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Traumatic brain injury in Picidae avian species: the neuropathology of woodpeckers

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TRAUMATIC BRAIN INJURY IN PICIDAE AVIAN SPECIES: THE NEUROPATHOLOGY OF WOODPECKERS

by

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DEDICATION

I would like to dedicate this work to my parents, without them, none of my success would be possible. Also, a large thank you to all the educators who have ever taught me up until this point. They have supplied me with the knowledge to succeed.
ACKNOWLEDGMENTS

First, I would like to acknowledge the Field Museum of Chicago, IL, specifically Mr. Ben Marks, the Head of Zoological Collections at the Field Museum for approving my project and allowing me to opportunity to complete this study. Also, the Ornithology Department at the Harvard University Natural History Museum, specifically Mr. Jeremiah Trimble, Curatorial Associate and Collection Manager and Dr. Scott Edwards, Curator of the Ornithology Department for also allowing me to collect specimens and use their facilities on numerous occasions. Secondly, to Drs. Donald Siwek and Peter Cummings for teaching me all the knowledge I needed for this project. Dr. Siwek was crucial in demonstrating the histological technique I needed and always had a solution for every problem. Dr. Cummings dealt with the frustrations of me when things were not going right and taught me to just chill sometimes. Also, many thanks to Dr. Doug Rosene and Ms. Samantha Calderazzo for allowing me and teaching me to use the Nikon microscope and camera used in this project. Lastly, to the Department of Anatomy and Neurobiology at the Boston University School of Medicine for funding this project.
TRAUMATIC BRAIN INJURY IN PICIDAE AVIAN SPECIES: THE NEUROPATHOLOGY OF WOODPECKERS

GEORGE FARAH

ABSTRACT

Woodpeckers can withstand 1200-1400 g of force during repetitive pecking. The forces a woodpecker’s skull and brain are subjected to warrants an in-depth investigation for the possible existence of neuro-trauma. Dr. Philip May and colleagues in 1976 published a paper titled “Woodpeckers and Head Injury” detailing two woodpeckers and one toucan control. The group utilized ferrocyanide staining, a general stain used for detecting iron deposits, on the sections. The results of these stains were not reported in Dr. May’s paper, yet he and his colleagues conclude that “clearly the woodpecker’s brain is protected somehow from impact and vibration injury.” 12 Close to 115 journal articles have cited this one paper as the standard for woodpeckers not incurring brain injury during pecking. Due to limited studies on the woodpecker brain and the fact the woodpecker is a model for advancing helmet technology, we set out to study the woodpecker’s brain for signs of injury. Taking 10 different ethanol preserved woodpeckers from all parts of the world in different climates, and five non-woodpecker, ethanol preserved red-winged black bird experimental controls, paraffin embedded sections were cut and stained. A piece of human Alzheimer’s disease cortex was also used as a positive control. We utilized Gallyas silver stain for the study of neurofibrillary tangles and tauopathies as well as anti-phospho-tau and anti-glial fibrillary acidic protein (GFAP) immunostaining to detect tau protein and GFAP respectively. The results
demonstrated perivascular silver-positive deposits in the superficial cortex and axonal tract injury of eight out of the 10 woodpeckers. The anti-phospho-tau immunostaining stained axonal tract injury in two of the three woodpeckers studied. The red-winged back birds demonstrated no positivity for all three stains. The Alzheimer’s positive control showed silver positive and phospho-tau positive staining as expected. This is the first study of this kind to discover and label potential brain injury in the woodpecker model. The negative staining of the red-winged black bird controls contrasted with the positive staining woodpecker sections suggest pecking in the woodpecker may induce brain injury. When addressing the development of safety equipment, the use of the woodpecker model should be approached with caution. Moving forward, research into different immunostaining molecular targets and an age controlled woodpecker and experimental control study should be performed to determine if the brain injury seen with our research is age-dependent.
TABLE OF CONTENTS

TITLE ......................................................................................................................i
COPYRIGHT PAGE ...........................................................................................ii
READER APPROVAL PAGE .................................................................................iii
DEDICATION ....................................................................................................... iv
ACKNOWLEDGMENTS ....................................................................................... v
ABSTRACT .......................................................................................................... vi
TABLE OF CONTENTS ...................................................................................... viii
LIST OF FIGURES ............................................................................................. ix
LIST OF ABBREVIATIONS ............................................................................... xi
INTRODUCTION ................................................................................................. 1
METHODS .......................................................................................................... 27
RESULTS ........................................................................................................... 33
DISCUSSION ...................................................................................................... 44
REFERENCES .................................................................................................... 51
CURRICULUM VITAE ......................................................................................... 56
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pathological Example of CTE</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>Sagittal Woodpecker and Toucan Anatomy Comparison from May et al., 1976</td>
<td>12</td>
</tr>
<tr>
<td>3</td>
<td>External Gross Anatomy of Woodpecker from May et al., 1976</td>
<td>13</td>
</tr>
<tr>
<td>4</td>
<td>The path of the woodpecker’s tongue</td>
<td>14</td>
</tr>
<tr>
<td>5</td>
<td>Comparison of Frontal Bone Trabeculae from Golden Fronted Woodpecker and Lark birds</td>
<td>15</td>
</tr>
<tr>
<td>6</td>
<td>Force direction during woodpecker drumming</td>
<td>16</td>
</tr>
<tr>
<td>7</td>
<td>Force diagram of Great Spotted Woodpecker against a force sensor</td>
<td>17</td>
</tr>
<tr>
<td>8</td>
<td>Alzheimer’s related pathology in great spotted woodpecker</td>
<td>18</td>
</tr>
<tr>
<td>9</td>
<td>Developmental divisions of the human brain</td>
<td>20</td>
</tr>
<tr>
<td>10</td>
<td>Developmental divisions of the woodpecker brain</td>
<td>21</td>
</tr>
<tr>
<td>11</td>
<td>Functional differences between the Older and Modern views of the avian brain compared to human brain</td>
<td>22</td>
</tr>
<tr>
<td>12</td>
<td>Older versus Modern views of the Human Brain compared to the Avian brain</td>
<td>23</td>
</tr>
<tr>
<td>13</td>
<td>Gallyas silver stain of an Alzheimer’s disease cortex</td>
<td>25</td>
</tr>
<tr>
<td>14</td>
<td>Gallyas Silver Stain Controls (F)</td>
<td>35</td>
</tr>
<tr>
<td>15</td>
<td>Perivascular and Axonal Degeneration Pathology of <em>Dryocopus lineatus</em> (H) and <em>Picoides pubescens</em> (F)</td>
<td>36</td>
</tr>
<tr>
<td>16</td>
<td>Axonal Tract Pathology of <em>Dryocopus lineatus</em> (H) and <em>Picoides pubescens</em> (F)</td>
<td>37</td>
</tr>
<tr>
<td>17</td>
<td>Anti-phospho-tau Immunohistochemistry Controls (F)</td>
<td>39</td>
</tr>
<tr>
<td>Page</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>Perivascular and Neuronal Anti-phospho-tau Immunostaining in <em>Dryocopus lineatus</em> (H)</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>Axonal Tract Anti-phospho-tau Immunostaining in <em>Dryocopus lineatus</em> (H)</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>Comparison of Gallyas silver staining and Anti-phospho-tau Immunostaining of <em>Dryocopus lineatus</em> (H) and <em>Picoides pubescens</em> (F)</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>Axonal Retraction Bulbs in Dryocopus lineatus (H)</td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>United States Tree Species Density Map</td>
<td></td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

CTE ................................................................. Chronic Traumatic Encephalopathy
GFAP ................................................................. Glial Fibrillary Acidic Protein
NCAA ............................................................. National Collegiate Athletics Association
NFL ................................................................. National Football League
NFTs ............................................................... Neurofibrillary Tangles
NINDS ......................................................... National Institute of Neurological Disorders and Stroke
TBI ................................................................. Traumatic Brain Injury
INTRODUCTION

