

ABSTRACT

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 OTOTOXIC DRUGS IN THE ZEBRA FINCH

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Aminoglycosides such as kanamycin and amikacin are ototoxic drugs that cause hair cell damage/loss that leads to hearing loss in humans and animals. Previous studies show both hearing loss and recovery following administration in birds. I assessed the effects of aminoglycoside treatment in the budgerigar, canary, and zebra finch using auditory brainstem response (ABR). The purpose of this study was to determine whether the ABR can accurately measure hearing loss following treatment, and to compare the effect of two aminoglycosides on zebra finch hearing sensitivity. After treatment, budgerigar and canary ABR audiograms were similar to those found through behavioral methods confirming the ABR as an efficient tool to measure hearing loss and recovery. Interestingly, zebra finches did not show the expected hearing loss but instead showed small threshold shifts across all frequencies. Overall, the zebra finch appears to be far less susceptible to aminoglycoside induced hearing damage than other birds.

ASSESSING HEARING LOSS DUE TO OTOTOXIC DRUGS
IN THE ZEBRA FINCH

By

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Preface

In humans and mammals noise overexposure and/or ototoxic drugs, such as aminoglycosides, cause permanent hair cell damage/loss and lead to hearing loss (Husmann et al., 1998). Birds are one of few species to show recovery of hearing after noise overexposure and/or ototoxic treatment. Studies show hair cell regeneration and subsequent recovery of hearing sensitivity, to near normal levels, begin to occur in all tested avian species as early as several days after aminoglycoside treatment (Husmann et al., 1998; Cotanche, 1999; Wooley et al., 2001; Dooling et al., 2006; Dooling et al., in prep).

The auditory brainstem response (ABR) technique has been used to assess hearing sensitivity in various avian species including budgerigars (*Melopsittacus undulatus*) (Brittan-Powell et al., 2002), Bengalese finches (*Lonchura striata domestica*) (Wooley and Rubel, 2002), eastern screech owls (*Megascops asio*) (Brittan-Powell et al., 2005), and zebra finches (*Taeniopygia guttata*) (Amin et al., 2007; Noirot et al., 2006). In Bengalese finches, Woolley et al. (2001) assessed changes in hearing sensitivity due to ototoxic drug exposure using the ABR.

The current study focuses on the zebra finch. Zebra finches are small birds that learn their song from a male tutor within the first few weeks of life, and then sing the same song throughout adulthood (Catchpole and Slater, 1995; Lombardino and Nottebohm, 2000). Because these birds, along with other songbirds and parrots, learn through auditory feedback, they serve as good models of vocal development and learning in humans (reviewed in Kroodsma and Miller, 1996).

The purpose of this thesis was two-fold. The first objective was to confirm that the ABR serves as a good measure of hearing sensitivity after aminoglycoside treatment in the budgerigar and canary by comparing results to previous behavioral data in the two species. The second objective was to compare the effect of two aminoglycoside treatments on hearing sensitivity in the zebra finch.

First, hearing sensitivity after aminoglycoside treatment was measured in the budgerigar and canary using the ABR technique. Subjects were administered a ten-day cycle of kanamycin (KM), and hearing sensitivity was measured at set time periods. After KM, ABR audiograms showed loss of sensitivity, but thresholds were higher than those found in the two species through behavioral methods. The general shift in sensitivity due to hair cell loss was similar despite method (behavior vs. ABR). Thus, the ABR was confirmed as an efficient tool to measure aminoglycoside induced hearing loss and subsequent recovery in the budgerigar and canary.

Next, hearing sensitivity after KM treatment was examined in the zebra finch. Due to the extreme toxic effects of the antibiotic, subjects were administered a slightly decreased dosage of the ten-day treatment. ABR audiograms did not display parallel results to those found in the budgerigar and canary. Instead of the anticipated high frequency loss, the zebra finch showed a very small loss in hearing sensitivity across all frequencies tested. Histological studies showed little to no change in the basilar papilla.

To assure that the modified dosage did not cause these unusual results, two budgerigars were also administered the decreased zebra finch dosage. ABR

audiograms were the same in budgerigars despite the difference in dose, thus both dosages are sufficient enough to cause hearing damage.

Finally, the effects of another aminoglycoside, amikacin, were examined in the zebra finch. Here, again, subjects were administered a ten-day dosage of the antibiotic. ABR audiograms displayed no change in hearing sensitivity at any of the time periods. Histological studies revealed little to no hair cell damage or loss in the basilar papilla. Overall, the zebra finch appears to be less susceptible to aminoglycoside induced hearing damage compared to other avian species.

Acknowledgements

As anyone who completes a thesis knows, there may only be one person on stage, but there are many people standing in the wings working behind the scenes and supporting you along the way. To all of those people, I thank you. I know that none of this would have been possible without your help.

I would especially like to thank Beth Brittan-Powell for taking me under her wing and teaching me everything I know about ABRs, injections and numerous other aspects of this thesis. I cannot express how thankful I am to have found such a great mentor and friend to turn to throughout this whole process.

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Table of Contents

Preface.....	ii
Acknowledgements.....	v
Table of Contents.....	vi
List of Tables	viii
List of Figures	ix
 Introduction.....	 1
 General Methods.....	 7
ABR Methods	7
Anesthesia.....	8
ABR Procedure	9
Calibration.....	10
Analysis.....	11
Histology.....	12
 Experiment 1	 13
Introduction.....	13
Methods.....	13
Subjects.....	13
Kanamycin Treatment.....	14
Results and Discussion	14
Hearing Sensitivity.....	14
 Experiment 2A	 17
Introduction.....	17
Methods.....	18
Subjects.....	18
Kanamycin Treatment.....	18
Results and Discussion	19
Hearing Sensitivity.....	19
Histology.....	23
 Experiment 2B	 25
Introduction.....	25
Methods.....	25
Subjects.....	25
Kanamycin Treatment.....	25
Results and Discussion	26
Hearing Sensitivity.....	26

Experiment 3	30
Introduction.....	30
Methods.....	31
Subjects	31
Amikacin Treatment	31
Results and Discussion	32
Hearing Sensitivity.....	32
Histology.....	34
General Discussion	34

List of Tables

- | | | |
|----|----------------------------------|---|
| 1. | Summary of four reviewed studies | 6 |
|----|----------------------------------|---|

List of Figures

1.	Photograph of a bird with electrodes in place	9
2.	Average ABR audiograms comparing methods of analysis in three species	12
3.	Average ABR audiograms for budgerigars (A) and the canary (B) treated with KM	15
4.	Average ABR audiogram for zebra finches treated with KM	19
5.	Bar graph showing species-specific threshold shifts after KM treatment	22
6.	Line graph showing time-dependent threshold shifts in zebra finches treated with KM	23
7.	Basilar papilla of a normal zebra finch (A), a zebra finch treated with KM (B) and a zebra finch treated with amikacin (C)	24
8.	Average ABR audiogram for budgerigars treated with a modified dosage of KM	27
9.	Bar graph showing threshold shifts at day 14 for budgerigars treated with the original dosage and the modified dosage of KM	29
10.	Average ABR audiogram for zebra finches treated with amikacin	33

Introduction

Aminoglycosides are antibiotics that cause profound toxic and functional effects in the kidneys as well as significant cochlear hair cell damage in humans, which manifests itself as impaired hearing in the high frequency range (Gulick et al., 1989; see review in Humes, 1999). In the United States alone, an estimated 4 million courses of ototoxic antibiotics, such as aminoglycosides, are distributed to patients each year (see review in Fischnel-Ghodsian, 1999). These antibiotics are typically prescribed for a duration of ten days (Cotanche, 1999), and are used around the world to treat such illnesses as tuberculosis, bronchitis and otitis media (see review in Fischnel-Ghodsian, 1999). Of those humans treated with aminoglycosides, approximately 2-5% develop clinically significant, permanent hearing loss (see review in Fischnel-Ghodsian, 1999).

Aminoglycoside induced hearing loss also occurs in many avian species such as the budgerigar, canary, Bengalese finch, and broiler chicken (Cotanche, 1999; Dooling et al., 1997; Dooling et al., 2006; Husmann et al., 1998; Woolley et al., 2001). In birds, the associated hearing loss is due to profound hair cell damage marked by hair cell loss and misorientation, primarily with hair cells in the basal end of the basilar papilla where high frequency sounds are encoded (Cotanche, 1999; Dooling et al., 2006; Dooling et al., in press; Husmann et al., 1998; Woolley et al., 2001). In the occasional instance of extended hearing loss (dependent upon time and/or dose), damage then travels through the mid-range frequencies and moves toward the apical end where low frequency sounds are encoded (Woolley et al., 2001; Woolley and Rubel, 2002).

