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The effect of early hearing loss on Bobwhites' responses to pure tones: A possible animal model of prelingual deafness

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The effect of early hearing loss on Bobwhites' responses to pure tones:

A possible animal model of prelingual deafness

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ABSTRACT

This study aimed to look at the behavioral responses of Bobwhite quail to pure tone stimuli by measuring peep suppression. We also considered if a duration of early hearing loss would affect Bobwhite's responsiveness to the tones. Bobwhites were tested individually at all different ages post hatch day. The pure tones were presented at several different intensity levels and peep suppression was calculated as a measure of responsiveness to the stimuli. Mock trials were conducted as a measure of control to determine the birds' typical peep rate. Some of the Bobwhite quail were injected with gentamicin 0-1 post-hatch day in order to simulate an early hearing loss. The control quail (those who did not experience a period of deafness) were found to be more responsive than the treated Bobwhites (those who experienced a period of deafness). The treated quail were less responsive than the control quail during their period of hearing loss but responsiveness did seem to improve once the hair cells were regenerated. However, the treated quail did not respond nearly as well as the control birds. A period of deafness does seem to have an effect on responsiveness to pure tone stimuli. There also seems to be something occurring, developmentally, at 15-21 days post-hatch. A follow up study would examine the responsiveness to species specific calls. And once accurate thresholds are attained with species specific calls, the effect of a duration of hearing loss on responsiveness on species specific calls can be measured. There is also hope that the quality of hearing following hair cell regeneration may be measured by altering the envelope and fine structure of the species specific call.

I. INTRODUCTION

Birds are often used as a model of human hearing due to many factors. The anatomy and functionality of bird and human auditory systems are very similar. The auditory system, in both birds and humans, develops rather early in embryonic development, and they both heavily rely on their auditory systems to understand their environments and to communicate.

Birds' hearing can also be measured with the same methods used to measure humans' hearing. Physiological measures such as otoacoustic emissions and auditory brainstem responses allow assessment of the functionality of certain structures, cells and pathways, but they do not provide a true measure of hearing sensitivity. The gold standard for audiological testing is behavioral testing. Gray (1987, 1992) found that psychometric functions of chicks could be generated by measuring peep suppression. Peep suppression is defined as the duration of time between the onset of a stimulus to the onset of the 2nd post-stimulus peep. Neonatal birds peep rather constantly but pause in their peeping when they detect auditory stimuli. Essentially, the longer the bird stops peeping, the more responsive that bird is to that stimulus (Gray, 1987; Gray, 1992; Gray & Jahrsdoerfer, 1986). Most avian species stop peeping after about 2 weeks following hatching, but Bobwhite quail continue to peep until they are about 30 days old. This fact about Bobwhites makes them particularly desirable for behavioral audiometry studies.

Now, it has been widely studied that birds are able to regenerate their auditory hair cells. It is understood that with the regeneration, functionality also returns, as does sensitivity, but with time. ABR studies on chicks have shown that the return to normal or near normal thresholds can take up to 20 weeks following the incident of damage (Girod, Tucci, & Rubel, 1991; Roberson & Rubel, 1995); yet hair cells are regenerated in just a

few days' time (Cotanche, 1999; Epstein & Cotanche, 1995). Since Bobwhites peep for so long, we can obtain behavioral measures of their hearing with peep suppression following hair cell damage and regeneration. The quality of hearing following regeneration is vital in order to determine if we should work towards inducing hair cell regeneration in humans.

Periods of hearing loss in humans can be detrimental to speech and language development, especially early in life. The critical period for developing speech and language has been determined to be between birth and about 5-7 years of age (Waltzmann & Roland, 2005; Yoshinaga-Itano et al., 1998); thus, a late diagnosis of congenital hearing loss can result in developmental delays in a child. While birds naturally regenerate their hearing, humans depend upon amplification options such has hearing aids and/or cochlear implants to improve their hearing sensitivity. But, it is unknown how an early onset of hearing loss would affect a bird's quality of hearing.

We know that the bird's anatomy would be capable of sending the signal to the brain, but would an early onset of hearing loss also alter the bird's responsiveness to sound? The only way to measure this would be to induce hearing loss soon after hatching and complete behavioral testing as their hearing returns.

Aminoglycosides are extremely ototoxic and have been proven to be effective at inducing hair cell damage in most species. Therefore, this study aims to induce significant hair cell damage across the basilar papilla with a single injection of gentamicin; measure the peep suppression to pure tones in Bobwhites who have experienced a period of hearing loss; and compare their suppression to normal

Bobwhites. We hypothesize that treated Bobwhites will not be as responsive to the stimuli as normal Bobwhites.

The purpose of this study is to assess the quality of "regenerated" hearing sensitivity in quail to pure tones and then eventually to complex stimuli such as species-specific calls (i.e. the maternal call).

II.   METHODS

A. Subjects

The test subjects were incubator-bred Bobwhite quail (*Colinus virginianus*) either

bred from mature quail in the lab or received as eggs from a commercial supplier. The

eggs were collected daily, numbered, and incubated in a Petersime Model I incubator for

20 days. They were removed from the Petersime on embryonic day 20 and then

transferred to a still-air incubator to hatch. The hatching incubators were monitored daily

and each hatchling received a unique numbered leg band.

The "treated" chicks received an intraperitoneal injection of gentamicin on their

hatch day (P0) or one day post hatch (P1) at a dosage of 200 mg/kg. The control birds

received no injection. All animals were treated in accordance with the National Institutes

of Health and Institutional Guidelines.

B. Equipment and Instrumentation

Each subject was tested individually, placed in a Plexiglas cylinder (140 cm ID

and 120 cm high) with wire screen top and bottom suspended inside a small Industrial

Acoustics Corporation double-walled sound booth (3'4" by 4' by 6'6" high) . The cage

was suspended 34 cm above a 14 cm diameter hole in a specially built speaker box. This

hole was about 16 cm above a 45° reflecting plate that was about 20 cm from a vertically

mounted JBL LE8T-H 14-cm diameter speaker which would play the different pure tones

via a Hafler P1000 amplifier. An Electrovoice Model 645N/D-B Dynamic Cardioid

microphone was suspended and centered above the cage to record the peeps of the

subject. An acoustical engineer had designed a specially built 43x58 x91 cm box with a

43x29 x 64 cm suspension system covered with sound absorbing insulation, with the goal

of producing as even as possible a sound field inside the Plexiglas cylinder that housed the freely moving subject.

The output of the microphone that picked up the peeps of the subject was connected to an audio monitor and a circuit designed to record the peeps (Severns, Gray, & Rubel, 1985). We refer to this circuit as the 'peep detector.' The peep detector was designed to accurately record/identify vocalizations of birds. The peep detector uses five different measures to identify peeps: frequency, bandwidth, amplitude, duration, and spacing. The bandpass filter controlled the output of the microphone around a center frequency that could range from 3000 to 5500 Hz. The peep would also have to be a certain amplitude in order to be recognized and analyzed for the proper duration and spacing for that specifies-specific peep. The proper settings were estimated and tested using two different sets of parameters called Loud/Insensitive or Soft/Sensitive, to determine the loudest intensity of the stimuli that would not trigger the peep detector. The method, described below would yield meaningless results if the stimulus triggered the peep-discriminator and was thus recorded as a response.

The Soft/Sensitive setting has a lower amplitude threshold than the Loud/Insensitive setting. This means the peeps did not need to be as loud in order to trigger the peep detector in the Soft/Sensitive stetting, yet, the stimuli could not be presented at higher intensities, and there were concerns the detector would be too sensitive and record the bird's movements. The Loud/Insensitive setting required the peeps to be louder in order to trigger the peep detector. However, there was concern that the Loud/Insensitive setting would be insensitive to some peeps, which could result in inaccurately low measures of recorded responsiveness, or even early termination of the

testing; the program self terminates when the quail did not peep for two minutes at any point during the test.   The Loud/Insensitive setting would also allow the stimuli to be presented at higher intensities which is preferable for testing hearing loss. Therefore, Loud can be used interchangeably with Insensitive and Soft can be used interchangeable with Sensitive in this paper.

A bird's typical response to stimuli is indicated by the amount of time between the onset of a stimulus and the onset of the second post stimulus peep which we will refer to as peep suppression. Gray (1987) had previously determined that the time to the 2nd post-stimulus peep was the most sensitive measure of stimulus detection in chicks. We tested the auditory thresholds of Bobwhite quail by measuring their peep suppression in response to pure tones at a range of different ages (anywhere from one post hatch day to 36). As many birds as possible were tested in one day. A bird could only be tested once a day but one bird could be tested at several different ages.

Birds were tested using the insensitive or sensitive peep discriminator settings. This choice determined whether they heard the Loud or the Soft stimuli, respectively. The peep detector parameters alternated between the Loud/Insensitive setting on odd days and Soft/Sensitive setting on even days.

C. STIMULI

The stimuli were pure tones: 250, 500, 1500, 4600, 5000, 6000, 7000, and 8000 Hz. These tones were presented as loud as possible given the Loud/Insensitive or Soft/Sensitive peep detector settings. As shown in Table 1, the intensities presented were 59, 34, 67, 64, 64, 70, 76, and 79 dB SPL for the Soft/Sensitive setting and 59, 34, 79, 73, 76, 82, 91, and 90 dB SPL for the Loud/Insensitive setting. The intensities of frequencies

above 1 kHz were set 2 dB lower than the maximum intensity allowed without triggering the peep-discriminator, and the frequencies below 1000 Hz were set 7 dB above estimated threshold from previous research (Gaven, J.M., Lickliter, R., Gray, L., 2009). The intensity at which each frequency was presented was well above (at least 10 dB) the estimated normal threshold of a quail (Dooling, 2002).

Table 1: Hz per Amplifier setting. Displays the dB SPL at which the frequency was presented for either the Loud/Insensitive or Soft/Sensitive setting.

Frequency (Hz)	Setting (dB SPL)	
	Loud/Insensitive	Soft/Sensitive
250	59	59
500	34	34
1500	79	67
4600	73	64
5000	76	64
6000	82	70
7000	91	76
8000	90	79

D. Procedure

Each bird was first acclimated to the chamber for at least a minute or whenever they began peeping regularly, whichever came last. A series of 25 trials began after this acclimation. At the start of each trial the computer made sure the bird was peeping, defined as at least 2 peeps in 2 seconds. A pulsing stimulus was then presented, 460 ms on 40 ms off, with 10 ms cosine squared rise and decay times. The pulsing stimuli continued until the 5th post-stimulus peep or 4.5 s, whichever came first. Some of these stimuli were maximally attenuated (-127 dB) and referred to as mock trials. All eight

frequencies were presented twice for a total of 16 stimulus trials plus 9 mock trials, all presented in random order to form 25 trials ($8*2 + 9 = 25$). The 9 mock (silent) trials were a control to determine the birds' typical peep rate (or 'noise' alone trial in the theory of signal detection). Peep suppression, the dependent variable, was the time in milliseconds from the onset of the stimulus to the second post-stimulus peep. After each trial there was a 90 second inter-trial interval before the computer started looking for 2 peeps in 2 seconds to begin the next trial. As many birds as possible were tested each day. Birds could be tested on different days but each bird could only be tested once a day. A total of 325 tests were completed.

E. Analysis

Peep suppression on each stimulus trial was converted into a z-score and is referred to as responsiveness. The peep suppression (in ms) on each stimulus trial was reduced by the average 'suppression' on mock trials and then divided by the standard deviation of the peep suppressions during the mock trials, when no stimulus was presented. This calculation was done separately for each test (group of 25 trials). Thus a responsiveness of 1 for (say) the 500 Hz tone means that the time to 2nd peep was one standard deviation greater than that expected over the 9 mock trials presented on that test (refer to Figure 1).

$$\frac{\{\text{peep suppression on a stimulus trial}\}-\{\text{mean peep suppression on 9 mock trials}\}}{\{\text{standard deviation of peep suppression on 9 mock trials}\}} = \begin{array}{c} \text{z-score} \\ \text{or} \\ \text{Responsiveness} \end{array}$$

Figure 1: The equation for Responsiveness.

Gleich & Langemann, 2011, estimated the pure tone thresholds of Bobwhites. We used these estimates to determine the dB above the expected Bobwhite threshold,

which we term dBQL, dB Quail Level, for each of our stimuli. We then plotted the

presentation levels of our stimuli against the estimated threshold to verify that our stimuli

were audible to normal-hearing Bobwhites (Figure 2). Figure 3 tabulates the dBQL for

each setting and plots the intensities by frequency for both the Loud/Insensitive and

Soft/Sensitive settings. The dBQL for the Loud/Insensitive setting were typically larger

than the dBQL for the Soft/Sensitive setting; however, they were the same for 250 and

500 Hz. Remember, the stimuli were presented as loud as possible without triggering the

peep discriminator except for the two lowest frequencies.

