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The acute effects of gentamycin ototoxicity on Bobwhite Quail Basilar Papilla and auditory brainstem responses

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The Acute Effects of Gentamycin Ototoxicity on Bobwhite Quail Basilar Papilla and Auditory Brainstem Responses

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Table of Contents

List of Tables

List of Figures

Abstract

The present study aimed to investigate the effectiveness of a single dose of gentamycin in elevating Auditory Brainstem Response (ABR) thresholds and causing inner ear hair cell loss or damage. A total of twenty-two Bobwhite quail were used (n=7 control, n=15 experimental); experimental group quail were injected with a 200 mg/kg dose of gentamycin within 24 hours of hatch (P0). Three to four days post-hatch or injection the quail underwent ABR testing. Thresholds were measured using a 1,000 Hz, 2,000 Hz, and 4,000 Hz tone pip and a click beginning at 90 dB nominalSPL. Resulting waveforms were analyzed using MATLAB (R2007) at each intensity level.

A subset of experimental and control group Bobwhite quail inner ears or basilar papillae (BP) were also analyzed histologically. Within 24 hours following ABR testing, basilar papilla were fixed by intralabyrinthine perfusion of 4% paraformaldehyde in 0.4M phosphate buffer. Photomicrographs were taken at overlapping intervals and combined to make montages of the entire basilar papilla. Montages were measured for an average length and divided into deciles for analysis of estimated hair cell loss (100% hair cell loss, >50% hair cell loss, <50% hair cell loss, 0% hair cell loss).

Electrophysiologic results showed absent or elevated thresholds in all experimental group quail. All of the quail in the subset analyzed histologically had total hair cell loss in the basal half of the basilar papilla; 83.3% (five out of six) had total hair cell loss across 60% or more of the length of the basilar papilla.

Previous studies in altricial and precocial birds have shown hair cell loss and threshold shift following a single dose of aminoglycoside antibiotics. However, no previous studies have shown consistent hair cell loss over an area greater than the basal

one-third of the basilar papilla. In this study, hair cells were reliably lost over the basal half of the basilar papilla and a subsequent profound hearing loss for frequencies above 1,000 Hz was measured. The Bobwhite minimal audibility curve indicates best hearing is between 1,000-3,000 Hz; therefore, we can conclude that the hearing loss induced represents a substantial disruption in the typical auditory world of these birds during early development.

1.0 Introduction

Birds offer a unique model for studying the influence of hearing loss and recovery on auditory perception. They are the only warm blooded mammals that naturally regenerate hair cells after damage. As a result, birds offer the possibility of studying the effects of a temporary hearing loss on the subsequent perception of complex auditory stimuli after hair cell regeneration and recovery. It is well known that early onset sensorineural hearing loss has significant consequences on humans' ability to learn and develop spoken language. The effects of early sensorineural hearing loss and restoration can be studied in humans and other mammals only with the use of artificial restoration (i.e. hearing aids or cochlear implants). Birds use hearing in a similar way to humans, to recognize, learn, and produce vocal song. Therefore, birds offer the rare opportunity to discover the perceptual consequences of early, and temporary, sensorineural hearing loss under a natural, although regenerated, state.

Previous studies have shown that these regenerated hair cells provide a return of auditory function within a matter of weeks (Janas et al., 1995). Birds also learn complex vocal calls and song patterns very early in life and several studies have shown that early experience influences their ability to recognize and/or produce species specific calls (Lickliter & Stoumbos, 1992). The current literature on the effect of hair cell regeneration on perception of complex species specific calls suggests that there are enduring effects of hearing loss and recovery through hair cell regeneration in adults, even after song learning has taken place. Dooling et al (2006) found that even though hearing sensitivity returned within weeks of hair cell regeneration, complex call perception remained affected for months, but that this deficit did not preclude learning to

recognize new calls. Birds, like humans, learn species specific vocal patterns very early in life, therefore, it seems reasonable to suggest that a temporary hearing loss might have long lasting influences on that perception (Lickliter & Stoumbos, 1992). Even though studies suggest that a temporary interruption in hearing does not preclude later learning in adults, we have no evidence for a similar affect in juveniles. That is, we don't know whether temporary hearing loss early in life would have long-lasting effects on auditory learning and vocal production. Previous studies of hair cell regeneration in hatchling chicks have shown that a single dose of an ototoxic drug (i.e. gentamicin and kanamycin) produces loss of all hair cells beginning at the proximal tip and extending to ~25-35% of length along the basilar papilla (Cotanche, 1999). This distance along the basilar papilla from the distal tip corresponds to frequencies above 2,500 Hz in the precocial place-frequency map of the avian basilar papilla (Gleich & Manley, 2000). Congruently, electrophysiological measurements (i.e. CAP, ABR, etc.) of hearing loss and recovery after hair cell damage and regeneration in hatchlings has shown primary hearing loss at frequencies above 2,000 Hz (Cotanche, 1999; Bhave et al., 1995 as cited in Cotanche, 1999; Dooling et al., 2006; Janas et al., 1995).

If we are to understand the influence of hearing loss prior to vocal learning on later complex perception we would optimally like to induce hearing loss across all audible frequencies, not just those above 2,000Hz. Further, such studies should take place in birds previously shown to rely on early experience for complex species-specific call recognition. The Bobwhite quail offers a model for this early experiential auditory perception. Bobwhite quail have been shown to recognize the maternal call within hours of hatching (Lickliter & Stoumbos, 1992). Further, studies suggest that the preference for

this call can be altered by early auditory experience (Lickliter & Stoumbos, 1992). An estimate of the Bobwhite quail audibility curve shows best hearing between 1-3kHz (Gleich and Manley 2000; Barton et al 1984; Gleich and Langerman 2011) and the spectrum of the maternal call encompasses frequencies both above and below this best frequency region. Thus, an alteration of auditory sensitivity within this best-frequency range would provide a substantial alteration in the normal auditory experience of developing Bobwhite quail.

The purpose of the present study was to determine whether a single dose of gentamicin, an aminoglycoside antibiotic known for its ototoxic effects, could cause hair cell loss for more distal regions along the basilar papilla than previously reported and result in a corresponding loss of hearing for frequencies as low as 1,000 Hz (within the best frequency hearing range of Bobwhite quail). If total loss of hair cells, and therefore hearing, can be accomplished at frequencies lower than 2,000 Hz we will have a unique and valuable model for future studies of the long-term effects of the temporary hearing loss on auditory perceptual development.

2.0 Materials and Methods

2.1 Animals

A total of twenty-two Bobwhite quail were used in the study (n=7 control, n=15 experimental). Quail in the experimental group received a single dose of gentamycin sulfate (IM, 200 mg/kg) within 24 hours of hatch (P0). Higher doses of 250 mg/kg such as used in studies of gentamicin ototoxicity in hatchling white leghorn chick (Janis et al 1995; Bhave et al 1995) resulted in unacceptable mortality rates (>50%) for hatchling Bobwhite quail. All quail were housed at James Madison University under the approved Institutional Animal Care and Use Committee protocol (IACUC).

2.2 Electrophysiology (ABR)

All quail underwent auditory brainstem (ABR) evoked potential testing three-four days post-hatch (P3-4). ABR's were performed under general anesthesia Ketamine/Xylazine (25 mg/kg Ketamine + 5 mg/kg Xylazine) and Ketamine/Diazepam (75 mg/kg Ketamine + 2.5 mg/kg Diazepam). Tucker-Davis hardware (System III) and software (BioSig) were used to generate the stimuli (click, 1kHz tone pip, 2kHz tone pip, and 4kHz tone pip), and collect data. All stimuli were presented in the sound field through a Tandy 7 speaker positioned 1.09 meters from the bird's left ear, birds were positioned on their side with the left ear up. Sound pressure levels (SPL) were monitored using a Brüel & Kjaer (B&K) Precision Sound Level Meter. Subcutaneous needle electrodes were placed behind the left ear (inverting), on the vertex (noninverting) and in the nape of the neck (ground) for recordings. Electrode impedance was maintained at $\leq$3 kOhms throughout testing. There were four presentations at each intensity (two rarefaction, two condensation) for each of the stimuli. Stimuli were presented at a rate of

11.1/sec. with a 10ms data acquisition window. Presentation levels decreased in 10 dB steps from 90 to 60 dB nominal SPL (nSPL) and in 5 dB steps from 55 to 30 dB nSPL; 100 acceptable sweeps were required before presentation level was decreased. Peak equivalent sound pressure levels for 90 dB nominal SPL (90 dB generated through BioSig/SigGen) were determined for each of the stimuli (click= 86 dB peSPL, 1,000 Hz=96 dB peSPL, 2,000 Hz=91 dB peSPL and 4,000=77 dB peSPL) using a Brüel and Kjaer pistonphone and sound level meter.