An important difference between birds and mammals is that avian species have the ability to recover hearing sensitivity through the regeneration of lost or damaged inner ear hair cells. For all birds tested to date, hair cell regeneration and hearing recovery, back to near normal threshold levels, begins in the basilar papilla as early as several days after aminoglycoside treatment (e.g. Cotanche, 1999; Dooling et al., 1997, Dooling et al., 2006; Dooling et al., in press; Husmann et al., 1998; Woolley et al., 2001). The end results are regenerated hair cells that are similar in appearance to normal, mature hair cells, but can be recognized by the misorientation of stereocilia bundles (Dooling et al., 1997; Dooling et al., 2006; Dooling et al., in press; Woolley et al., 2001).

Behavioral, physiological, and histological studies have successfully demonstrated and measured this high frequency hearing loss, followed by hair cell recovery and function of hearing in a variety of avian species (e.g. Dooling et al., 2006; Dooling et al., in press; Woolley et al., 2001; Woolley and Rubel, 2002). Two such studied aminoglycosides that are known to produce this predominately cochlear damage with subsequent recovery are kanamycin (KM) and amikacin (reviewed in Fischnel-Ghodsian, 1999). The two drugs are very similar in function, yet amikacin has far less toxic effects on the kidneys than kanamycin.

There are three studies examining the effects of aminoglycoside treatment in budgerigars and canaries (Dooling et al., 1997; Dooling et al., 2006; Dooling et al., in press). These studies use behaviorally trained birds to measure high frequency hearing loss, followed by regeneration of hair cells and recovery of hearing sensitivity. The birds are trained to peck at a light emitting diode (LED) when a tone

is presented and refrain from pecking the LED when the tone is absent; the presentation intensity level varies for each tone to establish the lowest intensity at which the bird can hear or the absolute threshold (Dooling et al., 1997; Dooling et al., 2006; Dooling et al., in press).

Dooling et al. (1997) examined auditory perception and vocalizations before, during, and after hair cell loss and recovery due to aminoglycoside treatment. Budgerigars were administered eight 200 mg/kg/day kanamycin injections. After six days of injections, the basal end of the basilar papilla was nearly devoid of all hair cells. Six days later (4 days post completion of injections) regenerating hair cells were found in the previously barren basal end of the basilar papilla. Regeneration and recovery of hair cells continued. Hair cell numbers were almost normal levels four weeks after km injections, and eight weeks later were within normal limits. Hearing sensitivity and vocalizations were both impaired with lost or damaged hair cells, but perceptual behaviors returned at a rate concurrent with hair cell regeneration (approximately 8 weeks after treatment), while vocal behaviors returned over a much shorter period of time (10-15 days after the completion of treatment).

A similar study (Dooling et al., (2006)) administered 8 days of kanamycin injections (Day 1: 100 mg/kg, Day 2-8: 200 mg/kg) to budgies and examined absolute hearing thresholds, auditory discrimination, and perception and recognition of complex vocalizations. Behavioral tests assessing hearing thresholds directly following KM treatment primarily showed a high frequency hearing loss with damage extending to all frequencies tested. By 20 weeks post injections, hearing thresholds improved to near normal levels (within 10-15 dB for low frequencies and 20-30 dB

for high frequencies). Tasks examining intensity and frequency difference limens showed that discriminations were relatively unaffected by exposure to the ototoxic drug. Call recognition tasks ascertained that after KM administration, previously familiar calls were no longer familiar to the birds, and remained so even three months later. Overall, this study established that while hearing sensitivity recovers to near normal levels, perception of vocalizations changes after hair cell loss and recovery.

Dooling et al. (In press) used behavioral methods to compare the effects of kanamycin on Belgian Waterslager (BW) and Non-Belgian Waterslager (non-BW) canaries (*Serinus canaria*). Kanamycin injections were administered over a period of ten days (Day 1: 100 mg/kg, Day 2-10: 200 mg/kg), and hearing sensitivity was assessed before, during, and after KM treatment. Results showed that while the two strains of canary both displayed high frequency hearing loss followed by recovery of thresholds, BW canaries were less sensitive to KM treatment than non-BW canaries. Most significantly, BW canaries have better hearing at high frequencies after KM treatment than they showed before KM injections.

Woolley et al. (2001) used the ABR technique and histological methods to assess hearing sensitivity after aminoglycoside treatment in Bengalese finches. Using alternating doses of 150mg/kg and 300mg/kg of amikacin for a period of seven days, they found profound hair cell destruction followed by hair cell regeneration and recovery of auditory sensitivity. One day after the completion of aminoglycoside treatment, no hair cells were present in the basal end of the basilar papilla. One week after aminoglycoside treatment showed larger areas of missing hair cells and the presence of regenerated hair cells and recovery of auditory sensitivity. Auditory

recovery peaked at four weeks post treatment, and full hair cell regeneration was complete by eight weeks post amikacin treatment.

All avian species tested to date show hearing loss followed by recovery after exposure to ototoxic drugs, but no work has yet been done on zebra finches (see Table 1). Zebra finches are small, Australian songbirds, that along with other songbirds and parrots are used to study vocal development and learning in humans. Like humans, these birds are constrained by a critical period for vocal learning and use auditory feedback to both learn and maintain vocalizations (Catchpole and Slater, 1995).

Zebra finches are a sexually dimorphic species in which males sing and females do not. Male zebra finches learn their songs within the first few weeks of life by imitating the songs of conspecific male tutors (Catchpole and Slater, 1995; Hough II and Volman, 2002; Kroodsma and Miller, 1996) and continue to produce the same vocalizations throughout adulthood (Lombardino and Nottebohm, 2000). Zebra finches are currently considered the best model system for studying vocal development and learning in humans. Because zebra finches, like all other avian species tested to date, also possess mechanisms of hair cell regeneration, they, unlike humans, are able to experience a temporary period of hearing impairment. For this reason, it is interesting to study the effects of aminoglycoside treatment on zebra finches so that in the future researchers can investigate the implications of temporary deafness on vocal learning.

SPECIES	AMINOGLYCOSIDE	METHOD	RESULT	REFERENCE
Canary	Kanamycin	Behavioral and Histology	High frequency hearing loss concurrent with hair cell damage after KM injections; regeneration of hair cells and recovery of hearing sensitivity followed.	Dooling et al., in press
Budgerigar	Kanamycin	Behavioral and Histology	Initial loss of hair cells and auditory sensitivity; hearing sensitivity recovers to near normal levels, perception of vocalizations changes after hair cell loss and recovery.	Dooling et al., 2006
Budgerigar	Kanamycin	Behavioral and Histology	Initial loss of hearing and vocal production abilities. Perceptual behaviors returned at a rate concurrent with hair cell regeneration; vocal behaviors returned much faster.	Dooling et al., 1997
Bengalese Finch	Amikacin	ABR and Histology	High frequency hearing loss followed by hair cell regeneration and recovery of hearing sensitivity which peaked at 4 weeks post treatment.	Woolley et al., 2001

Table 1: A brief summary of 4 studies that have successfully demonstrated and measured a high frequency hearing loss followed by hair cell recovery and function of hearing in birds.

In the following experiments, I assessed aminoglycoside induced hearing loss and recovery in budgerigars, canaries, and zebra finches using the ABR technique. The purpose of the first experiment was to confirm the ABR as an efficient tool to measure aminoglycoside induced hearing loss and subsequent recovery in the budgerigar and canary. The Experiment 2 & 3 examined the effects of aminoglycosides on zebra finch hearing sensitivity. Subjects were divided into two groups: one group received a ten day treatment of KM, the other received a ten day treatment of amikacin. Changes in hearing sensitivity and anatomy from the two groups were compared. The purpose of these experiments was to determine if these two aminoglycosides have similar effects on hearing in zebra finches and to compare hearing loss in zebra finches to published data on other avian species (such as the budgerigar, canary, and Bengalese finch).

General Methods

ABR Methods

Hearing sensitivity was assessed using the ABR at various times throughout the study. These were 1) prior to the administration of the aminoglycoside (predata), 2) after 6 days of aminoglycoside treatment (day 6), 3) three days after the completion of the injections (day 14), and 4) subsequently at weekly intervals following the administration of the full cycle of the aminoglycoside for a period of approximately two months.

It is important to note that data were not collected for all birds at each of the aforementioned time periods. Because of the extreme toxicity of the drugs, some

birds died prior to completion of the study. As such, the number of subjects assessed at each time stage is not consistent throughout the study, but will be noted in each experiment.

Anesthesia

Prior to all ABR recordings, subjects were anesthetized with an intramuscular injection of ketamine (25-50mg/kg) and diazepam (2mg/kg). Typically, this dosage is effective enough for the bird to remain sedated for approximately a period of one hour (Brittan-Powell et al., 2002).

If the standard dosage of anesthesia was not adequate, a second (half of the original) dosage of ketamine was administered. If the subject was still resistant, a third and final injection of one half of the original ketamine dosage was again injected. In the rare instance that after the third injection the bird was still not anesthetized, the experiment was terminated and the bird was then placed into a therapy unit to fully recover.