1.1 Traumatic Brain Injury

The human brain can be described as Jell-O like substance surrounded by a boney protective case. Jell-O is not the most robust of materials and neither is the human brain. According to the NINDS, TBI is defined as “a sudden trauma [which] causes damage to the brain.”\textsuperscript{19} During cranium high-impact forces, the brain can hit the skull and cause a plethora of microscopic and cognitive force related injuries. These include axonal shearing, alterations of cellular morphology, release of structural proteins, memory loss, and altered cognition\textsuperscript{19}. Diagnosis of TBI is medically complex, using several tests such as the Glasgow coma scale, speech and language tests, cognition and neuropsychology tests, and various imaging techniques\textsuperscript{18}. Even if a patient presents asymptomatically at first, TBI can show its effects weeks, months, even years after a traumatic blow. Patients diagnosed with a TBI are put into one of three levels; a mild TBI, moderate TBI, or severe TBI depending on the severity of the event (Table 1\textsuperscript{18}).
Table 1: The Three Levels of TBI

<table>
<thead>
<tr>
<th>Level of TBI</th>
<th>Symptoms</th>
</tr>
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<tbody>
<tr>
<td>Mild</td>
<td>• Person was not unconscious or was unconscious for less than 30 minutes</td>
</tr>
<tr>
<td></td>
<td>• Memory loss lasted less than 24 hours</td>
</tr>
<tr>
<td></td>
<td>• The Glasgow Coma Scale was 13 to 15</td>
</tr>
<tr>
<td>Moderate</td>
<td>• Person was unconscious for more than 30 minutes and up to 24 hours</td>
</tr>
<tr>
<td></td>
<td>• Memory loss lasted anywhere from 24 hours to 7 days</td>
</tr>
<tr>
<td></td>
<td>• The Glasgow Coma Scale was 9 to 12</td>
</tr>
<tr>
<td>Severe</td>
<td>• Person was unconscious for more than 24 hours</td>
</tr>
<tr>
<td></td>
<td>• Memory loss lasted more than 7 days</td>
</tr>
<tr>
<td></td>
<td>• The Glasgow Coma Scale was 8 or lower</td>
</tr>
</tbody>
</table>

Ultimately, the cellular damage TBI causes cannot be definitively determined until post mortem pathology analysis is conducted. Each human tolerates TBI differently, with different thresholds of force needed to cause injury. A 60 g force to the brain of one individual can cause completely different effects in another individual. These differences in TBI effects has been a focal point in a recently discovered form of TBI called chronic traumatic encephalopathy.

1.2 History of TBI and Concussions in Sports

The spectrum of TBI are placed into various categories by degrees of severity including concussions, contusions, diffuse axonal, and penetration injuries. Concussion, the most common of the injuries, is caused by an impact to the head or sudden
deceleration or rotational change. There are many theories as to how a concussion happens. Physical damage caused by a concussion is not entirely clear.

There are five main hypotheses that seek to explain the signs and symptoms seen with concussion. The first is the vascular hypothesis, where the loss of consciousness that can accompany a concussion is due to temporary cerebral ischemia. The second hypothesis is the reticular hypothesis which seeks to explain the disruption of autonomic functions such as heart functions, reflexes, and equilibrium due to forces that act on the brainstem. The third hypothesis is the centripetal hypothesis and is regarded as being null currently. It postulates that the rotational forces experienced by a concussion creates shearing strains on neurons and can cause disruptions in axonal tracts which could explain the amnesia and confusion with concussions. The fourth hypothesis is the pontine cholinergic hypothesis that is related to the reticular theory. Instead of depressing an active system like the autonomic system, the pontine cholinergic hypothesis says that concussions actually cause an activation of inhibitory systems located in the pons. One such depressor system is the parasympathetic system which uses acetylcholine as its primary neurotransmitter. The final hypothesis is the convulsive hypothesis that postulates that the seizures occasionally seen with concussions is due to an over-excitation of neurons in the brain leading to numerous and mixed signals in the brain.21

A contusion can be related to a concussion, as it is the hemorrhage of blood in and/or on the brain due to direct impact. Diffuse axonal injury is the umbrella term of any shaking or rotation of the head that causes brain structures to tear or shear. Finally, penetration injuries are the direct impact of an object to the cranium that causes hair,
skin, bone, and possible shrapnel from an object to enter the brain. With concussions being the most common TBI, research has focused into its prevention, specifically in high-impact sports such as hockey, boxing and football.

Concussions in sports is a relatively new issue, with the NCAA acknowledging the dangers of concussions in 1933 but actually drafting up concussion protocols as recently as 1994. In the past, concussions in professional football (such as the NFL) were viewed as “part of the profession,” with “the issue of knees, of drugs and steroids and drinking [as] a far greater problem.” While concussion protocols were being drafted at a collegiate and professional level, a Nigerian born forensic pathologist working in Pittsburgh, PA, by the name of Dr. Bennet Omalu, was slowly realizing there was another concussion related TBI injury to be discovered. He termed this form of injury chronic traumatic encephalopathy.

1.3 Chronic Traumatic Encephalopathy

While performing an autopsy on former NFL player and Hall of Famer Mike Webster in 2005 after a sudden unexpected death, Dr. Omalu realized profound neuronal degeneration in Mr. Webster’s brain. Numerous axonal structural proteins were found in the cerebral cortex, generally seen in professional boxers and not seen before in a football player. Dr. Omalu concluded this pathology was from repetitive episodes of mild traumatic brain injury and sub-concussive forces over a long period of time.

CTE, has since become a much discussed topic amongst athletes, coaches, researches, and fans alike. The interesting part of CTE is that individuals suffering from it
may never have had a history of a ‘full blown’ concussion. The cause of CTE is not fully understood; however, the moderate or severe concussions seem to be dwarfed by the small and repetitive un-noticed, sub-concussive hits. These hits are where the individual commonly does not notice the blow of a concussion due to it being small in force. CTE is not diagnosed until pathological post-mortem tests can be performed.

1.4 The Neuropathology of Traumatic Brain Injury and Chronic Traumatic Encephalopathy

The pathology of TBI and CTE are intertwined to a degree (Table 2\textsuperscript{15} and 3\textsuperscript{14}). Pathological findings for a TBI, and more specifically, diffuse axonal injury include axonal bulbs, small vascular hemorrhages, brain atrophy, activated microglia, and perivascular hyperphosphorylated tau protein in the form of NFTs and neurites. Axonal bulbs result from axons in white matter tracts shearing, while the proximal portion of the axon fuses and swells while the distal portion is lost.

Generally, in diffuse axonal injury, the bulbs and swellings are found diffusely throughout the corpus callosum, internal capsule, cerebral white matter, fornix, middle pons, medulla, and cerebellum.\textsuperscript{14} Microglia, the brain’s macrophage, are also found at the injury site in TBI and can be immunostained to show such an inflammatory response. Microtubule associated protein tau, or tau for short, stabilizes the neurons of the central nervous system for stability and flexibility to a certain point. During a TBI, the injury of neurons cause tau, in a process yet to be understood, to dissociate from the axons and become hyperphosphorylated to form insoluble tangles (NFTs).\textsuperscript{25}
Table 2: Summary of the Neuropathology Found in Diffuse Axonal Injury

<table>
<thead>
<tr>
<th>Diffuse Axonal Injury</th>
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<tbody>
<tr>
<td>• Axonal bulbs/swellings throughout the corpus callosum, internal capsule, cerebral white matter, fornix, middle pons, medulla, and cerebellum</td>
</tr>
<tr>
<td>• Small vascular hemorrhages</td>
</tr>
<tr>
<td>• Gross brain atrophy</td>
</tr>
<tr>
<td>• Activated microglia</td>
</tr>
<tr>
<td>• Perivascular, hyperphosphorylated tau protein in the form of NFTs and neurites</td>
</tr>
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</table>

