“The well-known spring and summer call of the Chickadee, consisting of three clear whistles, is uttered by both sexes. I am not aware that record has ever been made of this fact, which I determined some time ago by the judicious use of firearms.”

Jonathan Dwight, Jr., M.D., New York City.

From: Dwight, J., Jr. 1897. The whistled call of *Parus atricapillus* common to both sexes. Auk, 14:99.

University of Alberta

Differential effects of vocalization type, singer, and listener on Zenk immediate early gene response in black-capped chickadees (*Poecile atricapillus*)

by

Marc Thomas Avey ©

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Dedication

For the Birds

Abstract

Here I examined immediate early gene induction to vocalizations in the ascending auditory pathway of black-capped chickadees (*Poecile atricapillus*) to asses the impact that sex of the producer and perceiver has on ZENK induction. I manipulated the playback by both the vocal type (song/call) and sex of producer (male/female), and then presented these stimuli classes to either male or female black-capped chickadees. Neural response to the stimulus was quantified by the amount of protein of the immediate early gene ZENK in the caudal medial nidopallium and caudomedial mesopallium. ZENK induction varied depending on the sex of the producer, sex of the perceiver, the signal type, and brain area. These results demonstrated that sex is encoded in the signal and processed in auditory brain nuclei. Furthermore, dependent on sex of the perceiver and signal type the ZENK response varied with males having generally more ZENK induction than females.

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List of Abbreviations and Nomenclature

CMM	caudomedial mesopallium
CSt	caudal striatum In songbirds, Auditory information
ICC	immunocytochemistry
IEG	immediate early gene
M	molarity
Melospiza melodia	Song sparrow
MLd	nucleus mesencephalicus lateralis, pars dorsalis
NCMd	caudal medial nidopallium dorsal
NCMv	caudal medial nidopallium ventral
Ov	nucleus ovoidalis
PBS	phosphate buffered saline
PBS/T	PBS containing Triton X
Passeriformes	Oscine songbirds
Poecile atricapillus	Black-capped chickadee
Psittaciformes	Parrots
H_2O_2	hydrogen peroxide
RA	robust nucleus of the arcopallium
Sturnus vulgaris	European starling
Taeniopygia guttata	Zebra finch
Trochiliformes	Hummingbirds
zenk	gene (zif-268, egr-1, ngf-la and krox-24)
ZENK	Protein (zif-268, egr-1, ngf-la and krox-24)

1. INTRODUCTION

1.1 Songbirds

Songbirds (Passeriformes) are part of small group of phylogenetic orders that exhibit vocal learning (Jarvis, 2004). Vocal learners have the ability to learn vocalizations through imitation unlike auditory learners who do not learn their vocalizations. Parrots (Psittaciformes), hummingbirds (Trochiliformes) and oscine songbirds (Passeriformes) are the only branches on the avian phylogenetic tree that include vocal learners (Jarvis, 2004).

Songbirds have increasingly become an important model for studying the neurobiological basis of communication (Doupe & Kuhl, 1999). There are great similarities in the timelines for speech development in human infants and song development in songbirds (Doupe & Kuhl, 1999). Although there are differences in the time span of development between human infants both humans and songbirds have innate predispositions for the perception of species-typical signal, and periods of sensory learning followed by periods of sensory-motor learning (Doupe & Kuhl, 1999; Kuhl, 2003). The development of vocalizations from birth/hatch to adult requires tremendous energy resources, complicated interactions with other organisms, and self interactions such as auditory feedback. Initial vocalization in both human speech and birdsong are not like that of normally developed adults. Initial vocalizations in humans are termed ‘babbling’ and in songbirds ‘subsong’ (Goldstein, King, & West, 2003). Both of these initial vocalizations must be gradually moulded to become like a normal adult vocalization, resulting in a stereotyped set of vocal elements for an individual (Goldstein et al., 2003 for review see Doupe & Kuhl, 1999).

In birds, vocalizations are separated into one of two categories; songs or calls. Catchpole and Slater (1995) differentiate between songs and calls by the context in which the vocalization is produced and its complexity. Calls are described as generally short and simple vocalizations often used for contact and produced all year by both sexes of a species. Songs are produced almost exclusively by males during territorial disputes and mate attraction and described as long and complex vocalizations. These definitions while useful have their limitations. For instance, in many tropical species both male and female songbirds produce complex songs that are comparable in duration and complexity (Morton, 1996). Of the approximately 5 739 species of passerine birds (Sibley & Monroe, 1990), songbirds (oscines, suborder Passeri) represent nearly half of all extant species representing the largest radiation of birds (Nee, Mooers, & Harvey, 1992). Songbirds have diversified on all continents and inhabit almost all ecosystems (Barker, Cibois, Schikler, Feinstein, & Cracraft, 2004), which has resulted in wide ecological and behavioural diversity. Songbird vocal communication has also diversified in the songbird radiation making category definitions of behaviour difficult to generalize in such an overwhelming large number of species. However, the terminology introduced by Catchpole and Slater (1995) is useful when describing the behaviour of a species and provides a starting place to understand differences between species, sex, and their vocal behaviour.

Behaviour is the result of development, learning, and physiology which makes it an inclusive measure that can help guide researchers to compare between and within species (Gorissen, Snoeijs, Van Duyse, & Eens, 2005; Zala & Penn, 2004). Both between and within species comparisons have often focused on sexual differences in behaviour (Sherry, 2006). For instance, polygynous male meadow voles perform better than females in tests of spatial ability; whereas monogamous male pine voles have no advantage on tests of spatial ability over female pine voles (Gaulin & Fitzgerald, 1986).

Songbird vocal behaviour is an excellent model to study sex differences allowing between and within species comparisons. Songbirds produce and perceive vocalizations in their natural habitat and these behaviours play a crucial role in their ability to survive and reproduce (Smith, 1991). Songbird vocal behaviour is incredibly varied. For instance, some species are open-ended learners with the ability to modify existing and acquire new vocalizations and some are close-ended learners such that after song has crystallized little or no modification occurs. Differences between species vocalizations are numerous although no large scale phylogenetic comparison of vocalization has been done to date.

1.2 Black-capped Chickadees

One species of songbirds that has been studied extensively, and is the subject of the current research, is the black-capped chickadee (*Poecile atricapillus*; for a review see Smith, 1991). Black-capped chickadees learn their relatively simple two note *fee-bee* song via imitation of conspecifics

(Ficken et al., 1978; Shackleton & Ratcliffe, 1993). The *fee-bee* song of the black-capped chickadee is highly tonal in nature such that it only covers a narrow frequency bandwidth. The *fee-bee* song typically contains two note types (fee and bee), that are separated by a constant frequency ratio independent of the absolute frequency of first note (Ratcliffe & Weisman, 1985; Shackleton & Ratcliffe, 1994; Weisman, Ratcliffe, Johnsrude, & Hurly, 1990). Furthermore, both males and females produce their two-note song; however, the context and function differ between them (Smith, 1991). Males sing almost exclusively during the winter and spring (Smith, 1991; Dixon & Stefanski, 1970) and for mate acquisition and territory defense (Ficken, et al., 1978). In females, song has only been studied in the wild during the mating season and in this context appears to be used in the nest for communication with their mate (Dixon & Stefanski 1970; Odum, 1941; Odum, 1942).

The song given from a nesting female, as well as by the answering male, is lower in amplitude that that of normal male song, and is accordingly termed *faint fee-bee* (Smith, 1991). That said, recent laboratory-based studies have revealed that females can also produce song that resembles that of normal male song produced outside the specific context of mating and nesting (Lee, Charrier, & Sturdy, in preparation). The genomic response to female song in chickadees has never been tested. Indeed, ZENK induction has almost exclusively been studied using male signals (but see Vignal, Mathevon, & Mottin, 2004); although female signals are an important natural signal for songbirds (Freeberg, 2000). Even subtle cues from females can have important effects on male behaviour such as audience [e.g. Vicario, Naqvi, Raksin, 2001; Vignal et al., 2004) and these effects are not limited to songbirds (e.g. Range, 2005).