Frequency (Hz)	Setting (dB SPL)	
	Loud 🟥	Soft 🔺
250	59	59
500	34	34
1500	79	67
4600	73	64
5000	76	64
6000	82	70
7000	91	76
8000	90	79

Bobwhite Quail (*Colinus virgianus*)
Best Intensity (dB SPL) = 13.15
Best Frequency (kHz) = 2.00
Low Frequency Limit (kHz) = 2.13
High Frequency Limit (kHz) = 8.70
Center Frequency (kHz) = 1.35
30 dB Bandwidth (kHz) = 6.57
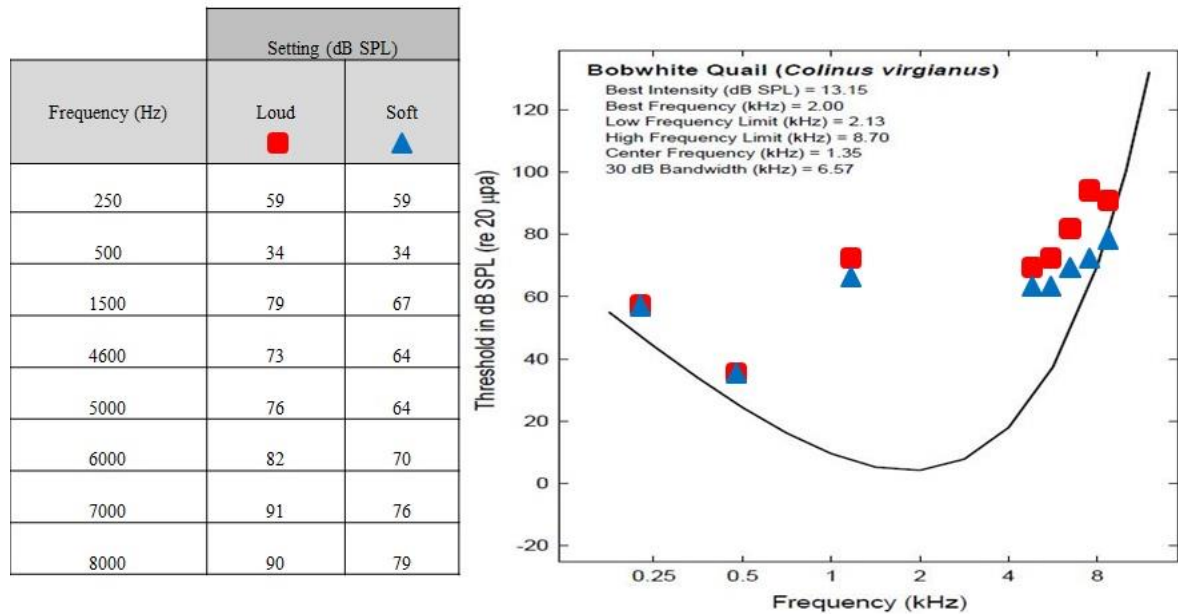
Figure 2. Estimated thresholds and stimuli presentation levels.

Hz	Loud dBQL	Soft dBQL
250	15	15
500	10	10
1500	74	62
4600	47	37
5000	43	32
6000	33	21
7000	31	16
8000	23	12
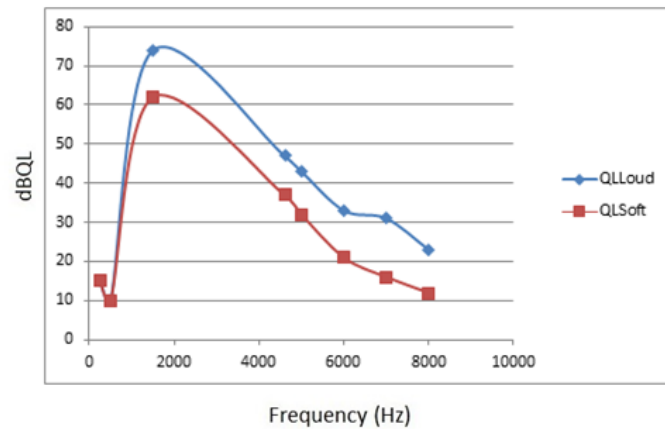
Figure 3: dBQL per Hz. dBQL represents the dB SPL above the estimated Bobwhite thresholds at which frequency that was presented for either the Loud/Insensitive or Soft/Sensitive setting. The graph depicts the intensities provided in the table.

Data were not analyzed if the animal completed less than 6 trials. Data were also discarded if a calibrated tone presented at the end of the test was outside the expected range (calstim <-65 dB), indicating that something was awry with the equipment (speaker, amplifier, or microphone) outside the computer. Subjects were then divided into equal quintile age ranges for analysis: 1-5, 6-9, 10-14, 15-21, 22-35 post-hatch days.

III. RESULTS

Probably the easiest analysis to understand, and perhaps the most interesting, is the responsiveness to the two lowest frequencies, 250 and 500 Hz. Conveniently for this analysis the intensities were the same on all tests (Soft/Sensitive and Loud/Insensitive detector settings).

Figure 4 exhibits the responsiveness of the normal and the treated Bobwhites to 250 Hz and 500 Hz across age ranges. At 250 and 500 Hz, the peep discriminator settings were the same for the Loud/Insensitive and Soft/Sensitive settings. 250 Hz was presented at 59 dB SPL and about 15 dB SPL above the estimated threshold (15 dBQL), and 500 Hz was presented at 34 dB SPL and about 10 dB SPL above the estimated threshold (10 dBQL) for each setting. We can see that the treated birds' responsiveness, or mean Z, is close to 0 at the youngest age ranges, and the responsiveness improves with age. Also, the normal Bobwhites are clearly responsive to the stimuli and it does not vary much with age. An ANOVA determined that there was a significant effect of treatment $F(1, 283)=8.3$, $p=.004$, partial $\eta^2=.03$ about half way between a small and medium effect size. There was no significant effect of age ($p=.9$) nor age-by-treatment interaction ($p=.995$). There were 80 and 213 measures of responsiveness from the treated and normal groups respectively.
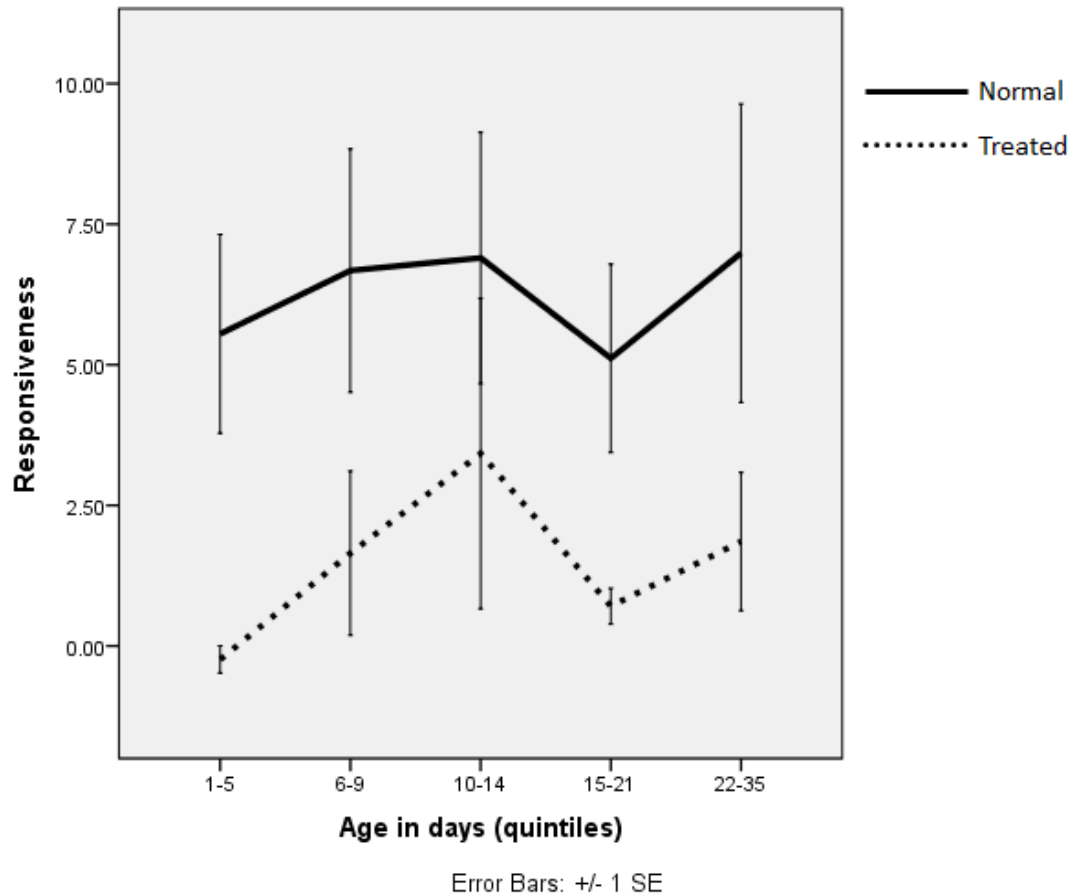
Figure 4: Age vs. Responsiveness for 250 and 500 Hz. A responsiveness of zero means the birds did not respond to the stimuli.

The high frequencies were more challenging to analyze. There is a larger spread of both frequencies and intensities. We chose to analyze the average responsiveness to the stimuli with relatively similar dBQL across the Loud/Insensitive and Soft/Sensitive settings. The range of dBQL we selected in the high frequency range was 31dBQL to 37dBQL which equates to 4600 Hz and 5000 Hz with the Soft/Sensitive setting and 6000 Hz and 7000 Hz with the Loud/Insensitive setting; see Figure 5. Again, for the treated Bobwhites, the two youngest age groups do not respond to the stimuli but responsiveness increases with the older age groups. Interestingly, for the normal Bobwhites, the responsiveness to this frequency range decreases with the increase in age. There was a

significant effect of treatment $F(1, 291)=8.9$, $p=.003$, partial $\eta^2=.015$ which is a small

effect size. There was no significant effect for age ($p=.3$).
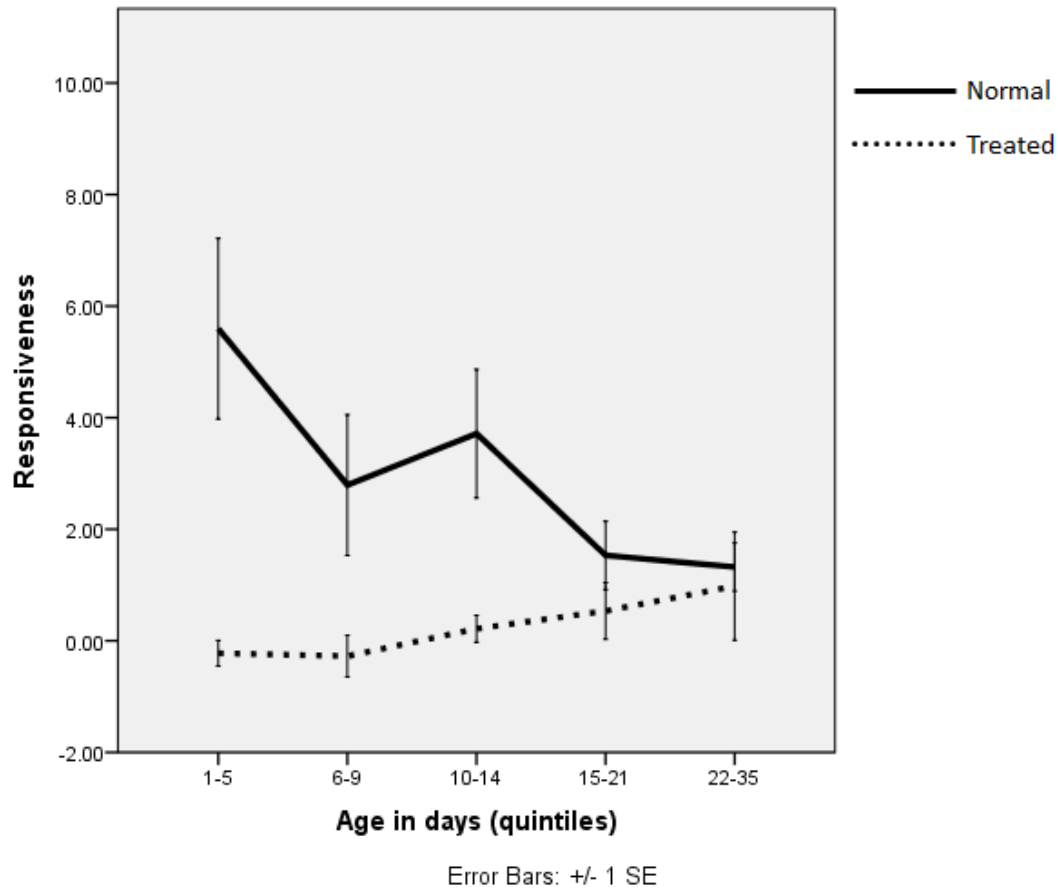
Figure 5: Age vs. Responsiveness to high frequencies in the 31-37 dBQL range.