Ketamine, like other anesthetics, may cause a multitude of side effects. Interestingly, zebra finches displayed extremely high hearing thresholds for approximately one hour beginning immediately after the injection, then with time, thresholds slowly lowered to normal. Additionally, in all three species, several birds experienced a severe allergic reaction and died immediately after the injection (per Dr. Doug Powell, UMCP Vet).

ABR procedure

Once anesthetized, each bird was securely wrapped in a small cloth to maintain warmth and hinder any movement. Electrodes were placed subcutaneously under the skin at the vertex (active), behind the ipsilateral canal opening (reference), and behind the canal opening of the contralateral ear (ground) (see Illustration 1).

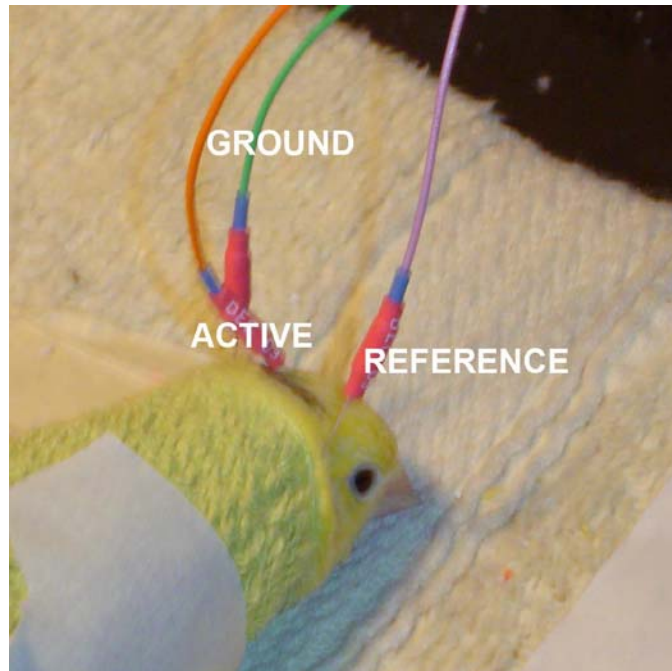


Figure 1: A sedated canary wrapped in a cloth with electrodes inserted. Electrodes are placed subcutaneously under the skin at the vertex (active), behind the ipsilateral canal opening (reference), and behind the canal opening of the contralateral ear (ground).

The stimulus presentation, ABR acquisition, equipment control, and data management were coordinated using a Tucker-Davis Technologies System 3 modular rack-mount system controlled by a F15 Gigabit interface module cable-linked 2.66-GHz Pentium4 PC containing a TDT P15 Gigabit interface PCI card and running TDT SIGGEN and BIOSIG software.

Sound stimuli in the form of tone burst trains were delivered using a JBL speaker model 2105H (James B Lansing Sounds Inc.) placed 30 cm from the bird's right ear. Individual tone bursts were 5 ms in duration with a 1 ms rise/fall and were presented at .5, 1, 1.5, 2, 2.86, 4, 5.7, and 8 kHz. Rectangular-pulse broadband clicks were 0.1 ms in duration with a 25 ms inter-stimulus interval. Each stimulus train consisted of nine single clicks or frequency tone bursts (a total of 230 ms in duration) that increased in intensity in either 5 or 10 dB steps, were presented at a rate of 4/s and acquired at 25 kHz (Brittan-Powell et al., 2002; Brittan-Powell et al., 2005).

Each ABR waveform represented the average of 300 alternating stimulus presentations replicated at each intensity level. During acquisition they were notch filtered at 60 Hz. After stimulus collection, each signal was filtered below 30 Hz and above 3000 Hz using the BIOSIG program.

Calibration

Sound stimuli were calibrated using a Larson Davis System 824 sound level meter. A ½" microphone connected to the sound level meter was placed 30 cm from the speaker, at approximately the same position as the bird's head. Eight hundred

millisecond tone bursts were played and the sound pressure level (Fast weighting dB A Scale), as displayed on the sound level meter, was recorded.

Analysis

ABR threshold was defined two ways. First, threshold was estimated through the visual detection method and defined as 2.5 dB below the lowest intensity at which a response could be visually identified (e.g., Brittan-Powell et al., 2002; Brittan-Powell et al., 2004; Brittan-Powell et al., 2005). Thresholds were determined as the arithmetic mean of 1) the lowest intensity level at which a response was detected and 2) the following intensity level in which no response was detected. Intensity levels decreased at a rate of 5 dB, therefore the average of two intensity levels resulted in a threshold that was estimated to be 2.5 dB below the lowest intensity detected.

The second estimate was based on the amplitude-intensity function for peak amplitude of wave 1 to the baseline. The distance from the base to the peak of wave 1 was measured and each data point was plotted. A regression analysis was performed and the line of best fit estimated the threshold.

To assure accuracy, thresholds were replicated approximately four to six times at each frequency tested. Thresholds that appeared more variable were replicated until a more consistent estimate was achieved. The arithmetic mean of the threshold estimates for each frequency tested was recorded.

Figure 2 shows a comparison of the two methods of analysis for determining thresholds. Predata ABR audiograms for the budgerigar, canary, and zebra finch show that similar thresholds were found through the two methods. A three- way

analysis of variance (ANOVA) showed that there were no significant differences found among species ($F[2,245]=0.931$, $p>.05$). More importantly, the interaction of species by method was not significantly different ($F[2,245]=1.693$, $p>.05$).

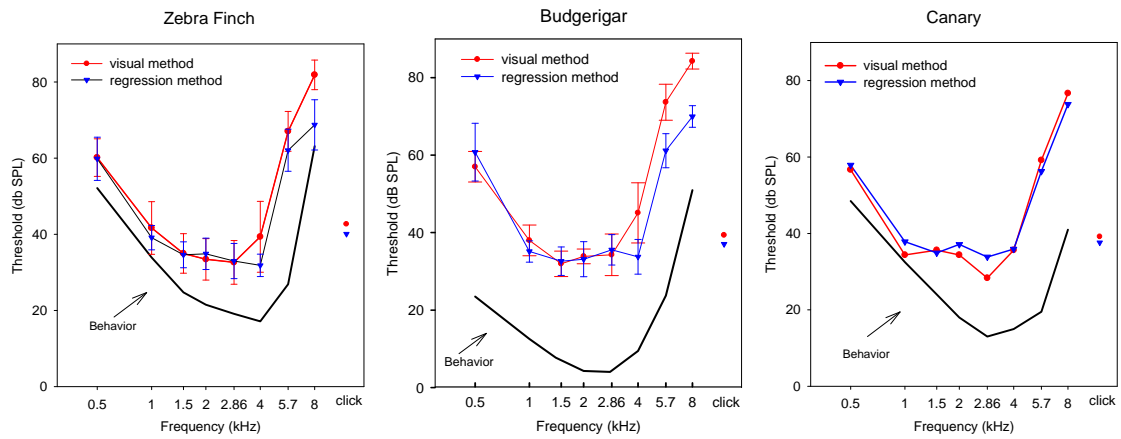


Figure 2: ABR audiograms before aminoglycoside treatment comparing the two methods of analysis in the budgerigar, zebra finch and canary. The red, closed-circle line displays thresholds determined by the visual detection method and the blue, downward-triangle line displays thresholds determined by the regression analysis method. The solid black line is the behavioral audiogram for each species.

Histology

All anatomical work was done in collaboration with Dr. Brenda Ryals at James Madison University. I assisted Dr. Ryals who kindly provided the expertise needed to perform the histological studies.

A total of five zebra finches (three administered KM treatment and two administered amikacin treatment) were used in histological studies. Birds were anesthetized with an overdose of Euthosol, sacrificed by decapitation, and a direct intra-labyrinthine perfusion of 4% paraformaldehyde/0.1% glutaraldehyde in 0.1 M

PO4 buffer at pH of 7.2 was performed bilaterally. The entire head was immersed in fixative and kept refrigerated overnight. Ears were dissected out and whole mounts were prepared for phalloidin staining and mounted on microscope slides in slow fade gold anti fade reagent.

Experiment 1 (Kanamycin in Budgerigars and Canaries)

Introduction

Behavioral studies showed a high frequency hearing loss, followed by regeneration of hair cells and recovery of hearing sensitivity after aminoglycoside treatment in both budgerigars and canaries (Dooling et al., 1997; Dooling et al., 2006; Dooling et al., in press). The purpose of this study was to measure the effects of kanamycin in these two avian species using the ABR technique. I hypothesized that ABR thresholds would show a change in hearing sensitivity that parallels those found in behavioral studies with budgerigars and canaries.

Methods

Subjects

Three budgerigars and one canary were used as subjects in this experiment. Each bird was given daily access to ample food, with the exception of the morning of ABR testing, and was housed in an avian vivarium at the University of Maryland, College Park. The Animal Care and Use Committee at the University of Maryland, College Park approved all care and use of the birds in this experiment.

Kanamycin Treatment

All birds were administered a ten-day cycle of the ototoxic drug, kanamycin (KM). The daily dosages of KM were Day 1: 100 mg/kg, and Day 2-10: 200mg/kg.