Another interesting aspect about black-capped chickadees is their most often used complex call (Avey, Quince, & Sturdy, submitted), the *chick-a-dee* call. The *chick-a-dee* call is learned via imitation and is used by both males and females (Hughes, Nowicki, & Lohr, 1998). The *chick-a-dee* is used all year but primarily in the autumn and winter (Avey et al., submitted; Smith, 1991), and appears to be used for flock mobilization, cohesion, alarm, and mobbing (Ficken et al., 1978; Templeton et al., 2006). Flock members converge in vocal characteristics which suggests that to some degree learning, at least of this call, is open ended in chickadees (Mammen & Nowicki 1981). Recent research has shown that the chick-a-dee call also conveys complex information such as the threat level of predators (Templeton, Greene, & Davis, 2005). The *chick-a-dee* call is relatively more complex than the *fee-bee* song. The *chick-a-dee* call has a four note type structure (A, B, C, D) that are always produced in the same order although notes can be omitted or repeated within a call (Charrier, Bloomfield, & Sturdy, 2004; Ficken, Ficken, & Witkin, 1978).

The fact that both male and female black-capped chickadees learn their vocalization types is atypical in temperate songbirds (Catchpole & Slater, 1995). Indeed, unlike highly dimorphic species such as the zebra finch (*Taeniopygia guttata*) that is also often studied in the lab and field, black-capped chickadees offer an interesting alternative for sex comparisons. Only male zebra finches learn and produce song (Immelmann, 1969; Price, 1979; Roper & Zann, 2006), and their relatively simple distance call (Runciman, Zann & Murray, 2005). Since, both male and female black-capped chickadees learn and produce vocalizations they are useful for comparative analyses of possible sex differences in black-capped chickadees. Furthermore, they may also be advantageous to make direct comparisons with human language acquisition and usage (Ball & MacDougall-Shackleton, 2001; Hartshorne & Ullman, 2006). Black-capped chickadees are also a useful model because the perception of conspecific vocalizations has been studied extensively in field (Charrier & Sturdy, 2005; Hailman & Ficken, 1996; Hailman, 1989; Hurd, 1996; Kershner & Bollinger, 1999; Shackleton, Ratcliffe, & Weary, 1992), and in laboratory studies (Bloomfield, Sturdy, Phillmore, & Weisman, 2003; Charrier, Lee, Bloomfield, & Sturdy, 2005; Weary & Weisman, 1991).

1.3 Immediate Early Genes

IEGs are an activity-dependent set of genes; thus, their expression in neurons is generally low unless there is some form of stimulation (Sheng & Greenberg, 1990). With stimulation, expression increases both rapidly and transiently leading a depolarization of the neuron (Morgan, & Curran, 1989). The expression of IEGs in the brain can indicate patterns of activation in response to stimuli or to particular contexts (Chaudhuri, 1997). Mello et al. (2004) note that the expression of activity-dependent genes in a neuron generally is evidence of the previous activation of that cell by the particular stimulus; expression of IEGs does not typically occur in the absence of depolarization. Thus, IEGs can typically be assumed to occur as the result of the stimulus and in relation to the depolarization of the neuron. However, Mello et al. (2004) provide a caveat to this typical scenario. Both the electrical activation of a neuron and the expression of IEGs require membrane depolarization; however, IEGs are also dependent on calcium entry and on the calcium signalling pathways (Sheng & Greenberg, 1990). Brain areas with insufficient calcium signalling pathways may not activate IEGs; this may explain regional differences in activation and differences in the levels of activation found for IEGs (Mello et al., 2004). These regional differences may be related to the activity patterns of neurons or groups of neurons. For instance, different activity patterns can lead to different distributions of intracellular calcium (Clayton, 2000), and the different distributions of intracellular calcium could be transduced into the signal for IEGs response (Clayton, 2000).

1.4 ZENK

The Zenk gene (also known as zif-268, egr-1, ngf-Ia and krox-24) encodes a transcriptional regulator that is sensitive to membrane depolarization (Jones, et al., 2001). The protein product of the Zenk gene, ZENK, is often quantified in localized brain regions to determine their functional relevance for a particular stimulus. For instance, in songbirds, conspecific song results in a rapid and robust transient increase in ZENK protein in discrete brain structures in the auditory pathway (Mello et al., 1992; Mello & Clayton 1994). Paradigms for ZENK expression have two important characteristics (Mello et al., 2004). First, individual neurons are visualized providing excellent identification of neurons that respond to song stimuli. Second, the paradigm allows the birds to be fully awake and unrestrained during stimulus presentation. Brain areas that express ZENK in response to stimulation undergo a plastic change that is experience dependent. ZENK as a transcription factor likely represents an early event in the gene regulation cascade that starts with cellular activation and results in long term cellular and synaptic modification (Mello et al., 2004). In other words, ZENK expression may represent the beginning of changes that result in changes of long term memory. In the case of songbirds, these changes occur in the auditory neurons/circuit in response to specific relevant stimulation.

Some authors have argued that ZENK playback allows for no interference with their natural behaviour (Clayton, 2000; Mello et al. 2004); however, there are limitations to the paradigm. Presentation of single modal or even multi-modal stimuli in an enclosed environment allows the birds to behave unrestrained but does not necessarily make the paradigm natural or ethologically relevant. For instance, the presentation of the stimulus may not be done in a natural manner (e.g. inter-song interval too long or too short). Selecting natural stimulus does not always equate to selecting relevant stimuli. For instance, with European starlings breeding context was not a relevant ethological variable but song length was (Duffy et al., 1999; Gentner et al., 2001). Responding to a particular stimulus, although unconstrained, is limited in its natural characteristics. Most ZENK expression experiments rely on presentation of auditory stimulation from a speaker to a bird housed in a cage. The ability of a bird to interact with a speaker is severely limited compared to interactions in a natural setting. Furthermore, there is no interaction between the recorded stimulus and the response of the bird in the chamber. These response problems may result in disconnect between the ZENK expression found in auditory regions and the expression of natural behaviour to the stimulus. For instance, birds may become behaviourally habituated to stimulus long before habituation of neuron excitability occurs. The only experiment to date investigating ZENK expression in freely ranging songbirds was conducted by Jarvis, Schwabl, Ribeiro, and Mello, 1997. Jarvis et al. (1997) found ZENK expression in the vocal pathway of freely ranging song sparrows in response to speaker playback in their territories.

The combination of multiple techniques such as behaviour, electrophysiology, and gene expression can be used to clarify results and interpretation of the functional significance of neurons and brain nuclei. Furthermore, modelling playback paradigms on ethological principles can help clarify results and the interpretation as well. For instance, Duffy et al. (1999) failed to find a difference in the expression of ZENK in European starlings in a reproductive state. However, Gentner et al. (2001) found that NCM did respond differently to different lengths of presented song, a confirmed behaviour preference of female European starlings (Gentner et al. 2000).

1.5 CMM and NCM

In songbirds, auditory information ascends from the midbrain (MLd: nucleus mesencephalicus lateralis, pars dorsalis) to the thalamus (Ov: nucleus ovoidalis) to the telencephalon where it projects to Field L, caudal medial nidopallium (NCM; Figure 2.), caudomedial mesopallium (CMM; Figure 2.), and the caudal striatum (CSt). Recently, neuroanatomical techniques have been applied to studying the neural systems underlying the auditory perception system including the use of markers for IEG activity (Mello & Clayton, 1994; Mello & Clayton, 1995). These techniques have also been recently applied to songbirds beyond zebra finches and canaries (e.g., house finches etc.) including black-capped chickadees (Phillmore, Bloomfield, & Weisman, 2003). In songbirds the auditory brain regions specialized for processing conspecific vocalizations were first identified by their increased expression of IEGs following exposure to conspecific song (Mello, Vicario, & Clayton, 1992). Regions including NCM and CMM exhibit selective increased expression of the IEG ZENK (ZENK is the transcription production, protein, of the gene Zenk) following exposure to conspecific song compared with exposure to other sounds such as heterospecific song, tones, and silence (Mello & Clayton, 1994; Mello & Clayton, 1995).

Both CMM and NCM have robust ZENK responses to ethologically relevant sounds in male and female birds who do not sing in response to stimulation. Activation of CMM and NCM in songbirds is higher when they hear species-specific sounds and during auditory learning (Chew et al., 1995; Gentner & Margoliash, 2003; Mello et al., 1992; Mello, Nottebohm, & Clayton, 1995). When a songbird hears itself sing or call however there is less ZENK activation in these areas compared to other conspecifics (Jarvis, Scharff, Grossman, Ramos, & Nottebohm, 1998). CMM and NCM are separated by the lamina hyperstriatica. CMM receives inputs from L1 and NCM (Jarvis, 2004), and NCM receives its main inputs from the thalamus, field L and CMM (Vates, Broome, Mello, & Nottebohm, 1996). CMM and NCM both receive inputs from field L (L2 specifically) and reciprocally from each other, while the L2 is putatively the analogue to mammalian layer 4 of primary auditory cortex. These properties make CMM and NCM similar to layers 3 and 4 of mammalian primary auditory cortex (Jarvis, 2004; Mello et al., 2004).

