

EFFECTS OF INSTRUMENTAL LEARNING ON AUDITORY REPRESENTATIONS
OF SOCIAL VOCALIZATIONS IN THE SONGBIRD FOREBRAIN

by

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ABSTRACT OF THE THESIS

Effects of Instrumental Learning on Auditory Representations of Social Vocalizations in
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Both humans and songbirds learn to vocalize by imitating conspecific tutors heard during development. Through imitation, juvenile male zebra finches develop a copy of their tutor's song. However, these copies are imperfect; each male produces a slightly different song that is unique and therefore useful for individual recognition. Although zebra finches can no longer learn to produce new vocal signals in adulthood, they remain able to show behavioral recognition of new songs they hear in social interactions that may involve reinforcement. Furthermore, neural memories of specific songs can be detected in the size and rate of adaptation of electrophysiological responses recorded in two auditory processing areas in the songbird brain, the caudomedial mesopallium (CMM) and caudomedial nidopallium (NCM), which may be analogous to a secondary auditory cortex.

The current experiment tests the effects of auditory discrimination training in a GO/NoGO operant paradigm on auditory responses in these areas, which can serve as models for neural representations of socially learned auditory objects. To do this, adult male and female zebra finches (n=16) were trained to peck in response to one of two

stimuli (GO) and to withhold responding from the other (NoGO). Prior to this conditioning, female subjects had cohabited with a male, heard his song in that social context, and produced a brood of offspring. After performance reached criterion, multi- and single-unit neural responses to operantly-trained, socially-relevant and novel song stimuli were obtained from multiple electrodes inserted bilaterally into NCM and CMM of awake, restrained birds.

The results show that both male and female subjects exhibited neural memories for operantly-trained auditory objects in forebrain auditory areas. The magnitude of neural responses and the rates of response adaptation for operantly-trained stimuli differed from those evoked by novel stimuli and also showed a different pattern of effects in NCM and CMM. In addition, when subjects were grouped by the number of days required to reach criterion during training, fast learners showed higher absolute responses and faster neuronal adaptation in CMM than slow learners, while, in NCM, fast learners showed absolute responses that were more strongly left-lateralized than slow learners. When females were also tested for neural memories of their mate's songs, the effects of operant and social conditioning on auditory responses were in different directions. Therefore, although both social exposure and operant conditioning induced neural memories for song in adult zebra finches, operant discrimination learning is not an appropriate model for studying the process by which individuals acquire the ability to recognize each other through song in social contexts.

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Animals that live in social groups have interactions with kin and non-kin on a near constant basis. In evaluating social exchanges with others, individual recognition plays an essential role. It can influence the probability of cooperation and sharing, especially with recognized kin, but also with others whom experience has shown to be worthy of reciprocal exchanges (Lode, 2008). Animals invest heavily in pair bonds and offspring, and must be able to recognize their mates and family if they become separated, using sensory feature(s), such as face, odor or vocal cue (Tate, Fischer, Leigh & Kendrick, 2006; Brennan & Kendrick, 2006; Belin, 2006). Humans recognize each other's hairless faces, but many other animals, including birds, are less visually unique. For example, it is known that birds recognize each other by their vocalizations, often used to communicate between individuals who hide to avoid predation. However, it is unknown how the familiarity of a unique complex signal is represented in the brain or how that representation is updated by social interactions. This study investigates how experience modulates the neural memory for signals used in individual recognition.

Songbirds learn their songs from adult tutors through a process of vocal imitation with many parallels to speech acquisition. The subject of this study, the zebra finch, learns a single song early in life that is used as a social and reproductive signal. In this species, song is only learned in males. Although the copies are good, they contain variations that make a song unique to an individual. Thus, they can be used as recognition signals, similar to the way humans use faces. This study investigated neural activity in forebrain auditory areas that appear to be specialized for the processing of individual songs: the caudomedial nidopallium, NCM, and caudomedial mesopallium, CMM. Neurons in these areas respond more strongly to conspecific vocalizations than other

sounds and undergo a process of stimulus- specific adaptation that is a form of long-term neural memory for individual songs (Mello, Vicario and Clayton, 1992; Mello, Nottebohm & Clayton, 1995; Chew, Mello, Nottebohm, Jarvis & Vicario, 1995; Chew, Mello, Nottebohm, Jarvis & Vicario, 1996; Chew, Vicario & Nottebohm, 1996; Terpstra, Bolhuis & den Boer-Visser, 2004). Behavioral studies also show that songbirds are able to recognize the songs of individual conspecifics that they have interacted with socially, e.g. a tutor or mate (Miller, 1979; Riebel, 2000; Vignal, Mathevon & Mottin, 2004). Further, songbirds can be conditioned to discriminate songs through operant training. Operant conditioning increases the expression of ZENK, an immediate early gene involved in learning and the formation of memories in NCM and CMM (Gentner, Hulse and Ball, 2004). In our study, birds were trained to recognize songs and their behavioral relevance through operant reinforcement, as a laboratory model for the way reinforcement of particular songs may occur during natural social interactions in the field. Then neural responses to reinforced, familiar, and novel songs were recorded in awake subjects. The results shed light on how reinforcement-predictive memories of the songs of other individuals are represented by sensory neurons in the brain, altered by experience, and accessed during recognition.

BACKGROUND

A. Function of Birdsong in a Social Context

I. Song Learning and the Development of a Recognizable Adult Song

Avian song learning is a widely used model of speech development, because, like human infants, young birds acquire their songs by listening to adult tutors through a

process of vocal imitation with many parallels to speech acquisition. During the juvenile period (35 to 90 days post-hatch) a male zebra finch (*Taeniopygia guttata*) imitates a tutor and develops a 'birds-own-song' (BOS). Each male learns one song from its tutor during development; that song becomes crystallized as the bird enters adulthood and does not change for the rest of the animal's life. Therefore, the period throughout development when these birds learn their song is referred to as a critical period; it parallels the plastic critical period for speech acquisition that humans exhibit at a young age, during which children are able to more easily learn language than is possible in adulthood (Immelmann, 1969; Tchernichovski, Mitra, Lints & Nottebohm, 2001). Although the copies of the tutor song produced by males during this period are good, they contain variations that make the song unique to the individual. Thus a male zebra finch's song can be used as a recognition signal, much as humans use faces.

The conclusion of the critical song-learning period coincides with the commencement of a male zebra finch's sexual maturity (Immelmann, 1969). The coincidence of these two events underlies the role that song plays in the social interactions of this species. In most songbirds (including the zebra finch), learned song is a male behavior, used as courtship and territorial defense signal; females do not sing. The consistent and unique (due to imperfect copying) nature of each male zebra finch's song makes it a likely candidate for individual recognition. In addition, male zebra finches learn their long distance call, while both males and females produce shorter, unlearned calls. A songbird's experience with conspecifics involves repeated exposure to both the songs and distance calls of those individuals. Exposure results in behavioral recognition of, and often preference for, the songs and calls of conspecifics that a bird is socially

associated with, such as a tutor or mate (Miller, 1979; Riebel, 2000; Vignal et al., 2004; Vignal, Mathevon & Mottin, 2008; Menardy et al., 2012). Although young female zebra finches do not learn to produce song as male juveniles do, they do form an auditory memory of their tutor's song (a form of sexual imprinting) and show the same behavioral preferences for tutor's song that males exhibit.

III. Role of Song in Mate Selection

Social interactions between conspecifics via song, in the field, have direct behavioral consequences, such as fighting, mating, cooperation and familial care. Although female zebra finches do not produce song, they do choose mates based on their song quality. Females learn their tutor's song 'template' during development through sexual imprinting, and later use that template to make mate selection decisions in adulthood (Riebel, 2000; Riebel, 2002). Female zebra finches choose to mate with males based on their songs, often choosing males that sing more complex songs, with longer durations that are sung at faster rates (Collins, 1999; Clayton and Pröve, 1989; Houtman, 1992). Therefore, interacting with a tutor and practicing often during the critical period in order to develop a good (complex, long and fast) song is vitally important for male zebra finches, as their song quality dictates directly how often females will choose to mate with them, and ultimately how many offspring they may be able to father.

Sexual selection theory suggests that the ability to produce an arbitrary signal (like song) will be only be selected for over the long term if it is an "honest signal" that is correlated with some desirable quality possessed by that male. For example, the nutritional stress hypothesis suggests that females choose mates based on song quality

because song complexity is a dependable indicator of male health and condition during the juvenile period. The quality of a male's song may be directly linked to his health as a juvenile because the song control system (the neural motor nuclei used for song production) develops after hatching in a young bird's life, at a time when he is susceptible to nutritional stress; if a juvenile zebra finch is not well nourished during this time period these brain nuclei are likely to under-develop, leading to poor song production (Nowicki, Peters & Podos, 1998, Spencer, Buchanan, Goldsmith & Catchpole, 2003). Females also tend to choose, as mates, male songbirds that spend more time singing, and this preference has been linked to direct behavioral outcomes such as territory quality, food availability and parental care quality (Alatalo, Glynn & Lundberg, 1990; Greig-Smith, 1982). Due to the fact that zebra finches form life-long pair bonds, females have extensive experience with her mate's song as it also used to establish contact and enable cooperation when mates have been separated, or are in a large group of birds. For this reason, it is unsurprising that female songbirds show behavioral recognition of their mate's songs, and both sexes show recognition of their mate's long calls (Lind, Dabelsteen & McGregor, 1996; Vignal et al., 2004; Vignal et al., 2008).

B. Operant Methods of Song Recognition

I. Operant Sensory Conditioning

Operant training, a method by which an experimenter utilizes reinforcement, punishment, or both, to train an animal to respond in a particular way to a sensory stimulus, has successfully been used by experimenters to teach animals of widespread species to recognize and respond to sensory stimuli (Kv, 1965; Kirsh & Erber, 1999; Sinnott, 1980; Takahashi, Funamuzu, Mitsumori, Kose and Kanzaki, 2010; Osmanski & Wang, 2011; Pulvermüller, Mohr, Schleichert & Veit, 2000). Experiments utilizing operant sensory conditioning are useful, not only for providing experimenters with information on how animals perceive stimuli of different modalities, but also for modeling how animals make associations between the sensory stimuli they encounter (in the field) and the behavioral outcomes of those stimuli.

One type of operant conditioning, in which both positive reinforcement and punishment are used to teach a subject to respond to two sensory stimuli in two different ways, is GO/NoGO training. In GO/NoGO training, most commonly, two stimuli from the same modality are presented to a subject; for one stimulus (GO) the subject must make a certain response to receive a reward (i.e. food), but for the other stimulus (NoGO) the subject must withhold responding or they are punished (i.e. shock). Thus, sensory GO/NoGO conditioning is a tool that can be used to test how different two sensory stimuli from one modality need to be in order to be discriminated, as well as what factors impair the discriminability of those stimuli. In addition, the number trials that are needed to achieve discrimination provides another measure of performance.

II. Operant Conditioning as a Model for Songbird Individual Recognition

Auditory GO/NoGO conditioning can be applied to the study of songbird song discrimination (Sinnott, 1980; Cynx & Nottebohm, 1992; Gess, Schneider, Vyas & Woolley, 2011). In songbird auditory GO/NoGO training, animals learn to peck for one song stimulus and to withhold pecking for another; this training method allows experimenters to investigate how easily songbirds can differentiate between two different song stimuli. This technique is particularly useful because songbirds learn their individual songs throughout their juvenile periods, and use those songs during adulthood in various social contexts (detailed above). Therefore, for songbirds, songs are auditory stimuli that are naturally associated with significant behavioral outcomes, even without operant training. In the field, songbirds are constantly differentiating between the songs of conspecifics and those of, possibly threatening, heterospecifics, as well as discriminating conspecific songs they hear. Further, songbird performance on operant conditioning tasks provides a measure of how well songbirds can recognize the differences between two songs. For instance, zebra finches are able to learn auditory discriminations between two conspecific songs more quickly than they can learn an auditory discrimination between two heterospecific songs (Benney & Braaten, 2000).