Waveforms were analyzed in MATLAB (R2007). Two averaged waveforms were displayed at decreasing intensity levels, each an average of one rarefaction and one condensation waveform. The examiner decided if the two averaged waveforms were congruent, and threshold was estimated at the midpoint between intensities judged to be congruent and incongruent. The latency of ABR Waves I and II were then picked from a grand average of all four recordings at each intensity presentation above threshold.

2.3 Histology

Six of the quail in the experimental group were euthanized three to seven days post-hatch (within 24-72 hours of ABR testing) and the basilar papilla (BP) fixed by intralabyrinthine perfusion of 4% paraformaldehyde in 0.4M phosphate buffer. Papillae were removed from the head, tegmentum vascularis and tectorial membranes removed and the BP stained with phalloidin (100:1 dilution). Photomicrographs were taken of whole mounted tissue at overlapping intervals along the length of each basilar papilla at 16x magnification and combined to make montages of the entire BP. To determine the position of hair cell loss along the BP the length of the individual BP (average of 3 measurements) was determined using NIH ImageJ (v5.1) and divided into deciles.

Starting from the proximal (basal) end of the papilla, hair cell loss in each decile was estimated as: 100% hair cell loss, >50% hair cell loss, <50% hair cell loss and 0% hair cell loss. While both ears were prepared for histological examination, only one ear (with the least artifact from dissesction) from each bird was used in the final analysis of hair cell loss. Previous studies have shown that systemic injection of aminoglycosides results in similar damage to both ears (Lippe, Westbrook, and Ryals, 1991).

3.0 Results

3.1 Electrophysiology

3.1a Normal Control

Figure 1 shows typical click ABR responses recorded from a 3-7 day old control group Bobwhite quail. As has been shown in auditory brainstem evoked potential responses (ABR) in other birds (Brittan-Powell, 2002), there were two prominent peaks in the quail ABR waveform. At the highest intensities both peaks are seen; as intensity decreases the latency of response increases and amplitude decreases. The first positive peak, Wave I, likely representing synchronous activity at the auditory nerve, was the most robust and its presence was used to determine threshold. The acquisition window for this experiment was 10 ms; it is possible that the second peak, Wave II, would have been visible for a longer time if the window had been longer. Average Wave I latency intensity functions for each stimulus are shown in Figure 2. As expected latency increased as intensity decreased and overall latencies were shorter for clicks and increased as frequency of stimulation decreased.

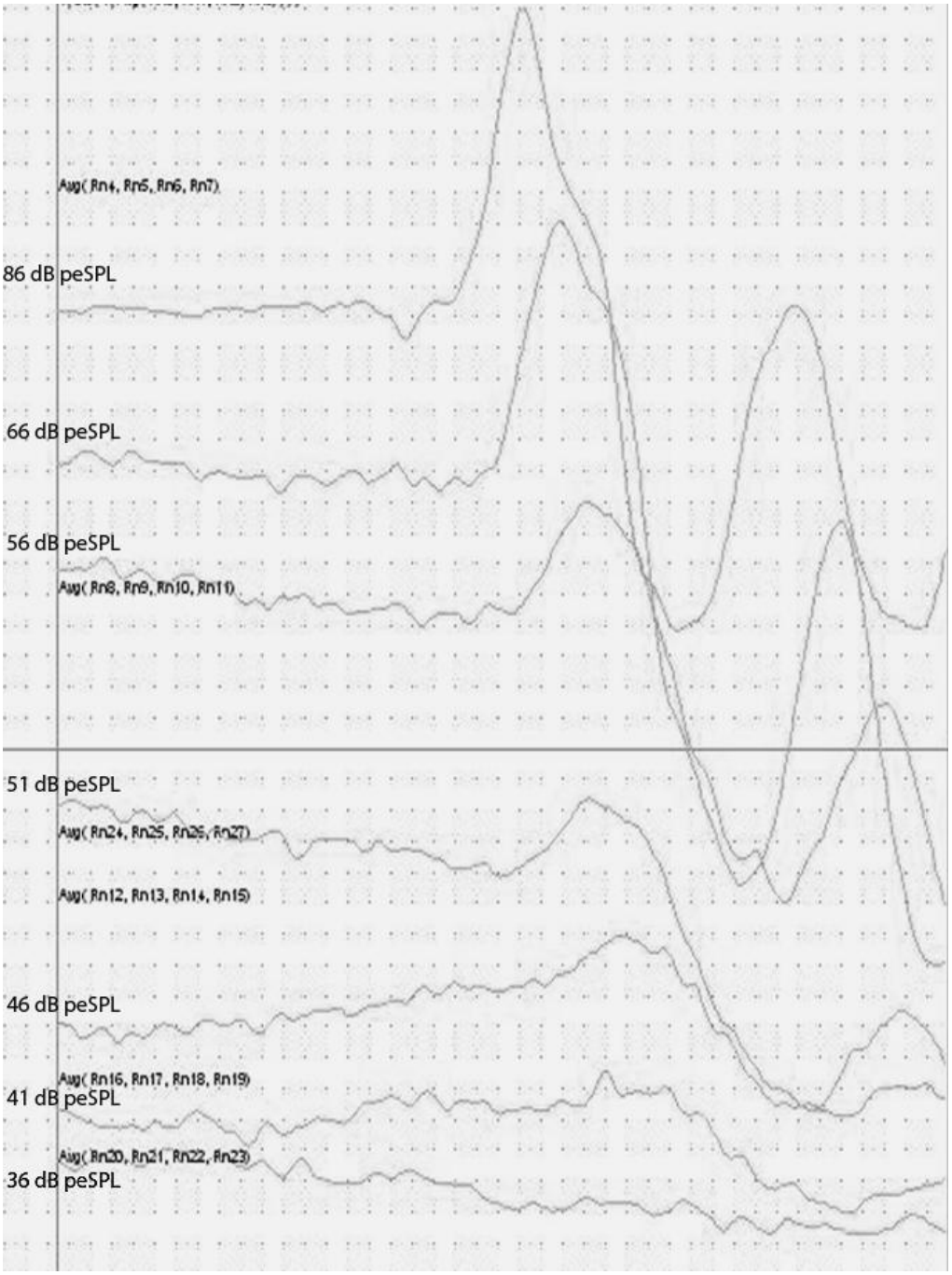

Figure 1. Click ABR responses from a control group Bobwhite quail. At the highest intensities two peaks are visible; however, as intensity decreases only one wave is present.