The main projections from field L are to CMM, NCM and the shelf of HVC (proper name; HVC is part of the song control circuit) and to the cup area adjacent to the robust nucleus of the arcopallium (RA; part of the song control circuit). Interestingly, electrophysical recordings have shown that as you progress away from field L toward CMM and NCM selectivity in response to complex stimuli (such as birdsong) increases (Sen, Theunissen, & Doupe, 2001). Both Jarvis (2004) and Mello et al., (2004) and others have suggested that this evidence suggests a hierarchical organization to the auditory processing system.

In contrast to the ZENK expression found in the auditory brain areas, there is no ZENK expression in response to song stimulation in the direct motor pathway nor is there ZENK expression in the anterior forebrain pathway of the song control circuit (Mello et al., 1992; Mello & Clayton, 1994). The song control circuit however shows robust response to song stimulation in electrophysical studies (Margoliash, 1997). ZENK expression however is studied in awake, unrestrained songbirds and electrophysical studies have been conducted in anaesthetized, restrained songbirds. Electrophysical studies in awake birds (Dave & Margoliash, 2000; Schmidt & Konishi, 1998) have found little or no activation of song nuclei by song auditory stimulation. If little electrophysical activity is associated with low ZENK expression in awake birds responding to auditory stimulation, then it is likely that the song control circuitry simply does not play a significant role in normal (awake) auditory responding. However, there is no conclusive evidence that rules out other possible explanations. In anaesthetized birds, electrophysical response in the song control circuit to auditory stimulation may be related to activation of cells involved in learning and memory for motor performance during sleep. Recent research with zebra finches has found that sleep ‘degrades’ performance from the previous day of song learning in juvenile songbirds (Deregnaucourt, Mitra, Feher, Pytte, & Tchernichovski, 2005). Connections from the auditory circuit could be activated by the unnatural presentation of the birds own song (or other songs) during sleep that causes activation of the song control circuit through projections from field L to the shelf of HVC and cup of RA. The question of the role of electrophysical responding to auditory stimuli in anaesthetized birds' song control circuit remains and open one until further evidence is found.

Differential gene regulation has proven an effective tool for determining the neural basis of behavioural responses in the ascending auditory pathway. Levels of ZENK expression in CMM and NCM (cerebrum) have been found to vary dependent on learning and memory (Bailey & Wade, 2003; Bolhuis, Zijlstra, den Boer-Visser, & Van der Zee, 2000; Hernandez & MacDougall-Shackleton, 2004; Jarvis, Mello, & Nottebohm, 1995; Kruse, Stripling, & Clayton, 2004; Leitner, Voigt, Metzdorf, & Catchpole, 2005; Sockman, Gentner, & Ball, 2002; Stripling, Kruse, & Clayton, 2001; Terpstra, Bolhuis, Riebel, van der Burg, & den Boer-Visser, 2006), sex (Avey, Phillmore, & MacDougall-Shackleton, 2005; Bailey & Wade, 2003; Gentner, Hulse, Duffy, & Ball, 2001, Phillmore et al., 2003; but see Duffy, Bentley, & Ball, 1999), stimulus-specific habituation (Chew, Mello, Nottebohm, Jarvis, & Vicario, 1995; Kruse et al., 2004), development (Bailey & Wade, 2003; Mello & Clayton, 1995; Stripling et al., 2001), neural organization (Chew et al., 1995; Mello & Ribeiro, 1998; Ribeiro, Cecchi, Magnasco, & Mello, 1998; Whitney, Soderstrom, & Johnson, 2003), hormones (Maney, Cho, & Goode, 2006), and behavioural saliency (Avey et al., 2005; Eda-Fujiwara, Satoh, Bolhuis, & Kimura, 2003; Gentner et al., 2001; Jarvis, et al., 1995; Leitner et al., 2005; Maney, MacDougall-Shackleton, MacDougall-Shackleton, Ball & Hahn, 2003; Park & Clayton, 2002; Vignal, Andru, & Mathevon, 2005). The mounting body of evidence has indicated that CMM and NCM are important neural regions for the perceptual processing of conspecific vocalizations (Mello, Velho, & Pinaud, 2004). Indeed, CMM and NCM both exhibit properties of response that implicate them in higher order auditory processing (Chew et al., 1995; Chew et al., 1996; Ribeiro et al., 1998; Stripling et al., 1997). Thus, CMM and NCM are likely brain regions to process higher order auditory information such as sex of the signaler.

1.6 Sex

To date research on sexual differences in response to vocalizations in songbirds has been limited to a few species. Duffy et al. (1999) found that playback of song to male and female European starlings (*Sturnus vulgaris*) did not result in different amounts of ZENK expression in CMM and NCM nor were there differences between females in different reproductive states. Further research by Gentner et al. (2001) on female European starlings found that although there were no differences in expression within CMM; the ventral portion of NCM responded differently dependent on the length of the song presented. Females exposed to longer songs had more ZENK expression which is supported by their behavioural preference for males with longer songs (Gentner & Hulse, 2000).

Similar to European starlings, adult female zebra finches exhibit ZENK expression in NCM that is significantly higher in response to conspecific song than following stimulation with heterospecific song, tones or silence (Mello, Vicario, & Clayton, 1992). This pattern is qualitatively similar to that seen in male zebra finches. Bailey and Wade (2003) found that in developing (day 30) male and female zebra finches, that ZENK expression was increased in CMM and NCM in males but not females. These results suggest that, in this highly dimorphic species, ZENK expression varies selectivity for conspecific song and may vary depending on the natural function of song (e.g. learning to produce in males versus learning to recognize in females). However, by day 45 these sex differences in zebra finches are no longer observed for ZENK (Bailey & Wade, 2005). Research in zebra finches is accelerating as more researchers study the song control circuit and ascending auditory pathway. Recent studies have examined sex differences in visual stimuli (Avey et al., 2005), brain areas in the song system (Bailey & Wade, 2006), and brain areas outside the song and auditory system (Heimovics & Riters 2007).

1.7 Black-capped Chickadees and ZENK

Research with songbirds has mainly focused on the zebra finch, a species that is sexually dimorphic in the production of its song. Here, I examined black-capped chickadees because their vocal repertoire is well studied (Ficken et al. 1978) and because both males and females produce a learned song (*fee-bee*: Gammon, Baker, & Tipton, 2005; Kroodsma, Albano, Houlihan, & Wells, 1995; Kroodsma et al., 1999; Shackleton & Ratcliffe, 1993;), and a learned call (*chick-a-dee*: Hughes et al., 1998; Clemmons & Howitz, 1990). Previous research with black-capped chickadees indicated that there were differences in ZENK expression depending on the type of vocalization presented and the sex of perceiver (Phillmore et al., 2003).

However, one aspect that was not controlled for in the previous research was whether or the sex of vocal producer affected the genomic response. The sex of the vocal producer is an important ethological variable for black-capped chickadees to identify and discriminate (Gammon, 2004; Otter, Chruszcz, & Ratcliffe, 1997; Otter & Ratcliffe, 1993). For instance, sex of the vocal producer may determine whether the appropriate response is aggression or courtship.

In contrast, black-capped chickadees have not been studied as extensively as either European starlings or zebra finches with IEGs. Phillmore et al. (2003) examined the effects of hearing *fee-bee* songs versus *chick-a-dee* calls on the amount of ZENK expression in CMM and NCM (dorsal and ventral) in male and female black-capped chickadees. Both *fee-bee* song and the *chick-a-dee* call induced ZENK expression in CMM and NCM, however, males only differed from females with a more robust response in CMM. There were no differences found between vocalization types in CMM and the ventral portion of NCM. In the dorsal region of NCM *fee-bee* songs did induce more ZENK expression. Phillmore et al. (2003) concluded that conspecific vocalizations as well as the sex of the listener may modulate the IEG expression in the ascending auditory pathway. However, the sex of black-capped chickadee producing the vocalization may also modulate the IEG expression in the ascending auditory pathway.