The learning advantage that songbirds show when discriminating between the songs of two of their conspecifics (rather than heterospecifics) may be due to the frequency with which they hear those songs, or may be an innate ability because of the importance of being able to discriminate between their kin and novel conspecifics. Animals invest heavily in pair bonds and offspring, and must be able to recognize their mates and family if they become separated, using sensory feature(s), such as face, odor or

vocal cue (Tate et al., 2006; Brennan and Kendrick, 2006; Belin, 2006). Humans recognize each other's hairless faces, but many other animals, including birds, are less visually unique. For example, it is known that birds recognize each other by their vocalizations, often used to communicate between individuals that are unable to see each other when hidden to avoid predation. In these instances, social interaction may act as a type of 'social-conditioning' in which auditory song stimuli are associated with natural behavioral outcomes (cooperation for resources such as food, mating, etc.) that direct an animal to behave in one way (or another) when they recognize that stimulus, much like operant conditioning. For this reason, operant sensory conditioning may be a good model for how social interactions affect the way the songbird brain experiences auditory stimuli by creating behavioral relevance for these social stimuli in a controlled experimental setting.

III. Tactics Used by Songbirds to Perform Auditory Discriminations

Songbirds (and other subjects) can use multiple different tactics to approach an operant conditioning paradigm, often using the simplest rule to accomplish the behavioral task rather than truly discriminating between auditory stimuli (Morisaka & Okanoya, 2008; Van Heijningen, De Visser, Zuidma & Cate, 2009). One of the tactics that songbirds often employ to perform operant auditory discriminations is simply memorizing one of the songs (whether it be the GO or NoGO stimulus) and its correct categorization and responding in the opposite way to all other stimuli (Morisaka & Okanoya, 2008).

To investigate which cognitive tactic a subject may be employing to perform an

auditory discrimination, additional trials in which novel unreinforced stimuli are played (probe stimuli) can be added to an operant paradigm. For example, if an animal has only learned to recognize and withhold pecking for the NoGO stimulus, it will respond to all probe trials by pecking, because any time these animals do not hear the NoGO stimulus they believe they should give a GO response. Therefore, high and low levels of responding to probe stimuli are indicative of a cognitive tactic in which only one stimulus category is truly learned by the subject during the discrimination learning. In this case, one song category is learned and familiar to subjects, while songs in the other training category are recognized and responded to no differently than novel stimuli. However, if a subject responds to probe stimuli at chance levels (50%) it is likely they have learned to recognize both the GO and NoGO stimuli and are therefore uncertain about the category to which a novel stimulus belongs.

C. Neural Representations of Meaningful Auditory Stimuli

I. NCM and CMM: Auditory Structures with Memory and Lateralization

Two avian auditory structures integrally involved in songbird auditory learning are the caudomedial nidopallium (NCM) and caudomedial mesopallium (CMM) (**Figure 1**). These auditory structures receive auditory projections from primary auditory areas (Field L) and may be analogous to mammalian secondary auditory cortex or to superficial layers of mammalian A1 (Vates, Broome, Mello & Nottebohm, 1996; Karten, 1991; Wang, Brzozowska-Prechtl & Karten, 2010). Neurons in these areas respond more strongly to conspecific vocalizations than other sounds, showing a response bias for stimuli that are behaviorally relevant to subjects (Chew et al., 1995; Chew et al., 1996a; Mello et al., 1992). In addition, during awake neurophysiological recordings, neurons in NCM and CMM undergo a process of stimulus-specific adaptation (SSA, **Figure 2**); responses are robust during the first few presentations of each stimulus and then decrease over subsequent presentations to reach an asymptote (Chew et al., 1995; Pierce, Phan, Shukla & Vicario, 2010). Other novel stimuli again elicit robust responses that in turn decrease with repetition. Therefore, the rate at which multiunit responses to song stimuli decrease over repeated presentations can be used to assess the familiarity of stimuli (Phan, Pytte & Vicario, 2006), and SSA can be thought of as a form of long-term neural memory for individual songs.

The avian auditory forebrain also shows lateralized neural responses to conspecific vocalizations (Phan and Vicario, 2010). This lateralization of neural activity is of specific interest because the human brain is also lateralized for language; both speech production and perception are predominately left hemispheric processes but the

reason for this lateralization is unclear. Data from our lab have shown that auditory responses in the NCM of zebra finches are lateralized (**Figure 3**), and further that the direction of lateralization can be affected by environmental changes (Phan and Vicario, 2010, **Figure 3**; Yang, 2012). In addition, lateralization in this area may be related to the quality of a songbird's auditory learning. When auditory information is blocked from reaching one hemisphere's NCM, CMM and Field L by lesioning the thalamic auditory relay nucleus of songbirds (nucleus ovidalis) birds show differential deficits in auditory discrimination learning according to which hemisphere received the lesion (Cynx, Williams & Nottebohm, 1992). In addition, birds that produce songs most similar to their tutor's song show increased neurogenesis in the left hemisphere NCM, when compared with the right (Tsoi et al., 2012). Therefore, successful operant auditory conditioning may depend more strongly on one hemisphere of the avian forebrain (NCM/CMM) than the other.

II. Representations of Social Stimuli in NCM and CMM

The behavioral recognition of the auditory cues of familiar conspecifics seen in songbirds is correlated with differential neural activity in auditory processing areas NCM and CMM in response to these cues. Studies of the induction of the immediate early gene ZENK (known as zif-268, egr-1, NGFI-A or Krox-24) and the electrophysiological firing of neurons in avian forebrain auditory structures have shown differential activity after playback of familiar and novel auditory stimuli (Mello et al., 1995, Chew et al., 1996a, Terpstra et al., 2004; Woolley and Doupe, 2008; Mernardy et al., 2012). Social interactions appear to train these birds to both recognize, and preferentially respond to,

socially-relevant cues, and this recognition is reflected in the neural firing of the auditory processing pathway (Menardy et al., 2012). For instance, a memory for the tutor song, a stimulus that is important not only socially, but also for sexual imprinting and song development, is held in the NCM (detectable through SSA) of both male and female zebra finches throughout adulthood (Phan et al., 2006; Yoder, 2012). In male zebra finches, the strength of the tutor-song memory is also correlated with how similar males' BOSs are to their tutors' songs, and, therefore, how well birds learned their tutors' songs. For males, NCM seems to be particularly important in holding and retrieving the tutor song memory, playback of the tutor song causes ZENK induction in this area and lesions of this area eliminate behavioral preference for the tutor song (Terpstra et al., 2004; Gobes & Bolhuis, 2007). In females, however, both NCM and CMM may be important for the storage of the tutor song memory; although SSA is slower for the tutor song than for novel songs in NCM, ZENK is expressed in CMM but not NCM after playback of the tutor song in females (Yoder, 2012; Terpstra, Bolhuis, Riebel, van der Burg & den Boer-Visser, 2006).

In addition to the behavioral preferences that zebra finch subjects show for their tutors' songs, males show a preference for their mate's long call and females show preferences for their mate's long call as well as his song (Vignal et al., 2004; Vignal et al., 2008; Lind et al., 1996). It is, therefore, likely that zebra finches show neural representations of their mate's song/call as well as the tutor's song in avian auditory processing areas. Although neural memories for mate's song have not yet been detected in the neurophysiological responses of NCM or CMM, recently it was shown that the neurons in NCM respond differently to a female's mate's call than they do to novel call

stimuli (Menardy et al., 2012). In addition, the playback of mate's song to females causes higher expression of the immediate early gene ZENK in NCM than the playback of novel songs (Woolley & Doupe, 2008). These recent data demonstrate that long-term neural memories do not only develop in NCM and CMM during song-learning and sexual-imprinting, but that memories can also develop for significant sounds heard throughout adulthood. Social experience and, specifically, mating are natural experiences which can cause these auditory stimuli (songs) to acquire significance. For this reason, female zebra finches are convenient models in which to compare socially and operantly-trained stimuli, to see if these different methods similarly train subjects to recognize songs, at least in part by creating neural representations in NCM and CMM.

III. Role for NCM and CMM in Auditory Discrimination Learning

Both NCM and CMM have been implicated in songbird auditory discrimination learning. The expression of ZENK, an immediate early gene involved in learning and the formation of memories, is increased during operant training in both the NCM and CMM of zebra finches (Gentner et al., 2004). After training has concluded, increased ZENK expression in CMM remains associated with playback of trained stimuli while ZENK expression in NCM is associated with the playback of novel stimuli (Gentner et al., 2004). Therefore, although both auditory areas are likely involved in auditory discrimination training, they may serve different roles; NCM may process stimulus familiarity while CMM processes the behavioral relevance of a stimulus.

In anesthetized starlings (another songbird species), after training is concluded, passive playback of operantly-trained songs increases neural firing in the CMM and

decreases firing in the NCM, in comparison to the playback of novel songs (Gentner & Margoliash, 2003; Thompson & Gentner, 2010). These results suggest that this training process (using food reward to model behavioral relevance in the field) not only changes how the bird (and the brain) reacts to a given stimulus, but that these changes are also long-lasting. Although passively familiar auditory stimuli also show differential neural responses in NCM due to SSA, slower adaptation to recently heard stimuli is maintained for a few (3-4) days only for these stimuli (Chew et al., 1996b). However, special status is given to stimuli that have been used in operant training, even when subjects are outside of the training environment, for more extensive periods of time (Gentner & Margoliash, 2003; Thompson & Gentner, 2010). The development of the neural memories for these stimuli is likely more similar to the development of tutor song neural memory than it is to the development of neural memories for passively familiar stimuli.

The neural responses to operantly-trained stimuli are not only distinct (in NCM and CMM) from responses to novel stimuli due to their reinforcement-predictive value, but are also affected by the valence of the reward (or punishment) with which they are reinforced. In the CMM of songbirds, the type of reinforcement (be it positive or negative) utilized in training has been shown to affect the magnitude of neural responses elicited by playback of stimuli (Genter & Margiolash, 2003). Consequently, these auditory structures may not only be important for recognizing which songs are familiar and important to an individual, but may also give insight into why an auditory stimulus is important to an individual.

D. Current Study

The purpose of this experiment was to investigate how the behavioral relevance of auditory stimuli is reflected in the neural activity of sensory processing areas.

Understanding the reinforcement-predictive value induced by training and its effect on neural activity in auditory processing areas may give insight into the neural correlates of behavioral relevance. In this study, we used operant training, in which a bird learns to recognize a song with an associated behavioral relevance, as a potential model for how songs become familiar through social interaction with conspecifics in the field.

Electrophysiological recording was used to investigate how the brain represents learned sensory stimuli, and how the use of positive or negative reinforcement during training affects those representations in different brain areas. To investigate the distinct status of operantly-trained stimuli by measuring neural responses to playback and how these change over time, electrophysiological experiments were conducted in awake birds that were able to process and adapt to the stimuli as playback occurred (Chew et al., 1995). The absolute response magnitudes (ARMs) and speed of neural adaptation of multiunit recording sites in response to auditory stimuli were simultaneously assessed at multiple sites bilaterally in NCM and CMM of trained subjects. In addition, single-units were isolated and their firing-rates and adaptation patterns were also analyzed to identify effects of learning on individual neurons in these auditory processing areas.

In addition to comparing the neural responses to trained and untrained stimuli, responses to trained stimuli were analyzed according to the valence of the reinforcement with which they were trained. In our operant paradigm, GO songs are positively reinforced and NoGO songs are negatively reinforced. Therefore, the neural

representations that develop for ‘GO’ stimuli are likely to be different from those of NoGO stimuli; and we expected that this difference would be detectable in the neurophysiological responses to these stimuli in auditory processing areas.

Trained songs were hypothesized to be more stimulating to the neurons of CMM (in terms of absolute multiunit activity as well as single-unit firing-rates) than novel songs. Further, responses to GO stimuli in this area were expected to be stronger than responses to NoGO stimuli. Likewise, GO songs were hypothesized to have stronger responses than NoGO songs in NCM. However, NCM neurons were expected to respond less (ARMs and single-unit firing-rate) to familiar stimuli than to those that are novel. In addition, trained songs were also expected to undergo slower adaptation (decrease in single- and multi-unit neural responding) than novel songs in both of these areas, as the neurons in NCM and CMM adapt more slowly to songs with which birds are familiar.