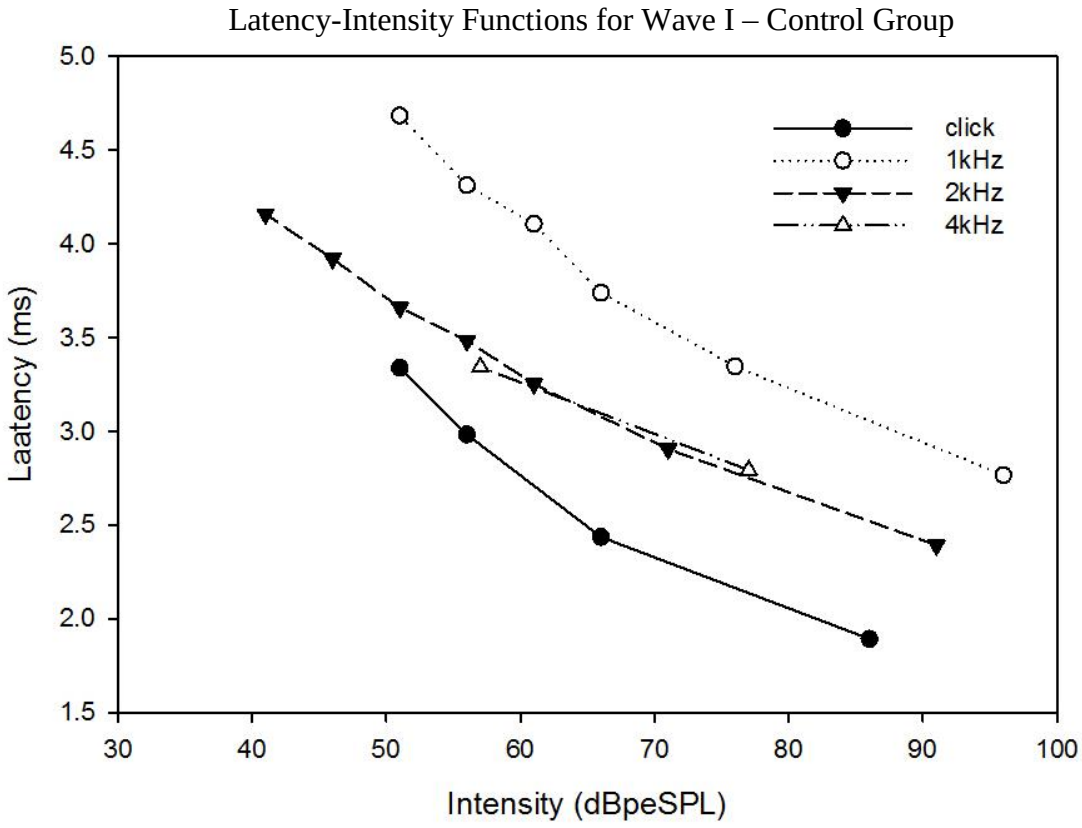

Figure 2. Average latency intensity functions for Wave I from the auditory brainstem evoked potential in normal control Bobwhite 3-7 days post-hatch (n=7). Standard errors have been omitted for clarity. Latency to Wave I was corrected for conduction delays between the sound source and the entrance of the ear canal of the animal (3.21 ms).

Average ABR thresholds for Wave I in normal, control 3-7 day old Bobwhite quail in the current study are compared to mature CAP thresholds in pigeon, chicken and canary (adapted from Gleich and Langemann, 2011) in Figure 3. ABR thresholds for hatchling Bobwhite quail are similar to mature CAP thresholds from 1,000-2,000 Hz in pigeon and chicken, both precocial birds. Thresholds for canary, an altricial bird, are generally better, especially above 1,000 Hz, than pigeon, chicken or Bobwhite quail. Behavioral auditory thresholds in hatchling chickens reach mature levels for frequencies above 1,000 Hz by 4 days post-hatch (Gray and Rubel, 1984). Bobwhite quail, like

chickens, are precocial and while the development of auditory sensitivity is not known at this point, it seems reasonable to expect that, like other precocial birds, threshold sensitivity for most frequencies have reached maturity within 3-7 days post hatch. ABR thresholds for hatchling Bobwhite quail at 4,000 Hz appear to be worse than canary but better than previously seen in adult pigeon and chicken.

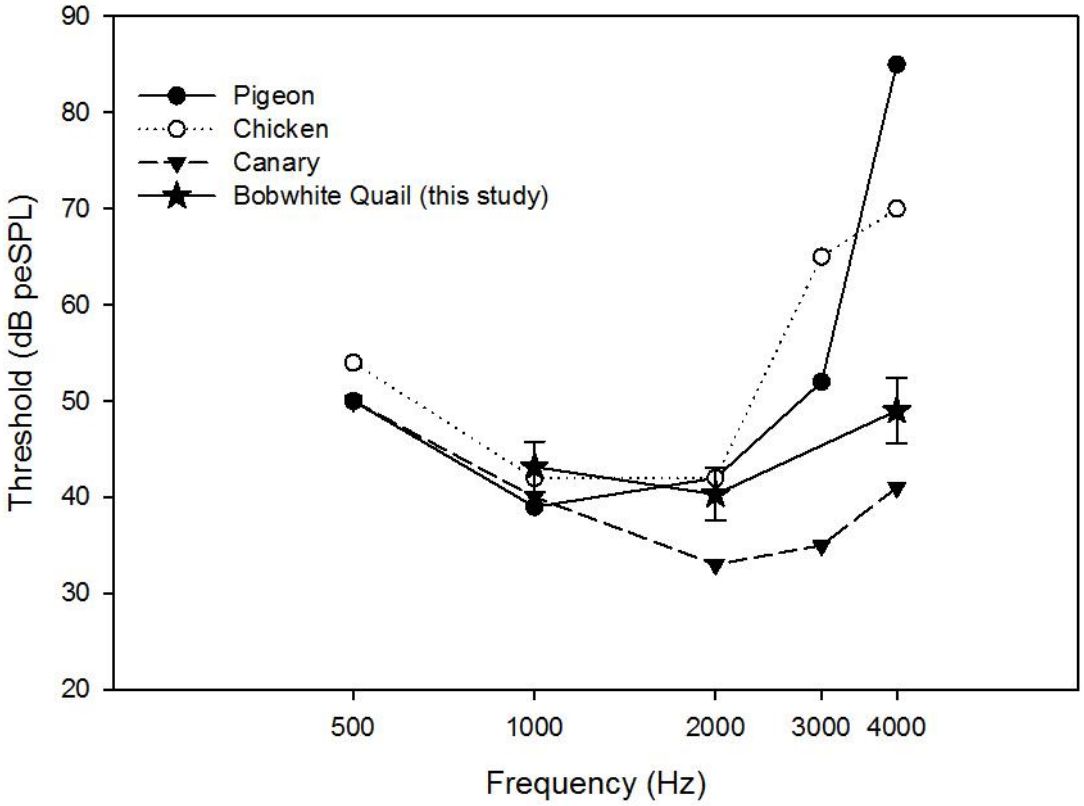

Figure 3. Comparison of compound action potential evoked responses from pigeon, chicken and canary (adapted from Gleich and Langemann, 2011). Compound action potential evoked responses compared with auditory evoked brainstem responses from 3-4 day old Bobwhite quail in the current study (n=7). It should be noted that compound action potentials from Gleich and Langemann (2011) were measured in dB SPL; ABR thresholds from this experiment were measured in dB peSPL.

3.1b Experimental

ABR thresholds were absent or elevated in all 3-4 day old Bobwhite quail injected with gentamicin at hatch. Waveform morphology, even at the highest intensities, was substantially poorer for the experimental group compared to the control group. In more than half of the birds (60%, 9 out of 15), no responses were seen for any of the stimuli at the maximum speaker output. Table 1 summarizes the threshold responses in the remaining six (40%) birds. No injected birds had reliable ABR responses at either 2,000 Hz or 4,000 Hz; however, 6 birds had present but elevated thresholds for clicks (n=3; average threshold = 73 dB peSPL ±12) or 1,000 Hz tone pips (n=4; average threshold = 91 dB peSPL ±10).

Bird number	**Click (dB peSPL)**	**1,000 Hz (dB peSPL)**	**2,000 Hz (dB peSPL)**	**4,000 Hz (dB peSPL)**
P71	66	76	NR	NR
Y92	NR	96	NR	NR
Y93	86	NR	NR	NR
R15*	NR	96	NR	NR
R16	NR	96	NR	NR
P55	66	NR	NR	NR

*also have histology results

Table 1. Thresholds for experimental birds. Individual thresholds for the six experimental birds (40%) where responses could be detected for a click or a 1,000 Hz tone pip. No responses were seen in any birds to stimuli presented at 2,000 Hz or 4,000 Hz. NR= no responses detected at maximum output of stimulus.