As TBI is typically diagnosed with fairly diffuse and evenly distributed pathology, CTE is known for its irregularities in pathology (Figure 1). According to Dr. Ann McKee and a panel of six other neuropathologists, CTE is defined as “an accumulation of abnormal hyperphosphorylated tau (p-tau) in neurons and astroglia distributed around small blood vessels at the depths of cortical sulci and in an irregular pattern.” Though the superficial, perivascular tauopathies (tau neuropathologies) are the hallmark for CTE, several other supporting pathology can aid in diagnosing CTE. These supporting pathologies include abnormal p-tau positive pretangles and NFTs in cortical layers II and III, hippocampal pretangles and NFTs mostly in the CA2 region as well as pretangles and dendritic swellings in the CA4 region. In the telencephalon, diencephalon, and mesencephalon, p-tau positive deposits surrounding neurons and astrocytes in subcortical nuclei including the mammillary bodies and adjacent hypothalamic nuclei,
amygdala, nucleus accumbens, thalamus, and midbrain structures such as the substantia nigra and raphe nuclei are also supporting pathologies of CTE. Another caveat to CTE is the fact that it is considered TBI, and therefore any number of TBI pathologies could be present in CTE patients. However, the unique position of perivascular tauopathies at a sulcal depth in the cortex of CTE is not found in other TBI injuries such as diffuse axonal injury.

Figure 1: Pathological Example of CTE
Phosphorylated perivascular tau neuropathology in the cerebral cortex of an 18-year-old football player with history of repetitive concussions. CP-13 anti-tau antibody immunostaining shows the irregular distribution of the tauopathies in the superficial cerebral cortex at a sulcal depth (A). A closer examination of (A) shows tau positive perivascular degeneration [white circles: blood vessels, black dots: tau positive degeneration] (B).
Table 3: Summary of the Neuropathology found in CTE

<table>
<thead>
<tr>
<th>Chronic Traumatic Encephalopathy</th>
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<tbody>
<tr>
<td><strong>Irregular</strong></td>
</tr>
<tr>
<td>• Accumulation of hyperphosphorylated tau (p-tau) in neurons and astroglia distributed around small blood vessels at the depths of cortical sulci</td>
</tr>
<tr>
<td><strong>Supportive diagnostic pathologies</strong></td>
</tr>
<tr>
<td>• P-tau positive pretangles and NFTs in cortical layers II and III</td>
</tr>
<tr>
<td>• Hippocampal pretangles and NFTs mostly in the CA2 region as well as pretangles and dendritic swellings in the CA4 region</td>
</tr>
<tr>
<td>• P-tau positive deposits surrounding neurons and astrocytes in subcortical nuclei including the mammillary bodies and adjacent hypothalamic nuclei, amygdala, nucleus accumbens, thalamus, and midbrain structures such as the substantia nigra and raphe nuclei</td>
</tr>
</tbody>
</table>

**1.5 Prevention of TBI and CTE in High-Impact Sports including the NFL**

Being able to diagnose patients with CTE while alive could lead to reduction in the severity of CTE and TBI. With the NFL officially declaring the Boston University CTE Center as its preferred brain bank, advancements in the mental health and wellness of professional football players can finally be front and center after years of denial from the NFL.  

Along with diagnosis of CTE and TBI in living football players, research has also been into preventing injury in general through policies that lead to less direct head contact as well as improvements in head protective-wear. A simple on-line internet search of “preventing CTE research” shows the current fascination with the big-horned ram and woodpeckers. Male big-horned rams slam heads together all day during breeding season while woodpeckers peck into hard surfaces such as trees for food. Logically,
these two animals would be excellent candidates for research into improving helmet technology seeing as though they experience extremely large quantities of force without any apparent brain injury.

2.1 The Woodpecker’s Mechanisms to Absorb Force

Many studies have looked into the woodpecker’s evolutionary advantage of how it withstands upwards of 1400 g of force and the consensus seems to be two fold: 1) numerous anatomical adaptations and, 2) the trajectory of the woodpecker’s beak while pecking, also known as drumming. The anatomical advantages include sharply pointed beaks which can move independently of the skull, a long tongue capable of bracing the skull and brain during impacts, and a tightly packed skull with numerous trabeculae in the skull to absorb force. What is regarded as the premier article on the trajectory of the woodpecker beak during drumming was carried out by Dr. Phillip May et. al in 1979. They determined that the drumming of a woodpecker is largely linear and not rotational, which the latter being regarded as the cause of most concussive injuries. Interestingly enough, Dr. May also produced, prior to his 1979 force article, a paper in the February 1976 issue of The Lancet detailing the anatomy of the woodpecker skull. It is thought to be one of the first papers into the anatomical advantages of the woodpecker. Both Dr. May’s 1976 and 1979 papers have been cited a total of 115 times combined, being relied upon heavily in this field of woodpecker research. Dr. May’s 1976 article, titled “Woodpeckers and Head Injury,” has repetitively been cited as the research declaring woodpeckers do not get brain injury due to their anatomical adaptations. However, his publication failed to demonstrate the lack of brain injury in woodpeckers.
2.2 Review of May et. al, 1976 “Woodpeckers and Head Injury”

Classic scientific method says to never accept one scientist’s results and conclusions as a theory until his or her results and conclusions have been repetitively confirmed through observation and experiment. However, many of the 115 articles cite May et. al, 1976 and May et. al, 1979 as fact that brain injury does not exist in woodpeckers. A read through “Woodpeckers and Head Injury” finds many flaws in the classic scientific method.

In the introduction alone of May et. al, 1976, assumptions and baseless statements are made. “Clearly, the woodpecker’s brain is protected somehow from impact and vibrational injury.”12 This statement is made after declaring that humans would never be able to withstand the forces and head-banging woodpeckers under-go. While it is probable that woodpeckers have neuronal protection from such forces, it is not entirely clear.

Reviewing the methods section, there are two woodpeckers of the Phloeoceastes guatemalensis species and one toucan, Aulacochunchus sp. used as a control. They are all fixed in formalin and are coronally, sagitally, and frozen horizontally cut, followed by ferrocyanide staining. From this, two very grainy and hard to interpret figures are presented as results (Figures 2 and 3)12. Four main conclusions are drawn from these two figures. 1.) The woodpecker has a very narrow subdural space, and therefore relatively little cerebrospinal fluid. 2.) The woodpecker’s brain is tightly packed by relatively dense, yet spongy bone, especially evident in the occiput, in the contre-coup position from the beak. In the toucan, the occiput is light and “almost frothy.” 3.) The woodpecker
has powerful protractor quadrati and protractor pterygoidei muscles, which are antagonistic muscles, that May hypothesizes could be a muscular shock absorber for the beak. Finally, 4.) The woodpecker’s skull is encircled by musculotendinous bands that extend from the floor of the mouth, up, and over the back of the head then anteriorly toward the right nostril. 12

Dr. May then goes on to address one of the major problems with the conclusions made in this paper, the small sample size. “The statistically punctilious will, quite properly, discern that an ‘n’ of two woodpeckers and one control subjected to an anatomical inspection that is innocently free from precise measurements, means, standard deviations, and tests for statistical significance should not be used as a basis for conclusions about all woodpeckers, let alone about all head injury.”12 Yet, in the first sentence of May et. al, 1979, May says “Observations on the morphology of the woodpecker indicate that the bird’s ability to pound its head repeatedly on a tree without apparently incurring concussion or head injury may be related to firm packing of the brain, powerful head-neck muscles, and a narrow subarachnoid space with relatively little CSF.”12 May has contradicted himself between the 1976 and 1979 papers, yet 115 articles cite these two papers as proof that woodpeckers do not get brain injury. Dr. May cannot proclaim in his 1976 paper that the conclusions from his research should not be used as the tell all about woodpeckers and brain injury. Yet, he cites his 1976 paper in his 1979 paper as the proof that woodpeckers do not have brain injury.
Figure 2: Sagittal Woodpecker and Toucan Anatomy Comparison from May et al., 1976
2.3 Woodpecker anatomy aiding in absorbing high-impact forces

