due to postprandial effects.<sup>18, 48</sup>

Increased activity of GGT showed significance at four time points (t=24, 60, 84, and 96) in the crush group. The graph of ln(GGT) indicated an initial trend of an early and substantial increase in GGT activity followed by a slow decline toward baseline. The significance of mean GGT in subgroup B at t=96 is questionable since there was an extreme outliner. If the outlier is excluded, the trend of mean GGT would have most likely followed a pattern of gradual decline. Usually GGT activity increases with biliary hyperplasia or cholestasis in domestic animals.<sup>32</sup> However, in this study, neither biliary hyperplasia nor cholestasis was observed microscopically. Therefore, the cause of the initial increase in GGT activity cannot be explained by theses mechanisms.

At only one time point (t=24) and in only one subgroup (B) was the mean GLDH significantly different from baseline. Focally extensive hepatocellular necrosis resulting from surgery may be the reason for the significant increase in GLDH. This enzyme has an extremely short biological half-life (< 1 hour) in racing pigeons.<sup>26</sup>. However, in Lierz *et al*,<sup>61</sup> GLDH peaked at 72 hours in wild raptors after liver injury. The subgroup B population was not examined at t=12 hours and therefore, may have demonstrated a more dramatic increase.

A markedly significant increase in mean SDH was observed at 12 hours after liver injury, but quickly returned to baseline at subsequent time points. This pattern of SDH activity is consistent with its biological half-life (< 12 hours) and suggests acute hepatocellular injury as the cause.<sup>18</sup> Interestingly, a significant decrease was noted at 48 hours; however, the reason for this decrease is unclear. Typically, decreases in hepatic enzymes do not have clinical relevance except in the cases of end-stage liver disease. Obviously, end-stage liver disease as a cause of decreased SDH in this study is unlikely.

# **Sources and Manufacturers**

- a. IsoFlo, Abbott Laboratories, North Chicago, IL
- b. Bickford PC-2B Non-Rebreathing System, A.M. Bickford Inc., Wales Center, NY
- c. Microtainer, Becton Dickinson, Franklin Lakes, NJ
- d. Hitachi 912 Chemistry Analyzer, Roche Diagnostics, Indianapolis, IN
- e. Rüsch Inc., Duluth, GA
- f. VT-5000, BAS Vetronics, Bioanalytical Systems Inc., West Lafayette, IN
- g. ETCO<sub>2</sub>/SpO<sub>2</sub> monitor, CO<sub>2</sub> SMO, Novametrix Medical Systems, Wallingford, CT
- h. Ultrasonic Doppler, Parks Electric Laboratory, Aloha, OR
- i. Precision Thermometer, Tandy, Fort Worth, TX
- j. 64018BSA, 67065C, 67161Z, 67161T, 69235106, 9219-B, 201320-20, 62501EK, Karl Storz Veterinary Endoscopy America Inc., Goleta, CA
- k. Monocryl, Ethicon, Somerville, NJ
- 1. CIDEX, Advanced Sterilization Products, Irvine, CA

Table 3.1	Condor	determination	n via and	locoonio	overminetion
Table 3.1	Genuer	ueterminatio	li via enu	loscopic	examination.

	Active reproductive organs	Inactive/underdeveloped reproductive organs
Crush group (n=10)	3 M	<b>4 M</b>
	<b>2</b> F	1 F
Biopsy sample population	5 M	1 M
(n=20)	<b>4</b> F	<b>0 F</b>
Total population (n=30)	15 M	5 M
	<b>9</b> F	1 F

 Table 3.2 Systemic abnormalities via endoscopic examination in crush group.

**Splenomegaly**<sup>a</sup>

<sup>a</sup>Identified in two birds

# Table 3.3 Systemic abnormalities via endoscopic examination in biopsy sample population.<sup>a</sup>

# **Mottled spleen**<sup>b</sup>

**Liver discoloration** (yellow)<sup>b,c</sup>

<sup>a</sup>Biopsy sample population (n=20) was a randomly assigned subset of the total parakeet population (n=30).

<sup>b</sup>Identified in one bird

<sup>c</sup>No hepatic abnormalities were noted via histopathology; however, the quality of the biopsy was poor.

Variables	95% Confidence Interval	Units
$ALP^1$	65.40-102.02	U/L
ALT <sup>2</sup>	8.32-13.78	U/L
AST <sup>3</sup>	181.56-282.49	U/L
CK <sup>4</sup>	681.63-981.09	U/L
LDH <sup>5</sup>	72.86-102.63	U/L
CHOL <sup>6,a</sup>	306.96-367.02	mg/dl
$BA^{7,b}$	13.87-30.37	µmol/L
GGT <sup>8</sup>	3.01-4.57	U/L
GLDH <sup>9,c</sup>	1.29-2.15	U/L
SDH <sup>10,d</sup>	7.21-11.94	U/L

Table 3.4 Biochemical analysis at time 0 for fasted Indian ring-necked parakeets (n=30).<sup>a-d</sup>

an=15

<sup>b</sup>n=23

 $^{c}n=27$ 

 $^{d}n=28$ 

 $^{1}ALP = Alkaline Phosphatase$ 

 $^{2}$ ALT = Alanine Aminotransferase

 $^{3}AST = Aspartate Aminotransferase$ 

 ${}^{4}CK = Creatinine Kinase$ 

<sup>5</sup>LDH = Lactate Dehydrogenase

 $^{6}$ CHOL = Cholesterol

 $^{7}BA = Bile Acid$ 

<sup>8</sup>GGT = Gamma-Glutamyl Transferase

<sup>9</sup>GLDH = Glutamate Dehydrogenase

<sup>10</sup>SDH = Sorbitol Dehydrogenase

# Table 3.5 Biochemical analysis of crush treatment group taken over time (0-120 hours) forIndian ring-necked parakeets.

<sup>1</sup>Abbreviations: ALP = Alkaline Phosphatase; ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase; CK = Creatinine Kinase; LDH = Lactate Dehydrogenase; CHOL = Cholesterol; BA = Bile Acid; GGT = Gamma-Glutamyl Transferase; GLDH = Glutamate Dehydrogenase; SDH = Sorbitol Dehydrogenase

<sup>2</sup>Units: ALP (U/L), ALT (U/L), AST (U/L), CK (U/L), LDH (U/L), CHOL (mg/dl), BA (µmol/L), GLDH (U/L), SDH (U/L)

<sup>a</sup>Mean

<sup>b</sup>95% confidence interval

<sup>c</sup>QNS = quantity not sufficient

<sup>d</sup>This result may be unreliable due to several missing baseline values.

\*Indicates a significant difference versus baseline (p < 0.05)

		Variables									
Time	Subgroup	ALP <sup>1,2</sup>	ALT	AST	СК	LDH	CHOL	BA	GGT	GLDH	SDH
0	Α	186.25 <sup>a</sup> (101.35-342.24) <sup>b</sup>	8.02 (4.33-14.86)	219.47 (144.78-332.69)	772.78 (535.55-1114.99)	64.64 (35.36-118.16)	QNS <sup>c</sup>	21.19 (11.25-39.90)	3.0 (0.90-5.10)	0.85 (0.05-2.67)	4.88 (1.97-9.09)
U	В	57.93 (50.78-66.08)	14.0 (7.90-24.66)	306.95 (225.02-418.68)	531.66 (345.47-818.19)	81.0 (56.05-117.07)	370.48 (285.97-479.96)	25.34 (7.82-82.08)	5.36 (2.21-8.51)	0.67 (0.14-1.57)	10.35 (5.32-17.04)
12	Α	131.47* (71.55-241.58)	13.70 (7.39-25.37)	511.83* (337.68-775.88)	4541.0* (3146.98-6552.49)	251.39* (137.52-459.53)	230.49 (205.41-258.63)	24.96 (16.59-37.56)	4.24 (2.14-6.34)	1.63 (0.66-3.02)	20.87* (14.16-28.89)
24	В	46.94* (41.14-53.54)	19.90 (11.26-35.15)	663.28* (486.29-904.79)	3549.28* (2306.30-5461.62)	148.84* (102.99-215.12)	271.89 <sup>d</sup> ,* (237.89-310.78)	9.21 (3.59-23.67)	7.48* (4.33-10.63)	2.19* (1.09-3.67)	9.0 (4.78-14.56)
36	Α	122.62* (66.73-225.32)	14.47 (7.81-26.79)	456.73* (301.30-692.36)	2308.61* (1599.91-3331.24)	101.30 (55.46-185.32)	263.35 (234.70-295.51)	26.74 (17.77-40.24)	3.88 (1.78-5.98)	1.14 (0.36-2.34)	2.0 (0.37-4.94)
48	В	51.36 (45.02-58.59)	12.20 (6.91-21.56)	496.86* (364.27-677.76)	1994.60* (1296.08-3069.59)	81.43 (56.34-117.68)	299.92 (231.78-388.08)	42.17 (6.05-294.04)	6.80 (3.65-9.95)	1.29 (0.49-2.48)	1.65* (0.22-4.41)
60	Α	134.12* (72.98-246.44)	12.10 (6.53-22.41)	257.01 (169.54-389.59)	1395.63* (967.29-2013.84)	73.64 (40.29-134.61)	265.10 (236.25-297.47)	39.35 (26.15-50.22)	5.44* (3.34-7.54)	0.40 (0.03-1.20)	6.82 (3.26-11.68)
72	В	47.30* (41.46-53.96)	12.10 (6.82-21.30)	383.29 (280.98-522.80)	1327.70* (862.74-2043.26)	53.51 (37.02-77.34)	293.54 (260.21-331.09)	31.47 (12.25-80.84)	5.82 (2.67-8.97)	0.77 (0.19-1.73)	5.82 (2.55-10.41)
84	Α	143.28* (77.97-263.28)	23.82* (12.86-44.12)	196.45 (129.59-297.76)	1071.81* (742.78-1546.58)	47.70 (26.09-87.19)	284.69 (247.42-327.54)	28.53 (18.96-42.93)	5.52* (3.42-7.62)	0.63 (0.11-1.58)	5.6 (2.43-10.06)
96	В	50.90* (44.62-58.07)	9.94 (5.62-17.56)	370.30 (271.48-505.12)	1054.48* (685.19-1622.79)	49.43* (34.20-71.45)	317.67 (263.91-382.41)	28.0 (9.92-79.03)	7.76* (4.52-11.0)	0.70 (0.12-1.76)	9.10 (4.85-14.68)
108	Α	145.40* (79.13-267.20)	13.11 (7.08-24.28)	209.33 (138.09-317.28)	843.87 (584.82-1217.68)	51.24 (28.03-93.66)	QNS	26.69 (15.85-44.93)	4.72 (2.62-6.82)	0.10 (0.04-0.70)	4.64 (1.81-8.76)
120	В	47.41* (41.56-54.09)	8.48 (4.80-14.98)	288.16 (211.26-393.07)	812.32* (527.90-1250.13)	44.76* (30.97-64.70)	304.42 (261.26-354.71)	33.33 (12.98-85.63)	5.40 (2.25-8.55)	0.72 (0.17-1.65)	6.36 (2.91-11.13)