Songbird subjects show variability in their speeds to acquire sensory discriminations (Guillette, Reddon, Hoeschele & Sturdy, 2011; Katsnelson, Motro, Feldman & Lotem, 2011; Range, Bugnyar, Schlögl & Kotrschal, 2006). We further hypothesized that individual differences in the subjects’ speed to reach criterion would be correlated to an individual animal’s ability to encode the meanings of salient cues, and therefore related to the strength of the resultant neural representations of learned songs. Specifically, we hypothesized that the animals that learned fastest would have the strongest differences between neural responding to learned and novel songs, and that this enhanced plasticity may have contributed to those animals’ enhanced performance. To test this, subjects were grouped in two categories, slow and fast learners (**Figure 7**). Electrophysiological responses were analyzed and compared between groups in order to

detect any relationships between speed of learning and neural responses to trained and untrained stimuli.

The female subjects used in training and neurophysiological testing were mated females that had produced a brood while cohabitating with a male throughout a full breeding period – this could be considered to provide a positive valence for the mate’s vocalizations. Because males sing to attract females, we expected to find the neural correlate of a memory for the mate’s song in the auditory areas of females (as assessed by adaptation rate). The female subjects were also expected to show a memory for their tutor’s song, which they learn during their juvenile periods through sexual imprinting (Miller, 1979). We therefore played the songs of both the mate and father of the female subjects’ during electrophysiological testing as socially salient stimuli that could be compared to the salient stimuli from operant training. We hypothesized that a neural memory would be detectable in the magnitude of the adaptation rates to the social songs in NCM and CMM, due to SSA. We also expected to observe stronger ARMs to socially-relevant songs than to novel songs in CMM. In addition, it was hypothesized that the memory (adaptation rate) for socially familiar stimuli would be closer (in magnitude) to operantly-trained stimuli than to novel or passively familiar stimuli in NCM.

It has been established that songbird subjects use multiple cognitive tactics to perform auditory discriminations. If some zebra finches memorize one song and its appropriate response and respond in the opposite fashion to all others, those birds should show a stronger memory for that song than other trained stimuli. If so, in electrophysiological testing, subjects should show a slower adaptation rate for the

memorized stimulus than others. In this experiment, as in Morisaka & Okanoya (2008), probe trials will be used at the conclusion of operant training to identify the cognitive tactic an individual subject has employed to perform the operant task. The correlation between the results of a bird's probe trials and that bird's valence-based difference in adaptation rates were quantified to analyze any relationship between cognitive tactics and the resulting neural representations. Subjects that responded to probe stimuli at chance levels (50%) were expected to show similar adaptation rates (in NCM) for GO and NoGO stimuli. However, if a bird responded to probe stimuli a majority of the time, we expected to find slower adaptation to NoGO stimuli than GO stimuli in NCM, and vice versa.

METHODS

Subjects:

The subjects of the experiment were 9 male and 5 female adult zebra finches (**Table 1**). Subjects were reared in an aviary, exposed to the songs of conspecifics, but naïve to discriminatory training. Female subjects were mated adults that had cohabitated with a male for the entirety of a breeding session and produced eggs. These females had all successfully produced offspring with their mates. Subjects were maintained on a 14:10 light:dark cycle and had access to cuttlebone and water at all times throughout training. Subjects were food deprived overnight on days prior to training sessions (during which food was used as a reward for correct responses) and then given food *ad libitum* at the end of the daily training session. For five days prior to operant training the birds were isolated and acclimated to a custom-built wire chamber (45.72 x 29.21 x 27.94 cm) inside of a sound-attenuated box (inside dimensions: 82.55 x 33.66 x 38.10 cm; outside dimensions: 91.44 x 40.64 x 48.26 cm), where they lived and trained the for duration of the experiment (**Figure 4A**). During weekends when the subjects were not training they were given food *ad libitum*, and females were allowed to cohabit with their mates.

Training:

Birds were trained 5 days a week (Monday through Friday) for 6 hours a day. After acclimation the subjects were shaped to peck a sensor, breaking an infrared beam, in order to stimulate a food reward (as in Gess *et al.* 2011) using the ARTSy program (David Schneider, Columbia University, New York, NY, U.S.A). After five days of shaping, the birds began operant GO/NoGO training, during which subjects had to peck the sensor to hear a stimulus, and then respond correctly to that stimulus based on

its GO/NoGO categorization (**Figure 4B**). The two stimuli used in each discrimination were novel 1-motif songs of unfamiliar male zebra finches; these stimuli were randomly assigned to the GO and NoGO categories. Each discrimination used a set of stimuli that were 50-70% different from one another, and of similar length (Tchernichovski, SAP 2011). During a trial, if the stimulus played was the chosen GO stimulus the subject's correct response was to peck again for a food reward, however if the stimulus was a NoGO stimulus the bird should have withheld pecking. Correct GO responses were rewarded with access to birdseed for 6 seconds and incorrect NoGO responses were punished with the chamber lights being extinguished for 16 seconds. Subjects were given 6 seconds to respond to stimuli before the trial was concluded and subjects were able to stimulate trials immediately after one another. Subjects were, therefore, be able to perform unlimited trials until they reached accuracy criterion, which was achieved by performing two subsequent sets of one hundred trials with at least 80% correct responses. Subjects were taught two discriminations in this way (**Figure 4C**). At the end of training, all song stimuli were played interwoven during operant training to ensure that both the first and second pair of stimuli were being accurately categorized and responded to by subjects immediately before electrophysiological recording.

Probe Trials:

After subjects reached accuracy criterion for two operant discriminations, correct GO responses and incorrect NoGO responses began to be reinforced only 80% of the time (rather than 100%) to prepare for probe trials. After this, two probe stimuli, which were each equally different (within 10% similarity, Tchernichovski, SAP 2011) from the

GO and NoGO stimuli, were added to the training trials. When these songs played as trial stimuli, the behavioral responses they stimulated were observed but not reinforced, either positively or negatively. If all probe trials were responded to as if the probe stimuli were GO stimuli, it could be inferred that the NoGO stimulus was better remembered by the subject than the GO stimulus, and vice versa.

Electrophysiology:

At the conclusion of operant training, two days prior to experimentation, subjects underwent partial craniotomies in preparation for testing and electrode placement. During this surgery the first layer of the skull was removed and a metal pin was cemented onto the skull of each subject while the bird was anesthetized under isoflourane. Such surgical pinnings are necessary to keep the subject's head stable while electrodes are placed in the forebrain during electrophysiological experimentation. Two days post-pinning, an awake electrophysiological experiment was performed, in which the activity of neurons in areas NCM and CMM were recorded (16 electrodes total, 4 in each area in each hemisphere) during playback of trained and novel stimuli. Trained stimuli included songs associated with a GO or NoGO valence in the operant behavioral paradigm, songs used as unreinforced probes in the operant behavioral paradigm, and songs socially-relevant to female subjects.

The subjects were kept awake and comfortably restrained (in a plastic tube) with the head pin clamped into a stereotaxic apparatus throughout the experiment. Once subjects were comfortably placed into the stereotaxic apparatus, the second layer of the skull was opened and a Microdrive was used to place 16 tungsten electrodes bilaterally

on the surface of the brain, near the bifurcation of the mid-sagittal sinus (**Figure 5A**).

The experiment was performed in a soundproof booth and stimuli were played through a speaker placed directly in front of subjects. The microelectrodes were initially lowered to a depth of 500 μm and then slowly lowered from this depth while a novel set of stimuli was played. While electrodes were being slowly lowered, experimenters listened for the neural responses indicative of NCM and CMM, through the amplifier. Once robust responses to song were located on all electrodes, the song stimuli were played and the multi-unit neural responses were recorded (at a gain of 19,000, band-pass filtered: 0.5-5 kHz; Spike 2 software, CED, Cambridge, England). All novel and trained song stimuli were equated for loudness (75 dB average, A scale; sampling rate, 44,444.4 Hz) and presented for 25 repetitions, in a shuffled order (with an 8s interval between stimuli). After a set of stimuli was heard by the subject at the initial position, the electrodes were advanced to a second depth (by driving down 500 μm), where the recording procedures were repeated with stimulus sets that included new novels.

Following this awake experiment, birds had 2-3 days to recover, during which they continued to train in the operant behavioral paradigm to keep discrimination at criterion. At the end of the recovery period, birds underwent a second electrophysiology experiment in which their neural responses to novel and trained stimuli were once again recorded at sites in each structure of interest. However, this second experiment was conducted while the birds were under urethane anesthesia. Subjects were injected with 10-13 μL of urethane anesthetic (10%, dose dependent on animal size) in three 3-4 μL injections spaced over an hour and a half. During the anesthetized experiments, multiunit activity was recorded while searching for single unit sites, as it is possible to find more

single-unit activity when the subject is not moving.

Histology

Once all recordings were done, lesions were made at recording sites by sending an electrolytic current through the electrodes, killing neurons and forming scar tissue that could be identified histologically (20 μ A for 12 seconds). Subsequently, subjects were anesthetized with Nembutal and then perfused with saline followed by 4% paraformaldehyde. Subjects' brains were removed, fixed and sectioned for histological review. Brains were sectioned into 50 μ m slices using a (Series 1000) vibratome, placed onto slides, and stained with cresyl violet. Sections were visualized under a light microscope to confirm electrode placement (**Figure 6**). Any data from electrodes placed outside the areas of interest were excluded from analyses.

Isolation of Single-units

All spike sorting was performed on multi-unit recording channels after data had been collected using Spike 2 software (CED, Cambridge, England). Channels in both NCM and CMM were visually inspected post-experiment and spike thresholds were set by an experimenter. All spikes that crossed this threshold were extracted into a new “wavemark” channel and the shapes of these extracted spikes were used to create waveform templates. The parameters were set so that a spike had to both match a template with a minimum of 80% points and deviate in amplitude by a maximum of 20%. After templates were established and used to sort spikes, principal components analysis and interval histograms were used to reclassify and group similar spikes together by their

voltage, shape, ISIs, etc. Due to the refractory period of these neurons, two spikes occurring within 2ms of one another were unlikely to come from the same unit; therefore only the units that showed inter-spike intervals longer than 2ms $\leq 2\%$ of the time were deemed to be true single-units. Units that were successfully isolated, with isolation maintained throughout an entire recording, were split onto different channels for individual analysis.

Data Analysis

The neural response of a multi-unit site to each stimulus repetition was quantified by subtracting the root mean square (RMS) of activity during a control period (0.5 s) before stimulus playback onset from the RMS of activity during stimulus playback (**Figure 5B**). Absolute response magnitude (ARM) was defined as the average neural response to a stimulus during a trial, for trials 2 to 6. The rate of adaptation of neural responses was also calculated for each stimulus at each multi-unit site, using the slope of decline in responses between trial 6 and 25, and dividing this slope by the ARM to normalize for the level of responding at a particular site. Sites were excluded from further analyses if: 1) they were not verified histologically within NCM or CMM (above); or 2) responses to 2 out of the 3 stimulus categories (GO, NoGO, novel) were not statistically different from responses during baseline

Single-unit responses were quantified similarly to multi-unit responses; baseline spike-rates were calculated by counting the number of spikes that occurred 0.5s before stimulus onset, and these values were subtracted from the spike-rates during stimulus playbacks to get a response spike-rate for each trial. The firing-rates used in single-unit

analyses are the average response spike-rates to the first 7 presentations of each stimulus. A single-unit's rate of response adaptation was also quantified for each stimulus by taking the slope of the regression line of spike-rate responses to trials 1 through 25. Finally, in order to compare single-unit responses to learned and novel stimuli, d-primes were calculated using a unit's average firing-rate and the trial-to-trial variance in spike-rates between presentations of a stimulus. A d-prime was computed by subtracting the average firing-rate (FR) response to one stimulus category (i.e. novel) from the average FR response to another stimulus type (i.e. GO), multiplying by 2, and finally dividing by the square root of the sum of the variances for those two measures (as below).

$$d'_{\text{Trials 1-7}} = \frac{2((FR_{GO}) - (FR_{Novel}))}{\sqrt{(\sigma_{GO}^2 + \sigma_{Novel}^2)}}$$

Three sets of d-primes were computed for both structures of interest. Each d-prime compared a unit's responses to: 1) GO stimuli to those of novel stimuli, 2) NoGO stimuli to those of novel stimuli; and 3) GO stimuli to those of NoGO stimuli. An additional set of d-primes was calculated, for each structure, by comparing single-unit spiking responses to multiple pairs of novel conspecific stimuli, to get a baseline measure of discriminability of arbitrary pairs of song stimuli by neurons in that area.