At approximately the same time every morning, each subject received an intramuscular injection of the antibiotic into the pectoral muscle. Each bird's chest/pectoral muscle was visually divided into four quadrants, and the daily injection site rotated among the divisions to help reduce bruising and scarring. In an effort to help maintain the health of the subjects, because KM has such an adverse effect on the kidneys (Gulick et al., 1989; reviewed in Humes, 1999), each bird also received a second injection of (1-2cc) Lactated Ringer's solution (Sarah Woolley, personal communication). The supplementary fluids were administered through a subcutaneous injection inserted dorsally between the wings.

The injection cycle occurred over a period of eleven days. The first six injections were administered on days one through six, and the last four were given on days eight through eleven. Subjects did not receive an injection on day seven. Instead, hearing sensitivity was assessed through the ABR after the sixth day of injections.

Results and Discussion

Hearing Sensitivity

Figures 3A and 3B show ABR audiograms for the budgerigar and canary at four time periods. Following kanamycin treatment, high frequency thresholds increased for the budgerigar ($F[33,54]=40.791$, $p<.01$) and canary ($F[3,28]=4.662$,

$p < .01$). and paralleled those found in the two species using behavioral methods

(Dooling et al., 1997; Dooling et al., 2006; Dooling et al., in press).

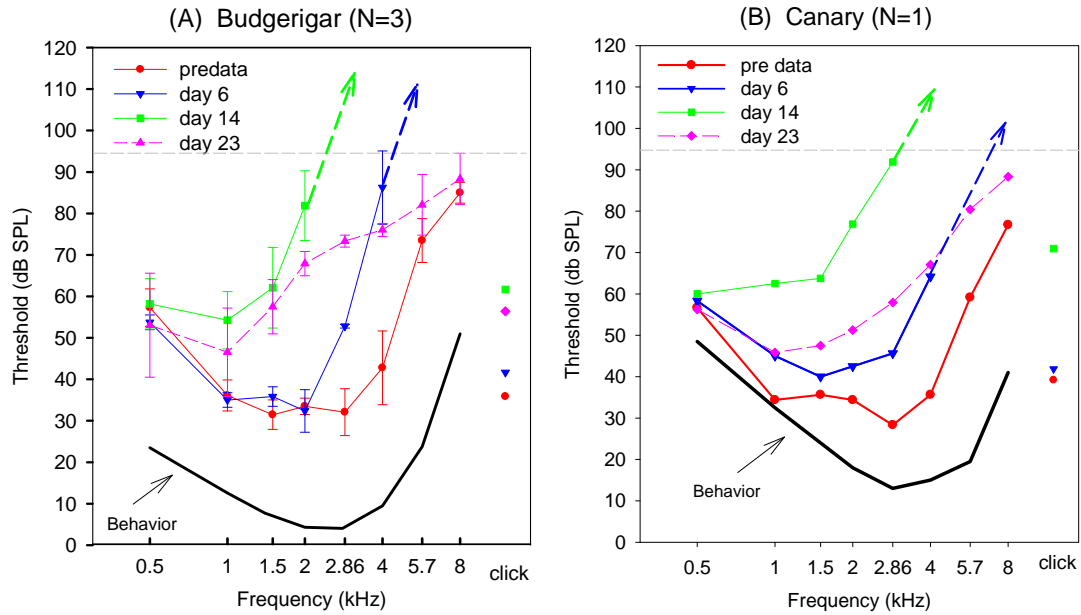


Figure 3: ABR audiograms before KM injection (red, closed-circle line), after 6 days of KM injections, or day 6 (blue, downward-triangle line), 3 days post injections, or day 14 (green, closed-square line) and 12 days post injections, or day 23 (pink, closed-diamond line) for the budgerigar (a) and canary (b). The solid black line is the behavioral audiogram for each species. Arrows project estimated hearing thresholds at the designated frequencies. Following six days of KM treatment, ABR audiograms of the budgerigar and canary hearing thresholds showed high threshold shifts similar to those found behaviorally due to KM administration. At three days post the completion of KM treatment, budgerigar and canary threshold shifts as a function of frequency show substantially larger differences than those displayed after 6 days of injections. By approximately 10 days post the completion of treatment, recovery of threshold shifts is evident in both species.

In both species, after six days of KM injections, ABR audiograms show extensive hearing loss in the high frequencies (i.e. 2.86, 4, 5.7, and 8 kHz). This loss in high frequency hearing sensitivity is represented by substantial threshold shifts at these frequencies as evidenced for example, from a 40dB shift at 4 kHz. The low and mid frequencies (i.e. .5, 1, 1.5, and 2 kHz) were relatively unaffected by the antibiotic. The budgerigar showed significant changes in thresholds as a function of treatment (day by frequency) ($F[21,54]=12.26$, $p<.01$). Post hoc tests showed significant differences in thresholds between testing day 6 and predata for the budgerigar ($t[3]=-5.48$, $p<.01$), but not for the canary ($t[3]=-1.78$, $p>.05$).

After the budgerigar and canary received the full ten-day administration of the KM treatment, the originally restricted high frequency hearing loss expands to affect all frequencies tested. Post hoc tests showed significant differences between testing day 14 and predata for the budgerigar ($t[3]=-18.58$, $p<.01$) and the canary ($t[3]=-3.74$, $p<.01$). Though all frequencies display a loss in hearing sensitivity, the greatest threshold shifts remain in the high frequencies. Threshold shifts reaching approximately 50 and 60 dB are recorded at select frequencies (i.e. at 4 and 2.86 kHz, respectively) in both budgerigars and canaries.

Recovery of hearing sensitivity began approximately ten days after the completion of the KM treatment. ABR audiograms display recovering thresholds at all frequencies tested for the two species (see Figures 3A and 3B). Post hoc tests showed significant differences between testing day 23 and predata for the budgerigar ($t[3]=-11.93$, $p<.01$), but not for the canary ($t[3]=-1.84$, $p>.05$). At day 23, thresholds decrease 20-30 dB back to near normal levels for both species. This provides

evidence of hearing recovery. With time, thresholds continue to slowly improve back to baseline levels. These results parallel those found through behavioral methods for both budgerigars and canaries (Dooling et al., 2006; Dooling et al., in press).

Experiment 2A (Kanamycin in Zebra Finches)

Introduction

The previous study showed that the ABR can be used as a valid measure of hearing sensitivity before, during and after aminoglycoside treatment. The ABR allowed successful demonstration of a high frequency hearing loss, followed by regeneration of hair cells and recovery of hearing sensitivity after kanamycin treatment in both budgerigars and canaries. Results from Experiment 1 mirrored behavioral results for the budgerigar and canary (Dooling et al., 2006; Dooling et al., in press).

To our knowledge, no work has yet been done on zebra finches. The purpose of this study was to measure the effect of kanamycin treatment in zebra finches using the auditory brainstem response and to compare the results to previous behavioral data in budgerigars and canaries. I hypothesized that zebra finch ABR thresholds (measured before, during and after KM treatment) would show a change in hearing sensitivity that parallels those found in ABR and behavioral studies with budgerigars and canaries.

Methods

Subjects

Nine zebra finches were used as subjects in this experiment. Six zebra finches were used to measure hearing sensitivity before, during, and after kanamycin treatment. Three zebra finches were used for histological studies. All procedures were the same as above, except where noted.

Kanamycin Treatment

All birds were administered a ten-day cycle of the ototoxic drug, KM. The daily dosages of KM for zebra finches were slightly lowered from the original dosage administered in Experiment 1. When conducting pilot experiments, I experienced an extremely high mortality rate in zebra finches with the standard dosage. Sixty percent (three out of five) of the subjects died as a direct result of the toxic effects of the antibiotic. Necropsy reports of the birds showed severe kidney damage, and the cause of death for all subjects was deemed renal failure. Therefore, the modified daily dosages of KM for zebra finches were: Day 1: 50mg/kg, Day 2: 100 mg/kg, Day 3-10: 200mg/kg.

As with Experiment 1, the injection cycle occurred over a period of eleven days. The first six injections were administered on days one through six and the last four were given on days eight through eleven. Subjects did not receive an injection on day seven. Instead, hearing sensitivity was assessed through the ABR after the sixth day of injections.

Results and Discussion

Hearing Sensitivity

Figure 4 shows an average ABR audiogram for six zebra finches at four different time periods. It is important to note that due to the extreme toxicity of the drug, all subjects did not survive until the final assessment. Therefore, the number of subjects at each measurement time period slightly varies. ABR results display a form of hearing loss due to kanamycin administration that is very dissimilar to other birds.