 Table 3.5 Biochemical analysis of crush treatment group taken over time (0-120 hours) for Indian ring-necked parakeets.

Variable	Subgroup A	Subgroup B
ln(ALP)	Decreases during $0 \le t^a \le 12$ and increases gradually during $12 < t \le 108$	Decreases $0 \le t \le 24$ and does not change significantly during $24 < t \le 120$
ln(ALT)	No significant change during $0 \le t \le 108$	No significant change during $0 \le t \le 120$
ln(AST)	Increases during $0 \le t \le 12$ , deceases during $12 < t \le 60$ , and does not change significantly during $60 < t \le 108$ .	Increases during $0 \le t \le 24$ , deceases during $24 < t \le 72$ , and does not change significantly during $72 < t \le 120$ .
ln(CK)	Increases during $0 \le t \le 12$ and decrease gradually during $12 < t \le 108$ ; there is no significant difference between t=0 and t $\ge 84$	Increases during $0 \le t \le 24$ and decrease gradually during 24< t $\le 120$ ; there is no significant difference between t=0 and t=120
ln(LDH)	Increases during $0 \le t \le 12$ , decrease during $12 < t \le 36$ , and does not change significantly during $36 < t \le 108$	Increases during $0 \le t \le 24$ , decrease during $24 < t \le 48$ , and does not change significantly during $48 < t \le 120$
ln(BA)	Does not change significantly during $0 \le t$ $\le 108$	Does not change significantly during $0 \le t \le 120$
ln(CHOL)	Increases during $12 \le t \le 36$ , and does not change significantly during $36 < t \le 108$	Does not change significantly during $0 \le t \le 120$
GGT	Does not change significantly during $0 \le t$ $\le 108$	Does not change significantly during $0 \le t \le 120$
GLDH	Does not change significantly during $0 \le t$ $\le 108$	Does not change significantly during $0 \le t \le 120$
SDH	Decreases during $0 \le t \le 12$ , decreases during $12 < t \le 36$ , and does not change significantly during $36 < t \le 108$	Does not change significantly during $0 \le t \le 120$

Table 3.6 Trend of each variable in crush treatment group over time (0-120 hours) for Indian ring-necked parakeets.

at = time in hours



Figure 3.1 Left flank approach for endoscopic evaluation of a bird with surgical preparation.



**Figure 3.2 Representative views obtained during an endoscopic examination of the reproductive tract.** A—immature/inactive ovary (o), left adrenal gland (a), and cranial pole of the left kidney (k). B—immature/inactive testicle (t), left adrenal gland (a), and cranial pole of the left kidney (k).



**Figure 3.3 Representative views obtained during an endoscopic liver biopsy procedure.** A—left liver lobe (l), membrane separating the cranial and caudal thoracic air sacs (a), proventriculus (p), and lung (lu). B—caudal edge of the left liver lobe (l) during biopsy specimen collection by use of 1.7 mm biopsy forceps (b) and confluent membranes of the left caudal thoracic air sac and the hepatic peritoneal cavity.



**Figure 3.4 Estimate of ln(ALP) for crush subgroups A and B over time (0-120 hours).** \*Indicates a significant difference versus baseline (p < 0.05)



\*Indicates a significant difference versus baseline (p < 0.05)



\*Indicates a significant difference versus baseline (p < 0.05)



Figure 3.7 Estimate of ln(CK) for crush subgroups A and B over time (0-120 hours). \*Indicates a significant difference versus baseline (p < 0.05)



**Figure 3.8 Estimate of ln(LDH) for crush subgroups A and B over time (0-120 hours).** \*Indicates a significant difference versus baseline (p < 0.05)



**Figure 3.9 Estimate of ln(CHOL) for crush subgroups A and B over time (0-120 hours).** \*Indicates a significant difference versus baseline (p < 0.05)



Figure 3.10 Estimate of ln(BA) for crush subgroups A and B over time (0-120 hours).



**Figure 3.11 Estimate of GGT for crush subgroups A and B over time (0-120 hours).** \*Indicates a significant difference versus baseline (p < 0.05)



**Figure 3.12 Estimate of GLDH for crush subgroups A and B over time (0-120 hours).** \*Indicates a significant difference versus baseline (p < 0.05)



**Figure 3.13 Estimate of SDH for crush subgroups A and B over time (0-120 hours).** \*Indicates a significant difference versus baseline (p < 0.05)

# CHAPTER 4

#### AFLATOXICOSIS

Aflatoxins (AFs) are highly toxic metabolites produced by *Aspergillus flavus*, *A*. *parasiticus*, *A. nomius*, and *Penicillium puberulum*.<sup>73,74</sup> *A. flavus* is the most common contaminants of many grains,<sup>75</sup> especially peanuts, peanut products, cotton seed, groundnuts, groundnut meal, and corn.<sup>76,77</sup> In 1961, over 100,000 turkey poults suddenly died from a suspicious disease, known as turkey X disease. Aflatoxins, from *A. flavus*, were identified as the cause of death.<sup>78,79</sup> In 1955, fatal hepatitis was observed in dogs and was temporarily diagnosed as hepatitis X; however, after the turkey X outbreak, it has now been linked to aflatoxin contaminated peanut meal in the diet.<sup>79,80</sup> In response, the United States Food and Drug Administration has set a tolerance limit of 20 parts per billion for corn, peanut products, cottonseed meal, and other animal feeds and feed ingredients.<sup>77,79</sup>

The production of AF is dependent on several factors which include high humidity ( $\geq$  85%),<sup>81-83</sup> high temperature (25-30 °C),<sup>81-83</sup> and moisture content of the substrate (18% for starchy cereal grains and 9-10% for oil-rich nuts/seeds).<sup>81</sup> Seventeen AFs have been identified, but only four (B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, and G<sub>2</sub>) fractions have been extensively examined.<sup>80, 81</sup> The designated letters represents their blue and green fluorescence in ultraviolent light.<sup>73, 81</sup> Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is the most acutely toxic to numerous species of animals and is considered a potent hepatotoxin and hepatocarcinogen.<sup>73, 75, 81</sup>

Animals are exposed to AFB<sub>1</sub> mainly by ingestion of contaminated feed;<sup>84</sup> however, inhalation<sup>85</sup> and direct contact<sup>76, 86</sup> have also been reported. A contamination rate of 1 ppm of AFB<sub>1</sub> is the most commonly reported contamination rate in commercial poultry rations.<sup>87</sup> The cytochrome P450 oxidative system in the liver converts  $AFB_1$  into a flatoxin  $M_1$  (AFM<sub>1</sub>) and other metabolites.<sup>88</sup> Aflatoxin M<sub>1</sub> is excreted in the urine, feces, and milk and is considered almost as potent as AFB<sub>1</sub>.<sup>81,88</sup> Although other organs (bursa of Fabricius, intestines, pancreas, heart, and spleen) may be affected with toxic exposure, the liver is the target organ for the toxic effects of AFB<sub>1</sub>; therefore, many functions of the liver such as protein synthesis can be impaired.<sup>81</sup> As a result, animals can present with neurologic signs (ataxia, convulsions, and opisthotonus) or with more nonspecific signs such as depression, ascites, inappetance, feather picking, reduced egg production, infertility, lameness, weight loss, and death.<sup>73, 76, 80, 83, 89, 90</sup> The extent of these clinical signs varies depending on the amount of toxin exposure and health and nutritional status, age, breed, and/or gender of the animal. Goslings, turkey poults, and especially ducklings are considered the most susceptible to aflatoxin exposure as opposed to chickens, bobwhite quail, Japanese quail, and the female rat being the least susceptible.<sup>73, 76, 81</sup> Birds with poor diets are more susceptible to lower doses of aflatoxins.<sup>76</sup>