The decrease in responsiveness of the normal birds to high frequencies is

puzzling. Since responsiveness is a measure of peep suppression on stimulus trials

relative to responsiveness on mock trials, we wondered if baseline peeping ('suppression'

on mock trials) might have changed over age. Figure 6 shows the peep suppression on

mock trials as a function of age and treatment group. We see that the peep rate for the

treated birds was slower than the normal subjects[2], but that there was no striking change

over age, nothing in the mock trials is seemingly sufficient to explain the puzzling

decreasing responsiveness as the non-treated birds get older.
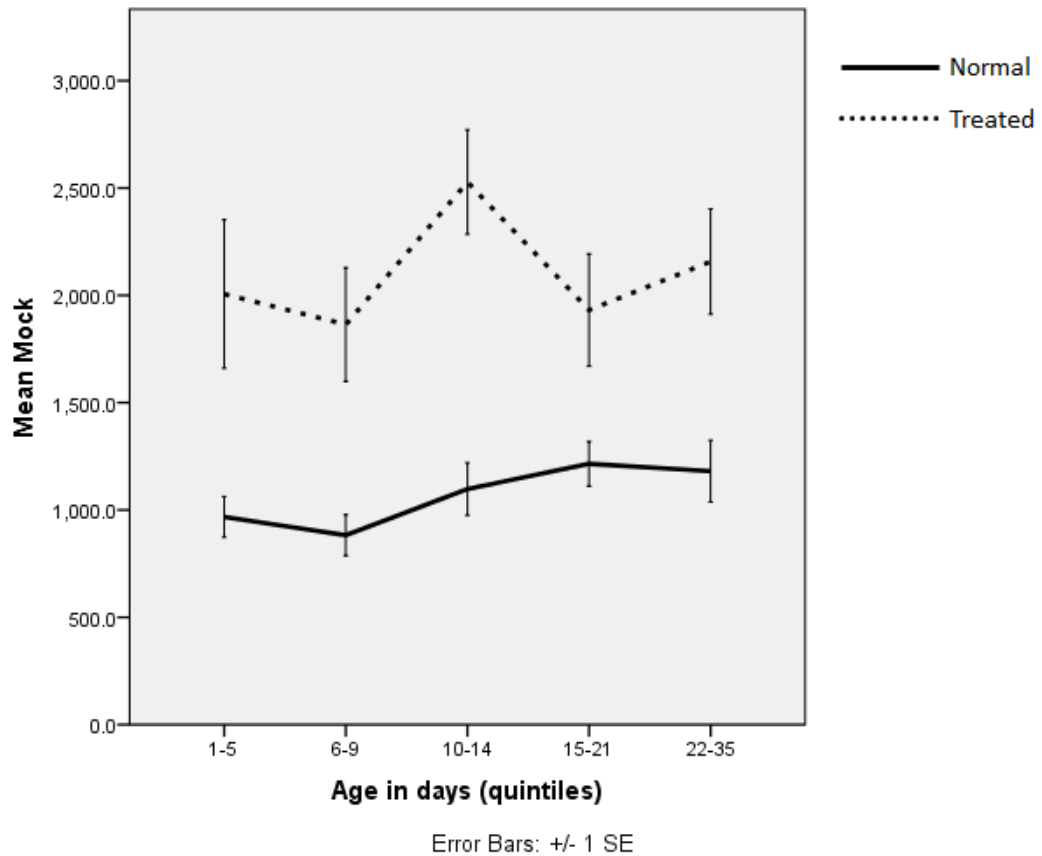
Figure 6: Age vs. Mean Mock peep rate (in ms) for normal and treated subjects.

We then analyzed the mean mock suppression for the normal birds only (Figure

7). The normals do slow their peeping with age, and it is significantly significant,

(p=.027) however, with a very small effect size (r-Square=.02).
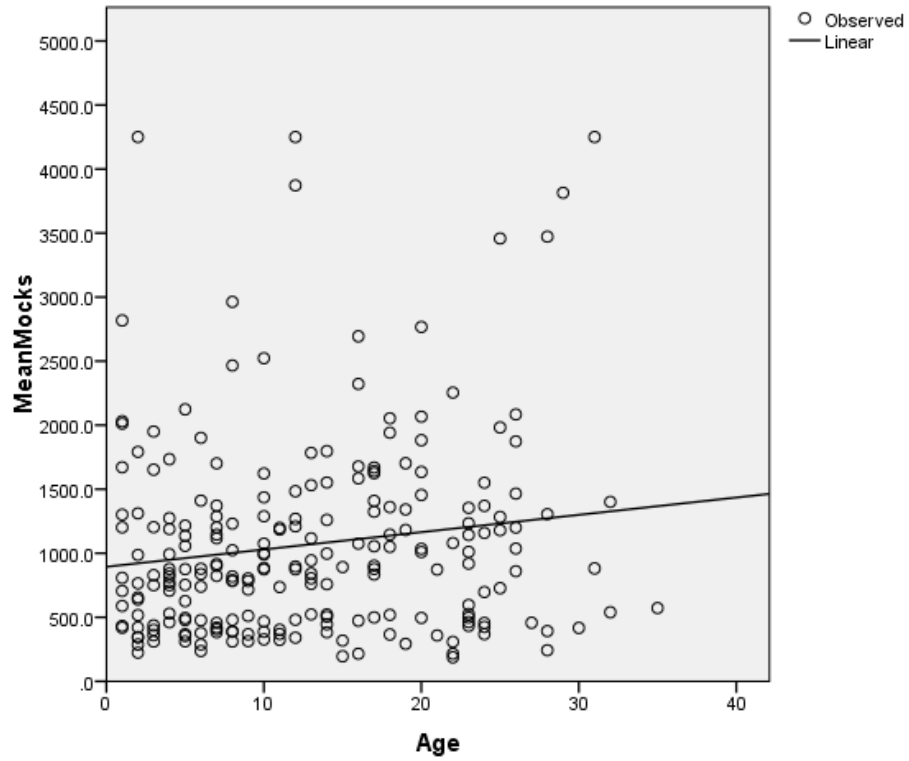
Figure 7: Age in days vs. average duration in ms of control trials (MeanMock) for each test of a normal Bobwhite. (r^2=.02, F[1, 224]=4.96, p=.027).

IV. DISCUSSION

A. Low Frequency Hearing Loss

The results obtained with the low frequency stimuli are rather interesting. Our results indicate that the young treated quail do not respond to the low frequency stimuli. This behavior, or lack of behavior/response, was induced by a single, early dose of gentamicin. Other studies have yet to indicate significant changes in hearing sensitivity to low frequency stimuli (below 1000Hz) with a single dose of gentamicin. The dose of gentamicin that would most likely induce such damage has typically resulted in nephrotoxicity and fatalities. (Roberson, Alosi, Messana, & Cotanche, 2000)

B. High Frequency Hearing Loss

The results obtained with the high frequency stimuli are rather puzzling. Again, during the period of hearing loss, the treated quail are not as responsive to the stimuli but as the hair cells are regenerating with age, responsiveness increases, slightly (statistically significant with a small effect size). So, the results seen in the treated birds are consistent with the literature; high frequency hearing loss occurs and recovery is minimal. However, the responsiveness of the normal quail decreases with age. In an attempt to understand this surprising finding, we further analyzed the peep rate in control and treated quail. We found that there was a difference between the groups but the groups' regular peep rate did not vary significantly with increasing age. When only analyzing the peep rate of the control quail, we see a significant decrease in their peeping as they age, yet the effect size is very small. So, we understand that peeping typically slows and ceases as fowl age, but we cannot explain why our results indicate that a normal decrease in responsiveness is more evident with high- than low-frequency stimuli.

A review of the literature of the function of hair cells following regeneration was conducted by Ryals, Dent, & Dooling (2012), and they surmised that following regeneration, a high frequency threshold shift persists. These results were obtained in young or adult birds, not neonates. It could be that the decline in responsiveness to high frequency stimuli is the norm with increasing age, such as the high frequency hearing loss found in Belgian waterslager canaries. Also, in both the low frequency and high frequency analysis, the graphs indicate a drop in responsiveness for both the treated and control birds in the 4th age group (15-21 post hatch days). Here the birds could be considered adolescents and could just be losing interest in the stimuli or some change could be occurring in their development.

C. Histology

Wilson (2013) conducted a corresponding study with Bobwhite quail, inducing hair cell damage with the same procedure as utilized in this study. She has provided the following images of the basilar papillae of normal Bobwhite quail and of Bobwhite quail who have been injected with an intraperitoneal injection of gentamicin on their hatch day (P0) or one day post hatch (P1) at a dosage of 200 mg/kg. Figure 8 features the basilar papilla of 4 day old, normal Bobwhite quail. Wilson used phallodin to stain the samples which results in stereocilia fluorescence. In the enlarged portion, there are numerous hair cells as indicated by the staining of stereocilia bundles; each red "dot" indicates a bundle of stereocilia atop an individual hair cell. Figure 9 is an example of a Bobwhite basilar papilla 4 days post hatch and post injection. There is significant stereocilia wipe out even in the distal, low frequency region of the papilla. Figure 10 depicts the same papilla 4 days post hatch and injection with a basal portion highlighted and enlarged. Figure 11 is