Phillmore et al. (2003) tested the function versus acoustic complexity hypotheses in black-capped chickadees. If function were more important than complexity then they predicted that *fee-bee* song would induce more ZENK protein in auditory brain regions than *chick-a-dee* calls. However, if acoustic complexity was more important than they predicted that *chick-a-dee* calls would induce more ZENK protein in auditory brain regions than *fee-bee* songs. Phillmore et al. (2003) found that function better predicted ZENK protein expression in the auditory brain regions in black-capped chickadees; however, they suggested that further research was required to determine what effect function and acoustic complexity played in the induction of ZENK. Here, I hypothesized that the amount of ZENK induction would not be related to acoustic complexity or function but to the sex of producer and sex of the perceiver. The sex of the producer is a salient feature that must be attended to. Moreover, the salience of the sex of the producer will vary dependent on the sex of perceiver. For instance, a male may respond aggressively to another male calling on his territory during spring where as a female may respond more positively. Thus, neither the acoustic complexity nor the function of the vocalization may be the most salient feature; that is, the feature that most strongly induces ZENK black-capped chickadees in natural conditions. First, I predicted that the amount of ZENK induction would be greatest to male stimuli (*chick-a-dee* call and *fee-bee* song) and that there would be no difference between the vocalizations types. Second, I predicted that the amount of ZENK induction would be greater in males than in females regardless of the type or sex of the stimuli presented because male black-capped chickadees use vocalizations more in their natural behaviour than females (Smith 1991).

Here I manipulated the playback by both the vocal type (*fee-bee*, *chick-a-dee*) and sex of producer (male/female), and then presented these stimuli classes to either male or female black-capped chickadees. Thus, there were five conditions played back to either a male or female black-capped chickadee: male song, male call, female song, female call, and silence. Response to the stimulus was quantified by the amount of protein of the IEG ZENK in CMM and NCM. A greater amount of IEG ZENK protein between conditions and/or brain areas was interpreted as an effect of the black-capped chickadees natural responding to the most relevant feature of the stimulus as presented.

2. METHODS

For the stimulus sets 12 black-capped chickadees (six males and six females) were captured from Kananaskis Research Station 13 to 26 months prior to the capture of the birds used in the experiment. For the experiment 28 black-capped chickadees were used (14 males and 14 females). Black-capped chickadees for the experiment were captured between six and seven months prior to the experiment at the Kananaskis Research Station using potter traps during the non-breeding season. All animals were housed individually in cages in a colony room with the natural seasonal light cycle for Edmonton (Latitude 53°34' N, Longitude 113°31' W). Food and water was provided *ad libitum* and colony room temperatures were maintained at about 20° C. All the birds were treated in accordance with standards set forth by the Canadian Council on Animal Care and the Biological Sciences Animal Welfare and Policy Committee at the University of Alberta.

2.1 Stimuli

Black-capped chickadees used for stimuli were not housed with black-capped chickadees used for playbacked to control for exposure to vocalizations. Black-capped chickadees were recorded individually between 0900 and 1700 by placing their home cage in a large (1.83 m × 1.83 m × 1.83 m) sound-attenuating chamber (Industrial Acoustics Corporation, Bronx, New York, USA). Birds selected for recording were not used in the experiment nor were birds in the experiment exposed to birds selected for stimulus recordings in captivity. Birds were recorded using an AKG C 1000S (AKG

Acoustics) condenser microphone (frequency response: 50 – 20 000 Hz; AKG Acoustics, Vienna, Austria), and a solid state recorder (Marantz PMD670). All vocalizations were bandpass filtered between 1000 Hz and 10,000 Hz to remove background noise and equalized using SIGNAL version 4.0 sound analysis software (Engineering Design 2003).

There were five stimulus categories: black-capped chickadee male song, female song, chick-a-dee male call, chick-a-dee female call, silence (Figure 1.). There were three male and three female chickadees in each group except for the silence condition that had only two male and two female birds. For each category there were two stimulus sets created excluding silence. Each stimulus set consisted of three vocalizations from different black-capped chickadees that were within the normal range of vocal production (Charrier, et al. 2004; Dixon & Stefanski, 1970; Ficken, 1981). The total duration of vocal stimulus was approximately equal in duration (Mello et al., 1992; Phillmore et al., 2003) such that exact measurements on the bioacoustic structure indicated 0.04 s average difference between male and female call stimulus, and 0.09 s average difference between male and female song stimulus. The total average duration difference between call stimulus and song stimulus was approximately equal with less than 0.26 s occurring between these which still allowed for natural vocalizations (Charrier, et al. 2004; Dixon & Stefanski, 1970; Ficken, 1981) to be selected and natural spacing between vocalizations to be used in the creation of stimulus. For both *fee-bee* song and *chick-a-dee* calls there was 16 seconds of stimulus followed by 44 seconds of silence and this was repeated 30 times. Stimuli were played back through a speaker (Realistic Minimus-7 Cat. No 40-2034; input 8 OHMS, 40W max) and amplifier (Cambridge Audio A300, London UK) with a compact disc player (Sony D-SJ301 S2 sports CD walkman). The amplitude was measured at the level of the perches and at the centre position of the cage. Playback amplitude was set to approximately 74 db with a sound level meter (Radio Shack 33-2055).

2.2 Playback

To control for differences in the natural cycle of vocal production and perception all playbacks occurred in August, a period known for low rates of both *chick-a-dee* calling and *fee-bee* song (Avey et al. submitted). The playback was conducted in one of two sound-attenuating chambers (1.83 m x 1.83 m x 1.83 m) Industrial Acoustics Corporation, Bronx, New York, USA). Individual birds were housed overnight in either chamber in a modified home cage which contained three perches at the level of the speaker and four water bottles and two food cups located evenly at either end of the cage. Playback was recorded using bullet cameras (Swann bullet-cam, SW-P-BCC) for both video and sound. Recording began at approximately 10:00 every day for 30 minutes with the lights illuminated but no playback. Following the initial 30 minutes the lights were turned off for 30 minutes to ensure the birds would not sing. Lights were then illuminated for 30 minutes and playback commenced. Following the 30 minute playback period the lights were again turned off for one hour.

2.3 Histology

Following the method just described, the bird was given an overdose 0.03ml of 100mg/ml ketamine and 20mg/ml xylazine intramuscularly (1:1) and then transcardially perfused with heparanized 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde. Following perfusion, the brain was removed and placed in 4% paraformaldehyde for 24 h then placed in 30% sucrose in PBS for approximately 24 h until saturated. The brains were then frozen in dry ice and stored at -80°C until immunocytochemistry (ICC) for ZENK protein was performed. For each bird, forty-eight 40 µm sagittal sections were collected for each hemisphere. Slicing started at the midline and proceeded laterally using a cryostat and sections were placed into PBS.

I processed brains in batches randomized across treatment groups and sex. Sections were washed in 0.1M PBS, incubated in 0.5% H_2O_2 for 15 min, and washed again in 0.1M PBS. Next, sections were incubated in 10% normal goat serum for 20 h, followed by incubation in the primary antibody (Egr-1, Santa Cruz Biotechnology, catalogue # sc-189) at a concentration of 1:10,000 in PBS containing Triton X-100 (PBS/T) for 24 h. Then sections were washed in PBS/T and incubated in biotinylated goat-anti rabbit antibody for 1 h (1:200 dilution in PBS/T). Next, sections were washed in PBS/T, incubated in avidin-biotin horseradish peroxidase (ABC Vectastain Elite Kit; Vector Labs) for 1 h and washed in 0.1M PBS. Finally, the sections were visualized using 3′,3-diaminobenzidine tetrachloride (Sigma FastDAB, D4418), mounted on gelatin-coated microscope slides, dehydrated in ethanol and protected with cover slips affixed with Permount (Sigma-Aldrich).

2.4 Analyses

ZENK immunoreactivity (ZENK-ir) was quantified for three auditory brain regions: the dorsal and ventral parts of the caudal medial nidopallium (NCMd, NCMv) and the caudomedial mesopallium (CMM; Figure 3.). The lateral ventricle defined the dorsal, ventral, and caudal borders of NCM, and Field L defined the rostral border. ZENK-ir in NCM was assessed at a dorsal and ventral location. ZENK-ir in CMM was quantified in the same sections used for NCM and was assessed in the most caudal area bounded by the lateral ventricle and the caudal-ventral boundary of the mesopallial lamina (LaM).

For each black-capped chickadee, eight sections per hemisphere were measured for ZENK-ir. Quantification began with the first section in which mesopallium was contiguous with the rostral portion of the nidopallium to ensure that the orientation of the neostriatum was correct. The section was then mounted in the correct orientation, and continued for the next seven sections moving laterally.