Gross lesions associated with AFB<sub>1</sub> may include atrophy (initially) or enlargement of the liver, enlargement of the spleen, kidney, and/or pancreas, atrophy of the bursa of Fabricius, and/or reduction in body fat deposition.<sup>77, 81, 91</sup> A red or yellow discoloration of the liver is usually evident and may result from congestion or fatty infiltration.<sup>73, 76</sup> Microscopic lesions associated with the liver can vary depending on chronicity, but are fairly consistent between species. Acute aflatoxicosis may result in venous congestion, anisocytosis, anisokaryosis, hepatic lipidosis, and severe periportal hepatocellular necrosis with focal nodular degeneration

and biliary proliferation.<sup>73, 80</sup> Hepatic fibrosis (mild in turkeys), hepatic lipidosis, anisokaryosis, diffuse biliary hyperplasia, nodular regeneration, lymphocytic hyperplasia, hepatoma or hepatocellular carcinoma, and reticuloendothelial cell hyperplasia are more common with chronic cases of aflatoxicosis.<sup>73, 80, 83</sup>

Because of the multi-organ involvement (in particularly the liver) with aflatoxicosis, numerous hematological and biochemical changes have been observed in the bird. Hemolytic anemia with bone marrow hyperplasia is possible and may result from increased destruction of erythrocytes and/or inhibition or defection of hematopoiesis.<sup>73, 90, 91</sup> Leukocytosis characterized by an immature heterophilia and a lymphopenia is often present.<sup>73, 92</sup> The lymphopenia is due to immunosuppression from the regression of the bursa and thymus and can lead to secondary infections and efflux of leukocytes.<sup>92, 93</sup> Unfortunately, in the face of AFB<sub>1</sub>, the bactericidal properties of heterophils are impaired.<sup>92</sup> The mechanism is unclear but thought to be a dysfunction of complement.<sup>94</sup> A prolonged clotting time can be observed and most likely due to the impaired synthesis of clotting factors II and VII in the liver.<sup>81</sup>

A decrease in serum total protein, albumin, triglycerides, and amylase can be seen and is directly attributed to the hepatotoxic effects of  $AFB_1$ .<sup>73, 91, 95-97</sup> Serum triglycerides is considered a sensitive indicator of hepatic dysfunction and can provide information concerning recovery from intoxication.<sup>98</sup> Pancreatic damage is also seen with aflatoxicosis and may result in an increase in amylase activity; however, Balachandran *et al*<sup>95</sup> believes the hepatic injury outweighs the damage to the pancreas and thus results in an overriding increase in amylase activity. The concentration of serum cholesterol is dose-dependent and therefore may be increased<sup>98</sup> or decrease<sup>91, 97</sup> with aflatoxin exposure. Serum calcium and phosphorus levels have been observed to significantly decrease due to inhibition of calcium absorption<sup>97</sup> and any increase in these

minerals should be considered a good prognostic indicator of recovery. <sup>98</sup> The serum activity of ALT and AST is inconsistently affected by AFB<sub>1</sub> and may reflect AF dose-dependency.<sup>82, 91, 95, 97.99</sup> An increase in GGT and ALP activity during aflatoxicosis have been observed and is result of enzyme induction from cholestasis and/or biliary hyperplasia.<sup>29, 90, 97, 98, 100</sup> Furthermore, Fernandez *et al*<sup>98</sup> reported that the increase in GGT was directly proportional to the dose of AF, duration of treatment, and severity of biliary hyperplasia. Activity of LDH in the serum has been found to be inconsistent and may increase<sup>87</sup> or decrease<sup>73, 98</sup> after AF treatment.<sup>91</sup> Serum SDH and GLDH also have inconsistent fluctuations in their activity.<sup>73, 75</sup>

Aflatoxicosis has been extensively studied in numerous species, in particularly gallinaceous birds; however, information concerning its hepatoxic effects in companion birds is lacking. Therefore, the purpose of this specific aim was to mimic the most common feed contamination dose of 1 ppm in order to evaluate changes in plasma hepatic biochemical parameters in acute avian liver disease caused by AFB<sub>1</sub> and to compare biochemical changes with microscopic lesions of the hepatic parenchyma.

### **Materials and Methods**

The study protocol was approved by the University of Georgia's Institutional Animal Care and Use Committee (IACUC No. A2003-10199-0). Thirty flighted, juvenile to young adult Indian ring-necked parakeets (*Psittacula krameri manillensis*) of unknown sex (later determined) were purchased from licensed breeders in Georgia and Florida. The 30 birds were housed in 6 cages (4 cages of 5 birds, 1 cage of 4 birds, and 1 cage of 6 birds) and maintained in a room with an ambient temperature of approximately 73°F at the University of Georgia College of Veterinary Medicine's Animal Resources. They were exposed to cycles of 12 hours of light

followed by 12 hours of dark. Their diet consisted of commercial parrot seeds with occasional fruit and millet seeds; water was available *ad libitum*. On arrival, all 30 birds were physically examined by licensed veterinarians and were found to be clinically healthy. The birds acclimatized to the research facility for approximately two months prior to the start of the study.

All 30 birds were to be subjected to phlebotomy with biochemical analysis and endoscopic liver biopsies. Ten of the thirty parakeets were randomly assigned to a group known as the aflatoxin group. No further data was collected from the aflatoxin group at this time.

On the day of surgery, food was withheld from all parakeets for approximately 12 hours before induction of anesthesia. The birds received Ketoprofen (2 mg/kg, IM) 15-20 minutes prior to induction of anesthesia. Based on the American Society of Anesthesiologists score, each bird was considered Class 1.<sup>62</sup> Anesthesia was induced with 3% to 5% isoflurane<sup>a</sup> in oxygen by use of an over-the-head mask that was connected to a Bickford PC-2 Non-Rebreathing System.<sup>b</sup> After induction, each bird was maintained at a surgical plane of anesthesia via the mask during the time necessary for phlebotomy and surgical preparation. Between 200-400 µl of blood was drawn from the right jugular vein (occasionally the left jugular, left brachial and/or right brachial veins) of all 30 parakeets with a 3-mL syringe and 25-gauge needle as described by Campbell<sup>14(p177)</sup> and placed into a lithium heparin Microtainer®<sup>c</sup> tube. Blood samples were stored on ice during the surgical procedures; plasma was harvested and biochemistry analysis was performed on an automated analyzer<sup>d</sup> at the end of the procedure. The birds were then intubated by use of an uncuffed endotracheal tube (internal diameter, 2 mm) with a Murphy eye.<sup>e</sup> During the surgical procedure, ventilation was assisted by the use of a pressure-limited mechanical ventilator;<sup>f</sup> adjustments were made to maintain an end-tidal CO<sub>2</sub> reading between 35 to 45 mmHg. A surgical depth of anesthesia was maintained in each bird by use of 1% to 3%

isoflurane in 100% oxygen (flow rate: 1 L/min). The depth of anesthesia was monitored by evaluating reflexes, end-tidal capnography,<sup>g</sup> cardiac Doppler ultrasonography,<sup>h</sup> and esophageal temperature measurement.<sup>i</sup> The risk of developing hypothermia was minimized by placing water circulating heating pads under each bird and maintaining the surgical room at an ambient temperature of approximately 75°F.