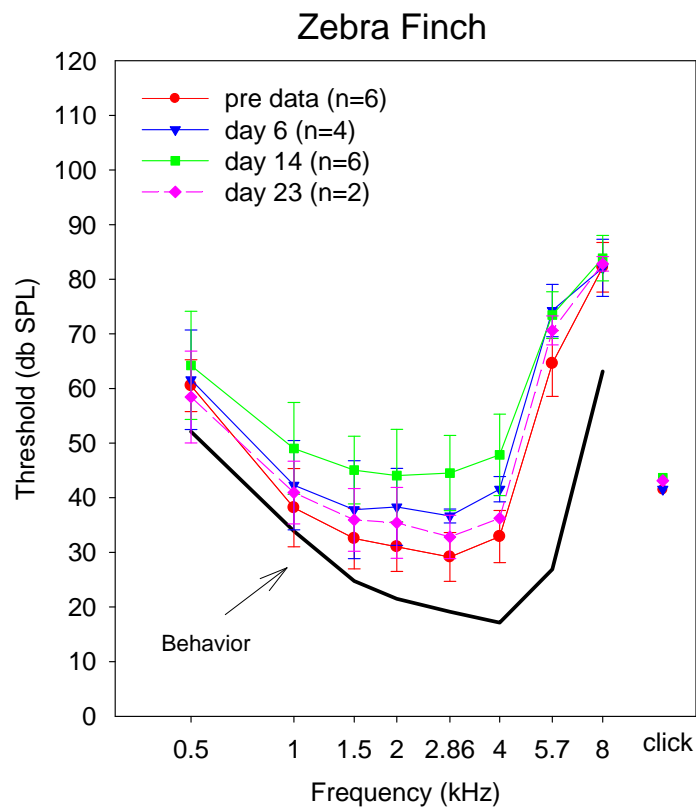


Figure 4: ABR audiograms before KM injection (red, closed-circle line), after 6 days of KM injections, or day 6 (blue, downward-triangle line), 3 days post injections, or day 14 (green, closed-square line), and 12 days post injections, or day 23 (pink, closed-diamond line) for the zebra finch. The solid black line is the behavioral audiogram for zebra finches. Following six days of KM treatment, ABR audiograms of hearing thresholds did not show a similar pattern of high threshold shifts similar to those found behaviorally due to KM administration. At three days post the completion of KM treatment, zebra finch threshold shifts as a function of frequency show only slightly increased threshold shifts as compared to those displayed after 6 days of injections. Approximately 12 days after the completion of treatment, recovery of threshold shifts is evident.

Following 6 days of KM treatment, 4 zebra finches showed a small threshold shift of approximately 5 dB across all frequencies, with the greatest shifts occurring in the mid frequencies (i.e. 1.5, 2, 2.86, and 4 kHz). A repeated measures ANOVA showed no significant difference between testing day 6 and predata across frequencies ($F[8,24]=1.188$, $p>.05$). Other avian species, under the same conditions, show a substantial high frequency threshold shift, leaving the mid and lower frequencies relatively unaffected.

Three days after the full ten-day KM course, zebra finches only show another slight increase of approximately 5 dB in threshold shifts across all frequencies. This sums to a total shift of approximately 10 dB at all frequencies tested. At this point again, the middle frequencies (1.5, 2, 2.86, and 4 kHz) appear to be most affected by the aminoglycoside. A repeated measures ANOVA showed a significant difference between testing day 14 and predata across frequencies ($F[8,40]=5.381$, $p<.01$). Although results show a statistically significant difference in threshold shift between day 14 and predata, the zebra finch still shows only a minimal change in contrast to results found in the budgerigar and canary.

The largest species-specific differences (due to KM administration) in hearing threshold shifts occurs 3 days post completion of treatment. A substantial difference in threshold shifts was seen at the mid to high frequencies (see Figure 5). The most extensive ranges among species occur at the high frequencies. For example, at 2.86 kHz, the budgerigar and canary show an approximately 50 dB threshold shift whereas the zebra finch shift was merely 15 dB. Six separate ANOVAs were performed to examine differences in threshold shifts among species by frequency – a Bonferroni correction was performed ($p < .008$). No significant differences were found at .5 kHz ($F[2,7] = 0.281$, $p > .05$) and 1 kHz ($F[2,7] = 4.328$, $p > .05$). A significant difference was found at 1.5 kHz ($F[2,7] = 15.449$, $p < .01$); post hoc tests showed a significant difference between the budgerigar and zebra finch ($t[2] = 5.239$, $p < .008$). A significant difference was found at 2 kHz ($F[2,7] = 18.612$, $p < .01$); post hoc tests showed a significant difference between the budgerigar and zebra finch ($t[2] = 5.785$, $p < .008$). A significant difference was found at 2.86 kHz ($F[2,7] = 37.886$, $p < .01$); post hoc tests showed a significant difference between the budgerigar and zebra finch ($t[2] = 7.865$, $p < .008$) and the canary and zebra finch ($t[2] = 5.357$, $p < .008$). A significant difference was found at 4 kHz ($F[2,7] = 15.998$, $p < .01$); post hoc tests showed a significant difference between the budgerigar and zebra finch ($t[2] = 4.941$, $p < .008$) and the canary and zebra finch ($t[2] = 3.765$, $p < .008$).

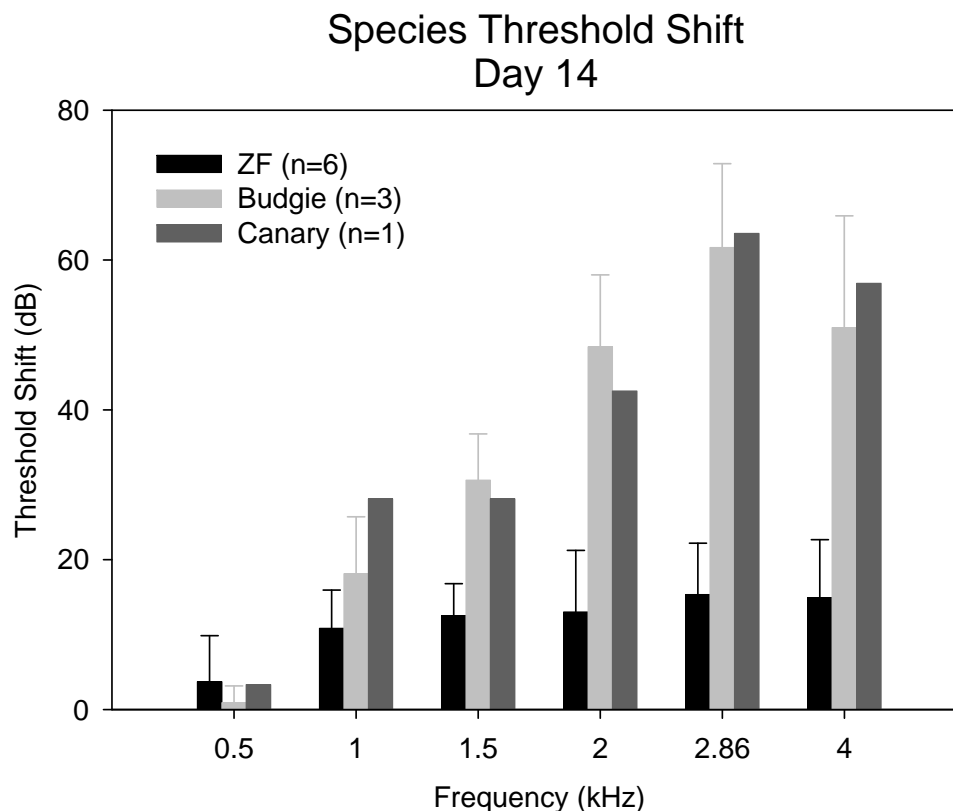


Figure 5: Species-specific threshold changes at low (.5, 1 and 1.5 kHz), mid (2 and 2.86 kHz), and high (4 kHz) frequencies following KM treatment in the zebra finch (black), budgerigar (light gray), and canary (dark gray). ABR thresholds were measured 3 days after the completion of KM treatment. The budgerigar and canary experience a similar threshold shift at each of the frequencies tested. Interestingly, the zebra finch shows a much smaller threshold shift at each frequency. The most substantial differences in threshold shifts are exhibited at the mid and high frequencies.

As with other avian species, recovery of hearing sensitivity in the zebra finch begins approximately ten days after the completion of KM treatment (see Figure 4A). At day 23, ABR audiograms show a 7 dB shift in thresholds back to baseline levels, signifying an improvement in hearing abilities. A repeated measures ANOVA showed no significant difference between testing day 23 and predata across frequencies ($F[8,8]=1.094$, $p>.05$). Over time, zebra finches continue to show slight

improvements in hearing sensitivity. By one month, recovery of hearing sensitivity was near normal, but not fully achieved (see Figure 6).