Dr. May’s findings he proposed in his 1976 and 1979 papers certainly have evolutionary advantages for the woodpecker. Firstly, the woodpecker’s tongue has many uses for the bird. Not only does it extract food but it also helps in the cushioning of the brain (Figure 4\textsuperscript{33}). The woodpecker’s tongue is intertwined with the hyoid apparatus, which most vertebrates possess. Starting in the right nostril, the bone splits in a y fashion to produce two hyoid horns. Wrapping around the posterior of the skull, the hyoid horns meet up inferiorly and assemble the posterior portion of the tongue. The hyoid bone and cartilage then extend anteriorly to the tip of the tongue. When the woodpecker needs to extend its tongue, the branchiomandibularis muscles contract which forces the hyoid
apparatus forward, therefore allowing the tongue to protrude from the mouth. While not in use, the tongue wraps around the orbit and provides some musculo-skeletal cushioning to the skull and brain.

**Figure 4: The path of the woodpecker’s tongue**
Starting with (A), the tongue is in resting position. This means that the tongue itself is resting around the right orbit and is able to cushion the brain and skull. In (B), the tongue is protracted which causes the tongue to come out of the right orbit and extension of the tongue outward.

Secondly, the woodpecker’s skull contains trabeculated portions that act as a cushion for force. Trabeculae are sponge-like holes in bone that reduces the weight of the bone but also allows the bone to flex more than compact bone. The cranium in the woodpecker contains varying layers of dense, compact bone plates as well as trabecular plates. The ratio of trabecular bone and compact bone changes depending on the location in the cranium. The occiput bone has the highest amount of trabecular bone in the woodpecker’s skull, more than the frontal or temporomandibular bones. However, the frontal bone of the cranium is also the thickest, with moderate trabeculae (Figure 5).
Therefore, although the occiput has the most spongy bone, the frontal bone is thicker and contains less trabeculae.

**Figure 5: Comparison of Frontal Bone Trabeculae from Golden Fronted Woodpecker and Lark birds**

The red square in (A) shows where bone in the SEM of (B and C) was gathered. In (B), the Golden Fronted woodpecker’s frontal bone is a mix of compact and trabecular bone with a plate appearance. The Lark (C), a non-pecking bird, shows more trabeculae in the frontal bone of the cranium.

Lastly, the woodpecker’s beak allows for some dissipation of force before hitting the cranium. The upper beak is slightly longer than the lower beak, which is also connected to the hyoid apparatus mentioned earlier. As the woodpecker pecks, the top beak contacts the surface first causing the bottom beak to slide forward and come into contact with the surface (Figure 6⁷). The hyoid apparatus bends as the lower beak is sliding to give some forgiveness in the skull.¹³
3.1 Previous Research

As noted previously, the majority of research investigating woodpecker brain injury has not involved the neurobiology but rather the forces involved during woodpecker drumming. May et al., 1979 paved the way for numerous studies into the force a woodpecker experiences as well as analysis of the woodpecker’s cranium. In Fan et al. 2011, the woodpecker’s drumming was studied with high-speed tape and force sensors as well as skull morphology using micro-CT. They found that the woodpecker’s skull is subjected to the most force during the initial hit of the upper beak while hitting an object (Figure 7). Skull morphology was also loosely studied by Fan et al. 2011 showing the difference in length of the upper beak versus lower beak.
Figure 7: Force diagram of Great Spotted Woodpecker against a force sensor

The initial spike of the beak$_{U}$ is the upper beak hitting the sensor. As the upper beak slides the force declines until the lower beak hits, showed by a spike in beak$_{L}$. At this initial impact of the lower beak, the orbit, anterior skull (skull$_{A}$), and posterior skull (skull$_{P}$) also show an increase in force. Finally, the upper beak slides back into position, after the peck is complete, in front of the lower beak shown by the gradual increase in force.

The Fan et al. group published a paper not even a month later in 2011 highlighting more of the skull morphology rather than the forces from their previous paper. In this second paper, a woodpecker’s skull and a lark’s skull (a non-pecking bird) were compared for bone type, thickness, and number of trabeculae found in the bone. They found that though the woodpecker’s skull had more trabeculae than the lark’s skull, the trabeculae hole size was actually smaller in the woodpecker than the lark. This is hypothesized to be for strength as trabecular bone is weaker than compact bone.  

30
As the Fan et al. group were looking into the forces and skull morphology, one group from the University of Tokyo were starting to tackle the neurobiology of the woodpecker. Doi et al. was interested in studying whether or not woodpeckers could develop Alzheimer’s disease related senile plaques and amyloid angiopathy. Taking a great spotted woodpecker that was 16 years old, they stained brain sections with Congo red for plaque identification and immunohistochemistry targeted towards human β-amyloid peptide (an Alzheimer’s disease protein marker). They found Congo red positive material in the cortex as well as β-amyloid peptide deposits upon immunohistochemistry staining (Figure 8\textsuperscript{17}). This was the first documented case of brain disease in a woodpecker species at the time.

![Figure 8: Alzheimer’s related pathology in great spotted woodpecker](image)

Congo red positive staining in the cortex showing fibrillary type structures (A). Immunohistochemistry reveals vascular β-amyloid deposits also in the cortex (B).

### 3.2 Current Woodpecker Neurobiology Research

Although the woodpecker is being heavily studied for its application in protective devices to prevent traumatic brain injury, no current research was found was into whether woodpeckers do or do not incur some sort of brain injury. While, in theory, the
woodpeckers should not have any brain injury due to all the protective mechanisms mentioned the question should still be answered. May et al., 1976 introduced the concept of studying woodpeckers for safety, though it failed to address specifically whether or not the woodpeckers had brain injury. With the increasing popularity of immunohistochemistry since Dr. May’s papers and the discovery of CTE in 2005, this project begs to answer the question of whether woodpeckers have brain injury.

4.1 Woodpecker Neuroanatomy

To fully understand woodpecker neurobiology research and how it relates to TBI, a comprehension of woodpecker gross brain anatomy is needed. Comparing the woodpecker brain to the human brain is difficult but the two are somewhat relatable. The major difference between the two brains are absence, size and presence of structures. In the human brain, there are the telencephalon, diencephalon, mesencephalon, metencephalon, and myelencephalon (Figure 9). The telencephalon is composed of the cerebrum (decision making/thinking), neocortex (upper level thinking), basal ganglia (control of voluntary motor functions, procedural learning, cognition, emotions), olfactory bulb (smell), and limbic system (emotions and drives). The diencephalon contains the thalamus and hypothalamus. The mesencephalon is the midbrain as a whole, which is associated with sight, hearing, motor control, sleep/wake cycles, and temperature regulation. The metencephalon is the cerebellum (motor coordination) and pons (spinal cord and brain signal communication). The myelencephalon is concerned with the medulla and controls the autonomic system of the central nervous system.
The divisions of the human brain include the telencephalon (white), diencephalon (green), mesencephalon (yellow), metencephalon (orange), and myelencephalon (red).

The woodpecker brain on the other hand has slightly different anatomy (Figure 10). They have a telencephalon that is comprised of just the cerebrum and olfactory bulb. The diencephalon is analogous to the human brain with the thalamus and hypothalamus present. The mesencephalon is different however, in the woodpecker this region is mostly concerned with sight and therefore referred to as the optic lobe rather than the midbrain. The metencephalon is also slightly different compared to the human as it is much larger in the woodpecker when compared to the brain as a whole. This is due to the bird having to coordinate more precise movements such as movement of their tongues in concert with drumming to eat. Finally, the myelencephalon is analogous to the human’s, where it controls the autonomic nervous system.
Figure 10: Developmental divisions of the woodpecker brain
The divisions of the woodpecker brain with the telencephalon (purple), diencephalon (red), mesencephalon (green), metencephalon (blue), and myelencephalon (yellow).