Each parakeet was positioned in right lateral recumbency with the wings secured dorsally and the left leg secured cranially; the surgical site was prepped by plucking the surrounding feathers and cleansed by alternating with povidone iodine and alcohol (refer to Figure 3.1). A small skin incision was made on the left flank with a No. 15 scalpel blade and the underlying tissue was bluntly dissected with small straight mosquito hemostats until the left caudal thoracic air sac was penetrated. A rigid 30°-viewing telescope<sup>j</sup> (2.7 mm X 18mm) housed within a 14.5-F (4.8 mm) operating sheath<sup>j</sup> was introduced through the incision at the junction of the caudal edge of the eighth rib and the flexor cruris medialis muscle.<sup>63</sup> The telescope was connected to an endoscopic video system<sup>1</sup> (xenon light source, camera, and monitor). Endoscopically-guided 1.7 mm scissors<sup>j</sup> were used to gently dissect through the coalescent membranes of the left caudal thoracic air sac and the hepatic peritoneal cavity (HPC). The endoscope was positioned at different angles and depths, as described by Taylor, <sup>63(p331)</sup> in order to evaluate the health of various coelomic structures including the gender of each bird (refer to Figure 3.2); the organ abnormalities and genders are listed in Tables 4.1 and 4.2. After which, two liver biopsies were collected from the lateral border of each liver by using 1.7 mm biopsy forceps<sup>j</sup> through the instrument channel of the operating sheath (refer to Figure 3.3). Each tissue specimen was gently transferred from the forceps to a biopsy cassette by a moistened cotton-tip applicator and placed in containers with neutral-buffered 10% formalin. Samples were routinely processed,

embedded in paraffin blocks, and thin sections placed on slides and stained for microscopic examination. Following the surgical procedures, the endoscopic instruments were removed, and the skin and muscle incisions were closed by use of a single, simple interrupted 4-0 MonocryI<sup>TM</sup> suture.<sup>k</sup> Subcutaneous fluids (8 ml of 2.5% dextrose) were administered in the right lateral flank prior to anesthetic recovery on 100% oxygen for approximately five minutes. All surgical instruments were sterilized by immersion in 2.4% alkaline glutaraldehyde solution<sup>1</sup> for 15 minutes and rinsed with sterile water between each surgical procedure. After recovery from anesthesia, the birds were returned to their enclosures and monitored for any change in behavior, food intake, or wound dehiscence; no abnormalities were noted.

Approximately two months following the initial liver biopsies, the birds were subdivided into groups A and B with 5 birds in each group. On day 0 of the aflatoxin study, weight was recorded and phlebotomy was performed on all 10 birds (Tables 4.3 and 4.4). Serial phlebotomy was performed for aflatoxin subgroups A and B on alternating days for a total of 10 days (Table 4.4). Between 200-400 µl of blood was drawn from the right jugular vein (occasionally the left jugular, left brachial and/or right brachial veins) of birds. The plasma was harvested from the lithium heparin tubes after each time point and analyzed on an automated analyzer<sup>d</sup> within 2 hours of phlebotomy. The 10 biochemical parameters analyzed were ALP, ALT, AST, CK, LDH, CHOL, BA, GGT, GLDH, and SDH.

The aflatoxin used in this study was produced through the fermentation of rice by *Aspergillus parasiticus* NRRL 2999 according to the method of West *et al.*<sup>101(p1018)</sup> The fermented rice was dried and ground to a fine powder and analyzed for the presences of AFB<sub>1</sub>. Each bird was orally gavaged with 0.30 mg of AFB<sub>1</sub>/gram of rice at 1 mg/kg of body weight on days 1-5.

Throughout the study, the birds were housed in the same manner as previously described. The health of the birds was observed and recorded each day. The birds in subgroup A and B were weighed on day 5 and 6, respectively (Table 4.3). On day 22, liver biopsies via coelioscopies were repeated on 9 birds by the previously described method; systemic abnormalities are listed in Table 4.1.

#### Statistical Analysis

Since the distribution of the data was highly skewed, an appropriate transformation (the natural logarithm denoted by ln) on the response variables was needed to normalize the error terms and stabilize the variance. To determine which variables needed to be transformed, a comparison was performed for each variable's Q-Q plot of the original scaled variable to that of the transformed variable to identify which scale had a distribution closer to normal. Transformation appeared to be appropriate for ALP, ALT, AST, CK, LDH, BA and CHOL but not for GGT, GLDH, and SDH. Therefore, changes were analyzed in ln(ALP), ln(ALT), ln(AST), ln(CK), ln(LDH), ln(BA), ln(CHOL), GGT, GLDH and SDH before and after liver damage in the aflatoxin study. All 10 variables of aflatoxin subgroup A and B were separately compared to the baseline data (t=0) by the use of repeated measure ANOVA. Values of p < 0.05 were considered significant.

#### Results

Bird handling, anesthesia, surgical procedures, and phlebotomy were all completed and well tolerated with minimal complications. Mild hemorrhage was observed at the site of some of the liver biopsies; however, no medical intervention was necessary. More severe abnormalities were noted during treatment with AFB<sub>1</sub>; general abnormalities included hematomas (most

consistent with venipuncture), depressed mentation, regurgitation, yellow-green urates, and weight loss (Table 4.3). One bird in subgroup B showed signs of disease with blood-tinged mucus on gavage needle, depressed mentation, ruffled feathers, prominent keel, enlarged liver, and ultimately, death on day 5 of the study. The bird's weight was recorded and a necropsy with histopathology was performed. On microscopic examination, AFB<sub>1</sub> effects were present in the liver; however, the proximate cause of death was bacteremia/septicemia.

The post-treatment data in the aflatoxin subgroups A and B was compared to its corresponding baseline data. A separate analysis was performed for the two aflatoxin subgroups over time (0-10 days) because the two groups were not similar to each other with respect to most of the 10 variables; consequently, estimates for days 1, 3, 4, 5, 7, and 9 were not compared to estimates for days 2, 4, 6, 8, and 10. The results are listed in Table 4.4.

To examine the trend of each variable over the experimental period of the aflatoxin study, the least squares means of the time points were plotted. The plots for aflatoxin subgroups A and B were different but with similar patterns for ln(ALP), ln(CK), and GLDH and quite different for other variables (Table 4.5; Figures 4.1-4.10). In Figure 4.10, the ln(SDH) value for subgroup B on the 4<sup>th</sup> day was substantially increased but not statistically relevant (p=0.0651). The lack of significance is most likely the result of small sample size (n=5) and one highly skewed value.

Biopsy procedures were successfully performed in all cases, with minimal hemorrhage. The liver biopsy specimens from each parakeet were routinely stained with hematoxylin and eosin (HE) stain (38 sections total). Sections were stained with acid-fast(AF) stain (10 sections total) and Perls' Prussian blue (PPB) stain (6 sections total) when deemed necessary. All sections were examined by light microscopy and scored with either ND (not determined), 0

(normal, 0-5%), 1+ (mild, 5-25%), 2+ (low moderate, 26-50%), 3+ (high moderate, 51-74%), or 4+ (severe,  $\geq 75\%$ ) based on the severity of histological changes. In the HE sections of the 10 birds prior to treatment with AFB<sub>1</sub>, crush artifact affected 0-5% of liver sections from 3 birds, 6-25% of liver sections in 3 birds, 51-74% of liver sections in 3 birds, and > 75% of liver sections in one bird. The histological changes in the 10 birds prior to treatment with AFB<sub>1</sub> included hepatocellular anisocytosis (ND in 7 birds, 1+ in 3 birds) and anisokaryosis (ND in 1 bird, 0 in 2 birds, 1+ in 6 birds, 2+ in 1 bird), hepatic lipidosis (0 in 8 birds, 1+ in 2 birds), hepatocellular necrosis (0 in 8 birds, 1+ scattered necrosis in 1 bird and focal necrosis in 1 bird), heterophilic hepatitis (0 in 2 birds, 1+ in 7 birds, 2+ in 1 bird), glycogen-laden hepatocytes (0 in 1 bird, 1+ in 9 birds), and iron-laden hepatocytes (0 in 5 birds, 1+ in 1 bird, 2+ in 3 birds, 3+ in 1 bird). Neither hepatic fibrosis nor bile duct hyperplasia was observed in any of the liver specimens. The AF stained sections were negative for acid-fast bacteria. In the HE sections of the 10 birds after treatment with AFB<sub>1</sub>, crush artifact affected 6-25% of liver sections from one bird, 26-50% of liver sections in 3 birds, 51-74% of liver sections in 2 birds, and > 75\% of liver sections in 3 birds. The microscopic changes in the 10 birds after treatment with AFB<sub>1</sub> included hepatocellular anisocytosis (ND in 9 birds) and anisokaryosis (1+ in 6 birds, 2+ in 3 birds), hepatic lipidosis (0 in 9 birds), hepatocellular necrosis (0 in 5 birds, 1+ scattered necrosis in 1 bird and focal necrosis in 3 birds), bile duct hyperplasia (ND in 3 birds, 0 in 5 birds, 2+ in 1 bird), heterophilic hepatitis with variable degrees of lymphocytic or histiocytic infiltrates (1+ in 8 birds, 2+ in 1 bird), glycogen-laden hepatocytes (1+ in 1 bird, 2+ in 3 birds, 3+ in 2 birds, 4+ in 3 birds), and iron-laden hepatocytes (0 in 7 birds, 1+ in 1 bird, 2+ in 1 bird). Hepatic fibrosis was not observed in any of the liver specimens. AF stain and PPB stain was not performed after treatment with AFB<sub>1</sub>.

# Discussion

To the author's knowledge, this was the first study conducted in companion birds that examined the effects of aflatoxin on certain biochemical parameters and its associated histological lesions in the liver. Liver biopsies and phlebotomy was performed on all 10 birds prior to exposure to AFB<sub>1</sub>. Microscopic examination of the liver biopsies indicated normal variance within clinically healthy birds. The biochemical values over the 10 day study were compared to the group's baseline values at t=0. Liver biopsies were repeated after treatment with aflatoxin and resulted in minimal signs associated with aflatoxicosis.