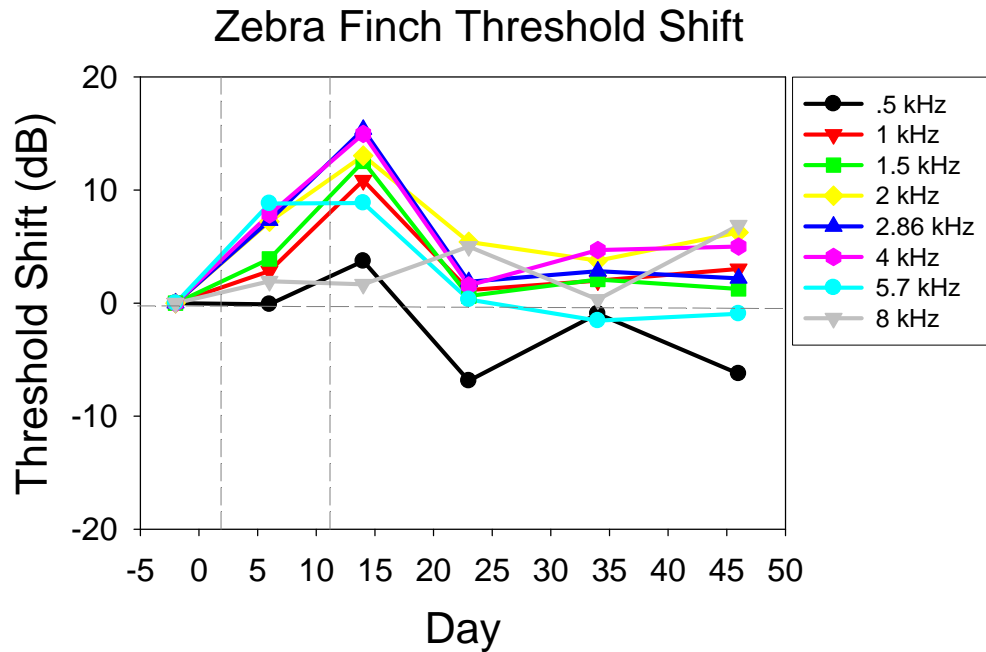


Figure 6: Graph showing threshold shifts and recovery over time at each individual frequency in the KM treated zebra finch. A substantial threshold shift is seen at day 14, and subsequent recovery back to near normal levels begins at day 23. Dashed gray lines denote injection period.

Histology

Examination of the basilar papilla of three zebra finches (0 days post KM course) using phalloidin staining showed no hair cell loss and little to no stereocilia misorientation among surviving hair cells in the basal and middle regions of the basilar papilla (see Figure 7B). In fact, the basilar papillae of the three zebra finches appeared quite similar to the papilla of a normal zebra finch (see Figure 7A). This is

quite unlike the massive amount of hair cell loss and destruction that is observed in other avian species.

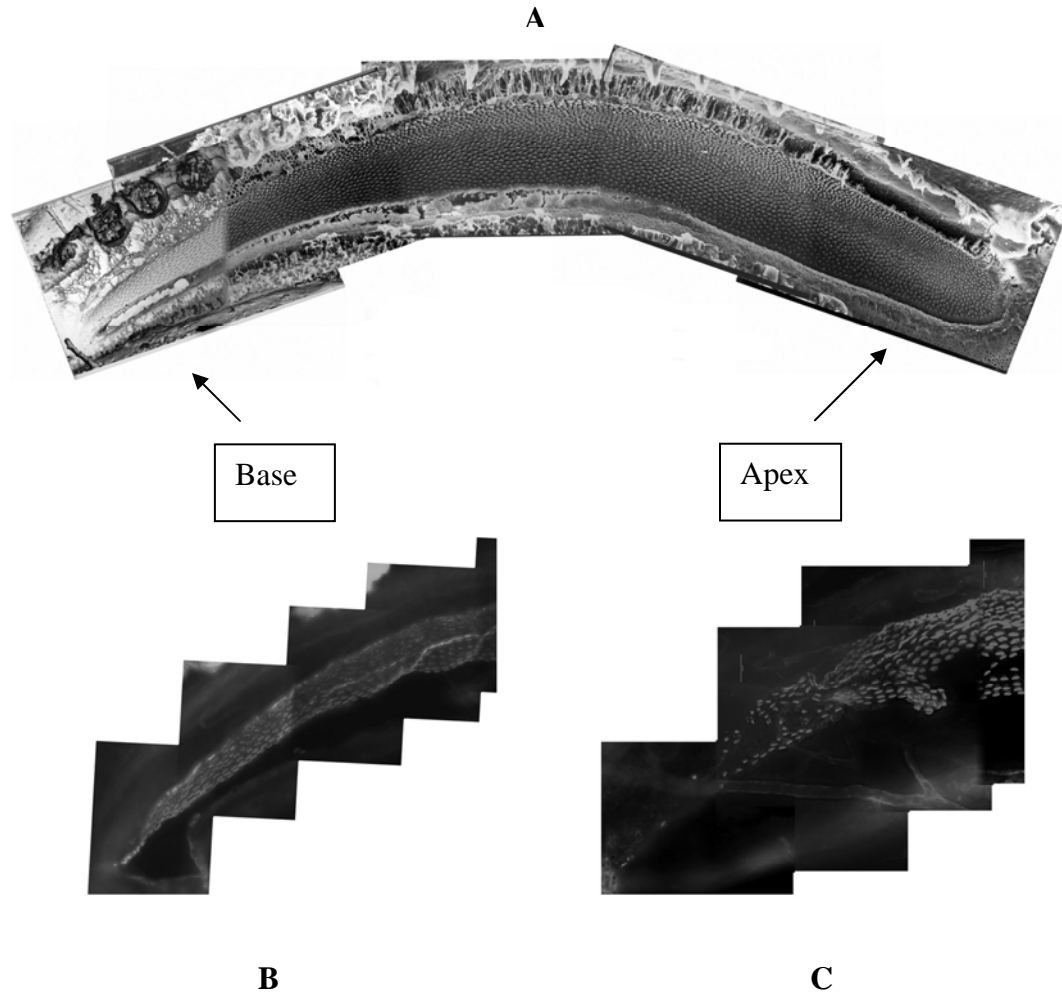


Figure 7: (A) Basilar papilla of a normal, control zebra finch after phalloidin staining. All hair cells are present, healthy and properly aligned. (B) Basilar papilla of a zebra finch at day 11 (zero days post the 10-day KM treatment course). The circled region shows an area of slight stereocilia misorientation among surviving hair cells in the basal end of the basilar papilla. There is very slight hair cell damage observed in the base in contrast to what is observed in other avian species. (C) Basilar papilla of a zebra finch at day 11 (zero days post the 10-day amikacin treatment course). The circled region shows the area of slight hair cell loss and stereocilia misorientation among surviving hair cells in the basal end of the basilar papilla. Only minimal hair cell damage is observed in the base in contrast to what is observed in other avian species.

Experiment 2B (Kanamycin (at the modified ZF dosage) in Budgerigars)

Kanamycin did not affect zebra finch hearing sensitivity the same way it did in budgerigars and canaries (Dooling et al., 2006; Dooling et al., in press). Because a lesser kanamycin dosage was used for the zebra finch, there was question as to whether the aberrant results were a consequence of the modified treatment. The purpose of this study was to measure the effect of the modified kanamycin treatment in budgerigars using the ABR, and to compare the results to ABR data in budgerigars receiving the original dosage. I hypothesized that budgerigar ABR thresholds (measured before, during and after the modified KM treatment) would show a change in hearing sensitivity that parallels those found in Experiment 1 with budgerigars.

Methods

Subjects

Two budgerigars were used as subjects in this experiment. All procedures are the same as above, except where noted.

Kanamycin Treatment

All birds were administered a ten-day cycle of the ototoxic drug, kanamycin (KM). The daily dosages of KM for budgerigars were slightly lowered to parallel those given to zebra finches in Experiment 2A. Therefore, the modified daily dosages of KM for budgerigars were: Day 1: 50mg/kg, Day 2: 100 mg/kg, Day 3-10: 200mg/kg.

As with Experiment 1, the injection cycle occurred over a period of eleven days. The first six injections were administered on days one through six and the last four were given on days eight through eleven. Subjects did not receive an injection on day seven. Instead, hearing sensitivity was assessed through the ABR after six days of injections.

Results and Discussion

Hearing Sensitivity

The two doses of kanamycin show a similar pattern of hearing loss and recovery (see Figure 8). Following the modified kanamycin treatment, high frequency thresholds increase and parallel those found in budgerigars in Experiment 1.