the basal portion enlarged (40x) with an arrow indicating a few small regenerating hair cells and no mature or normal hair cells present. Quantification of hair cell loss revealed that the treated quail had complete loss hair cells up to 60% the length of the basilar papilla.
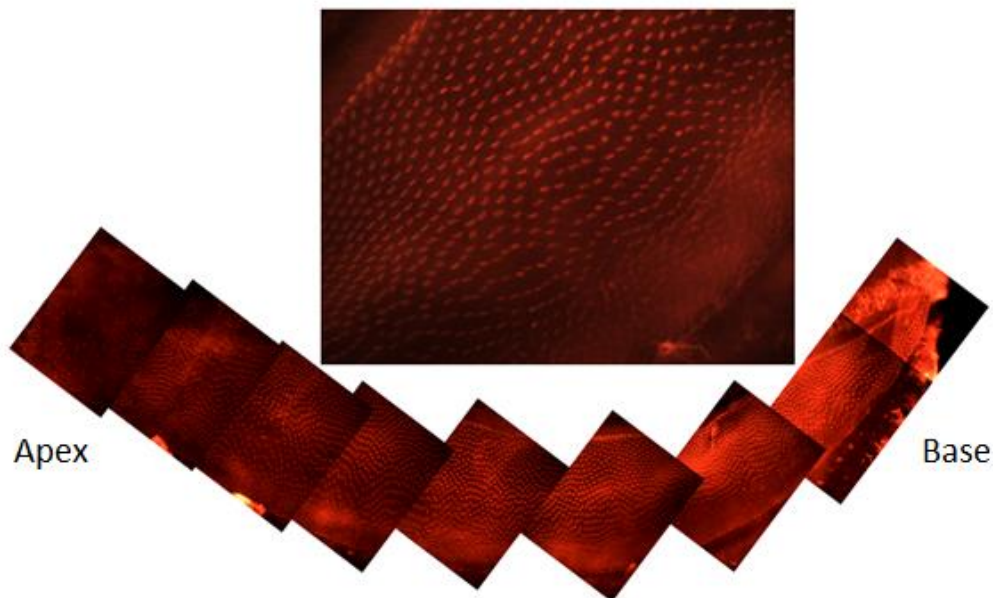
Figure 8: Normal basilar papilla and stereocilia (indicated by the dots) of a 4 day old Bobwhite quail.
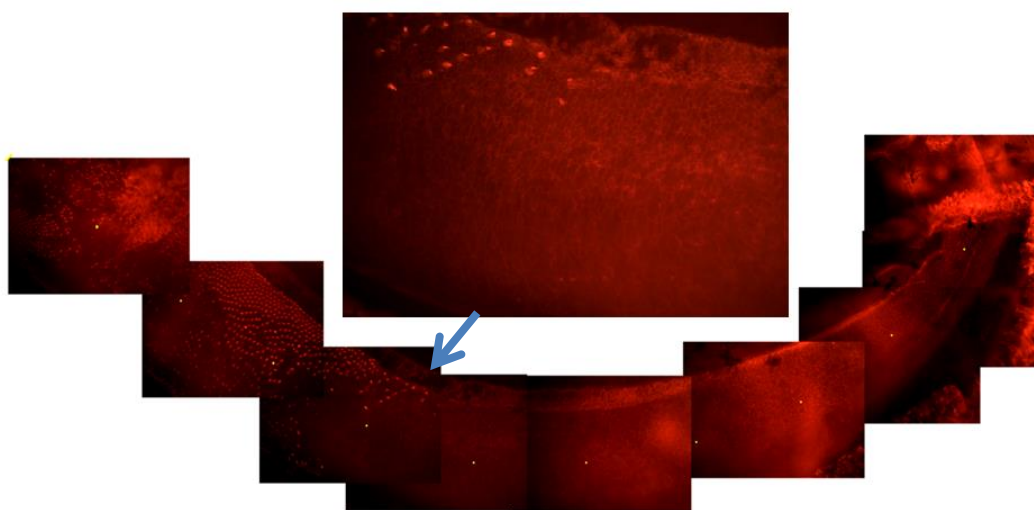
Figure 9: Basilar papilla 4 days post hatch and post injection. The arrow indicates distal, low frequency region where few hair cells remain.
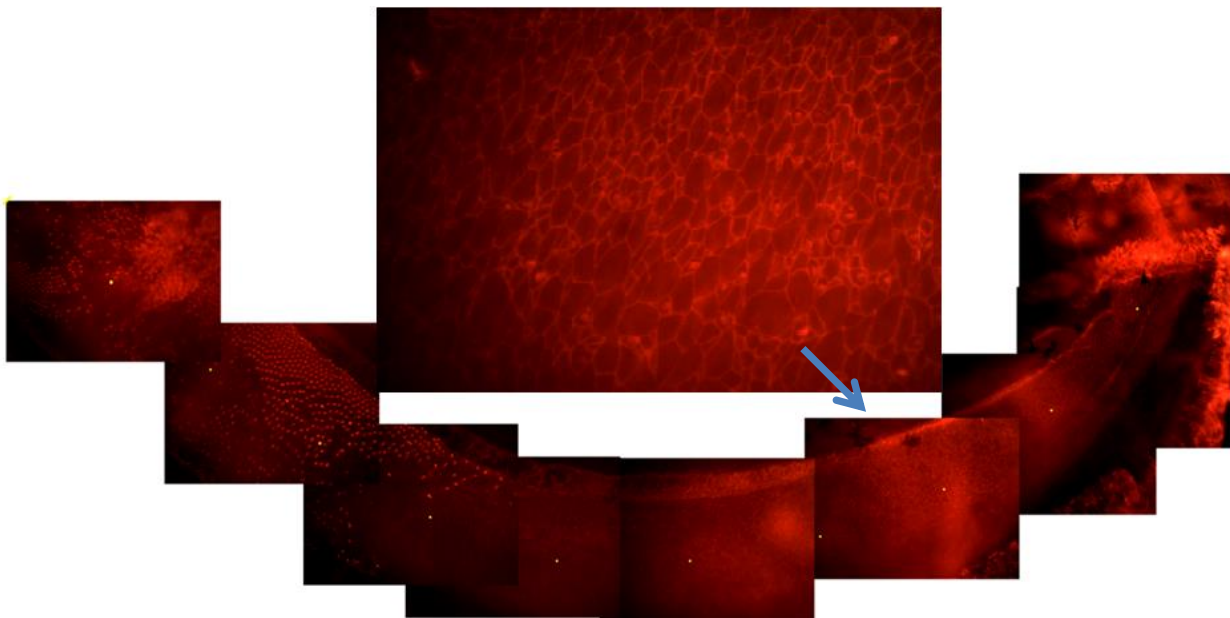
Figure 10: Basilar papilla 4 days post hatch and injection. The arrow indicates a basal portion which is enlarged.
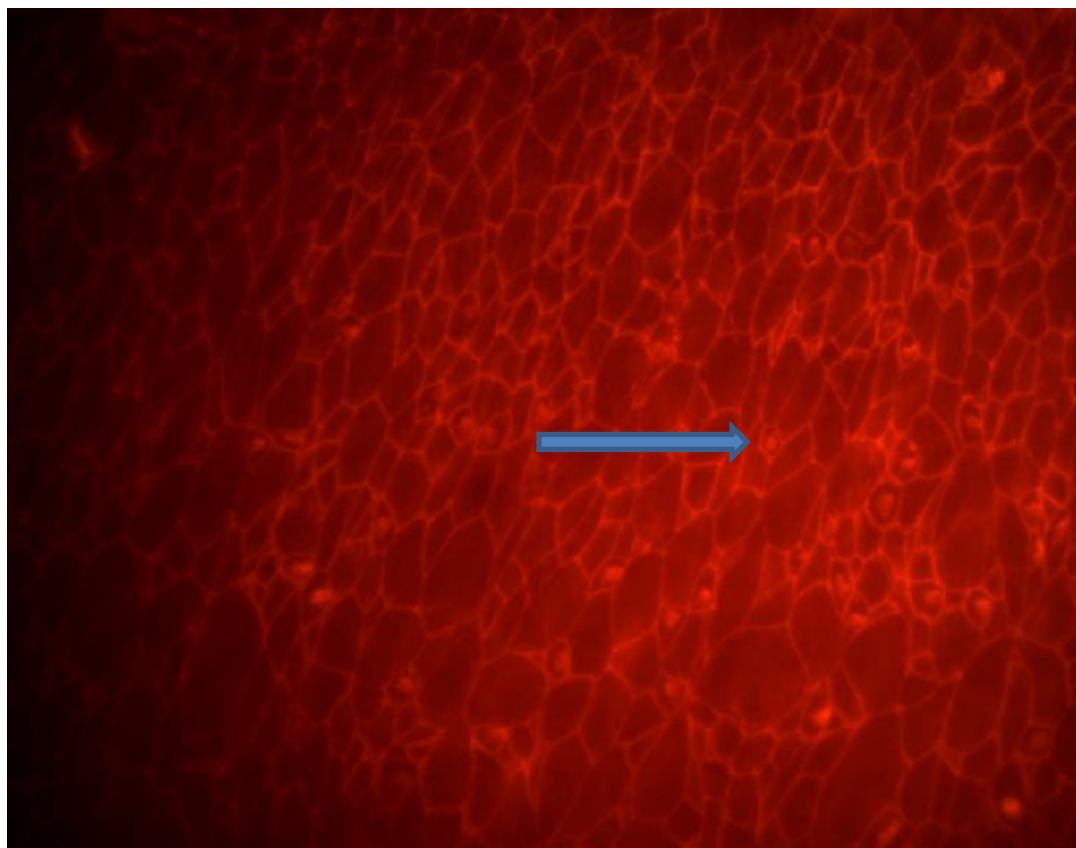
Figure 11: Enlargement (40x) of 4 day post hatch and injection basal portion of the papilla. Arrow indicates a few small regenerating hair cells.

Roberson, Alosi, Messana, & Cotanche (2000) assessed the histology of a chick's cochlea following a single, large injection of gentamicin. Again, hair cells at the basal end were completely lost and hair cells were present at the most apical end. Figure 12 was taken from their study, and Figure 13 compares the damage seen in the chick to the images above provided by Wilson. Clearly, the damage in the chick's cochlea does not extend as distally as in the Bobwhite's.
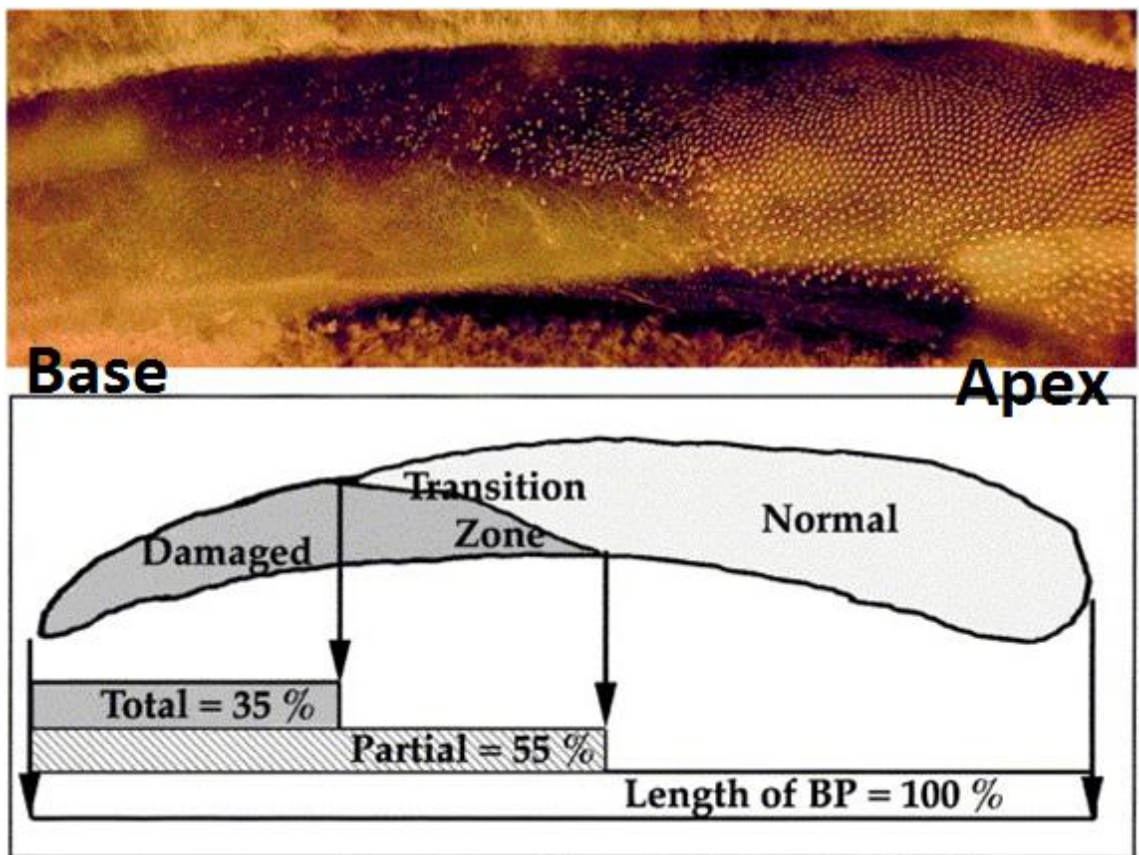
Figure 12: Basilar papilla of a chick following a single, large injection of gentamicin. Images taken from Roberson, Alosi, Messana, & Cotanche, 2000.
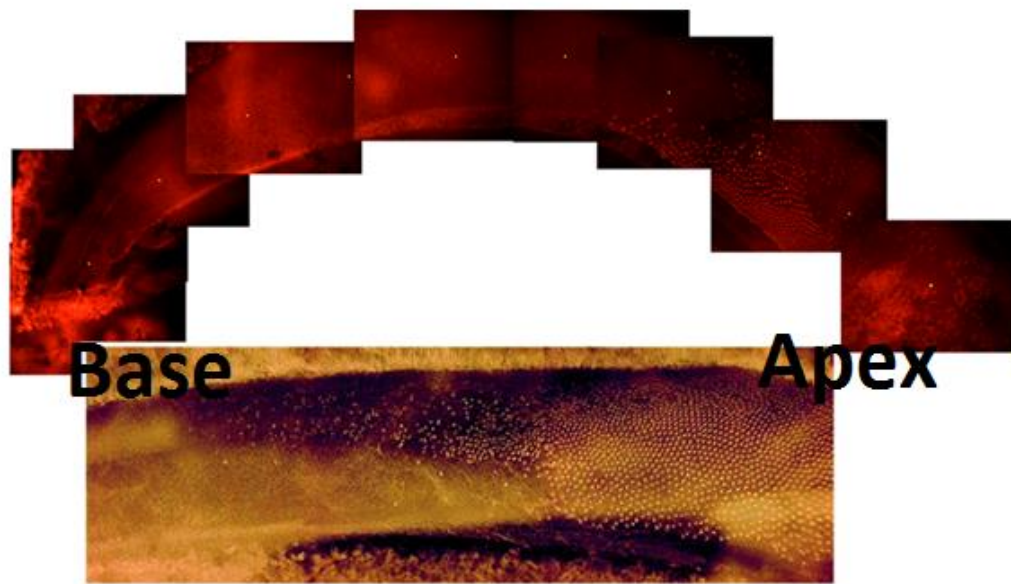
Figure 13: Compares hair cell damage in the Bobwhite, Wilson (2013) and Roberson, Alosi, Messana, & Cotanche (2000) studies.

D. Conclusions

The results obtained in this study were consistent with the current research on hair cell regeneration in avians. The pattern of damage was the same, clear hair cell loss in the high frequencies that somewhat regenerates over time. Some of our results were novel. We successfully induced a low frequency hearing loss in neonatal Bobwhite quail with a single injection of gentamicin. The results were evident in the behavioral measures and verified by histological analysis. Thus, the treated Bobwhite quail experienced a period of early deafness which somewhat resolved with age. The period of deafness reduced their responsiveness to pure tone stimuli that may have to some extent recovered at the high frequencies and improved at low frequencies but not to normal levels in a month.

There are several factors that may have affected our results: habituation, decreased peeping in adolescences, and visual stimuli. The subjects may have eventually habituated to the stimuli; some subjects may have just stopped peeping earlier or later than the other subjects; the subjects could have been more responsive if there was some sort of accompanying visual stimulus. We may also consider altering the recording equipment and parameters. These factors may be considered in follow-up studies.