The activity of ALP was significantly decreased in subgroup A on day 1 and 3 and increased in subgroup B on day 8 and 10. The reason for decreased activity of ALP in subgroup A is unclear. Increase in ALP activity, as seen in subgroup B, can be observed with osteoblastic activity, enteritis, aflatoxicosis, and hepatobiliary disease.<sup>29, 31</sup> In this case, the cause for the increase in ALP activity is likely unrelated to hepatic disease because plasma activity of GGT is not significantly different from baseline and the post-treatment liver biopsies for group B appeared unaffected by aflatoxin exposure When present, an increased in GGT activity is associated with biliary hyperplasia.<sup>98</sup> Plasma activity of SDH and LDH were also unaffected by intoxication; however, these enzymes have unreliable fluctuations with aflatoxicosis which may explain the lack of significance in this study.<sup>73, 75, 87, 91, 98</sup>

The activities of AST in subgroups A and B and ALT in subgroup B were significantly increased; however, there was always a concurrent increase in CK activity. In birds, these enzymes (AST and ALT) have a broad tissue distribution with the highest concentration in the skeletal muscle, liver, and kidneys; therefore, these enzymes can be increased with non-hepatic
disease.<sup>17, 33, 72</sup> CK is specific and sensitivity for muscle damage in birds and thus, should be examined simultaneously with the other enzymes. The increased activities of AST and ALT in the aflatoxin group were most likely the result of muscle damage rather than hepatic disease since there was concurrent increased activity of CK without significant change in the other variables measured.

A significant decrease in mean CHOL was noted on days 1, 3, 5, 6, 7, and 9. Hypocholesterolemia can be seen with altered lipid metabolism associated with liver damage<sup>91</sup> but is unlikely in this case because other biochemical parameters (LDH, GGT, and SDH) are unaltered. Instead, the reason is most likely due to inappetance.

Although the concentration of BA was found significant at t=5, it does not support liver disease because of the lack of other significant values. Instead, it is considered spuriously high because of postprandial effects.<sup>18,48</sup>

At three time points (t=4, 5, and 9), mean GLDH was significantly different from baseline; however, the data was skewed by one bird in each subgroup.

### **Sources and Manufacturers**

- a. IsoFlo, Abbott Laboratories, North Chicago, IL
- b. Bickford PC-2B Non-Rebreathing System, A.M. Bickford Inc., Wales Center, NY
- c. Microtainer, Becton Dickinson, Franklin Lakes, NJ
- d. Hitachi 912 Chemistry Analyzer, Roche Diagnostics, Indianapolis, IN
- e. Rüsch Inc., Duluth, GA
- f. VT-5000, BAS Vetronics, Bioanalytical Systems Inc., West Lafayette, IN
- g. ETCO<sub>2</sub>/SpO<sub>2</sub> monitor, CO<sub>2</sub> SMO, Novametrix Medical Systems, Wallingford, CT
- h. Ultrasonic Doppler, Parks Electric Laboratory, Aloha, OR
- i. Precision Thermometer, Tandy, Fort Worth, TX
- j. 64018BSA, 67065C, 67161Z, 67161T, 69235106, 9219-B, 201320-20, 62501EK, Karl Storz Veterinary Endoscopy America Inc., Goleta, CA
- k. Monocryl, Ethicon, Somerville, NJ
- 1. CIDEX, Advanced Sterilization Products, Irvine, CA

# Table 4.1 Systemic abnormalities via endoscopic examination in aflatoxin group pre-and post- treatment.

Liver discoloration

Mottled spleen <sup>a,b,c</sup>	Liver discoloration (yellow) <sup>a,b,c,d</sup>	Air sac granuloma <sup>e,f,g</sup>
<sup>a</sup> Pre-treatment		
<sup>b</sup> From the remaining population	of birds (n=20), 10 birds were ra	andomly assigned to the
aflatoxin group. 5 out of the 10 l	pirds in this group were previou	sly placed in the biopsy sample
population; therefore, the above	data is the same as Table 3.3.	
<sup>c</sup> Identified in one bird		
<sup>d</sup> No hepatic abnormalities were r	oted via histopathology; howev	ver, the quality of the biopsy was
poor.		
<sup>e</sup> Post-treatment		
<sup>t</sup> Identified in one bird		
<sup>g</sup> Both air sacs were biopsied and	stained with Periodic acid-Schi	ff (PAS) stain; results were

negative for fungal elements.

# Table 4.2 Gender determination via endoscopic examination.

	Male	Female		
Active reproductive organs	15	9		
Inactive/underdeveloped	5	1		
reproductive organs	5	1		

# Table 4.3 Weight in grams of birds pre- and post- aflatoxin B<sub>1</sub> treatment.

	Weight	Mean weight change (%)
Pre-treatment <sup>a</sup>	$112.9 \pm 14.5 (87.8-132.5)^{\circ}$	-6.47 (-5.3%)
Post-treatment <sup>b</sup>	$106.4 \pm 12.0 \ (85.1-123.6)$	

<sup>a</sup>Day 0

<sup>b</sup>Day 5—birds from subgroup A plus one bird from subgroup B that was found dead in cage.

<sup>b</sup>Day 6—remaining birds in subgroup B

<sup>c</sup>Mean  $\pm$  SD (range)

# Table 4.4 Biochemical analysis of aflatoxin treatment group taken over time (0-10 days)for Indian ring-necked parakeets.

<sup>1</sup>Abbreviations: ALP = Alkaline Phosphatase; ALT = Alanine Aminotransferase; AST = Aspartate Aminotransferase; CK = Creatinine Kinase; LDH = Lactate Dehydrogenase; CHOL = Cholesterol; BA = Bile Acid; GGT = Gamma-Glutamyl Transferase; GLDH = Glutamate Dehydrogenase; SDH = Sorbitol Dehydrogenase <sup>2</sup>Units: ALP (U/L), ALT (U/L), AST (U/L), CK (U/L), LDH (U/L), CHOL (mg/dl), BA (µmol/L), GLDH (U/L), SDH (U/L) <sup>a</sup>Mean <sup>b</sup>95% confidence interval \*Indicates a significant difference versus baseline (p < 0.05)

		Variables									
Time	Subgroup	ALP <sup>1,2</sup>	ALT	AST	СК	LDH	CHOL	BA	GGT	GLDH	SDH
0	А	94.90 <sup>a</sup> (83.10-108.35) <sup>b</sup>	8.41 (4.53-15.61)	143.47 (78.21-263.17)	557.97 (369.89-841.68)	80.12 (44.19-145.24)	343.68 (301.45-391.86)	62.65 (49.13-79.89)	5.82 (3.09-8.55)	0.28 (0.03-0.77)	4.85 (1.29-10.68)
U	В	47.96 (35.62-64.57)	3.31 (1.04-10.55)	194.90 (83.65-454.09)	500.15 (304.02-822.79)	58.05 (32.45-103.83)	290.18 (252.22-333.85)	64.72 (42.11-99.45)	5.06 (2.70-7.42)	0.87 (0.13-2.23)	8.13 (2.95-55.06)
1	А	76.55* (67.04-87.41)	9.05 (4.88-16.80)	249.46* (135.98-457.60)	1423.68* (943.79-2147.59)	64.46 (34.33-121.05)	313.75* (275.17-357.70)	81.27 (63.73-103.64)	5.65 (2.61-8.69)	1.01 (0.43-1.82)	5.16 (1.45-11.15)
2	В	38.51 (28.60-51.85)	16.60* (5.21-52.88)	533.74* (229.06-1243.52)	2707.82* (1645.99-4454.63)	104.40 (58.36-186.76)	267.09 (232.15-307.29)	86.92 (56.56-133.56)	5.18 (2.82-7.54)	1.42 (0.39-3.08)	15.86 (0.34-73.13)
3	A	74.45* (65.20-85.01)	5.49 (2.96-10.20)	234.0* (127.55-429.23)	1215.49* (805.77-1833.53)	73.23 (40.39-132.75)	306.13* (268.49-349.01)	54.30 (42.58-69.25)	6.9 (4.17-9.63)	1.24 (0.59-2.13)	8.63 (3.50-16.03)
4	В	39.22 (28.65-53.68)	25.29* (7.94-80.55)	786.74* (337.65-1832.98)	1457.68* (867.66-2448.91)	69.65 (36.89-131.51)	257.31 (221.69-298.63)	98.38 (61.25-158.0)	5.0 (2.36-7.64)	4.60* (2.50-7.33)	48.14 (5.61-132.42)
5	A	95.30 (83.45-108.81)	9.44 (5.09-17.52)	238.53* (130.02-437.55)	992.37* (657.87-1496.97)	73.12 (40.33-132.56)	282.45* (247.75-322.05)	87.42* (68.55-111.49)	6.98 (4.25-9.71)	2.72* (1.70-3.99)	7.70 (2.92-14.75)
6	В	46.25 (33.79-63.30)	30.98* (8.83-108.73)	241.19 (97.98-539.77)	967.0* (575.65-1624.57)	61.34 (32.49-115.82)	218.20* (188.01-253.23)	82.22 (51.19-132.07)	4.12 (1.54-6.81)	1.80 (0.51-3.89)	14.07 (1.29-74.62)
7	А	99.82 (87.42-113.98)	13.72 (7.39-25.46)	101.43 (55.30-186.08)	627.60 (416.05-946.72)	77.61 (42.81-140.70)	284.69* (249.71-324.60)	73.84 (57.90-94.16)	4.66 (1.93-7.39)	1.22 (0.57-2.11)	5.59 (1.69-11.77)
8	В	67.43* (49.26-92.30)	8.24 (2.35-28.91)	349.85 (142.11-861.18)	623.78 (371.30-1047.85)	65.5 (34.69-123.67)	260.81 (224.73-302.69)	63.21 (39.35-101.52)	4.6 (1.96-7.24)	1.27 (0.25-3.08)	12.35 (1.89-70.58)
9	Α	98.36 (86.14-112.31)	11.47 (6.18-21.29)	132.53 (72.24-243.11)	642.07 (425.64-968.55)	85 (46.89-154.08)	302.05* (264.91-344.36)	78.91 (61.88-100.62)	6.9 (4.17-9.63)	1.59* (0.83-2.58)	4.92 (1.32-10.78)
10	В	72.92* (53.28-99.81)	11.59 (3.30-40.67)	383.60 (155.84-944.26)	732.45* (435.98-1230.53)	83.40 (44.17-157.46)	265.92 (229.11-308.62)	84.67 (52.72-136.01)	5.63 (2.99-8.26)	2.50 (0.91-4.89)	15.59 (0.88-78.07)