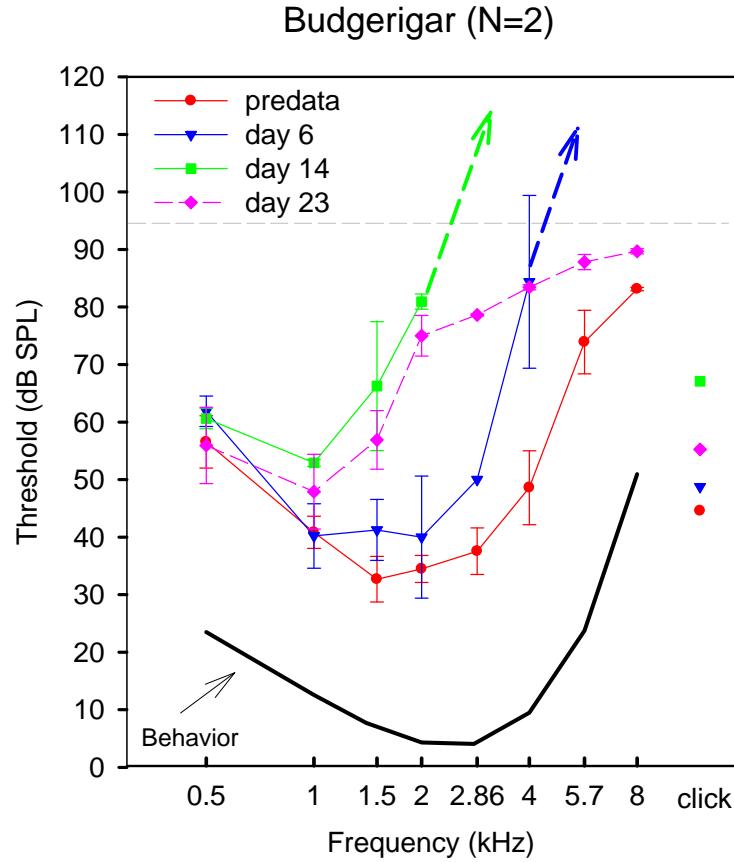


Figure 8: ABR audiograms before KM injection (red, closed-circle line), after 6 days of KM injections, or day 6 (blue, downward-triangle line) 3 days post injections, or day 14 (green, closed-square line), and 12 days post injections, or day 23 (pink, closed-diamond line) for the budgerigar at the modified KM dose. The solid black line is the behavioral audiogram for budgerigars. Arrows project estimated hearing thresholds at the designated frequencies. Following six days of KM treatment, ABR audiograms of the budgerigar hearing thresholds showed high threshold shifts similar to those found in budgerigars administered the original KM dosage. At three days post the completion of KM treatment, budgerigar threshold shifts as a function of frequency show substantially larger differences than those displayed after 6 days of injections. By approximately 10 days post the completion of treatment, recovery of threshold shifts is evident.

After six days of KM injections, ABR audiograms show substantial hearing loss in the high frequencies (i.e. 2.86, 4, 5.7, and 8 kHz). The low and mid frequencies (i.e. .5, 1, 1.5, and 2 kHz) are shown to be relatively unaffected by the

antibiotic. A least squares ANOVA showed a significant difference in the audiogram ($F[31,32]=35.57, p<.01$), with a significant treatment day by frequency effect ($F[21,32]=9.65, p<.01$). A main interaction effect between day and frequency was present. Post hoc tests showed significant differences between testing day 6 and predata for the budgerigar ($t[3]=-6.95, p<.01$).

After completion of the ten-day treatment cycle, the originally restricted high frequency hearing loss expands to affect all frequencies tested. Though all frequencies display a loss in hearing sensitivity, the greatest threshold shifts are in the high frequencies. Threshold shifts reaching approximately 57 dB are recorded at select frequencies (i.e. at 2.86 kHz) in budgerigars. As Figure 9 shows, at day 14, the threshold shifts exhibited by budgerigars receiving the modified daily dosage of KM, parallels the results of budgerigars administered the original dosage of KM. Post hoc tests showed significant differences between testing day 14 and predata across frequencies ($t[3]=-15.93, p<.01$).

Budgie Threshold Shift day 14

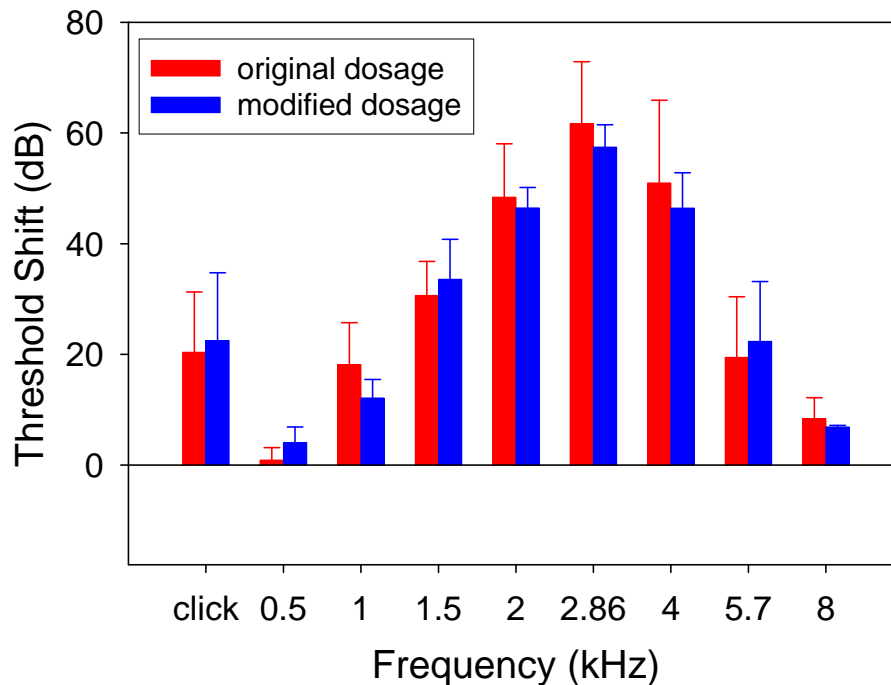


Figure 9: ABR audiograms before KM injection (red, closed-circle line), after 6 days of KM injections, or day 6 (blue, downward-triangle line) 3 days post injections, or day 14 (green, closed-square line), and 12 days post injections, or day 23 (pink, closed-diamond line) for the budgerigar at the modified KM dose. The solid black line is the behavioral audiogram for budgerigars. Arrows project estimated hearing thresholds at the designated frequencies. Following six days of KM treatment, ABR audiograms of the budgerigar hearing thresholds showed high threshold shifts similar to those found in budgerigars administered the original KM dosage. At three days post the completion of KM treatment, budgerigar threshold shifts as a function of frequency show substantially larger differences than those displayed after 6 days of injections. By approximately 10 days post the completion of treatment, recovery of threshold shifts is evident.

Recovery of hearing sensitivity begins approximately ten days after the completion of the KM treatment (see Figure 8). ABR audiograms display recovering threshold shifts at all frequencies tested. Threshold shifts back to near normal levels

reach approximately 16 dB at select frequencies, showing the beginning of hearing sensitivity recovery. Post hoc tests showed significant differences between testing day 23 and predata across frequencies for the budgerigar ($t[3]=-11.64$, $p<.01$).

Assessment approximately ten days later, day 32, displays further recovery of hearing sensitivity at all frequencies. At this time, budgerigar thresholds have improved on average an additional 10-15 dB from that recorded at day 23 (12 days post completion of administration). With time, hearing sensitivity continues to recover; by day 46, approximately one month after the completion of kanamycin treatment, all thresholds have nearly returned to baseline.

Recovery of hearing sensitivity was not fully achieved even by day 46. Instead, the budgerigar exhibited recoveries that culminated in near normal hearing levels. These results paralleled those found through both ABR methods and behavioral methods for budgerigars (see Experiment 1; Dooling et al., 2006).

Experiment 3 (Amikacin in Zebra Finches)

Introduction

Results evaluating the effect of kanamycin on zebra finch hearing sensitivity did not display a hearing loss pattern similar to those found behaviorally and physiologically in budgerigars and canaries (Dooling et al., 2006; Dooling et al., in press). Woolley et al. (2001) studied the effects of another aminoglycoside, amikacin, on hearing sensitivity in Bengalese finches. Results from the study showed a similar hearing loss pattern to those displayed behaviorally in budgerigars and canaries. The purpose of this experiment was to measure the effect of amikacin

treatment in zebra finches and to compare results to those found after kanamycin treatment in zebra finches and amikacin treatment in Bengalese finches. I hypothesized that zebra finch ABR thresholds (measured before, during and after amikacin treatment) will show a change in hearing sensitivity that parallels those found in Bengalese finches.

Methods

Subjects

Five zebra finches were used as subjects in this experiment to assess hearing sensitivity before, during, and after amikacin treatment. Two of the five subjects used to assess hearing sensitivity were additionally used for histological studies. Therefore, the number of subjects examined after predata and day 6 assessments decreased by two subjects. Additionally, one zebra finch died before the conclusion of the study from an allergic reaction to the ketamine injection given as an anesthetic before the ABR. All procedures were the same as above, except where noted.

Amikacin Treatment

All birds were administered a ten-day cycle of the ototoxic drug, amikacin. The daily doses of the antibiotic were Day 1: 50 mg/kg, Day 2: 100 mg/kg, and Days 3-10: 200 mg/kg. This dosage was a direct comparison with KM administered to zebra finches in Experiment 2A, but varied from the alternating 150mg/kg and 300mg/kg dosage of amikacin that Woolley and colleagues (2001) administered to Bengalese finches. As with Experiment 1, the injection cycle occurred over a period

of eleven days. The first six injections were administered on days one through six and the last four were given on days eight through eleven. Subjects did not receive an injection on day seven; instead, hearing sensitivity after six days of injections was assessed through the ABR.