3.2 Histology of Basilar Papilla

Histology was performed in a randomly chosen subset of six of the fifteen experimental group quail. Figure 4 shows fluorescent light microscopy images of basilar papilla in control (panel A; bird #R21) and experimental (panel B; bird #P03) quail 4-5 days post-hatch. Figure 4B shows a typical example of the basilar papilla in one of the experimental quail (P03) showing complete hair cell loss in the basal 50% of the basilar papilla and a visible transition zone of hair cell loss between 60-70% of length. The length of the basilar papilla was measured and sectioned into ten deciles, each decile was placed in one of the four categories and the estimated hair cell loss in each decile subsequently graphed (see Figure 5a). Of the six quail with histological results all had total hair cell loss in the basal half of the BP; five (83.3%) had total hair cell loss across the basal 60% of the basilar papilla. Figure 5 (b) shows the corresponding ABR thresholds measured for the six experimental group quail with histological results. Five of the six quail had no thresholds present at any stimuli. Bird R15 had one threshold, albeit elevated, at 1,000 Hz. Figure 5 (a) and (b) below show similar hair cell loss across the basilar papilla when compared to the other experimental group quail; however, R15 had a present threshold at 96 dB nSPL.

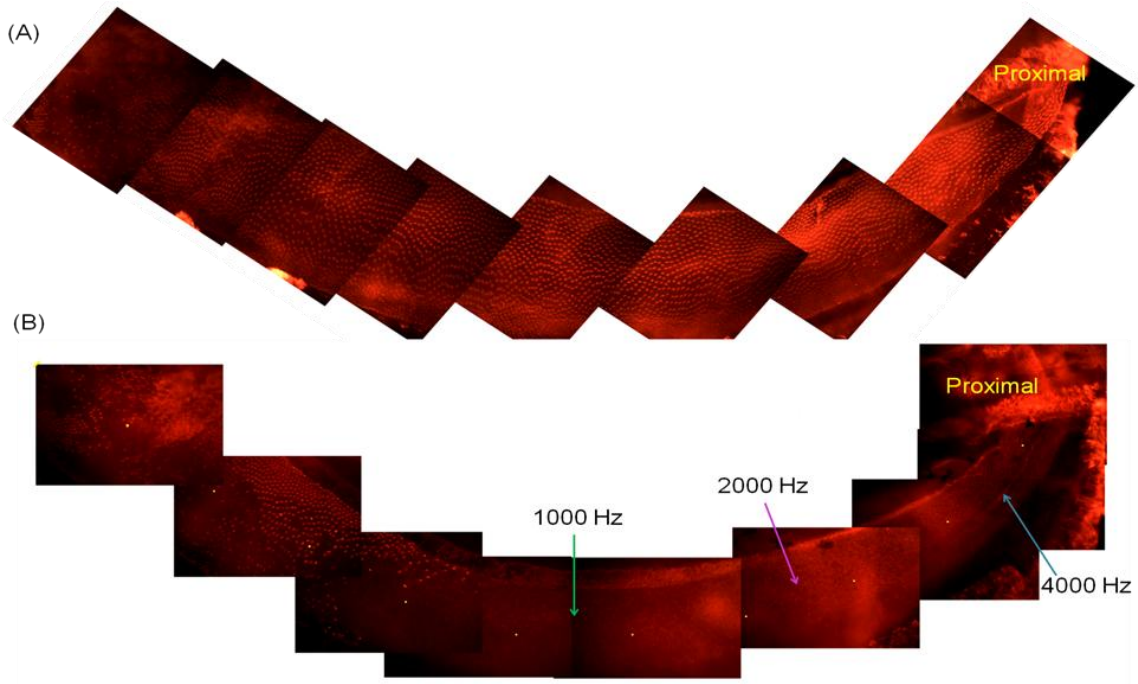

Figure 4. Fluorescent light microscopy images of the basilar papillae from a control and experimental Bobwhite quail (16X magnification). Figure 4(A) shows a control group (R21) basilar papilla with hair cells clearly present across the entire length of the basilar papilla. Figure 4(B) shows an experimental group (P03) basilar papilla with hair cells missing across approximately 60% of the basilar papilla. Each small yellow dot represents one decile, or 10% length of the basilar papilla. Three arrows are present and represent the approximate place-frequency for 4,000 Hz, 2,000 Hz, and 1,000 Hz as adapted from Dooling et al (2006).

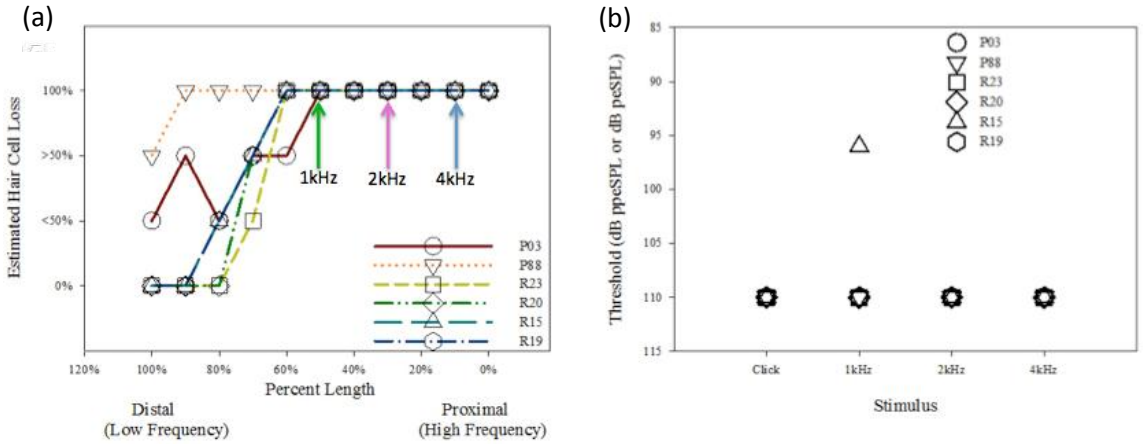

Figure 5. (a) The percent of hair cell loss that occurred according to length along the basilar papilla. Of the six quail with histological results, five had 100% hair cell loss up to 60% length, **(b) ABR thresholds for the six quail with histological results.** Place frequency is shown on figure (a), adapted from Dooling et al. (2006.)

4.0. Discussion

4.1 ABR Findings

Previous studies in both altricial and precocial birds have shown hair cell loss and subsequent threshold shift following a single dose of aminoglycoside antibiotics such as gentamicin. Those studies suggested that hatchling birds were more susceptible, meaning they incurred more hair cell loss at similar doses than adults. Those studies also suggested hair cell loss could reliably be predicted over the basal one-third of the basilar papilla within 3-5 days post-injection. No previous studies in precocial birds have shown consistent hair cell loss over an area greater than the basal one-third. The basal one-third of the basilar papilla generally corresponds to frequencies above 2,000 Hz. Predictively then, damage incurred over a greater length of the basilar papilla would correspond to frequencies lower than 2,000 Hz on the place-frequency map, encompassing more of the best frequency hearing of the Bobwhite quail.