# Table 4.4 Biochemical analysis of aflatoxin treatment group taken over time (0-10 days) for Indian ring-necked parakeets.

Variable	Sub-group A	Sub-group B
ln(ALP)	Decreases during $0 \le t^a \le 3$ , increases during $3 < t \le 5$ , and does not change significantly during $5 < t \le 9$	Decreases during $0 \le t \le 2$ and increases gradually during $2 < t \le 10$
ln(ALT)	Does not change significantly during $0 \le t \le 9$	Increases gradually during $0 \le t \le 6$ , and does not change significantly during $6 < t \le 10$
ln(AST)	Increases not significantly during $0 \le t \le 1$ , does not change significantly during $1 < t \le 5$ , then drops at t=7; no significant change during $7 < t \le 9$	Increases during $0 \le t \le 4$ , and does not change significantly during $4 < t \le 10$
ln(CK)	Increases during $0 < t \le 1$ , and then decreases during $1 < t \le 9$ ; there is no significant difference between t=0 and 5< $t \le 9$	Increases during $0 < t \le 2$ , and then decreases during $2 < t \le 10$ ; there is no significant difference between t=0 and $6 < t \le 10$
ln(LDH)	Does not change significantly during $0 \le t \le 9$	Does not change significantly during $0 \le t \le 10$
ln(BA)	Does not change significantly during $0 \le t \le 9$	Does not change significantly during $0 \le t \le 10$
ln(CHOL)	Decreases gradually during $0 \le t \le 5$ , and does not change significantly during $5 < t \le 9$	Decrease gradually during $0 \le t \le 6$ , and does not change significantly during $6 < t \le 10$
GGT	Does not change significantly during $0 \le t \le 9$	Does not change significantly during $0 \le t \le 10$
GLDH	Increases gradually during $0 \le t \le 5$ , and does not change significantly during 5< t $\le 9$	Does not change significantly during $0 \le t \le 10$
SDH	Does not change significantly during $0 \le t \le 9$	Does not change significantly during $0 \le t \le 10$

Table 4.5 Trend of each variable in aflatoxin treatment group over time (0-10 days) for Indian ring-necked parakeets.

at = time in hours



Figure 4.1 Estimate of ln(ALP) for aflatoxin subgroups A and B over time (0-10 days). \*Indicates a significant difference versus baseline (p < 0.05)



Figure 4.2 Estimate of ln(ALT) for aflatoxin subgroups A and B over time (0-10 days). \*Indicates a significant difference versus baseline (p < 0.05)



**Figure 4.3 Estimate of ln(AST) for aflatoxin subgroups A and B over time (0-10 days).** \*Indicates a significant difference versus baseline (p < 0.05)



Figure 4.4 Estimate of ln(CK) for aflatoxin subgroups A and B over time (0-10 days). \*Indicates a significant difference versus baseline (p < 0.05)



Figure 4.5 Estimate of ln(LDH) for aflatoxin subgroups A and B over time (0-10 days).



**Figure 4.6 Estimate of ln(CHOL) for aflatoxin subgroups A and B over time (0-10 days).** \*Indicates a significant difference versus baseline (p < 0.05)



Figure 4.7 Estimate of ln(BA) for aflatoxin subgroups A and B over time (0-10 days). \*Indicates a significant difference versus baseline (p < 0.05)



Figure 4.8 Estimate of GGT for aflatoxin subgroups A and B over time (0-10 days).



Figure 4.9 Estimate of GLDH for aflatoxin subgroups A and B over time (0-10 days). \*Indicates a significant difference versus baseline (p < 0.05)



Figure 4.10 Estimate of SDH for aflatoxin subgroups A and B over time (0-10 days).

### CHAPTER 5

#### CONCLUSION

A 95% confidence interval for AST, ALT, ALP, CHOL, BA, LDH, SDH, GGT, and GLDH was established in nonclinical fasted Indian ring-necked parakeets; gender was not significant. Following endoscopic liver biopsy, a persistent hypocholesterolemia was identified; the cause is unclear. In the traumatic liver injury via crush and biopsy, plasma ALP activity was consistently decreased (excluded one time point) throughout the study and is thought to be associated with isoflurane administration. Plasma GLDH activity was increased and rapidly declined which is thought to be due to acute focal hepatocellular necrosis. In the biopsy and crush/biopsy bird studies, an increase in plasma AST, ALT, and LDH were contributed to muscle injury since CK activity was concurrently increased. Bile acid concentration and GGT were not affected by liver biopsy or crush injury. Plasma SDH activity was the most specific indicator of liver injury in the groups (biopsy and crush/biopsy). After treatment with aflatoxin B<sub>1</sub>, no significant changes were observed in plasma activity of GGT, SDH, GLDH, and LDH. Hypocholesterolemia was persistent and possibly attributed to inappetance. Bile acid was unaffected after aflatoxin exposure. The microscopic lesions associated with aflatoxin treatment were minimal.

Ultimately, the liver damage induced by endoscopic biopsy, biopsy/crush injury, and aflatoxicosis was very minimal and therefore, provided little information regarding the recommended biochemical test with suspect liver disease. Repetition of the traumatic injury

78

study would be beneficial as long as more extensive liver damage was ensued. The dose of aflatoxin used in this study was minimally effective and may indicate a high LD50 in this particular species of bird. A dose-dependent aflatoxin study would hopefully answer this question.

### REFERENCES

- **1.** Harr KE. Clinical chemistry of companion avian species: a review. *Vet Clin Pathol.* 2002;31(3):140-151.
- 2. McLelland J. Digestive system. In: King AS, McLelland J, eds. *Form and Function in Birds*. Vol 1. London: Academic Press; 1980:69-181.
- **3.** Dror Y, Nir I. The relative growth of internal organs in light and heavy breeds. *Br Poult Sci.* 1977;18(4):493-496.
- **4.** Phelps PV, Edens FW, Christensen VL. The posthatch physiology of the turkey poult--I. Growth and development. *Comp Biochem Physiol A*. 1987;86(4):739-743.
- **5.** Lilja C. Postnatal growth and organ development in the quail (*Coturnix coturnix Japonica*). *Growth.* 1982;46(2):88-99.
- **6.** Lilja C. Postnatal growth and organ development in the goose (*Anser anser*). *Growth*. 1981;45(4):329-341.
- 7. Schmidt RE, Reavill DR, Phalen DN. *Pathology of Pet and Aviary Birds*. Ames: Iowa State Press; 2003.
- **8.** McLelland J. Digestive system. *A Color Atlas of Avian Anatomy*. Philadelphia: W.B. Saunders Company; 1991:47-65.
- **9.** Denbow DM. Gastrointestinal anatomy and physiology. In: Whittow GC, ed. *Sturkie's Avian Physiology*. 5th ed. San Diego: Academic Press; 2000:299-325.
- **10.** Schmidt RE, Reavill DR, Phalen DN. Liver. *Pathology of Pet and Aviary Birds*. Ames: Iowa State Press; 2003:67-93.
- **11.** Echols MS. Liver disease in birds. *North American Veterinary Conference. Proceedings of the North American Veterinary Conference.* Jan 1 2001;15:731-733.
- **12.** Doneley B. Treating liver disease in the avian patient. *Semin Avian Exotic Pet Med.* 2004;13(1):8-15.
- **13.** Dyce KM, Sack WO, Wensing CJG. Avian anatomy. *Textbook of Veterinary Anatomy*. 3rd ed. Philadelphia: Saunders; 2002:799-824.