Using the method described above, for each bird 16 images (0.20mm×0.15mm) of each brain region, 8 per hemisphere, were captured using a Leica microscope (DM LB2) with a 40x objective and Canon S40 (Canon Canada, focal length 21.3 mm) digital camera. Images were captured from locations used in Avey et al. (2005). For CMM, the image was captured from the most caudal part of the region. For NCM, a dorsal image was captured from the most dorso-caudal part of NCM and a ventral image was captured from the centre of the ventro-rostral area of relatively high immunoreactivity. This sampling method thus captured images from the areas with highest density of immunopositive cells within these auditory regions (Figure 4.).

For each image, I counted the number of immunoreactive cells following a semi-automated protocol using ImageJ (NIH, v.1.36b). Briefly, images were opened using ImageJ and they were automatically adjusted to grey scale, auto-contrasted and auto-thresholded. The observer manually adjusted the threshold to ensure that it was accurately representing the immunoreactive cells only. Cells were counted setting the range from 1000-3000 square pixels or 0.88 μm^2 – 0.264 μm^2 to ensure that objects too small and too large (eg. partial or overlapping nuclei) were not included and the raw number was entered into a spreadsheet for each brain slice. An observer blind to treatment condition and sex of the subjects performed all cell counts. In a pilot study I visually determined the average size of clearly labelled, individual cells (i.e., cells with nucleic ZENK-ir with no other nearby labelled cells) and compared visual counts to the semi-automated protocol using ImageJ and found them to be highly correlated ($r = 0.998$, $p = 0.01$).

3. RESULTS

CMM, NCMv, NCMd, and Field L were examined initially to determine if ZENK expression occurred only in auditory nuclei selective for conspecific vocalizations (CMM and NCM) or if expression was more general (Field L). However, no expression of ZENK was found in Field L indicating that ZENK expression was selective for conspecific vocalizations and not the result of a more general ZENK response. An analysis similar to Avey et al. (2005) was conducted to determine potential effects of sex, playback condition, brain region, hemisphere, and medial-lateral position. An omnibus mixed-model ANOVA including sex of responder and treatment group as between-subjects factors and brain region (CMM, NCMv or NCMd), hemisphere (left, right) and medial-lateral position (section numbers 1–8) as within-subject factors was conducted (Statistica 6.0). This analysis revealed significant main effects of playback group ($F(4, 18) = 31.6$, $p < 0.001$; male call, $\bar{X} = 65.21$; female call, $\bar{X} = 40.32$; male song, $\bar{X} = 58.40$; female song, $\bar{X} = 39.38$; silence, $\bar{X} = 1.44$), sex ($F(1, 18) = 13.1$, $p = 0.002$; male, $\bar{X} = 47.53$; female, $\bar{X} = 34.37$) and brain region ($F(2, 36) = 54.6$, $p < 0.001$; CMM, $\bar{X} = 52.64$; NCMd, $\bar{X} = 51.74$; NCMv, $\bar{X} = 18.48$). Neither hemisphere nor medial lateral position returned significant differences ($F(1, 18) = 0.24$, $p = 0.63$); ($F(7, 126) = 1.6$, $p = 0.143$). There were several significant interaction effects: sex×playback group ($F(4, 18) = 3.2$, $p = 0.036$), brain region×playback group ($F(8, 36) = 5.8$, $p < 0.001$), and brain region×slice ($F(14, 252) = 1.8$, $p = 0.042$).

Differences in ZENK-ir between the three auditory brain regions may result from a number of factors independent of the experimental manipulations of interest here such as specialization in processing of auditory information. Because of this, and because there were significant interactions between brain region and playback condition I conducted separate MANOVAs for each of these three brain regions (Avey et al., 2005). As well, because there was no significant main effect of medial-lateral position in our omnibus ANOVA I used the average number of ZENK-ir across the eight sampled sections for each hemisphere as the dependent measure in these separate sex×playback condition ANOVAs.

3.1 Results for the caudomedial mesopallium

In CMM there was significant variation in ZENK-ir between sex and group. A two-way ANOVA sex×playback revealed a significant main of sex (*F*(1, 18) = 10.4, p < 0.001, male $\overline{X}$ = 59.69, female $\overline{X}$ = 45.59), and playback group (*F*(4, 18) = 45.7, p < 0.005, male call $\overline{X}$ = 97.63, female call $\overline{X}$ = 46.59, male song $\overline{X}$ = 68.84, female song $\overline{X}$ = 47.50, silence $\overline{X}$ = 2.64). The interaction between sex and playback condition was also significant (*F*(4, 18) = 4.3, p = 0.013; males, male call $\overline{X}$ = 118.02, female call $\overline{X}$ = 51.98, male song $\overline{X}$ = 81.96, female song $\overline{X}$ = 43.38, silence $\overline{X}$ = 3.09; females: male call $\overline{X}$ = 77.23, female call $\overline{X}$ 41.19, male song $\overline{X}$ = 55.73, female song $\overline{X}$ = 51.6, silence $\overline{X}$ = 2.19).

In general ZENK-ir was greater in males than in females and the male call and male song conditions generally had the greatest amount of ZENK-ir

(Figure 5A.). Post hoc analyses (Tukey's HSD) revealed that in CMM all playback groups for males and females were significantly different from the control conditions (see Table 1. for p values; Figure 5A.).

The amount of male ZENK-ir differed significantly between conditions with the most ZENK-ir in the male call condition (see Table 1. for p values; Figure 5A.) followed by male song and female call conditions which did not differ significantly. ZENK-ir to female song the lowest; however, ZENK-ir to female calls and female song did not differ significantly.

For females, the male call condition resulted in significantly more ZENK-ir than the female call condition but did not differ significantly from either male or female song conditions (see Table 1. for p values; Figure 5A.). However, the female call condition did not differ significantly in the amount of ZENK-ir from either the male or female song conditions suggesting that male and female song were intermediate between male and female calls.

Differences in ZENK induction between males and females to the same conditions generally showed more ZENK-ir induction in males. Specifically, their was significantly greater ZENK-ir for males hearing male call than females hearing male call, female call, male song, or female song. Males hearing male song had significantly greater ZENK-ir than females hearing female call. Females hearing female call had significantly greater ZENK-ir than males hearing female song (see Table 1. for p values; Figure 5A.). Thus, within playback conditions only the male call condition demonstrated a significant difference between the sex of the playback recipient.

3.2 Results for the caudal medial nidopallium

In NCMd there was significant variation in ZENK-ir for sex and group. A two-way ANOVA (sex×playback) revealed a significant main effect of sex (*F*(4, 18)=15.014, $p < 0.01$; male $\bar{X}$ = 58.61; female $\bar{X}$ = 44.86), and playback group (*F*(1, 18) = 4.5, $p = 0.05$, male call $\bar{X}$ = 71.99, female call $\bar{X}$ = 57.90, male song $\bar{X}$ = 78.73, female song $\bar{X}$ = 49.09, silence $\bar{X}$ = 0.97) with no significant interaction between these factors. Thus, ZENK-ir was greater in males than in females and post hoc analyses (Tukey's HSD) revealed that all groups were significantly different from the control condition (male call, $p < 0.001$; female call, $p < 0.001$; male song, $p < 0.001$; female song, $p = 0.003$; Figure 5B.). Male song was significantly different from female song ($p = 0.047$) suggesting that male song induced the most ZENK-ir while male and female calling induced an intermediate amount and female song the least. In NCMv there were no significant main effects for sex (*F*(1, 18) = 3.9, $p = 0.063$; male $\bar{X}$ = 24.31; female $\bar{X}$ = 12.65) nor for playback condition (*F*(4, 18) = 2.3, $p = 0.102$) with no significant interaction between these factors.

4. DISCUSSION

Overall, presentation of conspecific vocal stimuli, that is *fee-bee* songs and *chick-a-dee* calls, produced by either males or females, led to higher amounts of ZENK-ir than silence in both male and female listeners. ZENK-ir was highest in males in both CMM and NCMd in response to call and song playback, whereas no significant differences were observed between males and females in the amount of ZENK-ir seen in NCMv. In CMM the amount of ZENK-ir varied according to the sex of the perceiver, the sex of the producer and vocalization type. In NCMd, too, the sex of the perceiver, producer and vocalization type influenced ZENK-ir however response was not influenced by the interaction of these factors as was observed in CMM. There were no significant differences observed in NCMv.