Statistical Analysis

To test for statistical differences between groups, non- parametric tests were performed when possible, and parametric ANOVAs were used only when repeated-measure and categorical factors (interactions) needed to be tested simultaneously. All statistical tests were run two-tailed with the criterion for statistical significance set at

alpha = 0.05. Male multi-unit ARM and adaptation rate values were analyzed in both NCM and CMM on a site by site basis, using repeated measures analyses of variance (ANOVAs), to isolate any differences in responding based on familiarity of stimulus, valence of stimulus and subject speed of learning. When overall significant effects were detected, post-hoc Bonferroni t-tests were calculated for comparisons of interest to detect which groups contributed to the significant difference. Non-parametric Friedman's tests were run on single-unit firing-rate and adaptation rate data to test for effects of training on auditory responses. Significant effects detected by Friedman's tests were further investigated with post-hoc Wilcoxon sign-rank tests (using Bonferroni-corrected p-values as criterion for significant effects). Effects of training on single-unit d-primes were tested using non-parametric Kolmogorov-Smirnov tests in order to detect both differences in the central tendencies as well as the variances of these values. Non-parametric Kruskal-Wallis ANOVAs and Friedman's tests were used to detect effects of social- and operant-conditioning on multi-unit auditory responses in NCM and CMM of female subjects. These tests were followed up by post-hoc Wilcoxon sign-rank and Kolmogorov-Smirnov tests to make informative comparisons and detect significant differences (using Bonferroni-corrected p-values as criterion for significant effects). In addition, a correlation was run to test the hypothesis that probe trial responsiveness was related to multi-unit rates of adaptation (familiarity) to trained stimuli.

Due to the great variance in subjects' latencies to reach behavioral criterion, subjects were median-split into two groups (slow and fast learners) according to their average speed to acquire the auditory discriminations (**Figure 7**). Neurophysiological data was analyzed with learning category as a factor and direct comparisons were made

between the two groups to investigate whether learning speed was related to neural responses to song in our two auditory processing areas of interest. Therefore, Kolmogorov-Smirnov tests were run to compare the multi-unit ARMs and adaptation rates of slow to those of fast learners, as well as to test for lateralization of ARM and adaptation rate measures within these groups.

RESULTS

Electrophysiological data was collected from 16 adult zebra finch subjects in this study, (11 males and 5 females). From these subjects a total of 228 responsive recording sites were histologically verified for placement in NCM or CMM. The analyses reported here focus on the 183 sites that were recorded during the awake state; analyses of the anesthetized data are not yet complete. Of these 183 sites, 84 were found to be in NCM and 99 were in CMM. The data from these sites were used to compute ARMs (trials 2-6) and multi-unit adaptation rate (trials 6-25) for further analysis. In addition, the multi-unit recordings from these sites were spike-sorted to enable analysis of single-unit activity. In all, 55 single-units were isolated and used for single-unit firing-rate (trials 1-7), d-prime (trials 1-7) and adaptation rate (trials 1 to 25) analyses of NCM (31 single-units) and CMM (24 single-units) activity.

Effects of Training on Male Multi-unit Auditory Responses in NCM and CMM

Stimuli that the birds heard in operant training elicited auditory responses during electrophysiological recording that differed from responses to stimuli that were novel to these subjects. Training had an effect on ARMs and multi-unit adaptation rates in NCM and CMM of both male and female (discussed in a separate section below) subjects. In males, repeated-measure ANOVAs were performed, with the categorical factor of learning speed, to analyze the effect of training on ARMs (trials 2-6) and adaptation rates (trials 6-25) in NCM and CMM, separately.

NCM

In the NCM of males there was a significant main effect of training on ARMs ($F_{(2, 30)}$).

$_{104}) = 3.12, p < 0.05$). Bonferroni post-hoc tests revealed that this effect was driven by a significant difference between the mean ARM to GO stimuli ($M = 84.04 \pm 7.11$) and the mean ARM to NoGO stimuli ($M = 78.43 \pm 6.74$) ($p < 0.05$, **Figure 8A**). In the NCM of male subjects, multi-unit sites responded more strongly to positively reinforced GO stimuli than negatively reinforced NoGO stimuli. However, differences between reinforcement-predictive and novel stimuli were not significant in this area. In male NCM there was also a significant main effect of training on multi-unit adaptation rates ($F_{(2, 98)} = 8.69, p < 0.001$). This effect was driven by the slow adaptation to NoGO stimuli. Bonferroni post-hoc tests revealed that NoGO stimuli were adapted to significantly more slowly ($M = -0.34 \pm 0.06$) than GO ($M = -0.45 \pm 0.07$) and novel stimuli ($M = -0.47 \pm 0.07$) were ($p < 0.01$, **Figure 8C**). Slower adaptation (in NCM) to NoGO stimuli would suggest that these stimuli were more familiar to subjects during electrophysiological testing than novel or GO stimuli, perhaps due to the cognitive tactics used by the subjects to perform discriminations.

CMM

In the CMM of males there was a significant main effect of training on ARMs ($F_{(2, 114)} = 11.93, p < 0.0001$). Bonferroni post-hoc tests revealed that this effect was driven by significant differences between the mean ARM to novel stimuli ($M = 72.99 \pm 5.19$) and the means of those stimuli that were reinforcement-predictive. Both GO ($M = 81.89 \pm 6.20$) and NoGO ($M = 83.00 \pm 5.98$) trained stimuli evoked higher ARMs, in CMM, than novel stimuli ($p < 0.001$, **Figure 8B**). In male CMM there was also a significant main effect of training on multi-unit adaptation rates ($F_{(2, 114)} = 3.90, p < 0.05$). As in NCM, this effect in CMM was driven by slow adaptation to NoGO stimuli. Bonferroni post-hoc

tests revealed that NoGO stimuli were adapted to significantly more slowly ($M = -0.37 \pm 0.08$) than GO ($M = -0.47 \pm 0.09$) stimuli were ($p < 0.05$, **Figure 8D**).

Effects of Training on Single-unit Auditory Responses in NCM and CMM

Due to the limited number of isolated single-units in each auditory structure, single-unit analyses were conducted by pooling male and female data. The 55 single-units (31 in NCM and 24 in CMM) were used to analyze effects of training on firing-rate, adaptation rate and stimulus discrimination (d-prime values).

Firing-Rates and Adaptation Rates

Repeated-measure non-parametric Friedman's tests were performed on single-unit firing-rates and adaptation rates to determine the effects of training on single-unit activity (**Figure 9**). The single-units in NCM, showed no effect of training on firing-rates ($\chi^2_{(2, N=31)} = 4.06$, $p = 0.13$). In parallel, single neuron adaptation rates were unaffected by training in NCM ($\chi^2_{(2, N=31)} = 1.35$, $p = 0.5$, **Figure 10A**). However, in CMM, although the responses to GO, NoGO and novel stimuli did not differ based on their firing-rates, single-units did adapt to trained stimuli significantly different than they adapted to novel stimuli ($\chi^2_{(2, N=24)} = 2.33$, $p = 0.31$; $\chi^2_{(2, N=24)} = 8.58$, $p < 0.05$). Post-hoc Wilcoxon sign-rank tests revealed that this effect was driven by fast adaptation to novel stimuli ($M = -0.118 \pm 0.101$) by CMM single-units. Novel stimuli adapted significantly faster than both GO stimuli ($M = -0.0072 \pm 0.104$) and NoGO stimuli ($M = 0.0223 \pm 0.0975$) in this area, reflecting familiarity ($p < 0.05$ & $p < 0.01$, respectively, **Figure 10B**). Although these data did show an effect of familiarity, it is important to note that the single-unit adaptation data from the isolated sites in both NCM and CMM were not distributed normally (**Figure 10**).

Although previous studies have shown that a majority of NCM sites show stimulus specific adaptation, the single units isolated in this experiment were heterogeneous populations of both adapting and anti-adapting neurons (Chew et al., 1995).

D-prime values

Although trained-stimulus firing-rates were not significantly higher or lower than responses to novel stimuli, d-primes were calculated from single-unit spiking activity (as described in Methods) to test how responses to reinforcement-predictive stimuli differed from novel (unreinforced) stimuli. Kolmogorov-Smirnov tests were performed to compare the baseline (novel to novel) d-prime values to those calculated by comparing responses to trained songs to those evoked by novel songs, in each structure. A Kolmogorov-Smirnov test was also performed for each structure comparing baseline d-prime values to those attained by comparing responses to GO stimuli to those evoked by NoGO stimuli. In NCM, firing-rate responses to trained stimuli (GO and NoGO) were no more discriminable from responses to novel stimuli than responses to two novel stimuli were from one another (GO: $D = 0.258$, $p = 0.216$, **Figure 8A**; NoGO: $D = 0.161$, $p = 0.778$, **Figure 11B**). Firing-rate responses to GO stimuli were also no more different from responses to NoGO stimuli than responses to two novel songs were from one another ($D = 0.226$, $p = 0.363$, **Figure 11C**).

In CMM, however, d-prime values calculated by comparing single-unit spiking responses to trained stimuli to responses to novel stimuli showed more variation than those calculated by comparing the neural responses to two novel stimuli. Neural responses to GO stimuli ($M = 0.980 \pm 0.4967$) were significantly more discriminable from responses to novel stimuli ($M = -0.0453 \pm 0.180$) than baseline in this structure ($D =$

0.500, $p < 0.01$, **Figure 11D**). In addition, neural responses to NoGO stimuli ($M = 0.852 \pm 0.649$) were also significantly more discriminable from responses to novel than baseline ($D = 0.458$, $p < 0.01$, **Figure 11E**). Although the reinforcement-predictive stimuli showed responses distinct from responses to novel in CMM, responses to GO stimuli were no more different from responses to NoGO stimuli than responses to two novel songs were from one another ($D = 0.167$, $p = 0.861$, **Figure 11F**).

A final Kolmogorov-Smirnov test was performed to test whether baseline d-prime values were significantly different in the two auditory structures of interest. Although the single-unit firing-rate responses to two novel songs were slightly more distinct from one another in NCM ($M = -0.326 \pm 0.326$) than they were in CMM ($M = -0.0453 \pm 0.180$), there was not a significant difference between the baseline d-prime values in these areas ($D = 0.258$, $p = 0.283$).

Multi-unit Neural Responses to Operantly-trained and Socially-relevant Auditory Stimuli in Female NCM and CMM

In female subjects the neural responses in NCM and CMM to both socially-relevant and operantly-trained stimuli were recorded in order to investigate the similarities between the neural representations of stimuli trained with these differing methods. Both socially-relevant and operantly-trained stimuli evoked neural responses significantly different from those evoked by novel stimuli. In NCM, there were effects of stimulus category on ARMs as well as multi-unit adaptation rates. In CMM, stimulus category had an effect on the speed of multi-unit neural adaptation but not absolute response magnitudes.

ARMs

When a non-parametric Friedman's test was performed on the data from multi-unit sites in NCM, a significant effect of auditory stimulus category was revealed ($\chi^2_{(5, N = 18)} = 33.87$ $p < 0.000001$). Post-hoc Wilcoxon sign-rank tests showed that this effect applied to both socially-relevant and operantly-trained stimuli. ARMs were significantly lower in response to tutor-song stimuli ($M = 32.47 \pm 8.26$) than they were to novel song stimuli (64.32 ± 5.31), suggesting that socially-relevant stimuli were recognized by the neurons in NCM ($p < 0.01$, **Figure 12A**). Probe stimuli ($M = 50.71 \pm 5.15$) also evoked weaker ARMs than GO (67.19 ± 5.28), NoGO (75.16 ± 5.90) and novel stimuli ($p < 0.01$, **Figure 12A**). In addition, in NCM ARMs were higher for NoGO stimuli than GO stimuli ($p < 0.01$, **Figure 12A**). However, neither trained song category evoked significantly different ARMs than novel stimuli did. Therefore, in NCM, there was an effect of valence but not reinforcement-predictive value on absolute responses to operantly-trained stimuli. A non-parametric Friedman's test was also performed on the multi-unit CMM ARMs data; the results of this test showed no effect of stimulus category (whether it was socially- or operantly-familiar) on auditory responses ($\chi^2_{(5, N = 19)} = 5.12$, $p = 0.40$, **Figure 12B**).