E. Future Directions

Further studies are warranted in order to continue research on the quality of hearing with regenerated hair cells. It would be best to clarify some of the unexpected findings in this study before moving on to assess the quality of hearing to complex stimuli. We may consider the use of multiple microphones to record the peeps; perhaps having microphones above and below the cage would allow us to create a differential recording system. This could then allow us to play stimuli louder and be able to better differentiate between the stimuli and the peeps for more accurate recording of vocalizations. Also, it could be beneficial in recording the health status of the subjects. If they are feeling ill or are having health issues during the experiment, it could affect the results. Additionally, studying the hair cells in mature Bobwhites could provide insight on their quality and/or ability to hear as they mature. This could then help us understand how their auditory system develops. Bobwhites may have a progressive high frequency hearing loss. Or, this period of hearing loss may alter how their auditory system develops. Once these issues are resolved, the quality of hearing to species-specific calls in Bobwhite could be then be likened to the quality of hearing to speech and language in

humans. The quality of regenerated hearing to complex signals would then be of importance if regenerating hair cells is eventually achieved in humans.

APPENDIX

Extended Review of Literature

Auditory and vestibular hair cell regeneration has been widely studied since its discovery in birds in the 1980s. Varying methods of causing hair cell damage have been assessed such as acoustic trauma with high intensity stimuli and treating subjects with known ototoxic drugs such as aminoglycosides (i.e. kanamycin, gentamicin, etc.). Damage can be seen in all species however, hair cell regeneration only occurs spontaneously in certain species. Vestibular hair cell regeneration has been seen in birds, amphibians, and mammals; yet, auditory hair cell regeneration has only occurred, organically, in avians (Cotanche, 1999; Ryals, Dent, & Dooling, 2012). A wide range of topics are related to the current study. Thus, many different topics are discussed below and indicated by their headings; one topic does not flow into the other but are all related to our research. The review of the literature aims to assess the current understanding of hair cell damage and regeneration with aminoglycoside treatment. If the goal of understanding hair cell regeneration is to implement it in mammals, then the functionality and quality of the regenerated hearing must be measured and deemed worthy of the efforts.

Hair Cell Regeneration

The first record of hair cell regeneration was published in the 1980s by Cruz et al (1987), Cotanche (1987), Ryals and Rubel (1988) as cited by Girod, Tucci, & Rubel, 1991, p.1139, and Corwin and Cotanche (1988). Cruz et al. (1987) and Cotanche (1987) published articles that alluded to the regeneration of hair cells in avian basilar papillae, but it was Ryals and Rubel (1988) and Corwin and Cotanche (1988) that discovered these

new hair cells were developing from cells that re-entered cell cycle and had undergone

mitosis. Hair cells were damaged in chicks and Coturnix quail with acoustic trauma.

Later, experiments were conducted with treatments of known ototoxic drugs. Further

experiments were conducted with different species of birds such as budgerigars, canaries,

European starlings, etc. to determine if the regeneration also occurred.

Ryals & Rubel (1988) described hair cells loss in Coturnix adult quail who were

exposed for 12 hours to 115 dB pure tone. The number of hair cells was then assessed 10

days following the trauma; there was clear damage/missing hair cells through the basal

and middle portion of the cochlea, with about 70% of hair cells lost in the middle portion.

Roberson & Rubel (1995) noted that unlike damage from acoustic trauma, ototoxic

damage does not cause damage to the surrounding structures such as the tectorial

membrane.

While humans may have more hair cells than birds, birds have the ability to

regenerate hair cells. So, when those sensory cells are damaged in humans, the damage is

irreversible and hearing sensitivity will be permanently altered. The only way humans

can overcome the loss is amplification with devices such as hearing aids or cochlear

implants. However, birds will regenerate what is lost in a few days' time. In humans

with hearing loss, their hearing will never be considered "normal" without amplification,

and it is believed that the birds' regenerated hearing sensitivity returns to normal. Much

research has been conducted in the hopes of understanding hair cell regeneration in the

hopes can it can be implemented in humans.

It has been stated that avians constantly regenerate their vestibular hair cells but

their auditory hair cells are only regenerated if damage has occurred (Edge & Chen,

2008). The hair cells regenerate by either cell cycle re-entry and proliferation or direct

transdifferentiation (Edge & Chen, 2008; Cotanche, 1999; Roberson & Rubel, 1995;

Ryals, Dent, & Dooling, 2012). Regeneration does not always follow the same pattern as

the damage, proximal to distal; rather the hair cells regenerate evenly across the basilar

papilla and reorganize themselves over time (Cotanche, 1999). Some supporting cells

appear to be potential hair cell progenitors and re-enter cell cycle to develop into hair

cells. However, it is unclear whether all supporting cells are capable of re-entry or if only

a subpopulation is capable. A population of the supporting cells surrounding the region

of damaged hair cells re-enters the cell cycle and divide; the younger the chicks, the

earlier the cells re-enter the cycle, but regeneration takes longer in older chicks. Since

younger chicks' cells are able to re-enter the cell cycle faster than the older chick it is

believed that the younger chicks' cells are not as deeply into quiescence as the older

chicks' cells which is why young chicks are able to regenerate hair cells more quickly

than older chicks. (Cotanche, 1999)

There are several requirements for cells to exit quiescence and re-enter the cell

cycle such as external growth factors and changes in the internal gene expressions. It

seems that the exposure to gentamicin stimulates many of the supporting cells to leave

quiescence and be prepared to enter the cell cycle, but only the cells located where the

damage occurs continue through the cycle. However, studies have shown that it takes

some time for the supporting cells to re-enter the cell cycle and divide. So, some of the

regenerated hair cells seem to arise without mitosis, via direct transdifferentiation. In

direct transdifferentiation the supporting cell changes its gene expression to become a

hair cell. Not all supporting cells can make this change, or else the basilar papilla would

lose its support since supporting cells are not continually renewed. The afferent and efferent innervations also repair themselves soon after treatment is discontinued, but it takes several weeks for them to completely normalize. Short hair cells in normal chicks typically have little to no afferent innervation, but regenerated short hair cells have multiple afferent terminals which dissipate after several weeks and are replaced with large efferent endings, mimicking embryonic innervation development. (Cotanche, 1999; Roberson & Rubel, 1995)

Again, the regenerated hair cells appear to function normally, and hearing is restored. Studies involving damaging avian hair cells and performing auditory brainstem responses following regeneration report a near complete recovery of the auditory system. Girod, Tucci, & Rubel's (1991) experiment involved avian subjects who were tested 20 weeks post treatment, and although the ABRs to low frequencies were normal there was still a mild high frequency threshold shift. They studied neonatal chicks that were administered gentamicin sulfate with 1 subcutaneous injection at a dose of 50 mg/kg for 5 or 10 days.   While the initial damage occurs at the basal, high frequency portion of the basilar papilla, the damage will spread to involve the mid and low frequency regions. At 20 weeks post treatment, the total number of hair cells was essentially back to normal but the mosaic pattern was still somewhat disorganized. The amount of damage between subjects did vary and there was a correlation between the physiological and anatomical results. The more damaged the anatomy, the poorer or more elevated the thresholds and near normal thresholds were seen in the subjects with the least amount of anatomical damage. Hair cells were considered damaged if their stereocilia were disorganized and if the surface of the hair cell was not smooth. New or regenerating hair cells were clearly

smaller with immature stereocilia and microvilli present on the apical surface of the hair cell. It was noted that there was no evidence of hair cell injury, loss, and, in turn, regeneration at the most apical portion of the basilar papilla in any subject. The hair cells at the basal end at 20 weeks were still not fully mature (and the mosaic pattern disorganized) while the regenerated hair cells in the other regions were mature. (Girod, Tucci, & Rubel, 1991)

Ryals, Dent, & Dooling (2012) also completed a review of the literature on the function of regenerated hair cells and concluded that for adult chickens, the cochlear microphonic recovers well, but not completely, by 11-14 weeks post injection which confirms some neural transduction recovery. Also, distortion product otoacoustic emission thresholds have partial to full recovery in time; however the highest frequencies do not seem to recover as well as the lower frequencies. These findings with the highest of frequencies are also consistent with ABR and CAP measures. Ryals, Dent, & Dooling (2012) also reviewed studies that measured behavioral audiograms measured after ototoxic drug administration in budgerigars, European starlings, and canaries. Again, for all the different birds, damage and hearing loss began and is the greatest at the high frequencies and proceeds apically. While the number of hair cells returns within one standard deviation of normal within 3 months following injection, behavioral thresholds still have a threshold shift and, again, are greatest in the high frequencies. The literature reviewed revealed that it is typically frequencies above 2 kHz and the corresponding regions on the basilar papilla that are damaged the most and earliest and have a permanent threshold shift following regeneration. Some suggested reasons include: multiple and/or abnormal stereocilia bundles and abnormal stereocilia bundle orientation,

some immature hair cells, and irregular pattern of hair cells; the reasons could also be due to changes in neural function and/ or basilar membrane mechanics.

Hair cell regeneration has also been discovered in some mammals. Edge & Chen (2008) report that the vestibular epithelium is capable of regenerating, but there has not been any evidence of auditory regeneration at that time. The overexpression of Atoh1 appears to be effective in hair cell regeneration. Roberson & Rubel (1995) are concerned that the only way to induce the regeneration in humans would require administering mitogenic substances that could be unsafe, especially in long-term effects. The reported incidents of hair cell regeneration have been seen in isolated sensory organs cultured in vitro. And this process begs questions about neural or systemic signals and what triggers the regeneration. The growth factors and genes that change within the supporting cells to exit quiescence need to be identified and regulated in order to be recreated in mammals (Cotanche, 1999).

Kawamoto et al. (2003) have data that indicate nonsensory cochlear cells in adult guinea pigs are able to become new hair cells with the overexpression of *Math1*. They inoculated the cochleae with the gene and found that it activated the cellular program that led to mature differentiated cells to recapitulate development and, in-turn, cause new hair cell production. Yet further studies needed to be conducted to determine if the stereocilia bundles would reach maturity, and this study also found the nerve terminals begin to lengthen and grow but the nerve terminals did not connect to the new hair cells within the 2 month analysis time. They propose that this therapy may also be used for other organs as well.

Mizutari et al. (2013) were also able to induce hair cell regeneration in adult mice. They discuss that Notch signaling controls the process of lateral inhibition that decides if a cell is a hair cell or a supporting cell. Notch signaling prevents supporting cells from differentiating into hair cells, and it increases after damage in the cochlea. And again, Atoh1 is necessary for regeneration and for allowing the γ-secretase inhibitor to affect Notch signaling. Mice were exposed to 800 to 16000 Hz octave band noise at 116 dB SPL which caused outer hair cell loss and hearing loss. New hair cells were formed after treatment with the inhibitor and were determined to have arisen by transdifferentiation of supporting cells. With regeneration also came partial reversal of hearing loss. Once again they found that the most damage occurred in the high frequencies, and regeneration and restoration of hearing was poorer in the highest frequencies. They discussed that it would probably be best for the long term if supporting cells were also replaced since they were being lost in order to generate new hair cells. Also, the inhibiting Notch signaling was difficult to maintain for a long time in order to allow the hair cells to regenerate thus this treatment may only be beneficial for the treatment of acute hearing loss, but may not be able to be maintained for long periods of time.

Peep Suppression as a Behavioral Measure

The gold standard for audiologic testing is considered to be behavioral testing. Other forms of assessment measure physiology or function, but behavioral testing allows for the evaluation of the entire auditory pathway and a subject's ability to hear and perceive sounds. However, the accuracy of the behavioral testing relies on the reliability of the test subject. While accurate tests have been widely studied and developed for testing humans, the same cannot be said for other species.

Severn, Gray, & Rubel (1985) developed "an objective, consistent and automated method for identifying vocalizations" which is valuable in a wide variety of studies. This electronic circuit can be adjusted to record different kinds of vocalizations and connected to a computer for analysis. The different variables of the vocalizations utilized by this circuitry are: frequency, bandwidth, amplitude, duration, and spacing. These variables can be adjusted to match the vocalizations which are to be studied. Interestingly, Bobwhite chicks peep until they are 30 days old. Other species of fowl stop peeping at about 2 weeks of age. Therefore, Bobwhite quail can be used for research with hair cell loss and regeneration.