4.2 Comparison of Human and Woodpecker Neuroanatomy

Previous ideology about woodpeckers, or any avian brain for that matter, says that the cerebrum was never a true and independent structure. Instead it was thought that the avian cerebrum was more related to the thalamus with primitive instincts and behaviors rather than decision making and thought in the human brain (Figure 11). This idea has changed recently however (Figure 12), where the avian cerebrum is actually a decision and thought center for the birds and more closely related to the cerebrum of humans. A way to envision the different embryonic divisions between the human and avian brain is the type of grey and white matter present. Pallium layers compose the neocortex and cerebrum, involved with thinking, decision making, and upper level thought. The
striatum layers are more associated with the midbrain and diencephalon structures. This includes reward centers, cognition, control of voluntary motor function, and the functions of the thalamus (but not including the thalamus itself). Finally, the palladium layers are exclusively connected to the basal ganglia. In humans, the palladium layers are the globus pallidus with the palladium layers in the avian brain being less understood but having similar functions to the globus pallidus.

**Figure 11: Functional differences between the Older and Modern views of the avian brain compared to human brain**

In (A), the classic view of the avian brain (left), the cerebrum is seen to be less concerned about complex thought and behavior and more about primal instinct. The modern view (B), shows more of a higher level though from the cerebrum of the avian brain (left), analogous to the cerebrum of the human brain (right).
In the classic view (A) of the avian brain (left) and human brain (right), the telencephalon in the avian brain is shown to consist of two parts, the cerebrum part (pallium layers, green) and the striatal layers (purple). The telencephalon in the human brain is shown to be the cerebrum, neocortex and olfactory bulb with the blue colored matter connecting the telencephalon to the striatal layers (purple). The metencephalon is shown in both the avian brain and human brain as the lighter blue color. In the modern view, the telencephalon of the avian brain is much more advanced and is comprised mostly of cerebrum (pallium layers, green) and olfactory bulb while the striatum layers (purple) is limited to just the thalamus region of the avian brain. The pallidum layers (orange) in the avian brain are less understood but are analogous to the basal ganglia structure, the globus pallidus, in the human brain.
5.1 Research Objectives

As mentioned earlier, to date there has been no project that specifically investigates whether or not woodpeckers have brain injury at a microscopic level. Though Dr. May et al. produced their “Woodpeckers and Brain injury” paper in 1976, it does not specifically study brain neurobiology or use any specific techniques that target common brain injury markers. These markers include tau protein pathology, GFAP (neuronal repair marker), and evidence of neuronal injury using a structural morphology stain. In the research presented, the overall goal of this project was to determine if woodpeckers develop histologic findings consistent with brain injury. The Gallyas silver stain was used, which is specific for showing neurofibrillary tangles or cytoplasmic inclusions (neuronal injury). The importance of the Gallyas stain is its specificity; instead of staining all structures with the silver particles as is common with silver stains, the Gallyas includes a blocking step. This induces conditions that are favorable for NFT and neuronal injury to be available for silver ion attachment while simultaneously blocking all other structures. The result is silver nitrate metal deposits on NFTs and cytoplasmic inclusions such as axonal bulbs (Figure 13).
Figure 13: Gallyas silver stain of an Alzheimer’s disease cortex
Cortex staining with Gallyas silver stain and a hematoxylin counter stain in an Alzheimer’s patient. The black staining indicates tau-like pathology surrounding the cell bodies (purple) that is typically found in Alzheimer’s disease.

After successful silver staining, the project shifted to immunohistochemistry to determine exactly what proteins were within the silver staining. As useful as the Gallyas silver stain is, it will not determine which proteins specifically are showing up in silver deposits. Immunohistochemistry uses antibodies whose antigen targets are specific for proteins. The first choice was tau protein, which is associated with not only TBI but CTE as well. Any tau immunostaining in the cortex with an absence of it in the midbrain structures is indicative of a TBI. While this goes for CTE as well, staining of perivascular tau at a superficial depth would specifically be the sign of CTE (Figure 1). The protein GFAP was used in conjunction to show evidence of neuronal repair in the brains which would be an obvious sign of brain injury.
Counterstains of both the Gallyas silver stain and immunohistochemistry were used to show cell bodies, in a Nissl stain-like fashion. Since the Gallyas silver stain uses a blocking step, general counter stains were not useful. This led to the use of toluidine blue, light green sf yellowish, and hematoxylin with each having their distinct advantages. The toluidine blue stains all basic structures a dark blue while acidic cell bodies are a lighter teal color. The light green sf yellowish is a textile dye which stains all tissue the same color green while cellular morphology can be seen as a circular shape. Hematoxylin is specific for acidic structures such as cell nuclei, staining them a dark purple/blue. The immunohistochemistry used hematoxylin as well as a counterstain. This was due to simplicity and availability in the lab.
METHODS

General Information

Avian specimens were all acquired within the United States and were previously deceased before experimentation started. No birds used in this project were harmed for the sake of research as they are protected animals. The woodpecker and red-winged blackbird specimens were obtained from the Field Museum in Chicago, IL and the Ornithology Department of the Harvard University Natural History Museum. Tissue visualization was performed using a Nikon E600.

Anti-tau (phospho S262) rabbit primary antibody was purchased from Abcam (ab64193) as well as anti-rabbit goat horseradish peroxidase secondary antibody (ab6721). Goat serum was purchased from Fisher Scientific (#16210064). 3,3’-diaminobenzidine (DAB) kit was purchased from Biocare Medical (DB801R). Tris and EDTA were both purchased from Sigma Aldrich (T1378 and E9884, respectively). Tween 20 and TritonX-100 were both purchased from Sigma Aldrich (P9416-50ML and X100-100ML, respectively). Mayer’s hematoxylin counter stain was acquired from Scytek (#HAQ999).

Lanthanum Nitrate, potassium permanganate, oxalic acid, tungstosilicic acid, ammonium nitrate, sodium acetate, and potassium iodide were all purchased from Sigma Aldrich (#331937, 399124, 75688, T2786, A9642, S5636, and 207969, respectively). Sodium hydroxide was acquired from Fisher Scientific (S318).
Tissue Processing and Embedding

Brain tissue was dissected from the birds in a craniotomy-style fashion. The skull cap was removed and the brainstem and surrounding nerves were severed to release the brain. The tissue was cut into cross sections, which loosely correlate to brain landmarks. Coronal cuts were made to separate the frontal pole from the rest of the cortex, and to separate midbrain and cerebellum from the rest of the cortex. The Field Museum and Harvard brains (which all were already ethanol preserved) were placed into 70% ethanol for three hours under vacuum (30 in. Hg), changing the ethanol one and a half hours into the three-hour incubation. The brains were then placed in 95% ethanol under vacuum (30 in. Hg), and the same three-hour incubation procedure was repeated. Following the 95%, the brains were placed into 100% ethanol under vacuum (30 in. Hg), for six hours, replacing the ethanol after hours two and four. The brains were removed and placed into Histoclear (xylene substitute) for six hours under vacuum (30 in. Hg), replacing the Histoclear after hours two and four. Finally, brains were placed into paraffin wax under vacuum (30 in. Hg) over night, and placed into reusable metal tissue molds to be sectioned on a rotary microtome.

Gallyas Silver Stain

Methodology for the Gallyas stain was adapted from Uchiara, 2007. Tissue samples were mounted and sectioned using a rotary microtome and disposable blades to a thickness of 12µm. Sections were floated onto 1% gelatin/deionized water at a temperature of 39°C. The sections were then adhered to gelatin coated slides and dried on
a slide warmer at 40°C overnight. The slides then went into a 37°C oven for one week before being used for staining.