Adaptation Rates

A non-parametric Friedman's test was also performed on the female NCM multi-unit adaptation rate data; results from this test showed a significant effect of stimulus category on multi-unit rates of response adaptation ($\chi^2_{(5, N = 18)} = 13.51$, $p < 0.05$, **Figure 13A**). Post-hoc Kolmogorov-Smirnov tests revealed that both socially- and operantly-conditioned stimuli evoked adaptation rates significantly different than those elicited by

novel stimuli. The socially-conditioned stimuli, female subjects' mates' songs ($M = -0.685 \pm 0.131$), were adapted to significantly faster than novel ($M = -0.450 \pm 0.0639$) and tutor ($M = -0.0327 \pm 0.155$) stimuli were ($p < 0.01$, **Figure 13A**). In addition, GO stimuli ($M = -0.283 \pm 0.0753$) were adapted to significantly more slowly than novel stimuli were by NCM multi-unit sites ($p < 0.05$, **Figure 13A**). A non-parametric Kruskal-Wallis ANOVA was conducted on the female CMM multi-unit adaptation data; this test also showed a significant effect of auditory stimulus category on neural responses ($H_{(5, N=183)} = 18.77$, $p < 0.01$, **Figure 13B**). Subsequently, post-hoc Kolmogorov-Smirnov tests were performed to isolate significant differences. Like in NCM, CMM multi-unit adaptation rates were faster for subjects' mates' songs ($M = -0.495 \pm 0.0694$) than for novel ($M = -0.225 \pm 0.0538$) stimuli ($p < 0.01$, **Figure 13B**). In addition to the pervasive effect of social conditioning on neural responses in this area, CMM neurons also showed an effect of operant conditioning. GO stimuli ($M = -0.0612 \pm 0.0806$) were adapted to, by these sites, faster than novel stimuli were ($p < 0.01$, **Figure 13B**).

Relationship between Familiarity of Reinforcement-predictive Stimuli and Probe Trial Responses

Subjects' behavioral responses to unreinforced probe trials were hypothesized to be related to how familiar those subjects were with the trained stimuli. Experimenters expected that subjects would respond to probe stimuli as GO stimuli if they had only truly learned to recognize the songs in the NoGO category (withhold pecking), and vice versa. Due to the established phenomenon of SSA, songbirds have historically been believed to have neural memories for (or familiarity with) auditory stimuli as shown by

NCM neurons that adapt significantly more slowly to familiar stimuli than they do to novel stimuli. Therefore, it was expected that the number of responses to probe trials would be related to the rate at which NCM (and CMM) neurons adapted to trained stimuli. To investigate this hypothesis a correlation was run between the difference in the speed of multi-unit adaptation to GO and NoGO stimuli and the percentage of probe trials that received responses (**Figure 14**).

To first test whether animals were differentially familiar with GO and NoGO stimuli, Wilcoxon sign-rank tests comparing multi-unit adaptation rates to GO and NoGO stimuli, were run for each subject that participated in probe trials. These statistical tests revealed that 5 of the 9 subjects that received probe trials showed significantly different multi-unit rates of adaptation to GO and NoGO stimuli ($p < 0.05$, **Figure 14A**). Although the direction of this effect was not consistent for all subjects, this result does suggest that trained songs did differ in familiarity based on their valence. Further, when the difference between GO and NoGO adaptation rates for each bird was plotted in conjunction with a bird's probe trial responsiveness, there was a trend to correlation between these two measures ($r = 0.606$, $p = 0.08$, **Figure 14B**). Unexpectedly, birds with slower adaptation to GO stimuli than to NoGO stimuli (more familiarity) also responded to probe trials more frequently. In the same vein, subjects that adapted to NoGO stimuli more slowly than GO stimuli responded to probe stimuli infrequently.

Relationship between Speed to Acquire Auditory Discriminations and Resultant Multi-unit Auditory Responses

Subjects were divided into slow and fast learners, based on a median split of the

speed of acquisition of behavioral discrimination (number of days/ number of trials, see methods). The neurophysiological results showed in both NCM and CMM showed an interesting pattern of differences in auditory responses between the learning groups.

NCM

Unexpectedly, the speed to acquire auditory discriminations showed a relationship with the direction in which auditory responses were lateralized in NCM. Kolmogorov-Smirnov tests were performed on NCM ARM and adaptation rate data to compare the responses recorded in the left hemisphere to those recorded in the right for both groups. Animals that learned faster showed significantly higher ARMs in the left hemisphere ($M = 76.01 \pm 3.93$) than were observed in the right hemisphere ($M = 58.63 \pm 3.08$) in response to song stimuli ($D = 0.238$, $p < 0.01$, **Figure 15**). On the other hand, animals that learned the discriminations more slowly showed stronger responses to song in the right ($M = 72.47 \pm 5.79$) than in the left hemisphere ($M = 71.29 \pm 3.57$) NCM, but the difference was not significant ($D = 0.207$, $p = 0.11$, **Figure 15**). Multi-unit adaptation rates did not show this type of hemispheric interaction in NCM.

CMM

In CMM, Kolmogorov-Smirnov tests did not show significant lateralization of ARMs or adaptation rates within the fast or slow learning groups. However, the fast and slow learners differed significantly from one another, in both their ARMs and multi-unit rates of adaptation. Absolute responses to song stimuli were stronger in faster learners ($M = 90.95 \pm 4.80$) than in slower learners ($M = 64.07 \pm 3.45$) ($D = 0.392$, $p < 0.0000001$, **Figure 16A**). In parallel, CMM multi-units adapted more quickly to song stimuli in

faster learners ($M = -0.379 \pm 0.531$) than they did in slower learning subjects ($M = -0.160 \pm 0.394$) ($D = 0.213$, $p < 0.05$, **Figure 16B**).

DISCUSSION

Auditory Responses to Operantly-trained Stimuli

To assess neural representations of the behavioral-relevance induced by training in two avian forebrain auditory structures, NCM and CMM, electrophysiological responses to operantly-trained songs were compared to those of novel songs, using absolute responses, firing-rates and adaptation rates. Both of these auditory processing areas showed effects of training on neural responses to song playback. These effects were detected in the ARMs, multi-unit adaptation rates, firing-rates and single-unit adaptation rates of both male and female zebra finch subjects. Therefore the general hypothesis that operant training produces and modulates long-term neural memories for reinforcement-predictive stimuli in sensory regions of the adult brain was correct. However, the patterns of differential neural activity for these stimuli differed between the two brain regions, between the sexes and also between hemispheres in different subject groups defined by learning performance.

Multi-unit Responses to Operantly-trained and Novel Stimuli

In NCM, absolute responses to operantly-trained stimuli were more driven by the valence of those stimuli than by their reinforcement-predictive value. In both males and females GO and NoGO stimuli induced significantly different levels of responding from one another ($GO > NoGO$ in males, $GO < NoGO$ in females), but neither category evoked higher or lower responses than novel stimuli. On the other hand, in CMM, training induced an increase in absolute responding to all reinforcement-predictive stimuli (GO and NoGO) with respect to novel stimuli in male subjects. In females, there were no significant differences in ARMs to reinforcement-predictive stimuli as compared

to novel stimuli. The male data agree with the results from Gentner and Margiolash, (2003) which showed stronger responses to reinforced stimuli than to novel ones in starling CMM (of both males and females). However, contrary to the results seen in Gentner and Margiolash (2003), the results from this experiment showed no significant differences between GO and NoGO stimuli ARMs in CMM.

As was expected, the training experience affected the neural responses to auditory stimuli in NCM and CMM in different ways, supporting the hypothesis that the two auditory areas serve different roles in auditory processing. It is possible that CMM activity is important for recognizing which stimuli are important to an individual (based on reinforcement history), while the neural responses in NCM reflect stimulus familiarity. Regardless, both areas seem to be important for auditory discrimination learning and it is likely that lesions to either area that would make the development of the neural representations of these stimuli impossible would also impair subject's ability to discriminate between two auditory stimuli.

The multi-unit adaptation data was less internally consistent than the results of the ARM analysis. Although there were effects of operant training on the speed with which NCM and CMM multi-neuron sites decreased responding over repeated stimulus presentations, the effects were very different in males and females. In males, NoGO stimuli were adapted to the most slowly, both GO and novel stimuli showed faster adaptation than NoGO stimuli in NCM and in CMM adaptation was significantly faster for GO stimuli than for NoGO stimuli. In female subjects however GO stimuli were adapted to significantly more slowly than novel stimuli, in NCM. In CMM, however, the significant difference lay between GO stimuli and probe stimuli.

In both sexes, the significant differences in multi-unit adaptation rate were driven by slow adaptation to one of the stimuli (either GO or NoGO); in females GO songs were adapted to slowly and in males NoGO songs were adapted to the most slowly. If these results are interpreted in terms of SSA relating to familiarity, the songs that are adapted to most slowly should be the songs that are the most familiar to subjects. Therefore, although it is hard to draw conclusions about this sex difference due to limited sample sizes, it is possible that males were more familiar with trained songs of negative valence (reinforced with punishment) and females were more familiar with trained songs of positive valence (reinforced with reward). This may be true if the sexes are differentially motivated by reward and punishment; if reward is, in fact, more motivating for females and punishment more motivating for male subjects, the pattern of results could be explained by motivational/attentional factors. Although why a motivational sex difference between male and female zebra finches would exist is unclear, sex differences in sensitivity to reward and punishment have been observed in other animals, including humans (van der Bos, Jolles, Knaap, Baars & Visser, 2012; Li, Huang, Lin & Sun, 2007).

Single-unit Responses to Trained and Novel Stimuli

Contrary to the hypotheses, single-unit firing rate responses to trained stimuli were neither faster nor slower than firing rate responses to novel stimuli in NCM and CMM. This may be because of the possibly lower sensitivity of the spike rate measure, the smaller sample, or because spikes that were isolable as single-units are not representative of the larger multi-unit population. However, when firing rates were used to calculate d-primes to compare responses to trained songs to those of novel songs, single-unit responses to GO and NoGO stimuli were more discriminable from responses

to novel stimuli than responses to two novel stimuli are from one another, at least in CMM. The responses to GO and NoGO stimuli were more variable, and somewhat stronger than responses to novel stimuli in this area. Interestingly, this effect was not seen in the d-prime data from NCM. In fact, neural responses to two novel conspecific songs were slightly (but not significantly) more different from one another (at baseline) in NCM than they were in CMM. This result is consistent with the results from Gentner et al. (2004) which suggest that NCM's role in auditory processing is primarily distinguishing and encoding the familiarity of conspecific vocalizations, while CMM's role is storing and retrieving behavioral relevance of conspecific vocalizations.

The analyses of single-unit adaptation rates also reflected effects of training on neural responses in CMM: novel stimuli were adapted to significantly faster than either GO or NoGO stimuli. In NCM, however, there was no effect of training on single-unit adaptation rates. The slight effects of training on single-unit adaptation rates in NCM and CMM are surprising because multi-unit adaptation rates had been strongly influenced by training as well as training valence. However, any effects of training on single-unit data may have gone undetected due, in part, to the low number of isolated units in each area (31 in NCM and 24 in CMM) as well as the fact that male and female data were collapsed in single-unit analyses (to increase n). The effects of training on multi-unit ARMs and adaptation rates were in different directions for male and female subjects, and therefore collapsing single-unit data across sexes may have hidden the single-unit effects that paralleled those multi-unit ones.