Results and Discussion

Hearing Sensitivity

Following amikacin treatment, zebra finch thresholds show no change in hearing sensitivity. Figure 10 shows an averaged ABR audiogram displaying hearing thresholds for predata (n=5), day 6 (n=5), day 14 (n=3), day 23 (n=3), and day 32 (n=2) for zebra finches administered a ten-day cycle of amikacin. As the audiogram displays, after 6 days of amikacin treatment, zebra finches do not show a substantial shift in hearing sensitivity at any frequency. A repeated measures ANOVA showed no significant difference between testing day 6 and predata across frequencies ($F[8,32]=1.916, p>.05$). Furthermore, three days post the completion of treatment, thresholds still remain relatively unaffected by the ototoxic aminoglycoside. A repeated measures ANOVA showed no significant difference between testing day 14 and predata across frequencies ($F[8,8]=1.730, p>.05$).

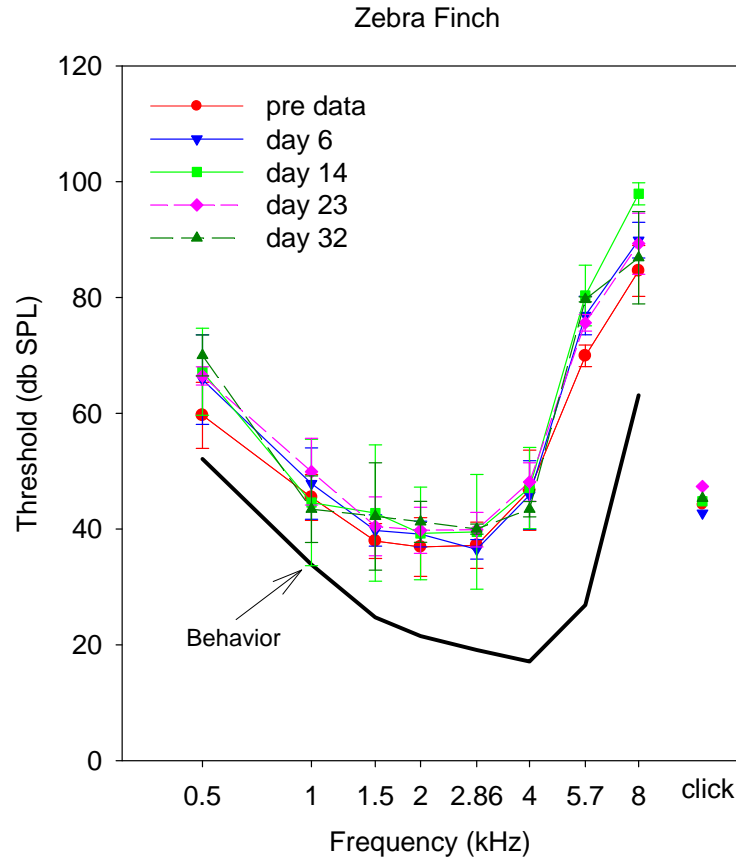


Figure 10: ABR audiograms before amikacin injection (red, closed-circle line), after 6 days of amikacin injections, or day 6 (blue, downward-triangle line) 3 days post injections, or day 14 (green, closed-square line), 12 days post injections, or day 23 (pink, closed-diamond line), and 22 days post injections, or day 32 (dark green, upward-triangle line) for the zebra finch. The solid black line is the behavioral audiogram for zebra finches. Following amikacin treatment, zebra finch ABR audiograms of hearing thresholds do not show a pattern of high frequency threshold shifts similar to those found in Bengalese finches. Interestingly, zebra finches show no hearing loss due to amikacin treatment.

Hearing sensitivity was monitored for nearly one month after the completion of amikacin treatment. There was no difference in thresholds at any frequency tested. A repeated measures ANOVA showed no significant difference between testing day 23 and predata across frequencies ($F[8,16]=1.660$, $p>.05$). As such, it appears that

the administered ten-day cycle of amikacin treatment has no effect on zebra finch hearing sensitivity. This is very dissimilar to results found in amikacin treated Bengalese finches which experience substantial threshold shifts especially at frequencies of 2 kHz and above (Woolley et al., 2001).

Histology

Figures 7A and C exhibit a normal zebra finch basilar papilla (7A) and the basilar papilla of a zebra finch treated with amikacin (7C). Examination of the basilar papilla of two zebra finches (0 days post amikacin course) using phalloidin staining showed little to no hair cell loss and damage in the basal end of the papilla (see Figure 7C). Of the two ears examined, one appeared normal, presenting no damage in the basal or middle regions of the basilar papilla. The second showed a very small area of hair cell loss and misorientation in the basal end of the papilla. There is exceptionally slight hair cell loss observed in the basal end of the papilla in contrast to what is observed in Bengalese finches (Woolley et al., 2001). Interestingly, the basilar papillae of a zebra finch treated with amikacin and a zebra finch treated with kanamycin both appear quite similar to the basilar papilla of a normal zebra finch (see Figures 7A, B, and C).

General Discussion

These experiments sought to measure the effect of aminoglycoside treatment on hearing in three avian species using the ABR technique. First, the effect of kanamycin treatment on the hearing of budgerigar, canary, and zebra finch was

measured. Budgerigar and canary ABR audiograms showed similar results to those found through behavioral methods in the two species (Dooling et al., 2006; Dooling et al., in prep). Both species experienced an initial high frequency hearing loss which over time extended to also affect the mid and low frequencies. Zebra finches did not show this typical high frequency hearing loss but instead showed only minor threshold shifts across all frequencies. Also, histological studies suggested that there was little to no change in hair cells within the basilar papilla of the KM treated zebra finch.

Next, the effect of a second aminoglycoside, amikacin, was examined in the zebra finch to determine whether the aberrant results seen with kanamycin were a consequence of that particular aminoglycoside, KM, or were a function of the species of bird itself. Here, again, the zebra finch displayed unusual results that were inconsistent with those found through physiological methods in other avian species. Zebra finch ABR audiograms did not display the typical high frequency hearing loss pattern that Bengalese finches showed after amikacin treatment (Woolley et al., 2001). Instead, they showed very little change in hearing sensitivity, even less than with kanamycin. Histological studies suggested that there was little to no change in hair cells within the basilar papilla of the amikacin treated zebra finch.

The effects of the two aminoglycosides on zebra finch hearing sensitivity suggest that the atypical results are not limited to a specific aminoglycoside. Overall, zebra finch threshold shifts as a result of aminoglycoside treatment are much smaller than those found in budgerigars and canaries. There are several explanations for this effect. A possible explanation for the irregular hearing loss pattern may be that zebra

finches possess a faster metabolism than other avian species. This may result in a diluted concentration of the aminoglycoside reaching the ear. Thus, in order for a robust enough concentration to affect the ear, a larger dosage may be required. Yet, because aminoglycosides such as kanamycin can lead to renal failure and subsequently, death, as was experienced with multiple subjects in this study, an effective dosage may not be possible.

It is interesting that the zebra finch also shows reduced hearing loss from acoustic overstimulation. Ryals et al., (1999) showed that zebra finches exhibited far less hair cell loss and damage and recovered hearing sensitivity at a much faster rate than budgerigars and quail that were exposed to the same duration and intensity of noise exposure. Additionally, hearing abilities were found to return to near normal levels with time, while budgerigars and quail suffered a permanent loss of approximately 20 dB. Taken together, these past results and our present findings using KM argue for a more resistant sensory epithelium in zebra finches.

In summary, the zebra finch appears less susceptible to hearing damage from ototoxic drugs than other avian species. However, the reasons for this reduced susceptibility are unclear. It might be interesting to examine the effects of a direct application method of KM, similar to that conducted by Husmann et al. (1998), in zebra finches. Their study compared the effects of systemic injections vs. direct application of another aminoglycoside, gentamicin. Broiler chickens were either administered doses of gentamicin through systemic injections or the direct application method in which sponges soaked in the antibiotic were placed directly onto the round window of the chickens. Results showed that the direct application method produced

a much larger amount of cochlear damage, with less toxic effects. The direct application method of KM should allow for a sufficient dosage of the antibiotic to be administered to the zebra finch without causing the toxic and potentially lethal effects that are experienced through systemic injections.

These experiments hope to lay the foundation for future research aimed at finding a means to reverse hearing loss in humans. Songbirds provide a model for vocal learning in humans, because, like humans, they learn through auditory feedback. Zebra finches are particularly popular models for vocal development and learning. It is too bad zebra finches are so resistant to damage from noise or ototoxic drugs because studies of the effects of temporary deafness on vocal learning would be valuable. Future researchers may want to find another songbird species for such studies. Findings from these experiments lead to many research questions on why the zebra finch is so protected from hearing damage.

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