4.1 Differential effects of playback condition and sex in CMM

In CMM, male black-capped chickadees had the highest ZENK-ir response to male call stimuli. This result differed from Phillmore et al. (2003), who found that male songs produced the most ZENK-ir. However, differences in the timing of the two experiments may account in part for these results. I conducted our playbacks during August, a known low time for both *chick-a-dee* calls and *fee-bee* song (Avey et al., submitted). In contrast, Phillmore et al. (2003) conducted their playbacks during October and November when *chick-a-dee* calling is high but *fee-bee* singing is low. The elevated response to *fee-bee* song observed by Phillmore et al. (2003) therefore may not only be attributable to the function of the signal but could also be related to the unusual or novel nature of hearing song at this time of year (i.e., the salience) and could have played a role in the differences observed between the two studies.

While inducing less ZENK-ir than male calls, male songs induced significantly higher levels of ZENK-ir than either female songs or silence. Female calls on the other hand, led to ZENK-ir of similarly high levels to that observed following male songs and significantly more ZENK-ir than silence. That female calls induced less ZENK-ir than male calls and that female song induced less ZENK-ir than male songs suggests that these effects are not simply related to complexity of the vocalizations (male and female calls are similar acoustically and male and female songs are similar acoustically) but rather suggests that there are particular acoustic features of the song and call that indicate sex and that these features are influencing the amount of ZENK-ir. Finally, female song led to the lowest ZENK-ir of all vocalization types presented. There are at least two possibilities that may have led to this finding. In the natural environment female song is relatively rare, and when used, occurs mainly during the breeding season (i.e., the spring and summer). Therefore, the low levels of ZENK-ir in response to female song could be related to the fact that (1) it was presented during a season when it may normally not be heard, thus leading to a low level of salience or (2) because its function is tightly linked to specific contexts (i.e., is used for mate-mate communication) and as such is not attended to by non-mates of either sex when presented outside of this context. Future research will be needed both on the basic behavioural biology of female song use as well as its perception, by the nervous system.

Female ZENK-ir response in CMM was highest to male calls and lowest to female calls with male and female songs producing intermediate response. These results suggest that for calls perceived by females, the sex of producer influences ZENK-ir. That said, there was no significant difference observed in ZENK-ir between male song and female song in female receivers. One possible explanation for this is that female song is relatively rare compared to male song in the natural environment, and females are less likely to hear another female's song than male's which may hear it if invading another males territory. Alternatively, females may have equivalent levels of ZENK-ir in response to both male and female song because they are not discriminating between the two; that is, they may not have learned or have a genetic predisposition to discriminate based on the sex of the producer for song because their natural environment does not require it. On the other hand, the novel nature of hearing another female's song may drive the ZENK-ir response greater than hearing a male's song (less novel) thus washing out any differences between ZENK-ir.

There was a general trend of higher male ZENK-ir induction in CMM than for females to playbacks across groups with the exception of female song where no difference was detected (Figure 5A.). This trend may be due to the fact that all conspecific vocalizations have greater salience/importance for territorial male chickadees (e.g. *fee-bee* song, gargle calls) and this may lead to greater stimulation and higher ZENK-ir following exposure to such stimuli.

4.2 Differential effects of playback condition and sex in NCM

In NCMd male ZENK-ir induction was higher overall than female ZENK-ir and male calls and male song led to the highest ZENK expression. Only male song differed significantly from female song with both male and female calls producing intermediate levels of ZENK-ir for both male and female listeners. There was no interaction observed between sex and playback condition suggesting that ZENK-ir in NCMd is more general and sex specific response occurs further upstream in CMM, an area previously implicated in higher-order auditory processing (Gentner et al., 2001; Gentner, & Margoliash, 2003; Jarvis, 2004). The general trend of ZENK-ir induction following playbacks in males was greater than female ZENK-ir induction which was similar to CMM with differences in all groups except for female song in both brain regions.

NCMv had limited ZENK-ir relative to NCMd and CMM and no differences in ZENK-ir induction between males and females or among stimulus types. This lack of differences in NCMv is consistent with the trend as we move from CMM to NCM and is in line with previous studies of songbird ZENK-ir (Phillmore et al., 2003).

4.3 Sex Differences

Differences found between male and female ZENK-ir can be explained by a variety of scenarios. Phillmore et al. (2003) outlined four possible hypotheses. First, cell densities in CMM and NCM are unknown for black-capped chickadees. Differences in these densities may result in different amounts of ZENK-ir that is related to the number of cells and not to the stimulus. Future research must address this although this hypothesis is unlikely to explain the differences as it has been shown that their robust responding in females in European starlings (Duffy et al., 1999; Gentner et al., 2001). Second, there is a sexual dimorphism between male and female black-capped chickadees. Male's learn their fee-bee song; where as females do not. However, both male and female chickadees learn their *chick-a-dee* call and differences are found for both vocalizations. Third, Phillmore et al. (2003) suggest that differences in ZENK-ir may be a result of differences in responding to the stimuli in the natural environment. For instance, male black-capped chickadees respond aggressively to *fee-bee* song even if it has been altered to have an unnatural frequency; however, females will not respond to this unnatural *fee-bee* song (Shackleton et al., 1992). The final problem raised by Phillmore et al. (2003) is that of seasonal variation. Male and female chickadees do not necessarily respond the same to vocalizations as the season shifts which can make it difficult to assess the response even when conducted at same time of year.

Although these problems still exist my research was able to determine that sex of both the producer and perceiver impact the ZENK-ir in black-capped chickadees. Previous researchers presented songs and calls of black-capped chickadees of unknown sex as stimulus (Phillmore et al., 2003). My results clearly illustrated that sex was encoded in the signal and processed at neurobiological level. Furthermore, my results also illustrated that sex of the perceiver impacted how the signal was responded to at a neurobiological level. The signal itself also impacted the type of responding that occurred. All of these factors impacted the ZENK-ir different depending on which auditory area was examined. These results clearly demonstrate that ZENK-ir is complex and can easily change dependent on which features are controlled for and manipulated in the design. Future research using auditory playback to songbirds should focus on ensuring that stimulus is ethologically relevant to the species studied.

4.4 Summary

Previous research on ZENK induction in the ascending auditory pathway (CMM & NCM) of black-capped chickadees explored the differences between the sex of the perceiver and the vocalization type. Here I showed that not only the vocalization type, sex of perceiver and brain region, but also the sex of the producer can influence the ZENK response. Further research exploring the functional and acoustic complexity hypotheses underlying these observed differences will be required to explore more factors. Indeed, little research on ZENK induction in black-capped chickadees has been done to date (Phillmore et al. 2003; Smulders et al. 2000). For instance, future research can further refine where in the ascending auditory pathway and what factors influence ZENK induction. Clearly, sex (producer and perceiver) is an important factor influencing ZENK-ir in chickadees, and so to could be season of playback, although not yet investigated systematically. Other factors such as state/situation (e.g. reproductive, flocked etc.), and salience (e.g. mobbing call vs. food call) of the vocalizations used in playback for ZENK-ir research will help us understand the natural factors that are important in the regulation of ZENK in the ascending auditory pathway.

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6. TABLES

Table 1. Playback Condition Post Hoc (Tukey's HSD) for CMM: p-values.

		Male				
Sex	Playback Condition	Male Call	Female Call	Male Song	Female Song	Control
Male	Male Call		0.000	0.028	0.000	0.000
	Female Call	0.000		0.100	0.993	0.005
	Male Song	0.028	0.100		0.016	0.000
	Female Song	0.000	0.993	0.016		0.028
	Control	0.000	0.005	0.000	0.028	
		Female				
Female	Male Call		0.028	0.428	0.224	0.000
	Female Call	0.028		0.849	0.976	0.036
	Male Song	0.428	0.849		1.000	0.002
	Female Song	0.224	0.976	1.000		0.005
	Control	0.000	0.036	0.002	0.005	
		Male				
Female	Male Call	0.010	0.239	1.000	0.045	0.000
	Female Call	0.000	0.970	0.010	1.000	0.043
	Male Song	0.000	1.000	0.202	0.934	0.003
	Female Song	0.000	1.000	0.093	0.995	0.006
	Control	0.000	0.004	0.000	0.024	1.000

7. FIGURE CAPTIONS

Figure 1. Representative spectrograms of black-capped chickadee songs and calls. A) male fee-bee song; B) female fee-bee song; C) male chick-a-dee call; D) female chick-a-dee call.