Slides were placed into Wheaton glass slide racks and deparaffinized using standard techniques of Histoclear (2x 3 min)→100% ethanol (2x 3 min)→80/20 95% ethanol/formaldehyde solution (1x 10 min)→70% ethanol (2x 3 min)→dH2O (2x 3 min). The 80/20 95% ethanol/formaldehyde solution aides in tissue adhering to the gelatin coated slides as according to Masson.

After the final water incubation, slides were placed into 0.25% potassium permanganate/dH2O solution for 15 minutes. Slides were then washed in 2% oxalic acid/dH2O for 5 minutes, followed by a five minute dH2O incubation at room temperature. After, slides went into a 0.4% lanthanum nitrate/2% sodium acetate block solution for 45 minutes at room temperature. Following incubation, slides were washed in dH2O for one minute before being placed into an alkaline silver solution for 4 minutes. The alkaline silver solution is comprised of 0.035% silver nitrate (using 1% silver nitrate/dH2O), 10% potassium iodide, and 4% sodium hydroxide. After the alkaline silver solution, the slides were placed into a 0.5% acetic acid/dH2O solution for 3x 1 minute. Following the last acetic acid wash, the slides are placed into physical developer. The physical developer was made as three separate solutions that were added together just before the developer step. Solution A is a 5% sodium carbonate solution, Solution B is a 0.2% ammonium nitrate/0.2% silver nitrate/1% tungstosilicic acid in dH2O solution, and Solution C is a 0.2% ammonium nitrate/0.2% silver nitrate/1% tungstosilicic acid/0.73% formaldehyde in dH2O solution. To make the developer, three parts of Solution B is
added to 10 parts of Solution A and mixed to be a clear solution. Then, seven parts of Solution C is added to Solutions A and B that were previous combined. The timing for the physical developer step was monitored until the human Alzheimer’s disease positive control showed signs of dark plaques in its cortex. Once the plaques appeared, the slides were placed into a 3x 1 minute 1% acetic acid/dH2O solution. After the acetic acid washes, the slides were placed into dH2O for two minutes before being put into the Mayer’s hematoxylin stain for two and half minutes. After staining, the slides were quickly washed in dH2O before going into a 0.1% sodium bicarbonate bluing solution for 30 seconds. Slides then were quickly placed into dH2O for one minute before going through the dehydration process. Starting with the 70% ethanol/dH2O solution, slides were incubated for 2x 2 minutes. Following the 70% ethanol/dH2O solution was a 1x 2 minutes 95% ethanol/dH2O solution incubation. Then, slides went into a 1x 2 minute 100% ethanol solution. Finally, the slides were placed into a 2x 3 minute Histoclear incubation. The slides were cover-slipped and mounted in permount.

**Immunohistochemistry**

The techniques used for this portion of the study was a combination of free-floating stain methods and traditional paraffin immunohistochemistry procedures. Tissue slices were cut to 25µm and placed into a 2cm in diameter steel-wire mesh container. The tissue slices were deparaffinized as described in the “Gallyas Silver Stain” section of the methods section by transferring the steel-wire mesh container from one solution to another. Once in water, the slices underwent antigen retrieval using a 90°C water bath
and glass beakers filled with 1x Tris/EDTA buffer pH 9 with 0.05% Tween 20. The sections were incubated for 20 minutes before being removed and cooled down to room temperature. Tissue sections were rinsed with 1x TBS buffer pH 7.4 with 0.025% Triton X-100, 2x 5 minutes. After the second rinse, the slides were blocked with 10% goat serum in 1x TBS buffer pH 7.4 under gentle agitation for two hours at room temperature. Once the two hours were over, the steel-wire mesh containers were placed onto Kim-wipes to drain as much goat serum block off the tissue as possible. Then the containers were transferred to small petri dishes with a 5µg/mL concentration of anti-tau (abcam) primary antibody in 1% goat serum 1x TBS buffer pH 7.4 and incubated overnight at 4°C with gentle agitation while in a humid container.

Following overnight incubation, the sections were removed from the primary antibody and washed with 1x TBS with 0.025% Triton X-100 for 2x 5 minutes. After washing, the tissue was transferred to a 0.3% hydrogen peroxide 1x TBS buffer pH 7.4 for 15 minutes. Sections were washed once again with 1x TBS buffer pH 7.4 0.025% Triton X-100 for 1x 3 minute. The sections were then put into a 1µg/mL concentration of horseradish peroxidase secondary antibody in 1x TBS buffer pH 7.4 for one hour at room temperature. Sections were rinsed in 1x TBS buffer pH 7.4 for 2x 5 minutes. The DAB chromagen was prepared according to manufacturer instructions and applied until desired degree of staining was achieved. The DAB reaction was stopped by submersion in dH20. Slices were rinsed for 1x 2 minutes in new dH2O and transferred to the Mayer’s hematoxylin for two and a half minutes. The slices were returned to dH20 before being placed into 0.1% sodium bicarbonate bluing solution for 30 seconds. Tissue was placed
back into dH20 for one minute before repeating the same dehydration procedure
described in “Gallyas Silver Stain” of the methods section. Once in the final Histoclear
step, the steel-wire mesh containers with the tissue inside were placed into a large
crystallization dish filled with Histoclear and removed from the containers to be free
floating within the crystallization dish. Gently, the tissue sections were placed onto glass
microscope slides while in the crystallization dish and removed on the microscope slides.
Slides were blotted to remove excess Histoclear and coverslipped using permount
mounting medium.
RESULTS

Avian Specimens

The 10 woodpeckers and five control red-winged black birds studied were collected from the Field Museum of Chicago, IL or the Ornithology department at the Natural History Museum of Harvard Museum of Cambridge, MA. Very detailed information was collected by the institutions related to the species of bird it is, where the specimens were found, and the date they were found. Details of the specimens and what pathological findings were found are outlined below (Table 3 + 4).

Table 3: Detailed Findings of each Experimental Control Studied

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Museum</th>
<th>Location Found</th>
<th>Date Found</th>
<th>Gallyas Silver Stain</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Agelaius phoeniceus</em></td>
<td>Field Museum</td>
<td>South Bass Island, OH</td>
<td>May, 1971</td>
<td>Negative</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Agelaius phoeniceus</em></td>
<td>Field Museum</td>
<td>South Bass Island, OH</td>
<td>April, 1971</td>
<td>Negative</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Agelaius phoeniceus</em></td>
<td>Field Museum</td>
<td>South Bass Island, OH</td>
<td>May, 1971</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><em>Agelaius phoeniceus</em></td>
<td>Field Museum</td>
<td>South Bass Island, OH</td>
<td>November, 1971</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td><em>Agelaius phoeniceus</em></td>
<td>Field Museum</td>
<td>South Bass Island, OH</td>
<td>May, 1971</td>
<td>Negative</td>
<td>N/A</td>
</tr>
</tbody>
</table>
Table 4: Detailed Findings of each Woodpecker Studied