Psychometric Function: Receiver Operating Curves

Soon after developing such a circuitry for recording species vocalizations, it was discovered that changes in vocalization patterns could indicate a response or acknowledgement of a stimuli. Gray (1987, 1992) discovered that neonatal chicks alter their peep pattern in response to auditory stimuli and signal-detection analyses could be applied to their behavior to measure psychometric functions in animals. The current study followed the same procedures and used similar instrumentation. It was established that the time until the second post stimulus peep would provide the largest or most significant difference between the control and stimulus trials. Neonatal fowl constantly peep, and briefly pause their vocalizations when they detect an auditory signal. Histograms were produced from the control and stimulus trials, and receiver operating characteristic (ROC) curves were generated from these two histograms. (Gray, 1987; Gray, 1992)

In previous signal-detection analyses a "yes" response was equivalent to a delay to the second post stimulus peep of greater than some variable value. The probabilities of hits and false alarms were derived from the percentage of delays longer than this cutoff on both stimulus and control trials. A receiver operating characteristic was then defined by defining the "yes" with a larger and larger delay. Gray analyzed tens of thousands of peeps and developed ROC curves which determined that the time to the 2nd post stimulus peep was the most sensitive to calculate delay, and the sensitivity increased with an increase in the intensity of the stimulus. Habituation is a legitimate concern but it can be controlled by varying the presentation of the stimulus (i.e. altering the interstimulus intervals, presenting varying frequencies, varying the intensity of the stimuli, etc.). (Gray, 1987; Gray, 1992)

Psychoacoustics is the study of the perception of acoustics or sounds. Much research has been conducted on humans in the hopes of understanding how we perceive sounds, but not much research has been done on other species.

One psychoacoustic theory is called signal detection theory. In this theory, there are two cases. One case is considered constant. We are constantly in "noise." There is, in this theory, no real quiet. The brain and auditory nerve are spontaneously active creating internal noise. In all environments, except deep space and absolute zero temperature, there is some external noise. In the other case, there is a signal mixed in with the noise. We want to be able to measure how sensitively and accurately we are at detecting the signal in the noise. Unfortunately, we will never be able to be 100% sensitive and 100% specific. There will be times when we will detect the signal when it

is not there; this is coined a false alarm. And, there will be times when the signal is there and we do not detect it; this is coined a miss.

As the subjects' criterion shifts, the probability of hits and false alarms will change. If the subject is behaving randomly, hits will equal false alarms. A conservative listener will have fewer false alarms and possibly fewer hits but more misses and correct rejections; conversely a lax observer could then have more false alarms and hits but fewer misses and correct rejections. In humans, criterion is changed by instructions or by various penalties for errors or rewards for hits or correct rejections. In chicks, the criterion can be varied by simply changing the definition of how much peep suppression is considered to be a 'yes' response. Given one such definition, the probability of hits (suppressions greater than this cutoff on signal+noise trials) and the probability of false alarms (suppression greater than this cutoff on noise alone trials) can be determined.

The ROC is a plot of hits versus false alarms over a range of different criteria. The ROC curve that yields a P(A) of 0.65 or a 65% level of sensitivity has been determined to be the measure of absolute thresholds in neonatal chicks and in human infants. (Gray, 1992)

In this study we took a different approach to the analysis of peep suppressions. ROCs are constructed from trials pooled over many different individuals. The approach taken here, the z-score measure of responsiveness, allows a conceptually similar measure of how much the bird's behavior differs when there is signal plus noise versus noise alone. Z-scores according to the formula used in this paper are equivalent to d' in signal detection theory. Both z=1 and d'=1 indicate that the response with signal added to noise is one standard deviation above the response expected from noise alone. A significant

advantage of the z-score measure of responsiveness is that this can be calculated separately each time an individual is tested.

Altering Birds' Behavior

Birds' behavior can be altered by experience. We know that humans are influenced by their environment while still in the womb (Pudir et al., 2012). Humans are able to hear the sounds in their prenatal environment and even develop a preference for the language that is spoken. Birds are similar to humans in that respect; they are able to use their auditory system to discern much of their environment prenatally. Lickliter, with several colleagues, has investigated influencing Bobwhites' preferences to different stimuli. They found that presenting altered Bobwhite maternal calls to the chicks prior to hatching resulted in the chicks being less responsive to the normal Bobwhite maternal call, successfully altering their postnatal auditory preferences (Lickliter & Stoumbos, 1992). Thus, one is inclined to believe that if birds are capable of learning preferences to auditory stimulation prenatally, and if birds experience hearing loss, then their auditory preferences and behavior could be altered by the deprivation of auditory stimulation.

Aural Development

Aural development and anatomy is similar in different species and develops rather early during gestation in fowl and humans. In avians, typically, the visual system is not developed until after hatching while the auditory system is functional before hatching, though vestibular and somatosensory systems can develop earlier. In humans, hair cell differentiation begins at about 10-12 weeks gestation in the cochlea, beginning with the inner hair cells and then the outer hair cells from base to apex. By 15 weeks, the

structures of the middle ear and cochlea are well formed. All-in-all, the system is functional approximately by 25 weeks gestational age. (Graven & Browne, 2008)

In humans and different avian species, the auditory system includes an ear canal, middle ear, and inner ear. Some of their characteristics are different and yet they have some similarities as well. Humans' inner ear houses the vestibular system and sensory cells of hearing. Humans have a cochlea and organ of Corti; avians have a basilar papilla. Here, both have hair cells of varying sizes; have tonotopic organization; and can be damaged from acoustic trauma or ototoxic drugs.

Depending upon the species of bird, the number of auditory hair cells located on the basilar papilla, a sickle-shaped structure, ranges from 3,000 to 16,000. Avian hair cells are differentiated across the width of the basilar papilla, classified as tall or short, and they have a functional distinction based on innervation patterns. Tall hair cells are primarily located towards the proximal portion/apex and the superior edge of the sensory curvilinear sensory epithelium while the short hair cells populate the distal portion/base and the inferior edge. Also, the tall hair cells' innervation pattern mimics the mammalian inner hair cells while the short hair cells' pattern mimics the mammalian outer hair cells (Girod, Tucci, & Rubel, 1991). Avian hair cells' tips also have stereocilia organized in a stair-step formation. The size, or length, of the basilar papilla varies and ranges from 2mm to 12mm in different species. The width and number of hair cells across the basilar papilla increases from the proximal/basal, high frequency end with 5 to 6 hair cells across the width to 30 to 40 hair cells across at the distal/apical, low frequency end. Weaved throughout the hair cells and around the supporting cells is a thin band of microvilli

which creates a mosaic on the surface of the epithelium (Girod, Tucci, & Rubel, 1991; Gleich & Langemann, 2011).

Aural development in humans has been widely studied. The auditory system development occurs early in gestation and is complete by the beginning of the third trimester (Cassidy& Ditty, 1998; Li, 2012; Pundir et al., 2012; Werner, 2007). The cochlea is small, about 1 cm wide and 5mm from base to apex and is broader near the base and narrows at the apex (Pickles, 2008). It is a spiral formation that contains many complex structures within its 2 ¾ turns that are critical for human hearing (Gelfand, 2007). There is about a total of 17,000 hair cells housed within the organ of Corti within the cochlea; human cochlear hair cells are classified as either inner or outer hair cells and are different in shape and innervation pattern (Edge & Chenz, 2008). Overall, there are more outer hair cells than inner hair cells. Also, outer hair cells are more differentiated than inner hair cells. It is also believed that OHC are more likely to die first since they are more exposed over the basilar membrane (CSD 512) and more populous in the apex. Similarly to the basilar papilla, the cochlea is tonotopically organized with the high frequencies located basally and the low frequencies apically.

Congenital Hearing Loss

Congenital hearing loss is defined as hearing loss that is present at birth and is the most common sensory defect (Bindu & Reddy, 2008; Van Egmond, 1954). In 2008, it was estimated that 3 out of 1000 individuals in the US are born with hearing loss (Parker, 2011); Weichbold, Nekahm-Heis & Welzl-Mueller (2006) report the prevalence of congenital hearing loss greater than 40dB HL to be about 1 in 1000 in the UK and .5 in 1000 in the US. It is caused by either genetic factors or environmental causes such as

viruses, trauma, or exposure to ototoxic drugs. The severity and configuration of the loss can vary widely. Now, early identification protocols such as universal newborn hearing screenings have allowed congenital hearing loss to be identified as soon as possible and have led researchers to believe that the incidence of congenital hearing loss to be closer to 2 or 3 per 1000 live births (Wrightson, 2007). This type of hearing loss can be the most detrimental to speech and language development if habilitation does not occur.

Critical Period for Speech and Language Development

We know that humans have a critical period for speech. If a person does not develop speech or language before this age, he/she may not be able to communicate normally. Researchers have found that the most critical period for developing these skills are from birth to approximately 5 to 7 years of age (Waltzman & Roland, 2005; Yoshinaga-Itano, Sedey, Coulter, & Mehl, 1998). Early hearing (re)habilitation optimizes speech and language development. Prior to universal newborn hearing screenings, the diagnosis of hearing loss could be delayed by several years; often, the red flag is a speech delay (Wrightson, 2007).

Hearing (Re)habilitation

Hearing aids and cochlear implants are viable treatments for hearing loss; however, these devices do not cure hearing loss. Hearing aids can only utilize the remaining, functioning portions of the auditory system. Cochlear implants provide a different method for hearing; the electrode array essentially replaces the hair cells and electrically stimulates the auditory nerve. Those who utilize these technologies may be able to hear within normal limits and develop normal speech and language.

The optimal outcomes with hearing aids or cochlear implants occur when the use of these devices are implemented as soon as possible. Studies have shown that in the incidences of congenital hearing loss, habilitation that occurs in the first 6 months of life "significantly increases the level of language development, speech intelligibility, and emotional stability as compared with children with later identification and intervention," (Waltzman & Roland, 2005); "Children whose hearing losses were identified by 6 months of age demonstrated significantly better language scores than children identified after 6 months of age" (Yoshinaga-Itano, Sedey, Coulter, & Mehl, 1998). Other studies have also found that with children who are diagnosed and receive cochlear implants, the earlier the better; the younger the child is implanted, the more likely the child will develop listening skills similar to or equal to that of children with normal hearing. Waltzman & Roland (2005) found that the children they tested with cochlear implants developed speech and language skills with a natural-sounding voice and those who were implanted early matched normal age targets.

However, some listening situations will always be more difficult for those with hearing loss; noisy environments, sound localization, and perceiving different pitches and changes in intonation is more difficult to translate through an electronic device (Hancock, Noel, Ryugo, Delgutte, 2010; Parker, 2011). Some nuances of sounds can only be appreciated with naturally, good hearing sensitivity.

Mimicking Congenital Hearing Loss in Avians: Focus on aminoglycosides

We are able to mimic a congenital hearing loss in avians by several different means: acoustic trauma and treatments with ototoxic medications such as aminoglycosides.

Aminoglycosides are antibiotics used to treat bacterial infections. Some examples of aminoglycosides are gentamicin, streptomicin, kanamicin, and tobramycin. They can pose a risk for renal, vestibular, and/or auditory toxicity. They are composed of highly polar cations which are not easily metabolized. The drug binds to the bacterial ribosome "[…] causing mistranslation and premature termination of protein synthesis. Decline in [protein] production might hence cause an increase in the production of reactive oxygen species (ROS), thereby damaging mitochondrial and cellular proteins, lipids and nuclear acids," (Bindu & Reddy, 2008). This damage results in the death of cochlear and vestibular cells and, in turn, hearing loss. (Bindu & Reddy, 2008)

In most studies involving gentamicin, it is administered in doses spread out over a period of several days, typically a 10-day treatment at dosages of 50 mg/kg (Cotanche, 1999). Hair cell loss begins in the proximal tip of the basilar papilla within the first 5 days and progresses distally with total hair cell loss at the proximal 25-50% of the basilar papilla while the distal damage is much more variable; both tall and short hair cells and their innervation patterns are damaged (Epstein & Cotanche, 1995; Cotanche, 1999). Regenerated hair cells are also noted by the 5th day of treatment. Gentamicin might have cumulative effects on those hair cells that were lost first and thus begin regenerating first resulting in multiple insults due to the repeated administration of the drug.

The effect of the drugs varies with the age of administration and method of administration. A larger systemic dose of gentamicin is required to cause the same amount of damage seen in a younger chick as in an older chick. However, as the dosage increases, the level of nephrotoxicity also increases. The dosage may be spread out over several days in older birds in order to decrease the risk of nephrotoxicity and fatalities.

Gentamicin can also be administered locally via the round window in order to reduce damage to the kidneys.

Chicks that were injected with 3 daily doses of 100 mg/kg gentamicin were examined at 5 days and 10 days post initial administration. There was clear damage to the hair cells at the proximal portion of the basilar papilla, up to about 75% along the inferior edge but only complete loss up to 20-50% of the basilar papilla by day 5. By day 10, hair cells were regenerated. The tallest stereocilia are actually embedded into the matrix of the tectorial membrane and following damage from gentamicin, the tectorial membrane becomes detached from the basilar papilla where the damage occurred. However, the tectorial membrane also appears to repair itself a rate matching that of the regenerating hair cells. (Epstein & Cotanche, 1995)

Although a daily regimen of gentamicin induces hearing loss, a single dose is most desirable to replicate hearing loss since there is a clear onset of damage and progression and the regeneration without the interference of repeated insult to the hair cells with subsequent injections. Again, with a single dose, damage begins at the very proximal tip soon after injection; however, it is more difficult to damage the more distal portions of the basilar papilla without causing nephrotoxicity and fatalities. (Cotanche, 1999)

Purpose of Study

Naturally occurring, good hearing sensitivity cannot be mimicked by amplification devices; however, if a human experiences hearing loss, amplification devices are the only option if they wish to try to improve their hearing sensitivity. Unfortunately, their hearing will never be considered "normal" again.