In the current study we were interested in determining whether we could obtain a consistently greater extent of hair cell loss after aminoglycoside ototoxicity in Bobwhite quail and whether threshold shifts for frequencies below 2,000 Hz could be correspondingly induced. To begin, a similar or higher dose level as had been reported to be ototoxic in hatchling white leghorn chickens (100-250 mg/kg) was assumed necessary to induce similar or greater hair cell loss. Surprisingly the dose used in hatchling chicks (250 mg/kg) resulted in an unacceptable mortality rate in Bobwhite quail. The dosing was thus reduced from 250 mg/kg to 200 mg/kg. This dose, slightly lower than used in previous studies, resulted in a much more extensive loss of hair cells along the basilar papilla, complete loss of hair cells in the basal 50%, than previously reported. Auditory

Brainstem Response threshold measurements confirmed that frequency sensitivity at 1,000 Hz was elevated or absent in all of the birds tested when compared to age-matched peers. Interestingly, the only animal examined histologically (P03) with some hair cells present in the areas of the basilar papilla associated with 1,000 Hz sensitivity had no response on ABR thresholds at the output limits of the system. Conversely, bird R15 had apparent hair cell loss in the same area sensitive for 1,000 Hz and had elevated, but present, ABR thresholds at 1,000 Hz. These correlational relationships between location on the basilar papilla and ABR threshold sensitivity could be interpreted to mean that the area along the basilar papilla important for 1,000 Hz is wider than a single percentage of length. Mentioned previously the length of the basilar papilla was measured, averaged and divided into deciles. Therefore, it is reasonable, given these results, to assume that the area of the basilar papilla that is sensitive to 1,000 Hz is longer than one decile or is in between more than one decile. In humans we know that frequency bands expand within the cochlea as frequency decreases; therefore, it is plausible that the same happens in the Bobwhite quail basilar papilla. These results suggest that hatchling Bobwhite quail are more susceptible to hair cell damage through ototoxicity than hatchling chickens and that airborne hearing sensitivity can be significantly reduced, or eliminated, for at least the first 4-5 days after hatch. The reason for this difference in susceptibility is unknown. Previous studies have found large species differences in susceptibility to hair cell loss in birds following acoustic trauma (Ryals et al., 1999). Further, variability in susceptibility to ototoxic drugs across adult avian species has been noted (Cotanche, 1999; Custer et al., 1979; Janas et al., 1995). One difference between white leghorn chickens and coturnix quail, the primary avian species used for studies of ototoxicity and hair cell regeneration,

and Bobwhite quail is genetic heterogeneity. White leghorn chickens and coturnix quail both have been bred for genetic homogeneity, generally to produce consistency in meat amount and quality and/or egg lying. Bobwhite quail, conversely, are generally found in the wild, or are raised on individual small farms where breeding is not commercially controlled. Laboratory studies reporting differences between wild type and laboratory-bred animals are well known and our results may represent such a difference.

We found histological evidence of hair cell regeneration in at least one quail (P03) 4-5 days after drug dose suggesting that the regenerative response was not eliminated by this extensive hair cell loss. Threshold estimation after hair cell regeneration was not a goal of this study and was not determined. Nevertheless, previous studies in precocial white leghorn chicks would suggest that thresholds return to a plateau level by about 3-5 weeks post ototoxic drug administration based on regeneration of hair cells matching those of age-matched peers (Janas et al., 1995). Thus, our results suggest that substantial sensorineural hearing loss can be produced in Bobwhite quail for the first several weeks of life. This is an important first step in developing an animal model for the study of the influence of early, profound, sensorineural hearing loss on auditory perception in adulthood.

In this study it has been shown that hair cells are reliably lost over the basal half of the Bobwhite basilar papilla and that this loss corresponds to profound hearing loss for frequencies above 1,000 Hz. The minimal audibility curve of the Bobwhite quail indicates that best hearing is between 1,000-3,000 Hz; therefore we can conclude that the hearing loss induced represents a substantial change in the auditory world for these developing birds. Further studies will be necessary to confirm the timeframe and the

extent of hearing recovery. Nevertheless, our results confirm that hair cells can be reliably destroyed and profound hearing loss induced in hatchling Bobwhite quail to an extent far greater than has previously been reported in other precocial birds. Future studies may not only look at how this disruption in hearing early in life can affect future perceptions of hearing but also address questions about whether perceptual patterns are learned, meaning they require auditory experience post-hatch, or are inherent.

Bobwhite quail have been shown to recognize the maternal call within hours of hatching (Lickliter & Stoumbos, 1992). It is well known that this early response to the maternal call can be altered by experience (i.e. lack of exposure, hearing loss, etc.); however, it is yet unknown whether there is a critical timeframe for experiential influences. As in humans, it may be that these birds experience a "critical period" in early development that is crucial for accurate perception of complex auditory signals and, further, production of these same auditory signals in adulthood. Defining the timeframe and extent of recovery in future studies will require further development of the currently proposed model and will give a window into the effects of early sensorineural hearing loss.

Appendix A. Literature Review

i. Aminoglycosides and Ototoxicity

Aminoglycosides have long been used to treat infections, specifically "aerobic gram negative bacterial infections" (Bindu and Reddy, 2008, pp. 703), gram positive bacterial infections (Steyger, 2011; Waguespack and Ricci, 2005) and various other pathogenic microorganisms (Custer et al., 1979; Steyger, 2011). These antibiotics are known to be ototoxic and are difficult to metabolize, thus they remain in the system for longer periods of time. These can lead to renal, vestibular or auditory disturbances; renal toxicity is typically reversible, while ototoxicity and vestibulotoxicity are not. Aminoglycosides bind to the ribosomal RNA of bacterial ribosomes and cause "mistranslation or premature termination of protein synthesis" (Bindu and Reddy, 2008, pp. 703). This process causes an increase in the reactive oxygen species (ROS) within the cochlea and damages the mitochondrial and cellular proteins, lipids and nuclear acids; thus, leading to hair cell death and/or possibly dysfunction of the neurons. (Bindu and Reddy, 2008; Waguespack and Ricci, 2005)

The toxicity caused by aminoglycosides has a known course as it moves along the length of the cochlea. It is a systemic response, meaning it occurs bilaterally, and begins in the base (proximal) and progresses distally to the apex. This is the same progression in all species, including humans and birds. (Janas et al., 1995)

Steyger (2011) found that gentamicin, specifically, overloads stria vascularis and causes a change in the ion composition of endolymph. The movement of gentamicin from stria vascularis into the marginal cells of the cochlea occurs via an active process between the intermediate and marginal cells within the cochlea. Once gentamicin is

within the marginal cells, it is able to passively diffuse into endolymph surrounding the hair cells. Once in the endolymph, ion channels open in hair cell stereocilia during excitation allowing flow of endolymph and aminoglycoside to enter the hair cells, where it has been shown to block the mechanotransducer (met) channel of sensory hair cells. The channel acts as a one-way entry for the aminoglycoside and allows for the buildup of the toxin within the cell. Subsequently, this buildup causes stereocilia and hair cell death. It has been suggested that treatments that open the met channels cause a greater deal of ototoxicity; conversely, those that close the met channels cause little to no ototoxicity. Steyger (2011) describes an increase in both ROS and intra-cellular calcium levels, which can be related to both apoptosis and/or necrosis; which form of cell death occurs, is linked to the duration and dose level of the aminoglycoside. Increases in both ROS and calcium levels are known to cause changes in threshold that be either temporary or permanent in nature. (Steyger, 2011; Marcotti et al., 2005, as cited in Waguespack and Ricci, 2005)

Similarly, aminoglycosides enter perilymph in both scala tympani and scala vestibuli. Aran et al. (1999, as cited in Steyger, 2011) found that the toxins did not cause any ill effects during the first 24 hours of being in the system. Steyger (2011) suggests that this shows aminoglycosides require a greater length of time within the cochlea in order to cross through to the endolymph-filled scala media and cause ototoxicity. Wang et al. (2009, as cited in Steyger, 2011) found higher doses of aminoglycosides within the perilymph were found to cause ototoxic effects quicker when compared to lower doses. This is important to note due to the variable dosing levels that have been shown to be ototoxic across aminoglycosides.