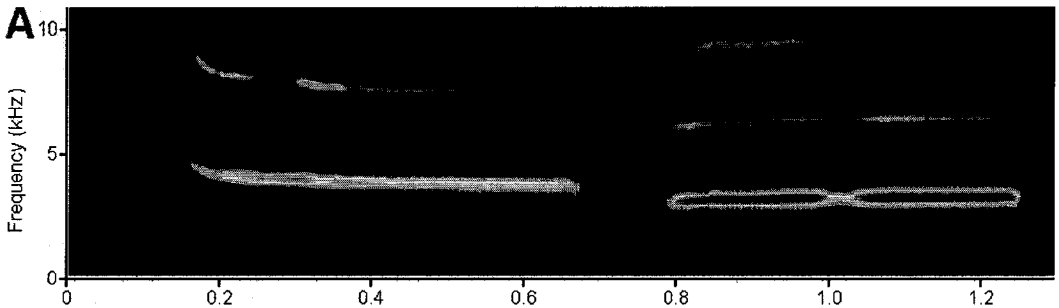
Figure 2. Schematic of a sagittal section section of songbird brain. CMM and NCM are depicted in rostral portion of the telencephalon.

Figure 3. Photomicrographs of a sagittal section of a male black-capped chickadee auditory forebrain. Sampling region used to quantify Zenk-ir in CMM (A), NCMd (B) and NCMv (C). Left is caudal and up is dorsal.

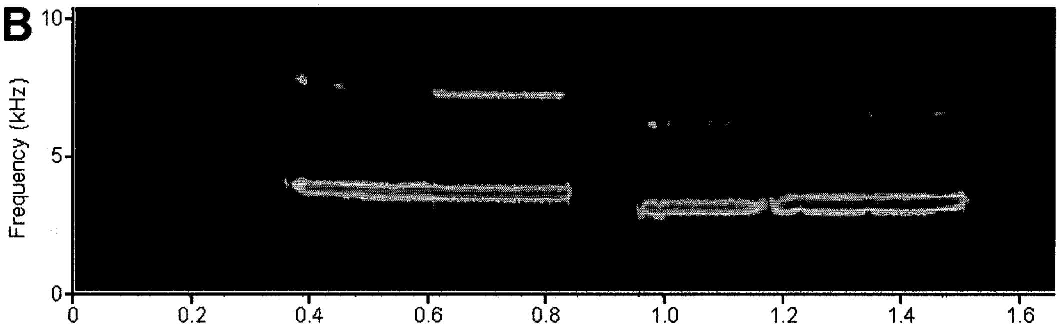
Figure 4. Example ZENK induction in the caudomedial mesopallium: (A) male hearing male call; (B) male hearing female call; (C) male hearing male song; (D) male hearing female song.

Figure 5. The mean number of ZENK positive cells per sampling window (0.20mm×0.15mm or 0.3mm^2) in the caudomedial mesopallium (A), the dorsal caudal medial nidopallium (B), and the ventral dorsal caudal medial nidopallium (C). Male (black) and female (white) black-capped chickadees were presented either male calls, female calls, male song, female song, or silence in the control condition. Black-filled star show and black solid line shows significant differences for male recipients compared to male recipients. White-filled star and black dashed line shows significant differences for female recipients compared to female recipients. The dotted line shows significant differences between male recipients and female recipients.

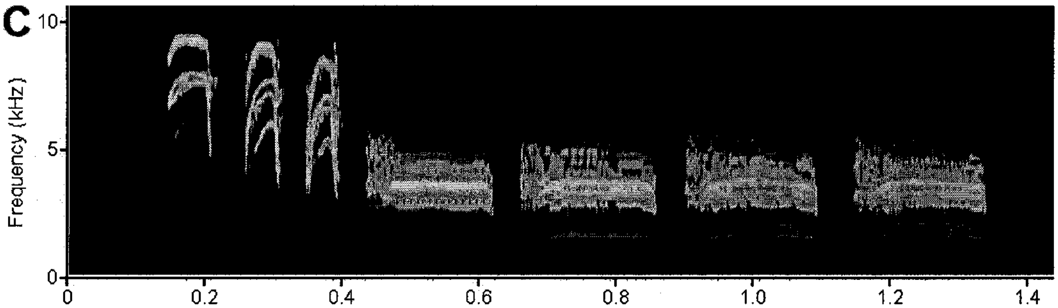
Figure 1.



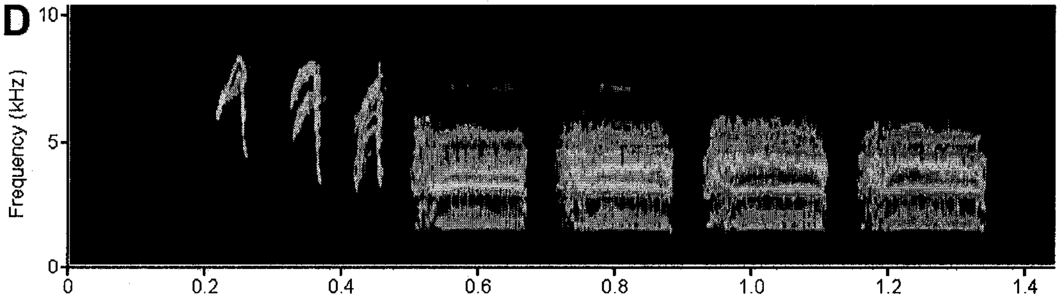
Male Fee-bee Song



Female Fee-bee Song



Male Chick-a-dee Call



Female Chick-a-dee Call

Figure 2.

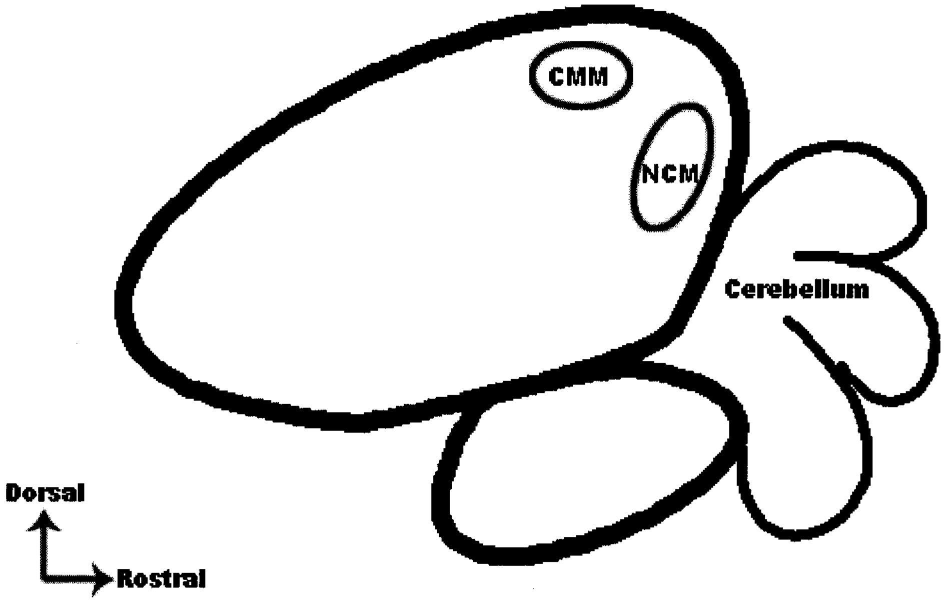


Figure 3.