<table>
<thead>
<tr>
<th>Species Name</th>
<th>Museum</th>
<th>Location Found</th>
<th>Date Found</th>
<th>Gallyas Silver Stain</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Picoides pubescens</em></td>
<td>Field Museum</td>
<td>St. Clair, MI</td>
<td>1972</td>
<td>Negative</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Field Museum</td>
<td>Monroe, MI</td>
<td>1961</td>
<td>Diffuse axonal streaks superficial and deep with tangles. Silver staining surrounding a few neuronal somas.</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>Field Museum</td>
<td>Ann Arbor, MI</td>
<td>1962</td>
<td>Diffuse axonal streaks superficially, with no tangles found. Perivascular deposits in superficial cortex.</td>
<td>Negative</td>
</tr>
<tr>
<td><em>Sphyrapicus varius</em> (Juvenile)</td>
<td>Harvard Natural History Museum</td>
<td>Fairfield, ME</td>
<td>2003</td>
<td>Diffuse axonal streaks superficially with no tangles or perivascular deposits.</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Picoides pubescens</em></td>
<td>Harvard Natural History Museum</td>
<td>Harvard, MA</td>
<td>1966</td>
<td>Negative</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Picoides pubescens</em></td>
<td>Harvard Natural History Museum</td>
<td>Vienna, VA</td>
<td>1984</td>
<td>Diffuse axonal streaks superficially with no tangles or perivascular deposits.</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Picoides pubescens</em></td>
<td>Harvard Natural History Museum</td>
<td>N/A</td>
<td>1956</td>
<td>Localized axonal streaks at a superficial depth, limited deep streaks found. A few perivascular deposits.</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Phloeaeatstes guatemalensis</em></td>
<td>Harvard Natural History Museum</td>
<td>Mexico</td>
<td>1956</td>
<td>Diffuse axonal streaks superficially and deep with no tangles. Numerous perivascular deposits in superficial cortex.</td>
<td>Anti-phospho-tau positive streaks in an organized fashion, analogous location to the streaks seen in the silver stain of this specimen.</td>
</tr>
<tr>
<td><em>Colaptes auratus</em></td>
<td>Harvard Natural History Museum</td>
<td>Lincoln, MA</td>
<td>1955</td>
<td>Diffuse axonal streaks superficially and deep with no tangles or perivascular deposits.</td>
<td>N/A</td>
</tr>
<tr>
<td><em>Dryocopus lineatus</em></td>
<td>Harvard Natural History Museum</td>
<td>Canada</td>
<td>1975</td>
<td>Diffuse axonal streaks superficially and deep with no tangles. Extensive perivascular deposits in superficial cortex.</td>
<td>Anti-phospho-tau positive streaks deep in an organized, thread-like fashion, similar location to the streaks seen in the silver stain of this specimen.</td>
</tr>
</tbody>
</table>
Gallyas Silver Stain

The aim of the Gallyas silver stain was to determine if there was any evidence of neuronal and/or white matter tract damage. Since the Gallyas stain has a high degree of specificity for neurofibrillary tangles and axonal injury, it was the first step of the project.

Figure 14: Gallyas Silver Stain Controls
An Alzheimer’s disease human cortex tissue sample was used as a positive control for the Gallyas silver stain. (A) is a sample of the Alzheimer’s disease positive control with degenerating neurons (yellow arrow, B) and a β-amyloid-like plaque (red arrow, C). (B) shows an increased magnification at the tip of the yellow arrow in (A) illustrating degeneration of an axonal cell body. (C) is an increased magnification at the tip of the red arrow in (A) shows a β-amyloid-like plaque. The pictures in the bottom of the figure (D, E, F) are all experimental control tissue sections from *Agelatus phoeniceus*, a non-pecking avian species from the Field Museum. No silver positive pathology was observed on any experimental control slides that were always stained alongside woodpecker and Alzheimer’s disease positive control slides. Counterstain is Mayer’s hematoxylin.
Figure 15: Perivascular and Axonal Degeneration Pathology of *Dryocopus lineatus* and *Picoides pubescens*

Perivascular Gallyas silver positive pathology was observed in many of the woodpecker species used. Here, (A) and (C) are from the same tissue section from a *Dryocopus lineatus* specimen. The blood vessel seen in (A) and (C) was from superficial cortex. The damaged neuronal cell body and axons in (B, red arrow) from *Dryocopus lineatus* was also observed in some of the woodpeckers studied. The perivascular silver positive pathology in (D) was from *Picoides pubescens* in a slightly deeper, but still superficial portion of the cortex when compared to (A) and (C). Experimental controls (E) and (F) from Figure 14 were included for comparison.
Figure 16: Axonal Tract Pathology of *Dryocopus lineatus* and *Picoides pubescens*

Possible diffuse axonal tract injury was observed in almost all of the woodpeckers studied using Gallyas silver stain. In (A), the *Picoides pubescens* stained Gallyas silver positive stemming from a structure that seemed analogous to the mammalian corpus callosum. The staining of (B), a *Dryocopus lineatus*, was very superficial. The most distal border of the cortex in (B) can be seen as the white background in the upper right corner. The axonal staining of (C) is in deeper parts of cortex from a different *Dryocopus lineatus* specimen.
Immunohistochemistry

With the Gallyas silver stain-positive tissue well established, the focus of this study shifted to determine if the lesions identified by the Gallyas stain was tau protein. As mentioned previously, the golden diagnostic tool of traumatic brain injury is post-mortem immunostaining.

A Dryocopus lineatus woodpecker was chosen for immunostaining after numerous Gallyas silver stain-positive pathology was observed in dozens of tissue sections of the same bird. Anti-phospho-tau antibody was used exclusively for this study because, although inflammatory responses can be important for brain injury, the immune system responses in the brain are only acute. Deposits of phosphorylated tau last a life-time as the body has no way to rid itself of accumulated phosphorylated tau.
Figure 17: Anti-phospho-tau Immunohistochemistry Controls
For the immunostaining portion of the study, the same Alzheimer’s disease human positive control was used in conjunction with the *Agelaius phoeniceus* experimental control. As seen in (A), there are numerous degenerating neurofibrillary pathology. At the tip of the yellow arrow is a textbook example of a degenerating axon “cone” which is phosphorylated tau not only around the neuronal soma but also down the axon itself giving a cone or pyramid shape. In (B), the neuronal cell soma is more clearly defined with the cytoplasm being almost translucent and the nucleus being a dark blue from the hematoxylin counter stain. Surrounding the soma is the phosphorylated tau which encompasses the soma. In (C) and (D), the same *Agelaius phoeniceus* tissue section is shown at 100x and 200x to demonstrate the clear non-staining observed in all experimental controls slides stained. The experimental controls were always stained along side the human Alzheimer’s disease positive control and the experimental (woodpecker) slides.
Figure 18: Perivascular and Neuronal Anti-phospho-tau Immunostaining in *Dryocopus lineatus*

The above figure is a 200x and 400x view of a cortex section from a *Dryocopus lineatus* specimen. The yellow and red arrows in (A) correspond to the yellow and red arrows in (B). Starting with the yellow arrow, perivascular tau staining is seen surrounding the lateral side of the vessel. The neuronal staining at the red arrow also is seen on the lateral side of the soma. Experimental controls (C) and (D) from Figure 17 were included for comparison.
Figure 19: Axonal Tract Anti-phospho-tau Immunostaining in *Dryocopus lineatus*

The most numerous pathology seen from the staining of *Dryocopus lineatus* sections was the appearance of these phospho-tau positive streaks going across the section in an organized fashion. In (A), the streaks are seen at a 100x magnification going in a superior-inferior direction. At 400x, (B) shows the organization of the streaks to be very thread-like and numerous. With (C) and (D), there is a horizontal organization to the streaks observed at 200x magnification.
Figure 20: Comparison of Gallyas silver staining and Anti-phospho-tau Immunostaining of *Dryocopus lineatus* and *Picoides pubescens*

The immunostaining of phospho-tau in (A) and (B) from the *Dryocopus lineatus* show streaks in an organized pattern. The Gallyas silver staining found in (C) and (D) from *Picoides pubescens* also show streaks of silver positive staining in an organized fashion.
Figure 21: Axonal Retraction Bulbs in *Drycopus lineatus*

The retraction bulbs seen at the red arrows of anti-phospho-tau immunostained (B) compared to the segmentation observed in the Gallyas silver stained (A) from the central cell soma.
DISCUSSION