It has previously been shown that adult starlings of both sexes show distinct neural representations of operantly reinforced stimuli in anesthetized electrophysiological

testing (Gentner & Margoliash, 2003; Thompson & Gentner, 2010). However, starlings are ‘open-ended’ learners; they incorporate new syllables into their songs post-crystallization. The current experiments were conducted in zebra finches and may differ because zebra finches are ‘close-ended’ learners. Members of this species sing a less dynamic song than starlings, consisting of stable motifs that do not change post-crystallization. Adult zebra finches are no longer able to learn to produce or incorporate new syllables in their own songs; therefore it is probable that they form auditory memories of songs in adulthood primarily in order to recognize others. Our results indicate that both male and female adult zebra finch subjects were able to form long-term neural representations of reinforcement-predictive stimuli in the auditory processing areas of interest. When zebra finches associate behavioral outcomes with auditory stimuli as they learn to perform behavioral auditory discriminations, these associations cause plastic changes in the way those stimuli are represented in sensory processing areas of the avian forebrain.

Auditory Responses to Socially-relevant Stimuli

To further assess neural representations of the behavioral-relevance induced by training, electrophysiological responses to socially-relevant songs were compared to those of novel songs, using multi-unit absolute responses and adaptation rates, to better understand the activity of neurons during recognition of behaviorally relevant stimuli. Once again, neural responses were recorded in NCM and CMM, but only in female subjects. Both of these auditory processing areas showed memory for the social stimuli, mate’s and tutor’s songs. Therefore, the hypothesis that social interaction (specifically

mating) produces long-term neural memories for reinforcement-predictive stimuli in adult female zebra finches was correct.

The data collected in this study replicated previous findings showing a neural memory for tutor's song in the NCM of females (Yoder, 2012). Absolute response magnitudes were significantly weaker in response to tutor song playback than they were in response to novel stimuli in NCM. However, multi-unit adaptation rates were no different for tutor song than they were for novel stimuli, contrary to what was found in the Yoder (2012) study. In addition, no effects of social-relevance were detected in the ARMs or adaptation rates of CMM sites in female subjects. This result was surprising because ZENK is induced in CMM during tutor song playback in the CMM of females (Terpstra et al., 2006). These deviations from prior results may be explained by the limited number of females recorded in our study; although we proposed to use 9 females, due to time constraints only 5 were trained and recorded from. Hence there may be a neural memory for tutor song in CMM as well as the adaptation rates of NCM that has not been detected here, but would be seen with a larger female sample size.

A novel finding of this study was the existence of a neural memory for females' mates' songs in both NCM and CMM. Although responses to novel and mate's songs did not differ in ARMs, multi-unit adaptation rates were significantly faster in response to mate's song than they were in response to novel stimuli in both NCM and CMM. In both of these areas the neural adaptation to females' mate's songs was the fastest adaptation observed among all of the stimuli presented. Although fast adaptation is typically associated with stimulus novelty in NCM, a subject's mate's song was clearly not novel to the subject, and in fact, had been heard many times. In addition, no other stimulus

class showed significantly faster adaptation than novel (other than mate's song) so this difference is unlikely due to unusual responses to novel stimuli. Therefore, this fast adaptation of neural responding must be explained in some other way than novelty. Mate's song was adapted to faster than novel songs in both NCM and CMM although neither area showed ARMs to mate's song that were significantly different than those to novel songs (mate's song evoked slightly lower ARMs than novel in NCM and slightly higher ARMs than novel in CMM). The fast degradation of neural responding to mate's song may be due to an extinction-like rapid loss of behavioral relevance of the subject's mate's song due to its playback in the electrophysiological apparatus, in a context where mate's song is no longer associated with the presence of the subject's mate. Regardless of the reason for the fast adaptation to mate's song, though, it is clearly held in a special class (by the neurons of these auditory processing areas) that distinguishes it from novel stimuli. In addition, in NCM, the mate's song was adapted to significantly faster than tutor song, suggesting that the neurons of this area recognize and respond to these two social stimuli in a fundamentally different way. The neural memory for mate's song detected in this study is supported by the Woolley and Doupe (2008) evidence showing that females express higher levels of ZENK, in NCM, after the playback of their mate's song than they do after the playback of novel song stimuli. The neural memories for tutor song that are formed during a subject's critical period through sexual imprinting may develop differently than the neural memories for the songs that become behaviorally relevant during adulthood.

From these results, we can conclude that social relationships change the way an individual's vocalizations are processed by their mate, in a monogamous species that

exhibits bi-parental care. Although neural recognition of mate's vocalizations have been shown in the NCM of females for long calls, this experiment further demonstrates that that the neurons in that area (and CMM) also recognize the songs of a subject's mate (Menardy et al., 2012). For female subjects, being able to quickly identify the song (or call) of their mate, is important not only for producing more offspring, but also for being able to care for their current clutch. The neural memories for mate's song that exist in NCM and CMM may be part of a larger individual recognition circuit that underlies behavior, and the behavioral preferences for song seen in females.

Relationships between Neural Responses in Auditory Areas and Operant Behavior: Probe Trial Responses and Learning Speed

To assess the relationship between learning behavior and the resultant neural representations of training stimuli, electrophysiological responses to GO, NoGO and novel stimuli were analyzed according to two independent behavioral measures: a subject's speed to acquire the auditory discriminations and a subject's probe trial response frequency. As hypothesized, learning speed did have an effect on the neural responses to song in both auditory processing areas NCM and CMM (see below). In addition, the pattern of behavioral responses to novel unreinforced probe songs showed a relationship to the comparative familiarity of the GO and NoGO stimuli (the difference between GO and NoGO multi-unit adaptation rates). Although this latter result demonstrates a relationship between cognitive tactics of auditory discrimination performance and neural adaptation rate to those stimuli, the result was in an unexpected direction.

Probe Trials

Probe trials were utilized during operant training to test whether animals had learned to recognize both or only one of the song categories, in an effort to identify the cognitive tactics subjects employed to perform auditory discriminations. In addition, multi-unit adaptation rate data was used to assess the familiarity of the song categories (measure as SSA). Then the neural data and probe trial data were analyzed together to reveal possible relationships. We hypothesized not only that the difference in familiarity of GO and NoGO stimuli would be related to the number of responses to probe trials individual subjects exhibited, but also that the subjects that responded to probe stimuli the most frequently (as GO stimuli) would show slower adaptation (greater familiarity) to NoGO stimuli as compared to GO stimuli. Subsequently, it was expected that the subjects that responded at chance levels to probe stimuli (50%) would have the most similar GO/NoGO adaptation rates.

A correlation concluded that the subjects who showed faster adaptation to GO songs than NoGO songs also showed more behavioral responses to the probe trials during training. Although this strong trend indicates that the neural and behavioral measures are fundamentally related to one another, as hypothesized, the relationship between these measures was in the opposite direction of what was expected. The stimulus category that showed the slowest adaptation (i.e. seemed the most familiar to the subject) was also the category into which subjects placed the novel unreinforced probe stimuli.

Animals that showed the slowest adaptation to GO stimuli responded to the probes most frequently. This finding goes directly against Morisako and Okanoya's theory that the animals that learn to recognize only one category of stimuli will place all

other (novel) stimuli in the less familiar category (2008). On the contrary, our results indicate that novel probe stimuli were placed into the category (GO or NoGO) with which subjects were more familiar. An explanation for this unexpected result may have to do with motivation and the internal salience of reward and punishment for individual subjects. If a subject is more motivated by food reward than the darkness punishment he/she would likely become more familiar with the song stimuli associated with that outcome than others. In the same vein, when a novel stimulus played for that subject, the animal would be more likely to attempt to peck and possibly get a food reward than they would be to withhold pecking in order to avoid possible darkness. Further experimentation that tests whether the length of the reward and punishment periods affect the frequency of probe responses, or perhaps even the multi-unit rates of adaptation to trained stimuli in NCM and CMM, may help to verify whether or not motivational variables affect the familiarity of GO and NoGO stimuli. However, even without a complete explanation of these results, we can conclude that learning, and the plastic neural changes that occur during the learning process, are specific to an individual and affected by (sometimes unforeseen) subject variables.

Learning Speed

The other behavioral measure that was analyzed was the speed with which subjects learned the auditory discriminations. Subjects were median-split into two groups, by their average speed to reach behavioral criterion (days to criterion) on the operant task. Then ARMs and multi-unit adaptation rates to song stimuli in both NCM and CMM were compared between groups. By grouping subjects into groups according to their speed to reach criterion and pairing discriminatory accuracy with the memory for

song in NCM and CMM, we sought to find a neurophysiological difference (in ARMs or adaptation rates) between subjects that master the task quickly and those that do not. In CMM, both ARMs and adaptation rates were affected by the learning group. In NCM, the degree to which ARMs were lateralized differed between the two learning groups.

The animals that learned faster exhibited higher ARMs (trials 2-6) and faster multi-unit adaptation (trials 6-25) in CMM than the animals that learned more slowly. The stronger neural responses to auditory stimuli and faster stimulus specific adaptation occurred for all stimulus classes (GO, NoGO, novel, probe, etc.) in this area. This parallel effect of learning speed on auditory responses may reflect enhanced neural plasticity in the faster learning individuals; these individuals not only showed stronger neural responses but also faster cessation of those responses. The neural differences between fast and slow learning subjects in ARMs and adaptation rates may, in fact, have contributed to how those individuals differed in their speed to reach criterion in the first place.

Auditory responses are lateralized in NCM and affected by environmental changes (Phan & Vicario, 2010; Yang, 2012). However, the effects of training were similar in the left and right NCM for the majority of the analyses conducted. However, fast and slow learning groups showed unexpected differences in the degree to which their ARMs were lateralized in the NCM data. In fast learners ARMs were stronger in the left NCM than in the right. Slow learners, however, showed no significant lateralization, and actually exhibited a trend for stronger responses in the right hemisphere than in the left. Therefore, either the experience of successful learning causes responses in the left hemisphere of NCM to increase while the responses in the right hemisphere of NCM

decrease or zebra finch subjects that are more left lateralized for auditory responses learn auditory discriminations more easily. Although the reason for this interaction between NCM lateralization and the speed of learning is unclear, this result does suggest that the two hemispheres of NCM serve different roles in auditory discrimination learning. In addition, this result may be consistent with previous results showing that birds with more neurogenesis in left NCM show better tutor song copying, and therefore superior song learning (Tsoi et al, 2012). Further study into the relationship between auditory (recognition) learning, lateralization and neurogenesis in the songbird may help to identify variables that produce successful processing of communication signals. Ultimately, this research approach may have valuable implications for understanding deficits in the processing of communication signals such as language aphasia seen in humans.

Exploring Birdsong in a Social Context: Using Operant Training as a Model

Although much of the songbird literature focuses on how animals learn to produce vocal communication signals by listening to and imitating a tutor, this part of the study was designed to investigate how receivers process the communication signals of the individuals with whom they socially interact and how they interpret the appropriate behavioral responses to those signals. The field of birdsong is uniquely advantageous for this type of study because the auditory cues exchanged between conspecifics can produce strong behavioral outcomes, such as fighting and mating. The female subjects used in our experiment were socially and operantly conditioned to associate male song with behavioral outcomes. After cohabiting with their mate and operant training with other

stimuli, female subjects' neurophysiological responses to reinforcement-predictive and mate's song stimuli were assessed. We expected that the absolute responses and adaptation rates of NCM/CMM responses would show similar patterns in response to these two types of stimuli, when compared to novel and passively familiar songs, supporting the use of operant conditioning as a model for pairing behavioral relevance with sensory cues that identify individuals as occurs in social interactions between animals. We found that, although subjects showed neural memories for both operantly and socially trained stimuli, the neural memories for mate's song were quantitatively different from the neural memories for the GO/NoGO songs. In fact, the effects of social and operant conditioning on neural responses to auditory stimuli were in opposite directions. Multi-unit adaptation rates were faster for mate's song than they were for novel stimuli, but GO and NoGO stimuli showed slower adaptation than novel stimuli in NCM and CMM. In addition, although responses to mate's song were significantly different from responses to novel stimuli in both NCM and CMM, GO stimuli showed responses significantly different from novel only in NCM, and NoGO responses were not different from novel at all. Therefore, although both methods were successful in inducing a neural memory for auditory stimuli in sensory processing areas of adult songbirds, operant and social conditioning clearly developed those memories in different ways. Furthermore, social conditioning was actually more successful in producing lasting, detectable representations of auditory stimuli. For this reason, one conclusion is that operant auditory discrimination training may not be a good paradigm for studying the neural correlates of social recognition of communication signals. This conclusion is consistent with recent neurophysiological studies of social auditory stimuli

(vocalizations) in bullfrogs, cats, monkeys, humans and mice which show different neural responses to natural vocal stimuli in ethological contexts than are evoked by: artificial auditory stimuli (Rieke, Bodnar & Bialek, 1995; Attias & Schreiner, 1998; Escabi, Miller, Read & Schreiner, 2003), species non-specific vocalizations (Romanski and Goldman-Rakic, 2002; Fecteau, Armony, Joannette & Belin, 2005) and behaviorally irrelevant stimuli (Liu, Linden & Schreiner, 2006; Liu & Schreiner, 2007). Thus, further experimentation into how neural representations of auditory stimuli used for individual recognition develop will need to focus on the social variables involved in songbird interactions.