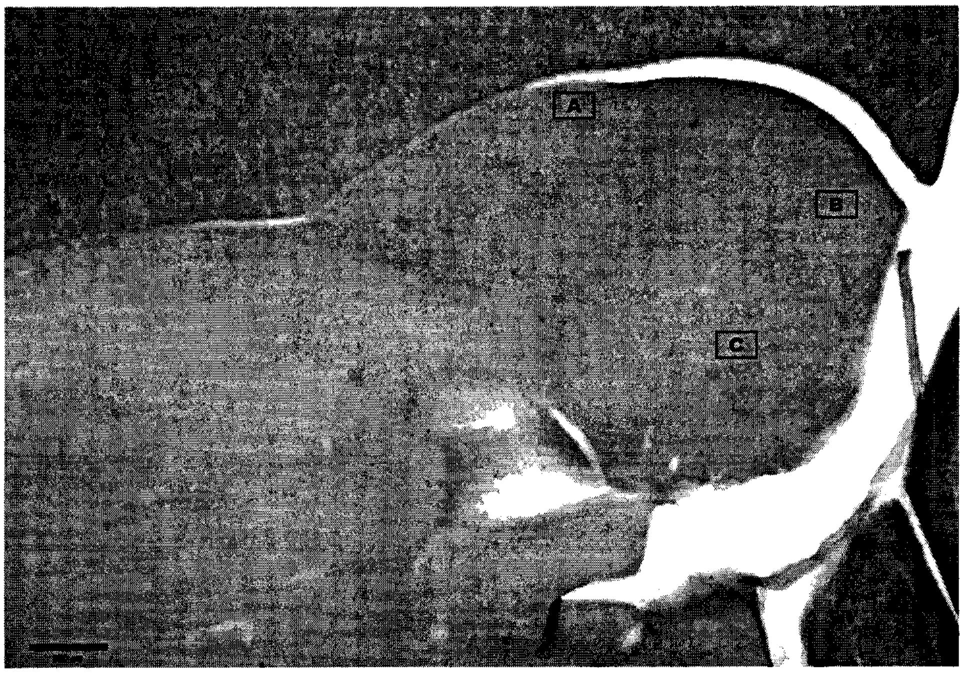


Figure 4.

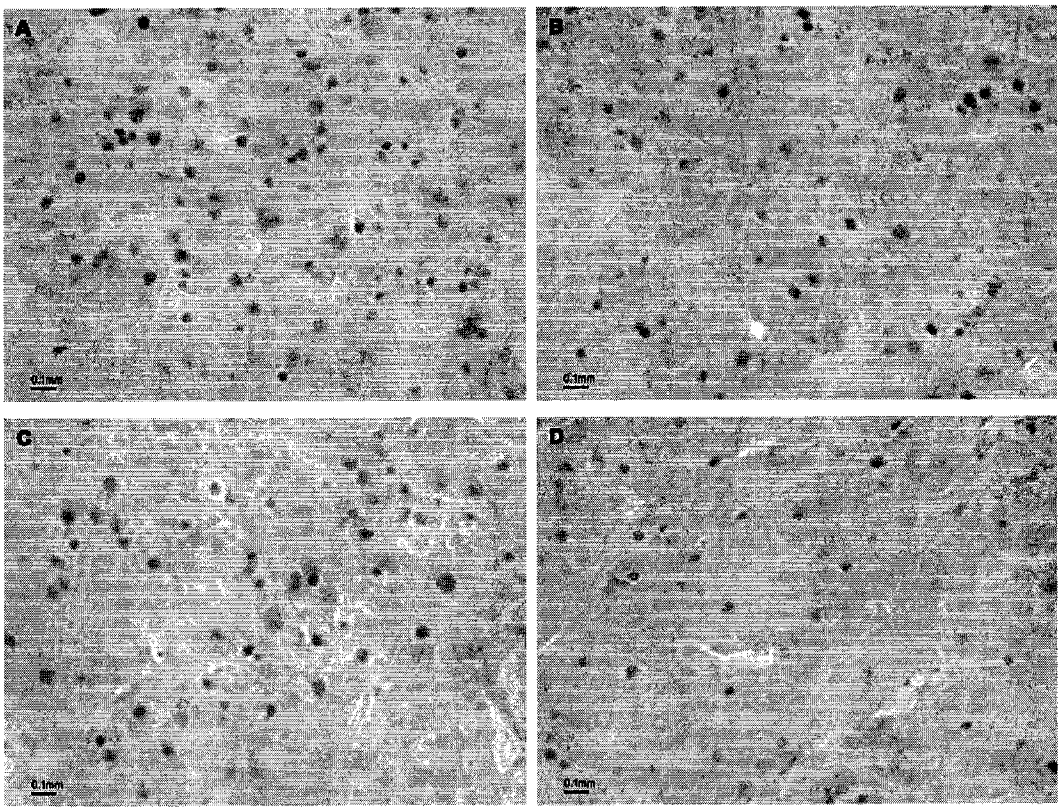
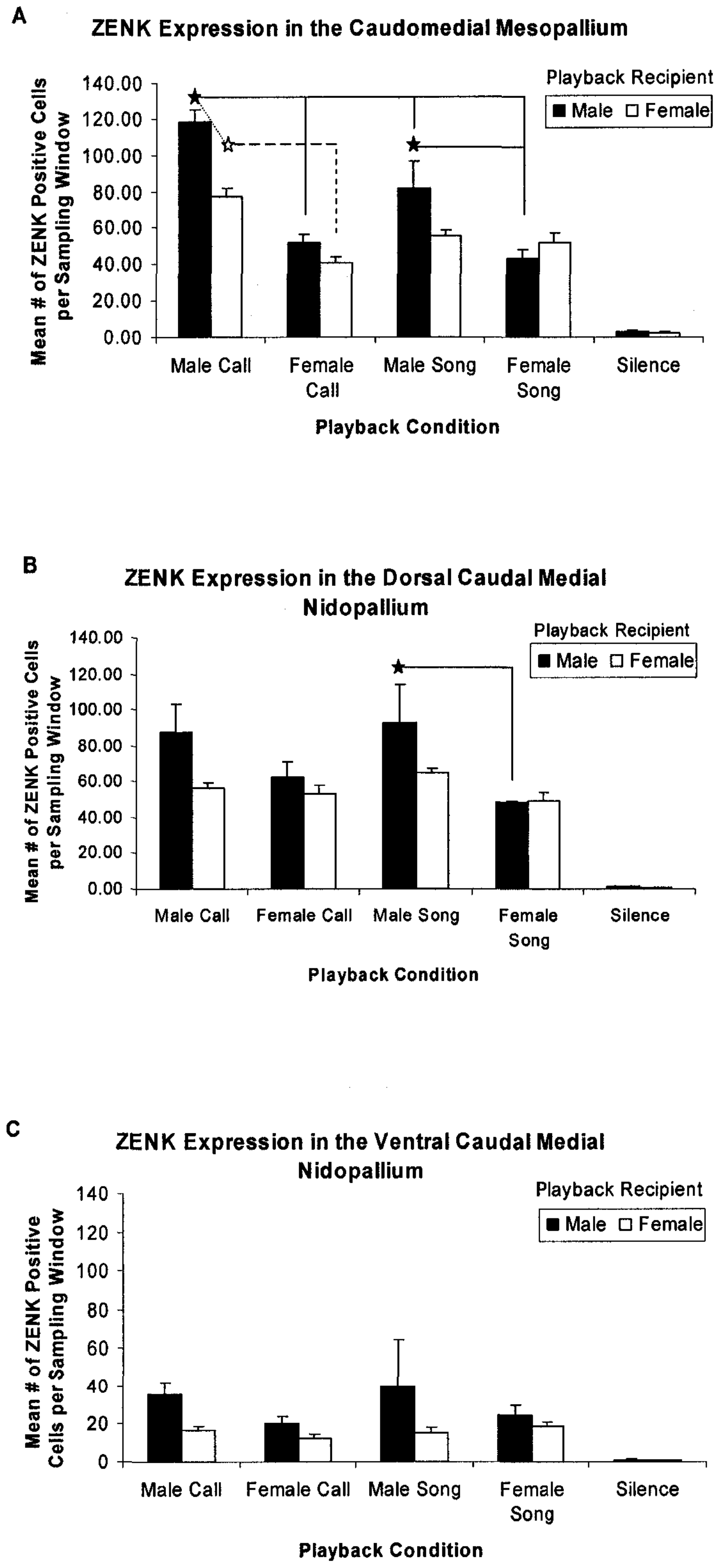


Figure 5.


A
ZENK Expression in the Caudomedial Mesopallium
Mean # of ZENK Positive Cells per Sampling Window
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Playback Recipient
■ Male □ Female
Male Call
Female Call
Male Song
Female Song
Silence
Playback Condition
B
ZENK Expression in the Dorsal Caudal Medial Nidopallium
Mean # of ZENK Positive Cells per Sampling Window
140.00
120.00
100.00
80.00
60.00
40.00
20.00
0.00
Playback Recipient
■ Male □ Female
Male Call
Female Call
Male Song
Female Song
Silence
Playback Condition
C
ZENK Expression in the Ventral Caudal Medial Nidopallium
Mean # of ZENK Positive Cells per Sampling Window
140
120
100
80
60
40
20
0
Playback Recipient
■ Male □ Female
Male Call
Female Call
Male Song
Female Song
Silence
Playback Condition