- **14.** Lumeij JT. Hepatology. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:522-537.
- **15.** Stalker MJ, Hayes MA. Liver and biliary system. In: Maxie MG, ed. *Jubb, Kennedy, and Palmer's Pathology of Domestic Animals.* Vol 2. 5th ed. Philadelphia: Saunders Ltd.; 2007:297-388.
- **16.** Bacha WJ, Jr., Bacha LM. *Color Atlas of Veterinary Histology*. 2nd ed. Philadelphia: Lippincott Williams & Wilkins; 2000.
- **17.** Hoefer HL, Orosz S, Dorrestein GM. The gastrointestinal tract. In: Altman RB, Clubb SL, Dorrestein GM, Quesenberry K, eds. *Avian Medicine and Surgery*. Philadelphia: Saunders; 1997:412-453.
- **18.** Bain PJ. Liver. In: Latimer KS, Mahaffey EA, Prasse KW, eds. *Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology*. 4th ed. Ames: Iowa State Press; 2003:193-214.
- **19.** Hochleithner M, Hochleithner C, Harrison LD. Evaluating and treating the liver. In: Harrison GJ, Lightfoot T, eds. *Clinical Avian Medicine*. Vol 1. Palm Beach: Spix Publishing; 2006:441-450.
- **20.** Davies RR. Avian liver disease: Etiology and pathogenesis. *Semin Avian Exotic Pet Med.* 2000;9(3):115-125.
- **21.** Harrison GJ, Ritchie BW. Making distinctions in the physical examination. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:144-175.
- **22.** Hochleithner C, Hochleithner M. Innovative approach to liver and kidney diseases in psittacine birds. *Exotic DVM.* 2000;2(3):84-86.
- **23.** Lumeij JT, Westerhof I. Blood chemistry for the diagnosis of hepatobiliary disease in birds. A review. *Vet Q.* 1987;9(3):255-261.
- 24. Webster CRL. History, clinical signs, and physical findings in hepatobiliary disease. In: Ettinger SJ, Feldman EC, eds. *Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat.* Vol 2. 6th ed. St. Louis: Saunders; 2005:1422-1434.
- **25.** McMillan MC. Imaging techniques. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:246-326.
- **26.** Lumeij JT, de Bruijne JJ, Slob A, Wolfswinkel J, Rothuizen J. Enzyme activities in tissues and elimination half-lives of homologous muscle and liver enzymes in the racing pigeon (*Columba Livia domestica*). *Avian Pathol.* 1988;17(4):851-864.

- 27. Tennant BC. Hepatic function. In: Kaneko JJ, Harvey JW, Bruss M, eds. *Clinical Biochemistry of Domestic Animals*. 5th ed. San Diego: Academic Press; 1997:327-352.
- **28.** AST [package insert]. Indianapolis, IN: Roche Diagnostics; 2007.
- **29.** Hochleithner M. Biochemistries. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:223-245.
- **30.** Engelking LR. *Textbook of Veterinary Physiological Chemistry*. Jackson: Teton NewMedia; 2004.
- **31.** Thrall MA. *Veterinary Hematology and Clinical Chemistry*. Philadelphia: Lippincott Williams & Wilkins; 2004.
- **32.** Stockham SL, Scott MA. *Fundamentals of Veterinary Clinical Pathology*. 2nd ed. Ames: Blackwell Publishing; 2008.
- **33.** Lumeij JT. Avian clinical biochemistry. In: Kaneko JJ, Harvey JW, Bruss M, eds. *Clinical Biochemistry of Domestic Animals*. 5th ed. San Diego: Academic Press; 1997:857-883.
- **34.** de Schepper J, van der Stock J, Capiau E. The characteristic pattern of aspartate aminotransferase and alanine aminotransferase in the bitch with the cystic hyperplasia-pyometra complex: Effect of medical or surgical treatment. *Vet Res Commun.* 1987;11(1):65-75.
- **35.** Sorbitol Dehydrogenase Assay [package insert]. Oxford, CT: Diagnostic Chemicals Limited; 2003.
- **36.** LDH [package insert]. Indianapolis, IN: Roche Diagnostics; 2007.
- **37.** Martins MJ, Negrão MR, Hipólito-Reis C, Azevedo I. Physiologic concentrations of bile salts inhibit rat hepatic alkaline phosphatase but not the intestinal isoenzyme. *Clin Biochem.* 2000;33(8):611-617.
- **38.** Latimer KS, Prasse KW. Leukocytes. In: Latimer KS, Mahaffey EA, Prasse KW, eds. *Duncan & Prasse's Veterinary Laboratory Medicine: Clinical Pathology*. 4th ed. Ames: Iowa State Press; 2003:46-79.
- **39.** ALP [package insert]. Indianapolis, IN: Roche Diagnostics; 2006.
- **40.** GGT [package insert]. Indianapolis, IN: Roche Diagnostics; 2007.
- **41.** Daeppen JB, Schoenfeld-Smith K, Smith TL, Schuckit MA. Characteristics of alcohol dependent subjects with very elevated levels of gamma-glutamyltransferase (GGT). *J Stud Alcohol.* 1999;60(5):589-594.
- **42.** CHOL [package insert]. Indianapolis, IN: Roche Diagnostics; 2005.

- **43.** Harr KE. Diagnostic value of biochemistry. In: Harrison GJ, Lightfoot T, eds. *Clinical Avian Medicine*. Vol 2. Palm Beach: Spix Publishing; 2006:611-629.
- **44.** Qureshi IA, Khan SA, Chaudhry ZI, Mian NA, Tipu MY, Rai MF. Effects of high dietary fat on serum cholesterol and fatty liver syndrome of broilers. *Pakistan Veterinary Journal*. 2004;24(3):153-154.
- **45.** Fudge AM. Avian liver and gastrointestinal testing. In: Fudge AM, ed. *Laboratory Medicine: Avian and Exotic Pets.* Philadelphia: Saunders; 2000:47-55.
- **46.** Rutgers HC, Stradley RP, Johnson SE. Serum bile acid analysis in dogs with experimentally induced cholestatic jaundice. *Am J Vet Res.* 1988;49(3):317-320.
- **47.** de Bruijne JJ, Rothuizen J. The value of serum bile acid and GLDH in the screening for canine liver function disorders. In: Blackmore DJ, Eckersall PD, eds. *Animal Clinical Biochemistry: The Future*. Cambridge: Cambridge University Press; 1988:175-180.
- **48.** Lumeij JT. Fasting and postprandial plasma bile acid concentrations in racing pigeons (*Columba livia domestica*) and Mallards (*Anas platyrhynchos*). *Journal of the Association of Avian Veterinarians*. 1991;5(4):197-200.
- **49.** Lumeij JT, Remple JD. Plasma bile acid concentrations in respose to feeding in peregrine falcons(*Falco peregrinus*). *Avian Dis.* 1992;36(4):1060-1062.
- **50.** Bile Acids-L3K [package insert]. Oxford, CT: Diagnostic Chemicals Limited; 2002.
- **51.** Grandage J. Functional anatomy of the digestive system. In: Slatter DH, ed. *Textbook of Small Animal Surgery*. Vol 1. 3rd ed. Philadelphia: Saunders; 2003:499-521.
- **52.** Martin RA, Lanz OI, Tobias KM. Liver and biliary system. In: Slatter DH, ed. *Textbook of Small Animal Surgery*. Vol 1. 3rd ed. Philadelphia: Saunders; 2003:708-726.
- **53.** Holt DE. Hepatic and splenic emergencies. In: Ettinger SJ, Feldman EC, eds. *Textbook of Veterinary Internal Medicine: Diseases of the Dog and Cat.* Vol 1. 6th ed. St. Louis: Saunders; 2005:440-442.
- **54.** Ritchie AH, Williscroft DM. Elevated liver enzymes as a predictor of liver injury in stable blunt abdominal trauma patients: case report and systematic review of the literature. *Canadian Journal of Rural Medicine*. 2006;11(4):283-287.
- **55.** Hennes HM, Smith DS, Schneider K, Hegenbarth MA, Duma MA, Jona JZ. Elevated liver transaminase levels in children with blunt abdominal trauma: a predictor of liver injury. *Pediatrics*. 1990;86(1):87-90.
- **56.** Risholm L, Sullivan L. Changes in the transaminase and phosphatase activity of the blood and the occurrence of haemobilia following experimental liver trauma in the rabbit. *Acta Chir Scand.* 1966;131(6):495-498.