Subsequent to these studies, as neural memories for operantly and socially trained stimuli have been documented in NCM and CMM, experiments exploring the mechanisms by which those neural memories are developed should be conducted. In starlings, roles for inhibition and attention have been implicated in the development of neural representations of operantly-trained stimuli (Thompson, Jeanne & Gentner, 2013; Knudsen & Gentner, 2013). When inhibition is blocked, neural responses to ‘GO’ and ‘NoGO’ auditory stimuli become more similar (in NCM) than they typically are (Thompson et al., 2013). In addition, the levels of estradiol in the brain, and more specifically locally in NCM, have also been established as modulators of auditory processing in zebra finches. These auditory areas of songbirds express estrogen receptors and estradiol levels increase in males during social interactions, including hearing song (Saldaha et al., 2011, 2000; Remage-Healey et al., 2008). Further, estradiol blockage within the avian forebrain auditory nuclei impairs neural discrimination and memory formation of song in males, perhaps by impairing inhibitory transmission in these areas

(Remage-Healey et al., 2010; Tremere and Pinaud, 2011; Yoder, Lu, & Vicario, 2012; Tremere, Jeong & Pinaud, 2009). Therefore, it is possible that further experiments that antagonize estradiol effects during social interactions between mates and during auditory discrimination training may block the neural memory formation and accurate song recognition seen in this study.

General Conclusions:

Although the results from this study indicate that operant conditioning is not likely to be a valid model for social recognition of vocalizations in zebra finch subjects, it is demonstrably useful for inducing neural recognition for otherwise irrelevant or unimportant auditory stimuli. Our results indicate that animals show different neural representations for those sensory stimuli that they can discriminate behaviorally. Furthermore, the effects of training on auditory responses (ARMs, adaptation rates, single-unit firing rates, d-primes), consistently differed between the regions of interest in this study: NCM and CMM. As NCM and CMM have reciprocal projections, it is likely that they interact heavily with one another, yet the results of our experiments reliably found that CMM responses were more often influenced by the reinforcement-predictive values of auditory stimuli, while NCM responses were driven by stimulus familiarity. GO/NoGO conditioning is a learning paradigm that causes plastic changes in the neural activity of the sensory areas that are involved in the perception of the trained stimuli. Chronic neurophysiological recording in the avian auditory forebrain during operant conditioning may, therefore, be useful for identifying neural correlates of sensory learning.

In addition, the neurophysiological differences between subjects that learn faster and slower provided insight into what factors produce better learners. Subjects with neural responses, in CMM, that changed more rapidly (starting at a higher magnitude and quickly adapting over repeated unreinforced stimulus presentations) also learned more quickly, showing both neural and behavioral plasticity. Interestingly, these fast-learning subjects also showed higher auditory responses in NCM of the left hemisphere, which is unexpected because most untrained zebra finches tested in the laboratory show higher responses in the right hemisphere (Phan & Vicario, 2010). Therefore, left-lateralized neural activity may also enhance accurate perception of sensory stimuli. This intriguing aspect of hemispheric asymmetry may also be reflected in a recent study in our laboratory showing that birds that make better copies of their tutor's song have higher levels of adult neurogenesis in left than in right NCM (Tsoi et al., 2012).

In addition, the effects of training on auditory responses detected in this experiment depended on the sex of the subject being tested. Published experiments of this type showed similar effects of training on auditory responses in male and female starlings (Gentner & Margiolas, 2003; Thompson & Gentner, 2010); however, zebra finches differ from starlings in that they have sex differences in song production: although male and female starlings sing, only male zebra finches produce song. This sex difference in production of learned vocalizations may produce a sex difference in auditory perception and memory for learned vocalizations (Vicario, Naqvi & Raksin, 2001; Terpstra et al., 2006). The effects of group differences (sex and learning speed) demonstrate that pre-existing subject variables can interact with the learning process, and affect the neural representations of learned auditory objects.

Finally, this study supports recent work showing that learned discriminations affect the neural responses of primary sensory areas of the brain, (Gilbert, Li & Piesch, 2009; Gilbert & Sigman, 2007), even extending out to the sensory periphery, (Kass, Moberly, Rosenthal, Guang & McGann, 2013). NCM and CMM are sensory areas only 3-4 synapses beyond from the avian auditory thalamus, yet their activity is modulated in a way that reflects both the history of exposure to a stimulus and its behavioral relevance. It is too soon to know how much of this modulation arises locally and how much originates from “top-down” processes. Nonetheless, the current results and those from other systems challenge the classical view that the role of sensory structures is simply to encode and process stimulus characteristics.

Appendix

	Types of Analyses	GO, NoGO and Novel Stimuli	Probe Stimuli	Mate and Tutor Stimuli
Male Subjects	Multi-unit	11 subjects	4 subjects	-
	Single-unit	8 subjects	-	-
Female Subjects	Multi-unit	5 subjects	5 subjects	5 subjects
	Single-unit	5 subjects	-	-

Table 1: Experimental Subjects and Analyses.

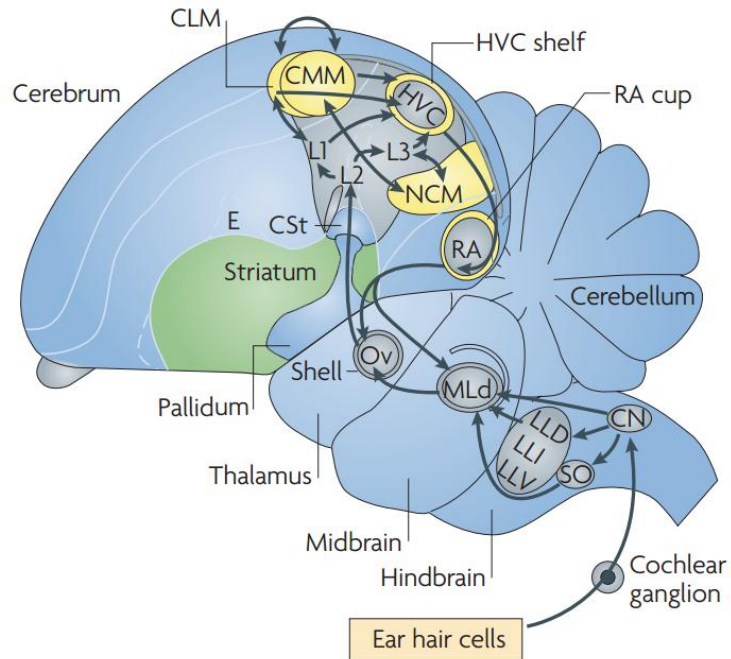


Figure 1. Anatomy of the Bird Song Auditory System. Primary auditory area Field L2 (probable homolog of A1, layer 4) projects to L3 and L1 which in turn project to higher auditory areas caudomedial nidopallium (NCM) and caudal mesopallium (CMM), shown in yellow. Diagram also shows projections into vocal motor areas not studied here. Figure from Bolhuis et al., 2010.

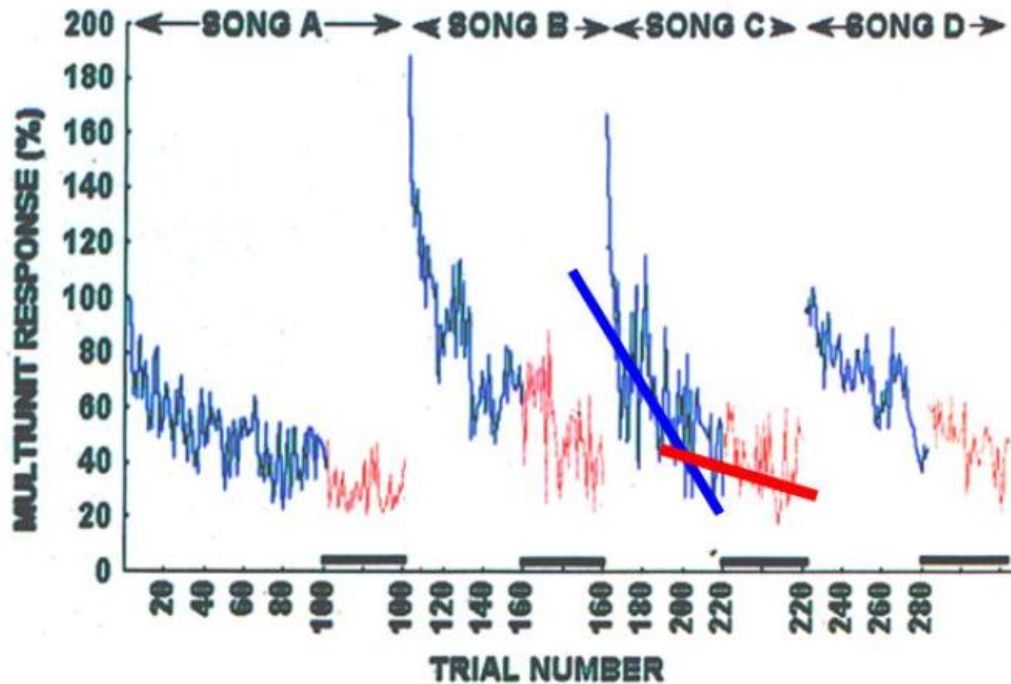


Figure 2. Stimulus specific adaptation (SSA). Stimulus-evoked multi-unit absolute response magnitudes decline over repeated presentations of an auditory stimulus. The reduction in neural responses to one stimulus does not cause an overall decrease in auditory responses to other stimuli, and is therefore called stimulus specific adaptation. This figure represents the SSA of a multiunit site in NCM to four different songs, presented sequentially. The blue traces show the initial multi-unit auditory responses to repetition of each song stimulus when it was novel. When each stimulus is again repeated (red traces) habituated responses are maintained. Adaptation is rapid when each stimulus is novel (blue line) and slow when it is familiar (red line). Thus, the rate of response adaptation reflects the novelty of an auditory stimulus and can be thought of as an index of a neural memory for each stimulus. In this study, multi-unit adaptation rates are calculated by dividing the slope of the regression line (over consecutive repetitions of a given stimulus) by the average ARM over those trials (to normalize for the response size of a recording site). Figure from Chew et al., 1995.

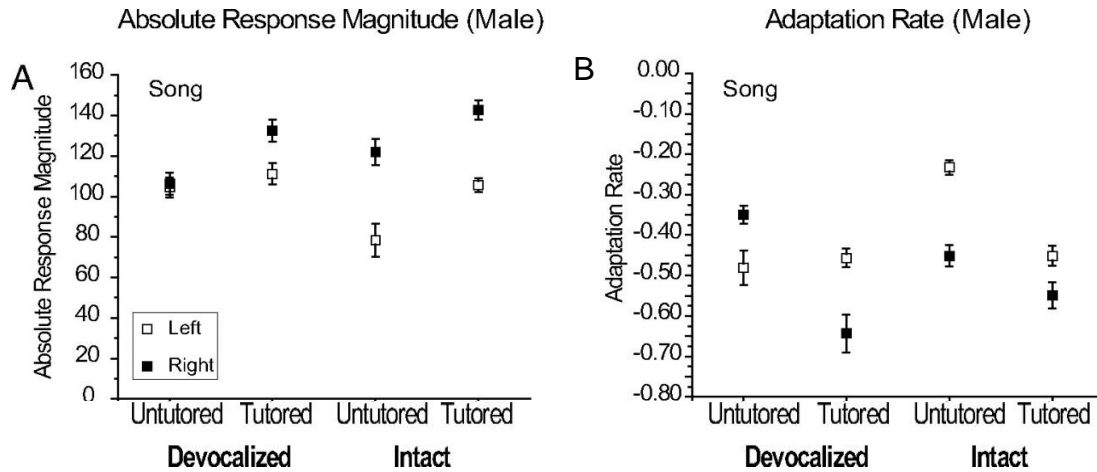


Figure 3. Lateralized auditory responses in NCM. ARMs and adaptation rates of multiunit responses to song in NCM of male zebra finches are lateralized. Males that have experienced song, either through tutoring or by hearing themselves vocalize, have: (A) stronger absolute responses to auditory stimuli in the right hemisphere (solid symbols) than in the left (open symbols) and (B) faster stimulus specific adaptation to auditory stimuli in the right hemisphere as compared to the left. Figure from Phan and Vicario, 2010.