Previous studies have traditionally looked at a 5-10 day regiment of aminoglycoside administration to produce ototoxic insult. This process more closely mimics the time course used in humans to treat infections. However, when using an extended administration time it is difficult to determine on which day hair cell loss occurs. Thus, it is important to find a single dose that may be administered within the first 24 hours following hatch to be sure at what point following the intra-muscular injection hair cell loss/damage occurs (Janas et al., 1995). This is particularly critical for studies where the onset of ototoxic insult and the onset of regeneration are important to defining the developmental period of hearing loss.

ii. Avian Basilar Papilla

Bobwhite quail are precocial birds, meaning that hearing develops prior to hatch. Vanzulli and Garcia-Austt (1963, as cited in Gottlieb, 1968) found that they were able to record cochlear microphonics in chickens at gestational day 13 in response to low frequency tones (100-250 Hz). They were able to record responses up to 3000 Hz later than gestational day 16 and by day of hatch they were able to record responses up to 4000 Hz. However, Gottlieb (1968) notes that these tests were done under lower than optimal temperature conditions. Sedláček (1964a, as cited in Gottlieb, 1968) was able to obtain responses at gestational day 16 in response to up to 3000 Hz under more optimal conditions. According to Gottlieb (1968), the sensory systems develop in the following progression: non-visual photic sensitivity, tactile sensitivity, vestibular sensitivity, proprioception, audition, and finally vision. Audition is one of the last systems to develop in the chick embryo; it occurs around 60% gestation, or day 12. Gottlieb (1968)

asserts that the little information known regarding duck and pigeon embryos supports the findings in the chick embryo. (Gottlieb, 1968; Gray & Rubel, 1981)

The anatomy of the bird basilar papilla differs in several ways when compared with the human cochlea. The basilar papilla extends proximal to distal in the inner ear, with high frequencies being located at the proximal end. Unlike human cochleae the basilar papilla does not have 2 ½ to 2 ¾ turns, it does not spiral at all; rather, only curves slightly Similar to humans, the basal or proximal end is tuned for high frequencies, while the apical or distal end is tuned for low frequencies. Gray & Rubel (1984) and Rubel & Ryals (1983) found, in avian species, sensitivity to low frequencies develops first, followed by high frequencies just 3 days later. The mammalian Organ of Corti develops similarly, with sensitivity to low frequencies occurring prior to high frequencies (Rubel & Ryals, 1983). They looked at differences in sensitivity between 1-day-old chicks and 4-day-old chicks; it was found that 4-day-old chicks were more sensitive, especially at 1,000 Hz and 2,000 Hz. (Gray & Rubel, 1984; Janas et al., 1995; Rubel & Ryals, 1983; Woolley et al., 2000)

Avian basilar papillae have short and tall hair cells that resemble human outer and inner hair cells in their innervation patterns and location across the basilar membrane. The short hair cells are located in the inferior edge of the basilar papilla, as is the case with human outer hair cells and are innervated predominately by efferent nerve fibers again similar to outer hair cells. Likewise, tall hair cells are positioned on the basilar membrane similarly to human inner hair cells and are largely innervated by afferent nerve fibers. Gleich and Manley (Hearing Organ of Birds and Crocodilia, 2000, in Dooling, R.J.; Fay R. R.; & Popper A. N.) state that there is no structural basis for differentiating

only two hair cell types on the basilar papilla. Rather they suggest a gradation of cell type from tall to short as one progresses along the length and width of the basilar papilla. There does appear to be a rough correlation between height of the hair cell and response frequency (Gleich and Manley, Hearing Organ of Birds and Crocodilia, 2000, in Dooling, R.J.; Fay R. R.; & Popper A. N.) The avian basilar papilla is, like the human basilar membrane, covered with a tectorial membrane. In order to perform histology on the avian inner ear, this must be removed. (Gleich and Langemann, 2011; Woolley et al., 2000)

The average length of the basilar papilla varies according to species ranging from 2 mm in small songbirds up to 12 mm in the barn owl. Gleich (2005) found a positive correlation between length of basilar papilla and body mass, meaning the larger the avian species the longer the basilar papilla. There are between 3,000 to 16,000 hair cells across the basilar papilla depending on species, with larger birds like the emu and barn owl having more. (Gleich and Langemann, 2011)

The best frequency for a particular bird varies depending on their species, for quail and other primitive land birds it has been shown to be at about 3000 Hz. Advanced land birds have a best frequency as low roughly 2100 Hz. The place-frequency map along the basilar papilla varies across species. However, it has been shown in chickens (*G. gallusdomesticus*), Starling (*S. vulgaris*), Rock Pigeon (*Columba livia*), and Emu (*Dromaiusnovaehollandiae*) that the first 10% length from proximal to distal is particularly coded to 4000 Hz, at roughly 30% length is 2000 Hz and at about 50% length is 1000 Hz (Gleich & Manley, Hearing Organ of Birds and Crocodilia, 2000, in Dooling, R. J.; Fay, R. R.; & Popper, A. N.) The number of hair cells across the width of the

papilla, or the number of stereovilli per hair cell and the maximum height all account for the place-frequency map along the basilar papilla. (Gleich and Langemann, 2011)

iii. Mammalian Organ of Corti

The dimensions of the human cochlea vary basally to apically, as do those of the avian basilar papilla. At the base, the human cochlea is close to 9 mm and it tapers as it winds its way towards the apex. It is approximately 35 mm in length and 5 mm high. At the center of the cochlea is the modiolus, which forms the axis of the spiral of the cochlea. The organ of Corti runs along the basilar membrane and is made up of a single row of inner hair cells and three rows of outer hair cells. There are as many as four to five rows of outer hair cells in the apical turn of the cochlea. (Gelfand, 2007; Pickles, 2008) The same pillar cells, with their tips ending in the reticular lamina, form the border between the scala media and scala tympani and do not allow the ions from both endolymph and perilymph to mix. (Clark, 2008; Pickles, 2008).

The organ of Corti is covered by the tectorial membrane; "a gelatinous and fibrous flap" (Pickles, pp. 28, 2008). Mentioned previously, the tectorial membrane is also present in the avian basilar papilla. It has been difficult to discern the actual ending of the lateral edge of the tectorial membrane because when it is preserved it tends to shrink and pull away from the reticular lamina. It is believed, however, that the tectorial membrane may be attached to the reticular lamina on its most lateral edge and in several other areas across the top. (Clark, 2008; Gelfand, 2007; Pickles, 2008)

The OHC are firmly embedded in the tectorial membrane by the stereocilia on the tops of each hair cell. The stereocilia are arranged in rows with the tallest farthest away from the modiolus. Stereocilia are formed of packed actin filaments, which make them

stiff; the same is true of the stereocilia present on the tops of hair cells within the avian inner ear (Gleich & Manley, Hearing Organ of Birds and Crocodilia, 2000, in Dooling, R. J.; Fay, R. R.; & Popper, A. N.) The stereocilia on top of a hair cell are linked together with tip links. The tip link begins at the top of a shorter stereocilia and stretches to the side of the next tallest stereocilia. These small fibrous links are surrounded by "amorphous material" (Pickles, 2008). They serve to join all the stereocilia together so they move as a bundle when one is pushed, usually the tallest. The tip links stretch as the stereocilia are bent away from the modiolus. Some of the actin filaments that form the stereocilia continue into the tops of the inner and outer hair cells and form rootlets. These rootlets anchor the stereocilia to the tops of the IHC and OHC. The rootlets of the stereocilia are embedded into the cuticular plate. The cuticular plate is made up of actin filaments, similar to the stereocilia; however, it forms a dense matrix in which each stereocilia rootlet is embedded. The cuticular plate has three rows of stereocilia embedded in it. There is also a noncuticular area in which the basal body of the rudimentary kinocilium resides. (Gelfand, 2007; Pickles, 2008)

The stereocilia of the IHC are not embedded in the tectorial membrane. It is hypothesized that the inner hair cells' stereocilia fit loosely into Hensen's stripe; a raised groove in the undersurface of the tectorial membrane. However, Hensen's stripe may be formed when the cochlea is preserved for examination. As stated before, the tectorial membrane shrinks as it is preserved and Hensen's stripe may be formed by the tectorial membrane forming around the IHC stereocilia. (Pickles, 2008)

iv. Ototoxicity in Humans

The variability of ototoxicity in humans has long been studied. According to Waguespack and Ricci (2005), between 2-5% of patients that receive aminoglycosides will also have permanent hair cell loss/damage. Bindu and Reddy (2008) have reviewed the link between genetics and ototoxicity; specifically aminoglycoside induced non-syndromic hearing loss (AI-NSHL). There have been several mutations linked to susceptibility to AI-NSHL located on the 12S rRNA gene (Bindu and Reddy, 2008, pp. 703-704). Mutations on this gene have been linked to many families that have non-syndromic hearing loss (NSHL) related to the use of aminoglycosides. (Bindu and Reddy, 2008)