- **57.** Nishida T, Fujita N, Nakao K. A multivariate analysis of the prognostic factors in severe liver trauma. *Surg Today*. 1996;26(6):389-394.
- **58.** Cogbill TH, Moore EE, Feliciano DV, Jurkovich GJ, Morris JA, Mucha P, Jr. Hepatic enzyme response and hyperpyrexia after severe liver injury. *Am Surg.* 1992;58(7):395-399.
- **59.** Miller K, Kou D, Sivit C, Stallion A, Dudgeon DL, Grisoni ER. Pediatric hepatic trauma: does clinical course support intensive care unit stay? *J Pediatr Surg.* 1998;33(10):1459-1462.
- **60.** Kaku N. Short-term and long-term changes in hepatic function in 60 patients with blunt liver injury. *J Trauma*. 1987;27(6):607-614.
- **61.** Lierz M, Ewringmann A, Göbel T. [Blood chemical parameters for wild raptor patients and their changes after liver biopsy]. *Berl Munch Tierarztl Wochenschr*. 1998;111(7-8):295-301.
- **62.** Sinn LC. Anesthesiology. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:1066-1080.
- **63.** Taylor M. Endoscopic examination and biopsy techniques. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:327-354.
- **64.** Darling JR, Murray JM, McBride DR, Trinick TR, Fee JP. Serum glutathione Stransferase concentrations and creatinine clearance after sevoflurane anaesthesia. *Anaesthesia.* 1997;52(2):121-126.
- **65.** Nishiyama T, Yokoyama T, Hanaoka K. Effects of sevoflurane and isoflurane anesthesia on arterial ketone body ratio and liver function. *Acta Anaesthesiol Scand*. 1999;43(3):347-351.
- **66.** Jones RM, Diamond JG, Power SJ, Bajorek PK, Munday I. A prospective study of liver function in infants and children exposed to daily isoflurane for several weeks. *Anaesthesia.* 1991;46(8):686-688.
- **67.** Topal A, Gül N, Ilçöl Y, Görgül OS. Hepatic Effects of Halothane, Isoflurane or Sevoflurane Anaesthesia in Dogs. *J Vet Med Ser A*. 2003;50(10):530-533.
- **68.** Hikasa Y, Yamashita M, Takase K, Ogasawara S. Prolonged sevoflurane, isoflurane and halothane anaesthesia in oxygen using rebreathing or non-rebreathing system in cats. *Zentralbl Veterinarmed A*. 1998;45(9):559-575.
- **69.** McEwen MM, Gleed RD, Ludders JW, Stokol T, Del Piero F, Erb HN. Hepatic effects of halothane and isoflurane anesthesia in goats. *J Am Vet Med Assoc.* 2000;217(11):1697-1700.

- **70.** González Gil A, Silván G, Illera JC. Pituitary-adrenocortical axis, serum serotonin and biochemical response after halothane or isoflurane anaesthesia in rabbits. *Lab Anim.* 2007;41:411-419.
- **71.** Dressen PJ, Wimsatt J, Burkhard MJ. The effects of isoflurane anesthesia on hematologic and plasma biochemical values of American kestrels (*Falco sparverius*). *J Avian Med Surg.* 1999;13(3):173-179.
- **72.** Lumeij JT. Enzyme profiles and biochemical changes due to liver disease in the racing pigeon. In: Blackmore DJ, ed. *Animal Clinical Biochemistry: The Future*. Cambridge: Cambridge University Press; 1988:161-174.
- **73.** Hoerr FJ. Mycotoxicoses. In: Saif YM, ed. *Diseases of Poultry*. 11th ed. Ames: Iowa State Press; 2003:1103-1132.
- **74.** Gathumbi JK, Usleber E, Ngatia TA, Kangethe EK, Märtlbauer E. Application of immunoaffinity chromatography and enzyme immunoassay in rapid detection of aflatoxin B<sub>1</sub> in chicken liver tissues. *Poult Sci.* 2003;82(4):585-590.
- **75.** Quezada T, Cuéllar H, Jaramillo-Juárez F, Valdivia AG, Reyes JL. Effects of aflatoxin B(1) on the liver and kidney of broiler chickens during development. *Comp Biochem Physiol C Toxicol Pharmacol.* 2000;125(3):265-272.
- **76.** Dumonceaux G, Harrison GJ. Toxins. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:1030-1052.
- 77. Brue RN. Nutrition. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:63-95.
- **78.** Bradburn N, Coker RD, Blunden G. The aetiology of turkey 'X' disease. *Phytochemistry*. 1994;35(3):817-817.
- **79.** Böhm J, Razzazi-Fazeli E. Effects of mycotoxins on domestic pet species. In: Diaz DE, ed. *The Mycotoxin Blue Book*. Nottingham: Nottingham University Press; 2005:77-91.
- **80.** Newberne PM, Butler WH. Acute and chronic effects of aflatoxin on the liver of domestic and laboratory animals: a review. *Cancer Res.* 1969;29(1):236-250.
- **81.** Dalvi RR. An overview of aflatoxicosis of poultry: its characteristics, prevention and reduction. *Vet Res Commun.* 1986;10(6):429-443.
- **82.** Beers KW, Glahn RP, Bottje WG, Huff WE. Aflatoxin and gluathione in domestic fowl (*Gallus domesticus*)—II. Effects on hepatic blood flow. *Comp Biochem Physiol Part C Toxicol Pharmcol.* 1992;101(3):463-467.

- **83.** Olsen JH. Anseriformes. In: Ritchie BW, Harrison GJ, Harrison LR, eds. *Avian Medicine: Principles and Application*. Lake Worth: Wingers Publishing; 1994:1235-1275.
- **84.** Sudakin DL. Dietary aflatoxin exposure and chemoprevention of cancer: a clinical review. *J Toxicol Clin Toxicol*. 2003;41(2):195-204.
- **85.** Jakab GJ, Hmieleski RR, Zarba A, Hemenway DR, Groopman JD. Respiratory aflatoxicosis: suppression of pulmonary and systemic host defenses in rats and mice. *Toxicol Appl Pharmacol.* 1994;125(2):198-205.
- **86.** Rastogi S, Dogra RK, Khanna SK, Das M. Skin tumorigenic potential of aflatoxin B1 in mice. *Food Chem Toxicol.* 2006;44(5):670-677.
- **87.** Amer HA, Awad ET, el-Batrawi AM. Effect of aflatoxin B1 on alkaline phosphatase and lactic dehydrogenase levels in liver and serum of broiler chicks. *Arch Exp Veterinarmed*. 1988;42(4):595-602.
- **88.** Gallo A, Moschini M, Masoero F. Aflatoxins absorption in the gastrointestinal tract and vaginal mucosa of lactating dairy cows. *Ital J Anim Sci.* 2008;7(1):53-63.
- **89.** Klein PJ, Van Vleet TR, Hall JO, Coulombe RA. Biochemical factors underlying the agerelated sensitivity of turkeys to aflatoxin B<sub>1</sub>. *Comp Biochem Physiol Part C Toxicol Pharmcol.* 2002;132(2):193-201.
- **90.** Chattopadhyay SK, Taskar PK, Schwabe O, Das YT, Brown HD. Clinical and biochemical effects of aflatoxin in feed ration of chicks. *Cancer Biochem Biophys.* 1985;8(1):67-75.
- **91.** Huff WE, Kubena LF, Harvey RB, Corrier DE, Mollenhauer HH. Progression of aflatoxicosis in broiler chickens. *Poult Sci.* 1986;65(10):1891-1899.
- **92.** Chang CF, Hamilton PB. Impaired phagocytosis by heterophils from chickens during aflatoxicosis. *Toxicol Appl Pharmacol.* 1979;48(3):459-466.
- **93.** Singh H, Singh B, Roy KS. Histochemical/histoenzymic studies in broiler chicks fed aflatoxin, ochratoxin and inoculated with inclusion body hepatitis virus singly and in concurrence. *Mycopathologia*. 1994;125(2):119-128.
- **94.** Ibrahim IK, Shareef AM, Al-Joubory KMT. Ameliorative effects of sodium bentonite on phagocytosis and Newcastle disease antibody formation in broiler chickens during aflatoxicosis. *Res Vet Sci.* 2000;69(2):119-122.
- **95.** Balachandran C, Ramarkrishnan R. Influence of dietary aflatoxin on certain serum enzyme levels in broiler chickens. *Mycopathologia*. 1988;101(2):65-67.
- **96.** Maurice DV, Bodine AB, Rehrer NJ. Metabolic effects of low aflatoxin B<sub>1</sub> levels on broiler chicks. *Appl Environ Microbiol*. 1983;45(3):980-984.

- **97.** Shukla SK, Pachauri SP. Blood biochemical profiles in induced aflatoxicosis of cockerels. *Br Poult Sci.* 1995;36(1):155-160.
- **98.** Fernandez A, Verde MT, Gascon M, et al. Variations of clinical biochemical parameters of laying hens and broiler chickens fed aflatoxin-containing feed. *Avian Pathol.* 1994;23(1):37-47.
- **99.** Gawai KR, Vodela JK, Dalvi PS, Dalvi RR. Comparative assessment of the effect of aflatoxin B1 on hepatic dysfunction in some mammalian and avian species. *Comp Biochem Physiol C.* 1992;101(2):415-418.
- **100.** Raju MVLN, Devegowda G. Influence of esterified-glucomannan on performance and organ morphology, serum biochemistry and haematology in broilers exposed to individual and combined mycotoxicosis (aflatoxin, ochratoxin and T-2 toxin). *Br Poult Sci.* 2000;41(5):640-650.
- **101.** West S, Wyatt RD, Hamilton PB. Improved yield of aflatoxin by incremental increases of temperature. *Appl Microbiol*. 1973;25(No.6):1018-1019.