Birds are capable of regenerating their hair cells and it is understood that their hearing sensitivity also returns. But the quality of hearing has only been assessed using physiological measures and histology assessments. Therefore the purpose of this study is to assess the entire pathway of hearing with a behavioral measure during the period of deafness and following complete hair cell regeneration. We will measure the behavioral response by analyzing peep suppression in "congenitally" deafened Bobwhites and analyzing the suppression as the hair cells are regenerating. The peep suppression measures in the Bobwhites who have experienced a period of deafness will then be compared to the normal Bobwhites.

Hypothesis:

Many studies have shown that different species of birds are able to lose their hair cells by several different methods and following regeneration, auditory brainstem responses match age-matched normal thresholds. However, physiological measures only tell us if the structures are functioning, not whether the subject actually perceives and responds to the stimuli. We hypothesize: Bobwhites, after hair cell regeneration, will be less responsive than the normal hearing Bobwhite.

Future Implications

This study has provided unique insight into the amount of hair cell damage possible with a single injection of gentamicin, and the functionality of the regenerated hair cells to pure tones in a species that is heretofore not typically used for hair-cell regeneration studies. A future study could focus on the quality of regenerated hearing in response to complex stimuli such as maternal calls. It is hoped that the follow-up study might allow us to understand how these early deafened subjects would respond to species

specific calls which could then be related to speech and language development in humans.

We attempted a pilot study of responsiveness to the maternal alarm call {??}; unfortunately, our subjects would no longer peep. And if you recall, since our behavioral measure requires the subject to be peeping, our measure of responsiveness could not be completed. Several factors could have led to this. Some of our subjects may have been too old and had reached the age at which they stop peeping. We even tried altering the test environment in the hopes that would elicit more peeping which is discussed further, later. Alas, this particular group would not peep. Perhaps the maternal alarm call, which is the ecologically relevant stimulus to stop peeping, was too effective.

Lickliter & Hellewell (1992) attempted to train Bobwhite embryos to prefer the maternal call of an individual Bobwhite hen. They found that they could train the embryo to prefer the call for at 24 hours following hatching but not for 48 hours. They also found that environments for training and testing should be similar since they do affect the birds' response; training and testing should be done either in groups or individually. But it is clear that Bobwhites have an early auditory learning capacity that is heavily dependent on context and experience.

Roberson & Rubel (1995) piqued an interesting thought. They noted that the innervation patterns of the newly generated hair cells do not normalize for several months. Their work found that the afferent terminals degenerate for up to 3 months following damage and at 6 months the innervations are still "ultrastructurally different from control animals." It would have been interesting to see if the mature quail's

responsiveness further improved; however, the same issue arises –quail stop peeping as they mature and cannot be tested using the current procedure.

Current Study Calibrations

      Calibrations were taken of the intensities of each test frequency at 15 different locations within the chamber that the birds were tested. A B&K Model 4176 ½-inch calibrated microphone and pre-amp were suspended in one of 15 different locations (where a quail's ear might reasonably be). The microphone was connected through the walls of the booth through a B&K cable to a B&K Model 2235 Precision Sound Level Meter. The AC output of this meter was input to an Agilent model 35670A Dynamic Signal Analyzer, which reported the intensity and frequency of the input. The areas were labeled as Center, North, East, South, or West at levels of 1cm, 3 cm, and 5 cm above the cage floor, see Figure 14.

      Table 2 and Table 3 contain the same information organized differently (the intensity levels measured at each location and frequency) and include the average intensity level and standard deviations. The variability in several of the frequencies was larger than expected ($s > 5$ dB). The majority of the frequencies with the large range of intensity levels was 5000Hz and above, as expected because calibrations of high frequencies are generally more variable than those of low frequencies. These frequencies' intensities levels may have been more variable due to standing waves. However, 1500 Hz did have a larger range of intensities and, in turn, a larger standard deviation than we would have expected.
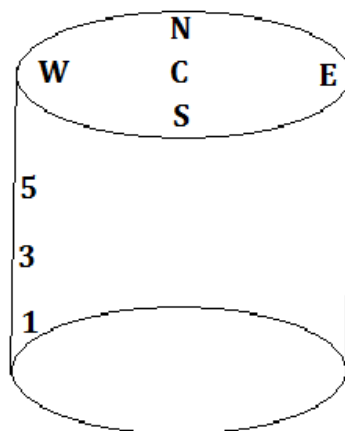
Figure 14: Depicts the cage and calibration locations. C, Center; N, North; E, East; S, South; W, West; 1, 1 cm; 3, 3 cm; 5, 5 cm.

Table 2: **Contains the measured intensities in dB SPL for each location, frequency and setting. C, Center; N, North; E, East; S, South; W, West; 1, 1 cm; 3, 3 cm; 5, 5 cm; I, insensitive setting; S, sensitive setting.**

Frequency (Hz)	Center (dB SPL)						North (dB SPL)						East (dB SPL)						South (dB SPL)						West (dB SPL)					
	Loud			Soft			Loud			Soft			Loud			Soft			Loud			Soft			Loud			Soft		
	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm	5 cm	3 cm	1 cm
250	58	58	60	59	59	59	59	59	59	60	59	60	60	60	60	60	60	60	60	59	60	60	59	59	60	59	59	60	60	59
500	33	33	36	33	37	37	33	35	36	33	35	37	33	34	36	32	35	35	32	34	35	33	34	35	32	35	36	32	35	36
1500	75	81	83	62	69	71	78	81	83	66	69	72	73	79	82	62	67	71	74	77	81	61	65	70	77	81	83	65	69	72
4600	71	77	72	64	68	62	74	74	75	65	64	66	75	74	75	64	63	64	74	70	73	64	60	63	73	73	69	64	62	60
5000	77	78	78	67	66	66	77	71	78	64	59	65	74	76	76	62	64	64	77	76	77	66	64	66	75	74	73	63	63	61
6000	85	84	87	75	72	75	78	80	83	66	68	70	81	82	83	69	71	71	81	81	84	69	69	72	81	79	80	68	67	68
7000	95	95	94	80	80	78	87	92	92	70	77	76	90	92	92	74	76	77	90	91	93	74	76	78	88	90	88	73	74	74
8000	96	97	91	86	88	80	89	92	88	77	81	76	90	91	88	79	79	76	89	92	88	78	80	77	86	90	87	74	78	75

Frequency (Hz)	Average dB SPL	Standard Deviation
250	59	1
500	34	2
1500	73	7
4600	68	5
5000	70	6
6000	76	7
7000	84	8
8000	85	7

Table 3: Contains the measured intensities in dB SPL for each location, frequency and setting. L, Loud/Insensitive setting; S, Soft/Sensitive setting.

5 cm	Center (dB SPL)		North (dB SPL)		East (dB SPL)		South (dB SPL)		West (dB SPL)			
Freq. (Hz)	L	S	L	S	L	S	L	S	L	S	Avg (dBSPL)	StDev
250	58	59	59	60	60	60	60	60	60	60	60	1
500	33	33	33	33	33	32	32	33	32	32	33	1
1500	75	62	78	66	73	62	74	61	77	65	69	7
4600	71	64	74	65	75	64	74	64	73	64	69	5
5000	77	67	77	64	74	62	77	66	75	63	70	6
6000	85	75	78	66	81	69	81	69	81	68	75	7
7000	95	80	87	70	90	74	90	74	88	73	82	9
8000	96	86	89	77	90	79	89	78	86	74	84	7

3 cm	Center (dB SPL)		North (dB SPL)		East (dB SPL)		South (dB SPL)		West (dB SPL)			
Freq. (Hz)	L	S	L	S	L	S	L	S	L	S	Avg (dBSPL)	StDev
250	58	59	59	59	60	60	59	59	59	60	59	1
500	33	37	35	35	34	35	34	34	35	35	35	1
1500	81	69	81	69	79	67	77	65	81	69	74	7
4600	77	68	74	64	74	63	70	60	73	62	69	6
5000	78	66	71	59	76	64	76	64	74	63	69	7
6000	84	72	80	68	82	71	81	69	79	67	75	6
7000	95	80	92	77	92	76	91	76	90	74	84	8
8000	97	88	92	81	91	79	92	80	90	78	87	7

1 cm	Center (dB SPL)		North (dB SPL)		East (dB SPL)		South (dB SPL)		West (dB SPL)			
Freq. (Hz)	L	S	L	S	L	S	L	S	L	S	Avg (dB SPL)	StDev
250	60	59	59	60	60	60	60	59	59	59	60	1
500	36	37	36	37	36	35	35	35	36	36	36	1
1500	83	71	83	72	82	71	81	70	83	72	77	6
4600	72	62	75	66	75	64	73	63	69	60	68	6
5000	78	66	78	65	76	64	77	66	73	61	70	7
6000	87	75	83	70	83	71	84	72	80	68	77	7
7000	94	78	92	76	92	77	93	78	88	74	84	8
8000	91	80	88	76	88	76	88	77	87	75	83	6

Various Attempts at Graphical Representation and Analysis

Presenting our data has proven to be an arduous task since we have several

dimensions to our study: normal versus treated subjects, Loud/Insensitive versus

Soft/Sensitive setting of the peep detector, frequency of stimuli, intensity of stimuli, age

of subjects, and responsiveness. We attempted presenting our results in several different

ways; Figure 15 includes examples of these efforts. Figure 15A dBGrp vs. AgeGrp vs.

Mean Z was an attempt to represent the responsiveness of the different ages and

intensities for the treated and control subjects. Figure 15B features several different line

graphs. Each graph was an attempt to look at the changes in responsiveness across the

intensity levels that the stimuli were presented for each age group with either the

Sensitive or Insensitive setting and either for the Normal or Treated Bobwhite. Figure 16

is our data analyzed with a multiple regression only looking at the effect of treatment.

Here, our data nicely shows the responsiveness of the normal quail does not alter too

much over time (except for that dip at the 4th age group as seen in all our data) while the

responsiveness of the injected birds increases over time. With a closer look at this data,

the responsiveness to high frequencies (>2500 Hz) is still poor in the normal birds

overall. It looks as though Bobwhite just respond better to low frequencies. Figure 17 is

an analysis of all the high frequencies tested with just the Soft/Sensitive setting. Once

more we see that decrease in responsiveness for both the normal and treated birds at the