Woodpecker Axonal Injury

As reported by Dr. May in his 1976 paper, the woodpecker’s skull has adaptations that aid in force absorption such as a tightly packed subdural space and varying skull bone morphology. While this may prevent injuries that involve the brain directly striking the skull, the anatomical features may not prevent axonal injury caused by a change in directional forces. With the 1400 g of force a woodpecker can subject itself to while drumming, the sudden acceleration/deceleration of each peck could be a potential source of brain injury. The results presented in this study show that even though, anatomically, the woodpecker has protective mechanisms in place, attempting to protect against such a high amount of force is difficult. Despite the adaptations of the woodpecker skull, our histological findings demonstrated probably brain injury. In this study, we found brain injury with patterns of both diffuse axonal injury and CTE like features. Perivascular Gallyas silver staining and tau immunohistochemistry staining patterns seen in this project are suggestive of CTE-like lesions in the woodpecker brain. The majority of sections from multiple woodpecker specimens demonstrated diffuse axonal-like injury within white matter tracts of the brains.
The role of tau protein and TBI

Tau protein is a structural protein that surrounds microtubules, primarily in axons. It is mostly found in the distal portion of axons near the axonal button where varying degrees of axonal. With neuronal injury, the tau protein is hyperphosphorylated by an unknown mechanism, leading to accumulations of phosphorylated tau. With six different isoforms of tau and a combined 85 phosphorylation sites, understanding the complex nature of this protein has been a challenge. In humans, there is no known mechanism for the brain to dispose of these abnormally phosphorylated tau accumulations and this is believed to be a possible cause of some neurodegenerative diseases and the impairments seen with TBI. In this study, the Gallyas silver stain positive axonal “streaks” seen in the woodpeckers, are evidence suggestive of axonal tract brain injury. The presence of hyperphosphorylated tau protein within the axonal streaks was confirmed by immunohistochemistry.

Woodpeckers and Tau protein

A total of 10 woodpeckers and five red winged black birds were used in this study. Eight out of the 10 woodpeckers had Gallyas silver positive staining and no positivity was seen in the red winged black bird controls. The silver positive lesions were either perivascular deposits indicating possible CTE-like pathology; or black “streaks” in white matter tracts of the woodpeckers suggestive of diffuse axonal injury (Figure 20). It is pertinent that the silver deposits in a Gallyas silver stain are independent of β-amyloid-like plaques as the silver deposits may be from old age rather than brain injury. With
age, tau has been shown to accumulate in NFTs much like brain injury in the cortex, but coincide with β-amyloid plaques. Since the Gallyas silver stain observed in the woodpeckers was independent of β-amyloid plaques (verified with the presence of silver positive plaques in the human Alzheimer’s positive control), it can be said with confidence that the Gallyas silver stain was not due to age. This statement is further verified by the positive Gallyas silver staining in the only juvenile woodpecker specimen of the whole study. The juvenile woodpecker had the same diffuse axonal injury-like black streaks as seen in the adult woodpecker specimens, without having any perivascular silver deposits. The tau immunohistochemistry was performed on three woodpeckers, with two demonstrating positive staining.

As mentioned previously, tau immunostaining is the gold standard for diagnosis of TBI. Though only two of the three woodpeckers studied with immunostaining showed positive lesions, none of the red-winged black birds demonstrated any tau positivity. The sharp contrast between the woodpeckers and the red-winged black birds in regards to pathology in both the Gallyas silver stain and the tau immunostaining led us to believe that the variable of pecking was the main difference between the two groups of birds.

The Possible Role of Climate and Environment on Woodpecker Brain Injury

The climates and environments of the northeast, Great Lakes region, mid-Atlantic, and Mexico offer possible variations in the type of trees that each bird is more likely to peck. With a softer wood, the woodpecker would meet less of a resistive force than hardwood. The northeast is dominated mostly by Oak, Maple, Beech, and Birch
trees which are classified as hardwoods. The Great Lakes region is populated by mostly White/Red/Jack Pine, Spruce, and Fir trees with them all being softwoods. The mid-Atlantic region is predominantly Oak, Hickory, and Loblolly/Shortleaf Pine trees with the first two trees being hardwoods and the last tree being softwood. Finally, Mexico is predominantly pine and oak trees with the pine tree being a softwood and the oak tree being a hardwood. It is interesting that, though eight out of the 10 woodpeckers studied had some sort of brain injury pathology, the wood they were most likely surrounded by varied in wood strength. More research would be needed to determine if wood strength had a definite affect on brain injury.

Climate may also be a potential variable. At night, specifically during cold months, often the woodpecker creates a cavity to sleep in by pecking a hole into a tree. The longer winter months of the northern United States could cause a woodpecker in that region to incur more brain injury than a woodpecker in a warmer climate simply by having to create more holes to sleep in. Another increase in woodpecker tree cavity pecking occurs during breeding season when the bird creates a nest. With wood strengths varying by region, the basic nesting needs of a woodpecker could also contribute to brain injury.
The density of tree species across the United States may be linked to amount of brain injury incurred by woodpeckers. Woodpeckers that live in a predominantly hardwood area of the United States may peck against harder surfaces more frequently than woodpeckers that live in predominantly softwood areas.

The Role of the Woodpecker with Helmet Technology Research

With both the United States Army and football helmet manufacturer Riddell looking to improve helmets to reduce the amount of force subjected to the frontal cortex, they have turned to the woodpecker as a model to study. While the woodpecker does have many advantageous anatomical adaptations for the reduction of forces, the data from this research warrants a re-evaluation of the woodpecker. Specifically, the thought...
that the small subdural space of the woodpecker reduces the likelihood of brain trauma. Though there is a significantly smaller subdural space when compared with humans, the frontal bone of the woodpecker is not as forgiving as previously thought. The frontal bone of the woodpecker is not comprised of as much trabecular bone compared to non-pecking birds, which is known to provide cushioning. Actually, the frontal bone in the woodpecker is denser than other birds, which leads to less cushioning of the brain from the frontal bone. Although, in theory the woodpecker is an excellent model to study, a more cautious view before studying woodpeckers is needed until woodpecker TBI research is thoroughly explored.

**Future Research**

Unfortunately, we were unable to fully conduct in-depth immunohistochemistry research. Ideally, all 10 woodpeckers and five experimental controls would have been immunostained for phospho-tau, however only three of the woodpeckers and two of the experimental controls could be studied. In addition, a more diverse panel of antigen targets should be studied such as CD45 (a microglial marker) and a total tau/phospho-tau western blot to determine the ratio of phosphorylated tau to native tau.

Along with changes in antigens studied, an age dependent study of woodpeckers targeting phospho-tau would be ideal. As it stands, the research presented in this thesis reveals evidence of TBI; however the age of these woodpecker specimens prior to death is unknown. The only woodpecker in this study that was determined to be juvenile based off of skull ossification (by Jeremiah Trimble, Ornithology department at the Harvard
Museum of Natural History) was the *Sphyrapicus varius* specimen. While the juvenile woodpecker did indeed show brain injury, a larger scale study on how age affects the brain injury of these birds is warranted.

**Conclusion**

With the link between high-impact professional sports and TBI growing increasingly stronger, the need to develop new protective equipment for athletes is at an all-time high. Mentioned by Dr. May in his 1976 paper regarding previous research using non-human primates, “a neck collar protects the monkey brain against impact injury…”

Our findings should caution researchers studying how nature deals with high-impact forces with the eventual goal of improving helmet technologies. Though woodpeckers are, in theory, an excellent candidate for helmet research, the model needs to be approached with skepticism.

More research is needed to confirm the findings of this research. The evidence is clear, the non-woodpecker red-winged blackbirds showed no evidence of silver stain or immunostain while the woodpeckers showing silver positive axonal damage both around vasculature superficially as well as deep within white matter tracts. The woodpeckers also showed positive anti-phospho-tau immunostaining within white matter tracts of the cortex. Although the cure for TBI is far in the future, its prevention can be in the present, possibly a little help from the woodpecker.
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