Other possible risk factors were studied by Moore et al. (1984) in 135 patients at The Johns Hopkins Hospital, Baltimore who were receiving aminoglycoside IV antibiotics. Bedside audiometry was done on all patients and risk factors were assessed for their likelihood to contribute to ototoxicity. Of the 135 patients, ototoxicity occurred in thirty patients, or 22.3% of patients. According to Moore et al.'s (1984) criteria, all patients needed to have hearing loss at ≥1000 Hz. Of the thirty that were deemed as having ototoxicity, twenty-nine of them had a loss at either 4000 Hz or 8000 Hz; and seventeen of the thirty had a loss at two or more frequencies. Following the end of treatment, twenty-two patients had follow-up audiological testing and 50% had partial or complete recovery of thresholds within 24 hours to 14 weeks. The average threshold change during treatment was 20 dB; there were two patients that had ≥50 dB threshold shift during treatment. Of these patients, there were four significantly contributing risk factors for ototoxicity: "total aminoglycoside dose, duration of aminoglycoside therapy,

the initial peak temperature of the patient, and bacteremia" (Moore et al., 1984, pp. 25-26).

It was previously thought that all patients receiving aminoglycoside treatments would incur permanent hearing loss; Fee (1980, as cited in Girod et al., 1991) found that roughly 55% of patients regained some hearing sensitivity between 1 week and 6 months following the end of treatment. Dulon et al. (1993, as cited in Hashino et al., 1995) found the progressive removal of gentamicin from the mammalian inner ear is quite slow given that the half life of gentamicin is roughly 5-6 months following the final treatment. (Dulon et al., 1993, as cited in Hashino et al., 1995; Girod et al., 1991)

The recommended schedule for administering gentamicin to humans is a concentration of 1 to 1.7 mg/kg every 8 hours. In humans the drug half life is between 2-3 hours; this is much shorter than that for amphibians and longer than that of Japanese quail (*Coturnixcoturnix*). For Japanese quail, Custer et al. (1979) found the half life to be approximately 42 minutes. These differences in half life of the drug are believed to be a result of varying metabolic rates; reptiles have a slower metabolic rate than humans, while humans have a slower metabolic rate relative to birds. This may also be a contributing factor in the differences of ototoxic effects seen within a species (i.e. Bobwhite quail, Japanese quail, or humans). (Custer et al., 1979)

v. Ototoxicity in birds

Gentamicin and other aminoglycosides have been used as a preventative measure for newly hatched chicks or to treat bacterial infections in avian species. However, given the known ototoxic effects of aminoglycosides, the dosing used is of great concern. According to Custer et al. (1979), there is a fine line between therapeutic dosing and oto-

or nephro-toxic effects. Peak concentration levels of the drug in plasma have been studied to find the effects of various dosages of gentamicin. Custer et al. (1979) found that a peak concentration level of 12 to 15 µg/ml is ototoxic. Thus, for it to be therapeutically effective, without being ototoxic, the peak concentration levels must be kept below 12-15 µg/ml. (Custer et al., 1979)

As mentioned previously, the rate at which gentamicin is absorbed and processed varies with species. Custer et al. (1979) found that even within a species, the concentration levels of gentamicin were variable between individual birds. As would be expected, larger doses of the drug produced greater concentration levels and were detectable for longer periods of time in the plasma (Custer et al., 1979).

According to Cotanche (1999) and Janas et al. (1995), many of the studies involving ototoxicity in birds have used gentamicin or kanamycin with a 5-10 day progressive introduction of the drug to the system. Traditionally, when administered to a patient, aminoglycosides are given as a 5-10 day treatment regimen, thus it has been used as the dosing pattern in birds to mimic how ototoxicity occurs in humans. Differences in the lasting effects of kanamycin versus gentamicin have been hypothesized using patterns of regeneration. Birds injected with kanamycin tended to show damage and regrowth of hair cells in the proximal region of the basilar papilla; however, birds injected with gentamicin tended to have damage first in the proximal region of the basilar papilla and this area was the last to produce mature hair cells following injections. Cotanche (1999) has postulated that gentamicin has a cumulative effect because these basilar hair cells matured more slowly after gentamicin as compared to kanamycin (Cotanche, 1999).

Historically, the use of kanamycin or gentamicin produced total hair cell loss along the first 25-50% length of the basilar papilla, starting at the most proximal portion (Cotanche, 1999, pp. 277). Cotanche (1999) found that damage incurred due to the use of aminoglycosides occurs in the basal, or proximal, region first; thus effecting high frequency thresholds. The damage occurs across the entire width of the basilar papilla and spreads distally; the extent to which it spreads to the low frequency region of the papilla is grossly dependent on the dose (Cotanche, 1999; Hashino et al., 1995; Woolley et al., 2000). Damage tended to begin four to five days post injection and continued to occur as far after as the tenth day. More recently, the effects of a single injection of gentamicin have been studied. Total hair cell loss has been recorded across the proximal 25% of the basilar papilla when using a single injection at a dose of 100 mg/kg in 1-day-old chicks (Janas et al., 1995). Similarly, Bhave et al. (1995; as cited in Cotanche, 1999, pp. 277) used a dose of 250 mg/kg of gentamicin in 3-day-old chicks and found total loss through the proximal 45% of the basilar papilla. Cotanche (1999) states that a single dose of gentamicin appears to be able to cause total hair cell loss along the first 25-50% of the basilar papilla. However, as chicks age a higher dose is needed in order to produce consistent, total hair cell loss (Cotanche, 1999, pp. 278). As the goal of this research project is to look at hair cell loss to delay the onset of air-conducted hearing, chicks were injected within 24 hours of hatch.

Janas et al. (1995) looked at the efficacy of a single, subcutaneous injection of 100 mg/kg given to white leghorn chickens within 24 hours of hatch. These were divided into three survival groups: 3-5 days post injection, 2 weeks post injection and 5 weeks post injection. Those sacrificed at 3-5 days post injection had variable amounts of

damage along the length of the basilar papilla. The greatest amount of damage occurred in a single chick that was sacrificed at 3 days post injection. Damage extended proximal to distal up to 45-50% of length. The least amount of damage occurred in one of the chicks sacrificed at 5 days post injection; damage extended proximal to distal up to 18-20% length. The other chicks studied had damage occurring between the two at variable amounts. (Janas et al., 1995)

vi. Using Ototoxicity to Delay the Onset of Air-Conduction Hearing

A study looking at the degree of hearing loss incurred and the consistency of hearing loss needs to be completed before the perceptual effects of delayed onset air-conduction hearing can be looked at. It is necessary to find the degree and how consistent hearing loss is before looking at how it effects the development of complex auditory perception that is so important in avian species. Gottlieb (1968, pp. 166) asserts that the absence of auditory stimulation should alter the development of auditory discrimination and perception, but should not alter the rate at which the system develops. An experiment conducted by Gottlieb (1968) found that communally incubated Mallard ducklings were able to discriminate a species specific maternal call; however, those that were isolated during the incubation process from Day 23 on were unable to discriminate a species specific maternal call. Similarly, Lickliter et al. (1992) studied the effects of prenatal perceptual experience in Bobwhite quail. In this experiment, Bobwhite quail eggs were kept in isolation with only the sounds of their broodmates and their own embryonic and postnatal vocalizations. In the first part of this experiment chicks were exposed to either a normal presentation rate of the maternal call or an increased presentation rate prenatally. Their responsiveness to the maternal call at a normal

presentation rate was tested at 12 and 24 hours post-hatch. Those chicks exposed to the increased presentation rate responded typically at 12 hours but nearly half did not respond at all at 24 hours post-hatch. Similarly, those exposed in a second experiment to the increased presentation rate preferred the increased presentation rate over the typical presentation rate of the maternal call. This shows that normally occurring auditory stimulation is important to the development of auditory discrimination during the gestational period. A follow-up study showed that there was a temporal lag in the development of auditory discrimination, rather than a complete lack thereof. (Gottlieb, 1968; Gray &Rubel 1981; Gray &Rubel, 1984; Lickliter et al., 1992)

Appendix B.