4th age group suggesting something is occurring at that time in the birds' development.
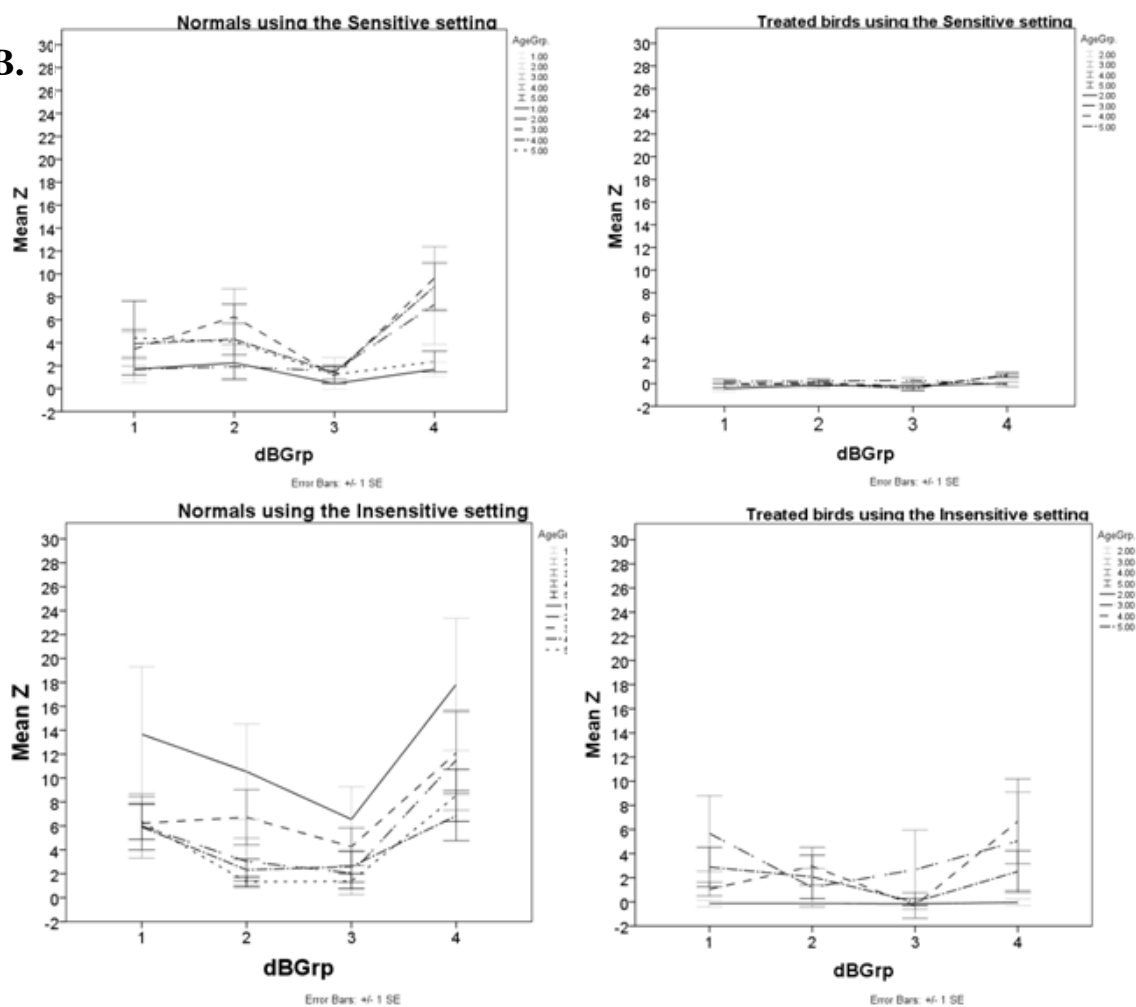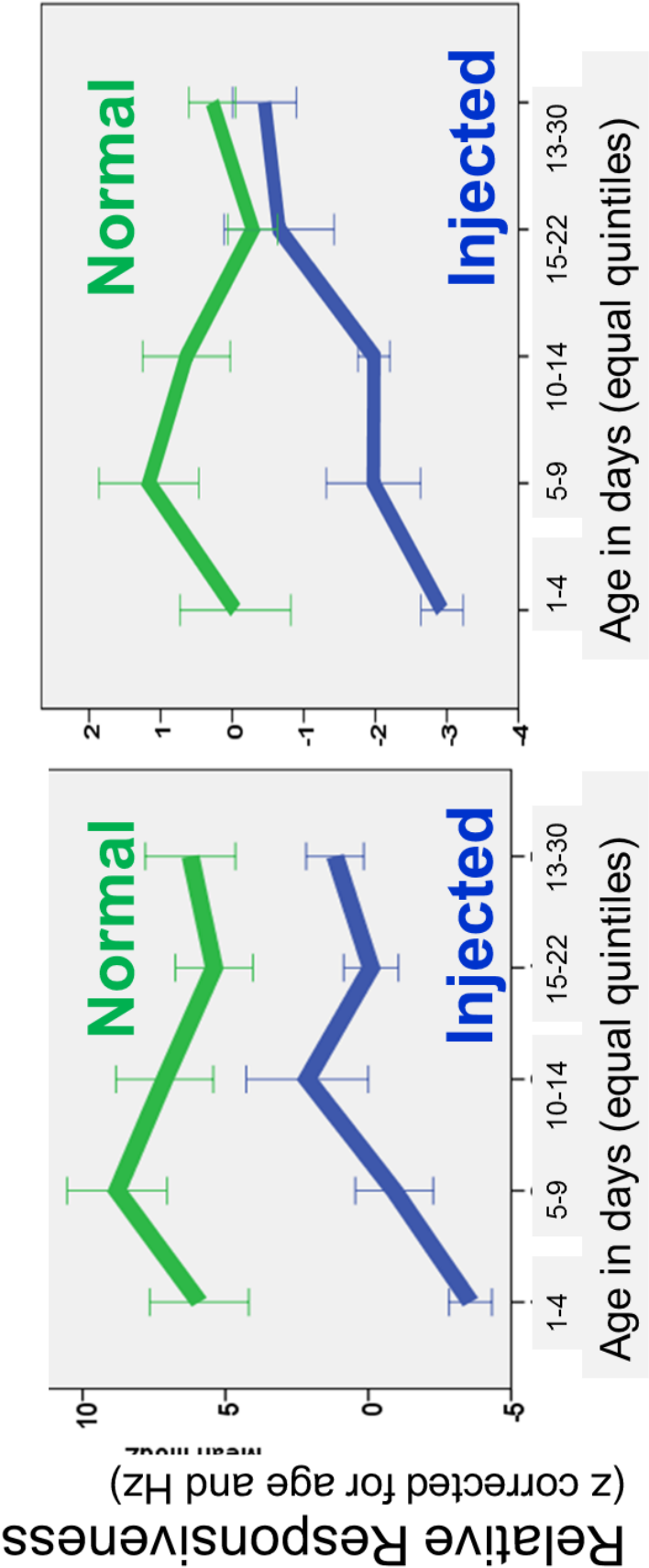
A.
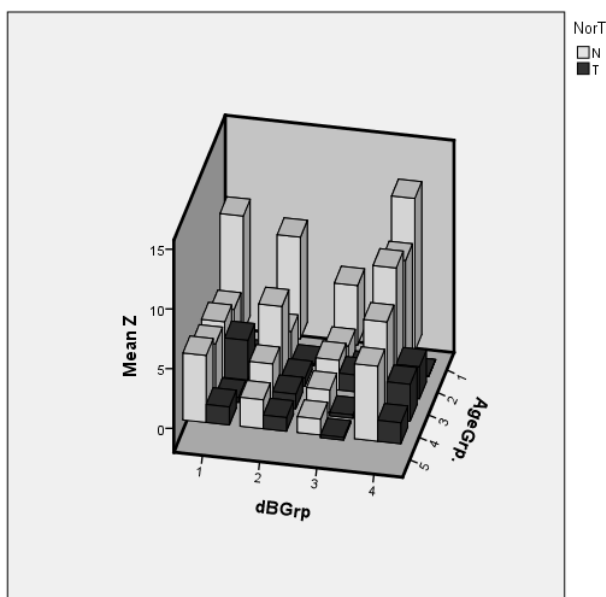
B.

Figure 15A: dBGrp vs. AgeGrp vs. Mean Z. Figure 15B: Composite of dBGrp vs. Mean Z for the different detector settings and for the treated and control birds. Previous attempts at presenting our data graphically.

Figure 16: Different Plots. Here, we took out the effect of age and the effect of frequency and are looking at just the effect of treatment.
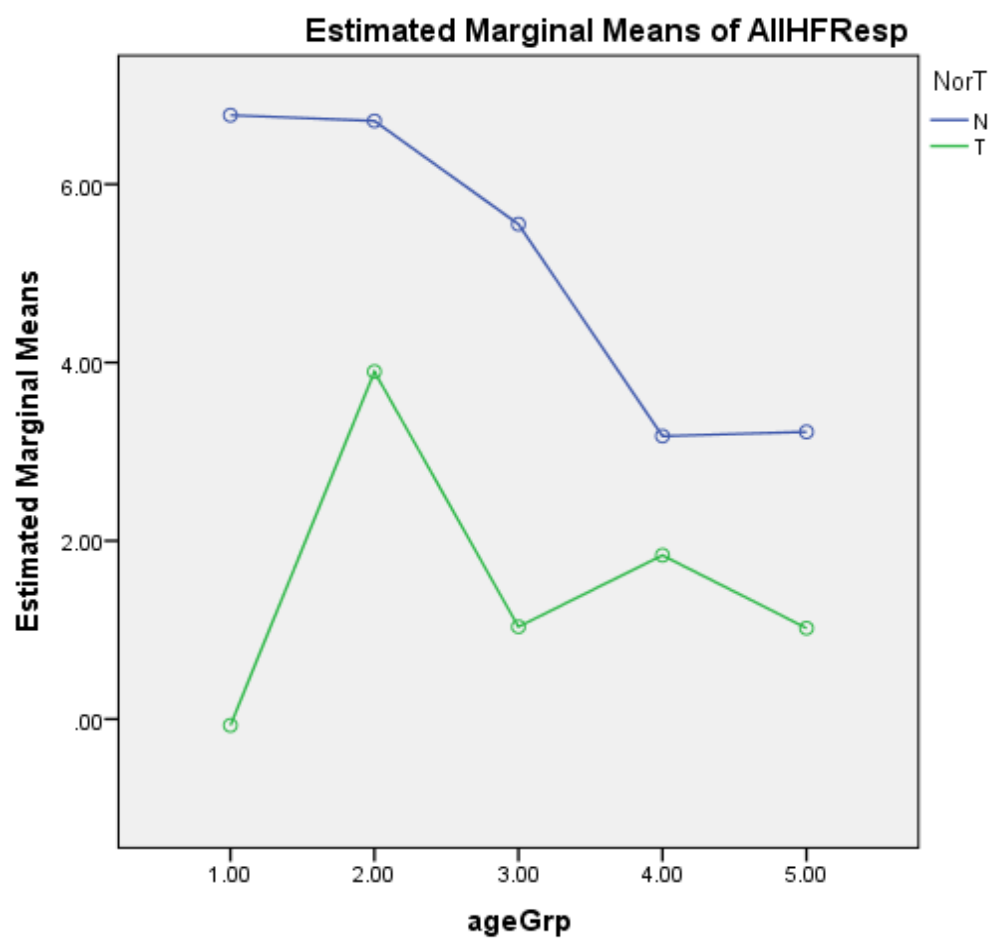
Figure 17: All high frequency responsiveness with the Soft/Sensitive setting only.

Further Studies

The current study yielded interesting results with pure tone stimuli and further research was warranted to see the effect of regenerated hearing sensitivity on species specific call. Therefore, we then tested the auditory thresholds of Bobwhite quail and Coturnix quail by measuring their peep suppression in response to the Bobwhite alarm call. Gray and Jahrsdoerfer (1986) showed that the use of species specific calls led to lower and more consistent auditory thresholds in peep suppression in mallards. Thus, we expected that the Bobwhite would be more sensitive and consistent in responding/reacting to the maternal alarm call than the Coturnix.

Bobwhites and Coturnixs were tested at all different ages post hatch day ranging from one post hatch day to 36. Two different alarm calls were presented at 40, 50, 60 and 70 dB(A) for a total of eight different stimulus trials, each presented twice for a total of 16 stimulus trials, and peep suppression was calculated as a measure of responsiveness to the stimuli. Peep suppression is the amount of time in milliseconds measured from the onset of the stimulus to the onset of the second post stimulus peep. There were also a total of 9 mock (silent) trials conducted as a measure of control to determine the birds' typical peep rate. A total of 25 trials were presented in random order. A one-way ANOVA was run on all the data: duration of peep suppression, the number of the last trial that bird completed, mean mock, standard deviation of mock, responsiveness, intercept and slope of the linear best fit to responsiveness as a function of intensity – estimated parameters of the psychometric function derived from each test. We also tested Bobwhites who had a period of "congenital" deafness with the same protocol as used with the normal hearing Bobwhite and Coturnix. A total of 161 tests were conducted with

the three groups.   A one-sample T-test was then run on the responsiveness of the normal Bobwhite, the Bobwhite with hearing loss, and the Coturnix in order to indicate if each group did hear the alarm calls and responded appropriately.

Another follow-up study altered the environment in which the birds were tested. We continued to work with Bobwhites, but instead of testing each bird individually within the sound booth, we tested the birds in groups who were approximately the same age in the "bird room" within the brooder. The bird room is the room in which the incubators and bird brooders were located. In the previous experiment, we noticed that the birds did not peep as much in the sound booth as they did in the brooder with their peers. In these conditions, it was found that the birds, in fact, peeped and more frequently than recorded with the previous conditions. The alarm call was presented and the birds were tested for a total of 1000 trials.   No assembly call was used in this study; instead, the program ran until it reached 1000 trials. The alarm call was still presented at the four different intensity levels (40, 50, 60 and 70 dB(A)) and each stimuli was presented an equal number of times, but there was a higher number of mock trials than stimulus trials. The trials were divided into blocks; there were 20 blocks and 25 trials in each block. The 25 trials were composed of a random order of stimuli and mock trials and the order was scrambled for each block.

We attempted to measure the groups of birds within the sound booth but found that the birds still did not peep. Apparently the maternal alarm call, the species-typical signal to stop peeping/'shut up' did just that – more than we had hoped. So effective was the peep-suppression from the maternal alarm call that we couldn't measure it. We then decided to measure the birds in the setting that they were most familiar with, inside the

brooder that was within the "bird room." We found that the birds did peep in this setting. In order to be sure that we were, in fact, recording the peeps of the birds that were within the brooder and not the peeps of older and further away birds, we measured the brooder without any birds within it. The results confirmed that we were just recording the peeps of the birds within the brooder. We also looked at changing the measurement settings to the Loud/Insensitive setting from our first study. We found that the Soft/Sensitive setting was the most appropriate setting.

Unfortunately, our subjects would no longer respond to the stimuli and our research could not be completed. However, we are most interested in how the birds respond to the species specific calls since it could relate to language acquisition in humans. Further research is necessary to facilitate the continuation of measuring the responsiveness of quail to species specific calls with normal hearing and regenerated hearing sensitivity.

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