Table 2. ABR thresholds for all birds. All ABR Threshold measures for both the experimental and control group. Listed across the top are stimuli and down the right side are leg band numbers for each individual bird.

Bird	Click	1kHz	2kHz	4kHz	Injected
P03	NR	NR	NR	NR	Experimental
P88	NR	NR	NR	NR	Experimental
P02	NR	NR	NR	NR	Experimental
P55	66	NR	NR	NR	Experimental
P59	NR	NR	NR	NR	Experimental
P71	66	76	NR	NR	Experimental
P26	NR	NR	NR	NR	Experimental
Y91	NR	NR	NR	NR	Experimental
Y92	NR	96	NR	NR	Experimental
Y93	86	NR	NR	NR	Experimental
R15	NR	96	NR	NR	Experimental
R16	NR	96	NR	NR	Experimental
R19	NR	NR	NR	NR	Experimental
R20	NR	NR	NR	NR	Experimental
R23	NR	NR	NR	NR	Experimental
P66	51	36	41	57	Control
P63	NR	51	56	57	Control
P06	51	51	41	NR	Control
R08	36	46	36	47	Control
R05	46	46	36	42	Control
R06	41	36	36	42	Control

R18	46	36	36	NR	Control

Resources

1. Barton, L., Bailey, E. D., & Gatehouse, R. W. (1984). Audibility curve of bobwhite quail (Colinus virginianus). *Journal of Auditory Research*, 87-97.

2. Bindu, L. H., & Reddy, P. P. (2008). Genetics of aminoglycoside-induced and prelingual non-syndromic mitochondrial hearing impairment: A review. *International Journal of Audiology*, 702-707.

3. Brittan-Powell, E. F., & Dooling, R. J. (2002). Auditory brainstem responses in adult budgerigars (*Melopsittacus undulatus*). *Journal of Acoustical Society of America*, 999-1008.

4. Chang, J., Yang, J. Y., Choi, J., Jung, H. H., & Im, G. J. (2011). Calcium imaging in gentamicin ototoxicity: Increased intracellular calcium relates to oxidative stress and late apoptosis. *International Journal of Pediatric Otorhinolaryngology*, 1616-1622.

5. Clark, W. W., & Ohlemiller, K. K. (2008). *Anatomy and Physiology of Hearing for Audiologists.* New York: Thomspon Delmar Learning.

6. Cotanche, D. A. (1999). Structural Recovery from Sound and Aminoglycoside Damage in the Avian Cochlea. *Audiology and Neuro-Otology*, 271-285.

7. Custer, R. S., Bush, M., & Carpenter, J. W. (1979). Pharmacokinetics of Gentamicin in Blood Plasma of Quail, Pheasants, and Cranes. *American Journal of Veterinary Research*, 892-895.

8. Dooling, R. J., Ryals, B. M., Dent, M. L. and Reid, T. L. (2006) Perception of Complex Sounds in Budgerigars (*Melopsittacus undulates*) with Temporary Hearing Loss, *Journal of the Acoustical Society* 119(4):1-9.

9. Epstein, J. E. & Cotanche, D. A. (1995). Secretion of a new basal layer of tectorial membrane following gentamicin-induced hair cell loss. *Hearing Research*, 31-43.

10. Gelfand, S. A. (2007). *Hearing: An Introduction to Psychological and Physiological Acoustics (4th Edition Revised and Expanded).* New York: Informa Healthcare USA, Inc.

11. Girod, D. A., Tucci, D. L., & Rubel, E. W (1991). Anatomical Correlates of Functional Recovery in the Avian Inner Ear Following Aminoglycoside Ototoxicity. *Laryngoscope 101*, 1139-1149.

12. Gleich, O., & Langemann, U. (2011). Auditory capabilities of birds in relation to the structural diversity of the basilar papilla. *Hearing Research*, 80-88.

13. Gleich, O., & Manley, G. A. (2000). Hearing Organ of Birds and Crocodilia. In R. J. Dooling, R. R. Fay, & A. N. Popper, *Comparative Hearing: Birds and Reptiles.* New York, NY: Springer-Verlag New York, Inc.

14. Gleich, O., Dooling, R. J., & Manley, G. A. (2005). Audiogram, body mass, and basilar papilla length: correlations in birds and predictions for extinct archosaurs. *Natur Wissenschaften*, 595-598.

15. Gottlieb, G. (1968). Prenatal Behavior of Birds. *The Quarterly Review of Biology*, 148-174.

16. Gray, L. & Rubel, E. W (1981). Development of Responsiveness to Suprathreshold Acoustic Stimulation in Chickens. *Journal of Comparative and Physiological Psychology;* 95:1, 188-198.

17. Gray, L. G. & Rubel, E. W (1984). Development of absolute thresholds in chickens. *Journal of the Acoustical Society of America,* 1162-1172.

18. Hashino, E., TinHan, E. K., & Salvi, R. J. (1995). Base-to-apex gradient of cell proliferation in the chick cochlea following kanamycin-induced hair cell loss. *Hearing Research*, 156-168.

19. Janas, J. D., Cotanche, D. A., & Rubel, E. W (1995). Avian cochlear hair cell regeneration: stereological analyses of damage and recovery from a single high dose of gentamicin. *Hearing Research*, 17-29.

20. Jones, S. M., & Jones, T. A. (1995). The tonotopic map in the embryonic chicken cochlea. *Hearing Research*, 149-157.

21. Köppl, C. (2011). Birds - same thing, but different? Convergent evolution in the avian and mamalian auditory systems provides informative comparative models. *Hearing Research*, 65-71.

22. Lickliter, R., Stoumbos, J. (1992). Modification of prenatal auditory experience alters postnatal auditory preferences of bobwhite quail chicks. The Quarterly Journal of Experimental Psychology Section B: Comparative and Physiological Psychology 44(3-4): 199-214. DOI: 10.1080/02724999208250612

23. Lippe, W. R., Westbrook, E. W., & Ryals, B. M. (1991). Hair cell regeneration in the chicken cochlea following aminoglycoside toxicity. *Hearing Research, 56,* 203-210.

24. Mann, Z. F., & Kelley, M. W. (2011). Development of tonotopy in the auditory periphery. *Hearing Research*, 2-15.

25. Marcotti, W., van Netten, S. M., & Kros, C. J. (2005). The aminoglycoside antibiotic dihydrostreptomycin rapidly enters mouse outer hair cells through the machano-electrical transducer channels. *The Journal of Physiology, 567*, 505-521.

26. Moore, R. D., Smith, C. R., & Lietman, P. S. (1984). Risk Factors for the Development of Auditory Toxicity in Patients Receiving Aminoglycosides. *The Journal of Infectious Diseases*, 23-30.

27. Pickles, J. O. (2008). *An Introduction to the Physiology of Hearing.* Bingley, BD: Emerald.

28. Ryals, B.M, Dooling, R.J., Westbrook, E., Dent, M, Linzenbold, A., Larsen, O. (1999). Avian Species Differences in susceptibility to noise exposure. *Hearing Research*, 131:71-88.

29. Rubel, E. W & Ryals, B. M. (1983). Development of the Place Principle. *Science*, 219:4584; 512-514.

30. Steyger, P. S. (2011). Mechanisms Involved in Ototoxicity. *Seminars in Hearing*, 217-228.

31. Waguespack, J. R., & Ricci, A. J. (2005). Aminoglycoside ototoxicity: permeant drugs cause permanent hair cell loss. *Journal of Physiology*, 359-360.

32. Woolley, S. M., Wissman, A. M., & Rubel, E. W (2001). Hair cell regeneration and recovery of auditory thresholds following aminoglycoside ototoxicity in Bengalese finches. *Hearing Research*, 181-195.