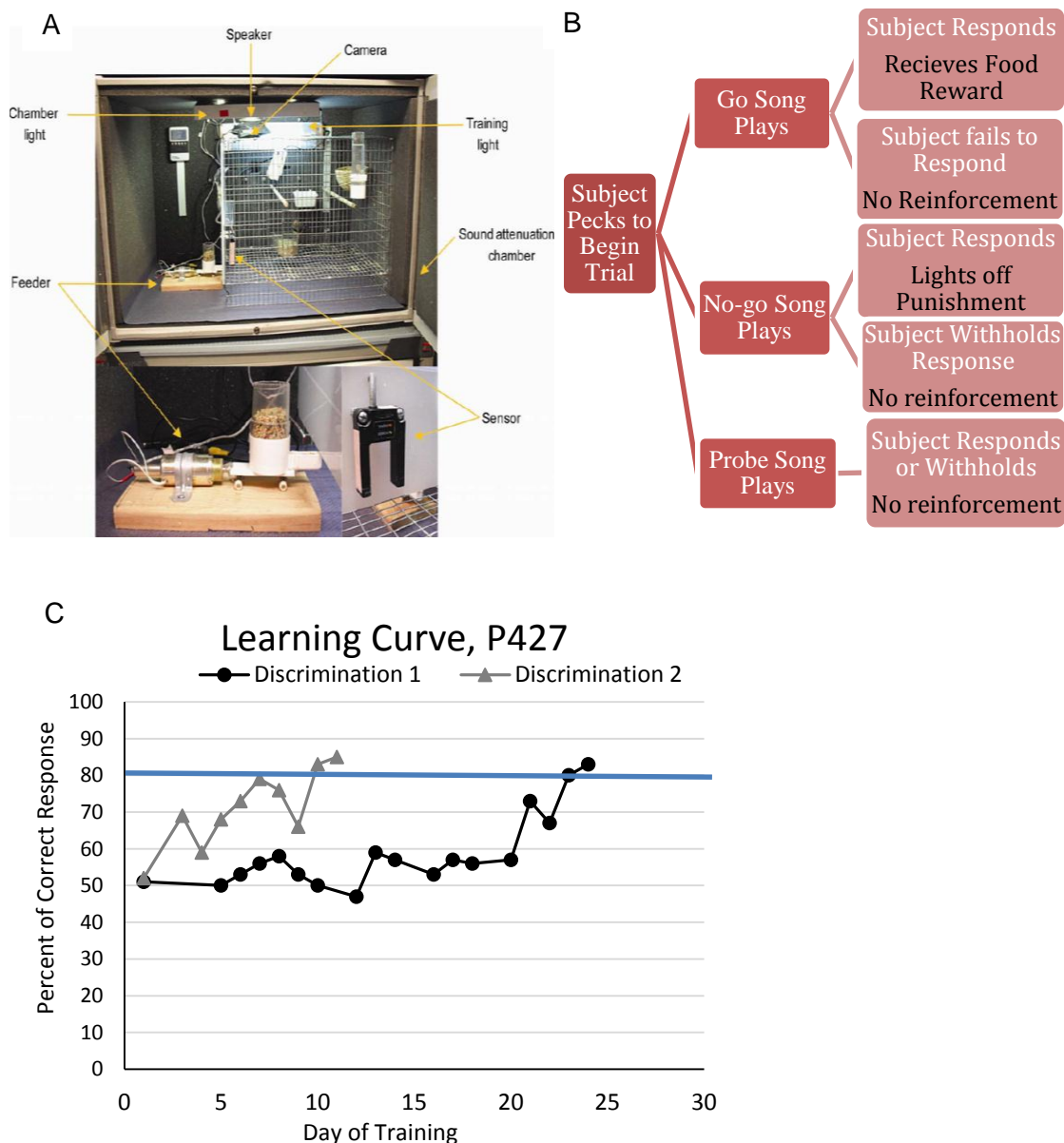


Figure 4. Operant Conditioning Paradigm, Apparatus and Learning Curve. (A) The apparatus used for operant conditioning, including: infrared sensor, feeder, chamber light, speaker, camera, training light and sound-attenuated chamber. Figure from Gess et al., 2011. (B) The operant conditioning GO/NoGO paradigm, including GO, NoGO and probe trials. Subjects were expected to respond for GO stimuli, and withhold pecking to NoGO for NoGO stimuli. Correct GO responses were positively reinforced and incorrect NoGO responses were punished. Responses to probe stimuli were observed but unreinforced. (C) The learning curve of a male subject, P427, for auditory discriminations 1 and 2. Subjects took on average (24.6 ± 13.9) days to reach behavioral criterion (2 sets at 80% accuracy) for their first discrimination, and (15.0 ± 9.27) on average to reach criterion on their second discrimination.

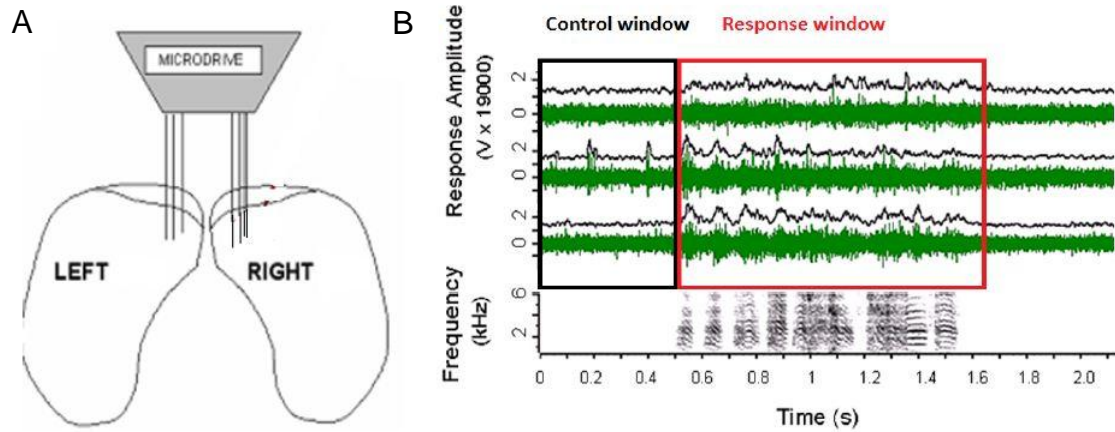


Figure 5. Electrophysiological apparatus and multi-unit recording. (A) Sixteen micro-electrodes are placed bilaterally in the avian auditory structures NCM and CMM (4 in each structure in each hemisphere). (B) Multi-unit recording of neural activity in NCM shown for 3 channels. Multi-unit recordings were quantified by taking the RMS of the response window during stimulus presentations and subtracting from that the RMS of the control window (500ms before stimulus onset). Multi-unit recordings were also spike-sorted to quantify single-unit activity.

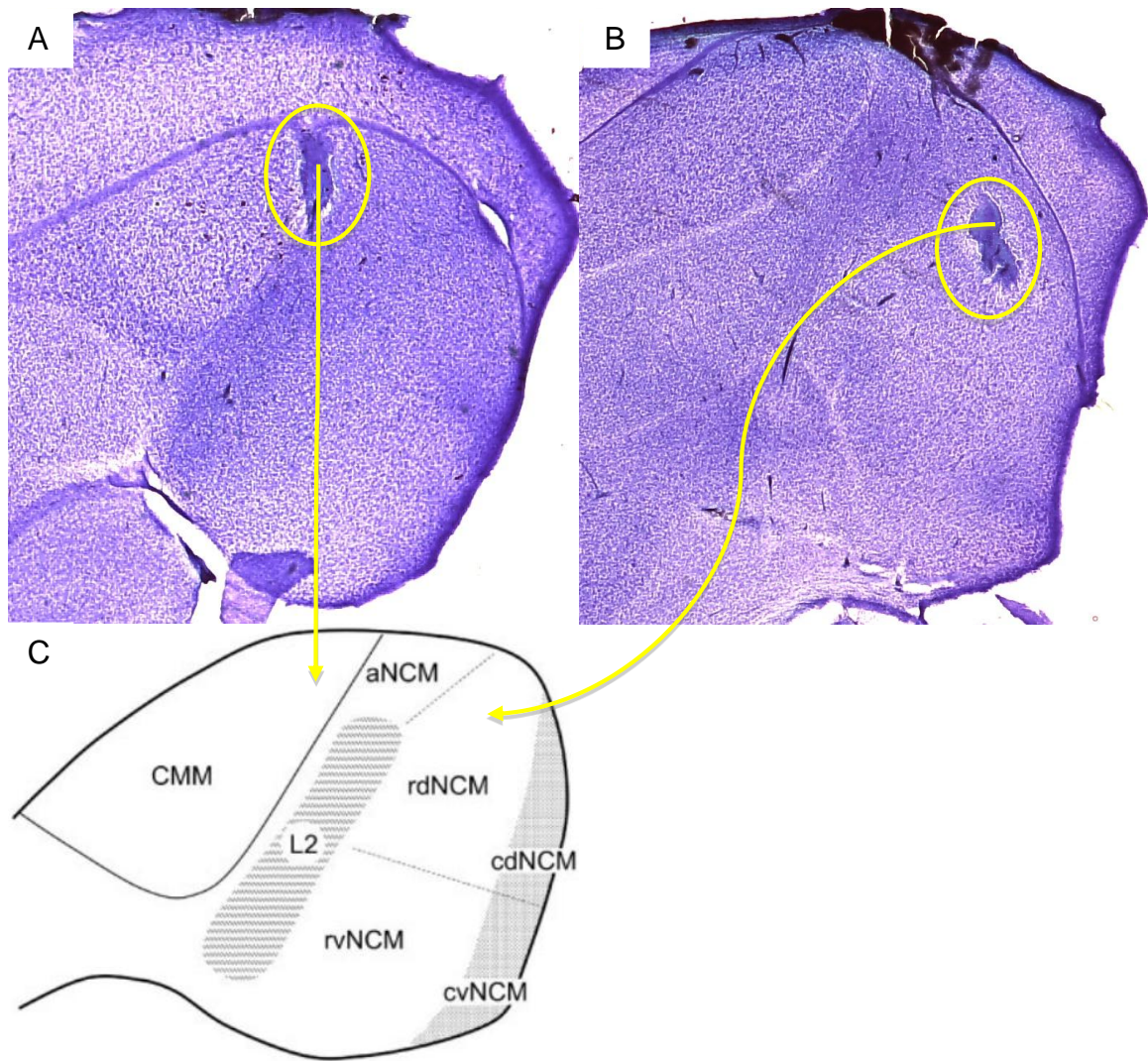


Figure 6. Histology and Electrode Placement in NCM and CMM. Sites used for data analyses were confirmed to be in either (A) CMM or (B) NCM by sending electrolytic current (20 μ A for 12 seconds) through electrodes at the conclusion of the electrophysiological experiment to produce lesions. Brains were then sectioned and slices were stained with cresyl violet and visualized under a light microscope to confirm placement. (C) Figure showing the boundaries of avian auditory areas (NCM, L2, CMM) from Sanford, Lange & Maney, 2010.

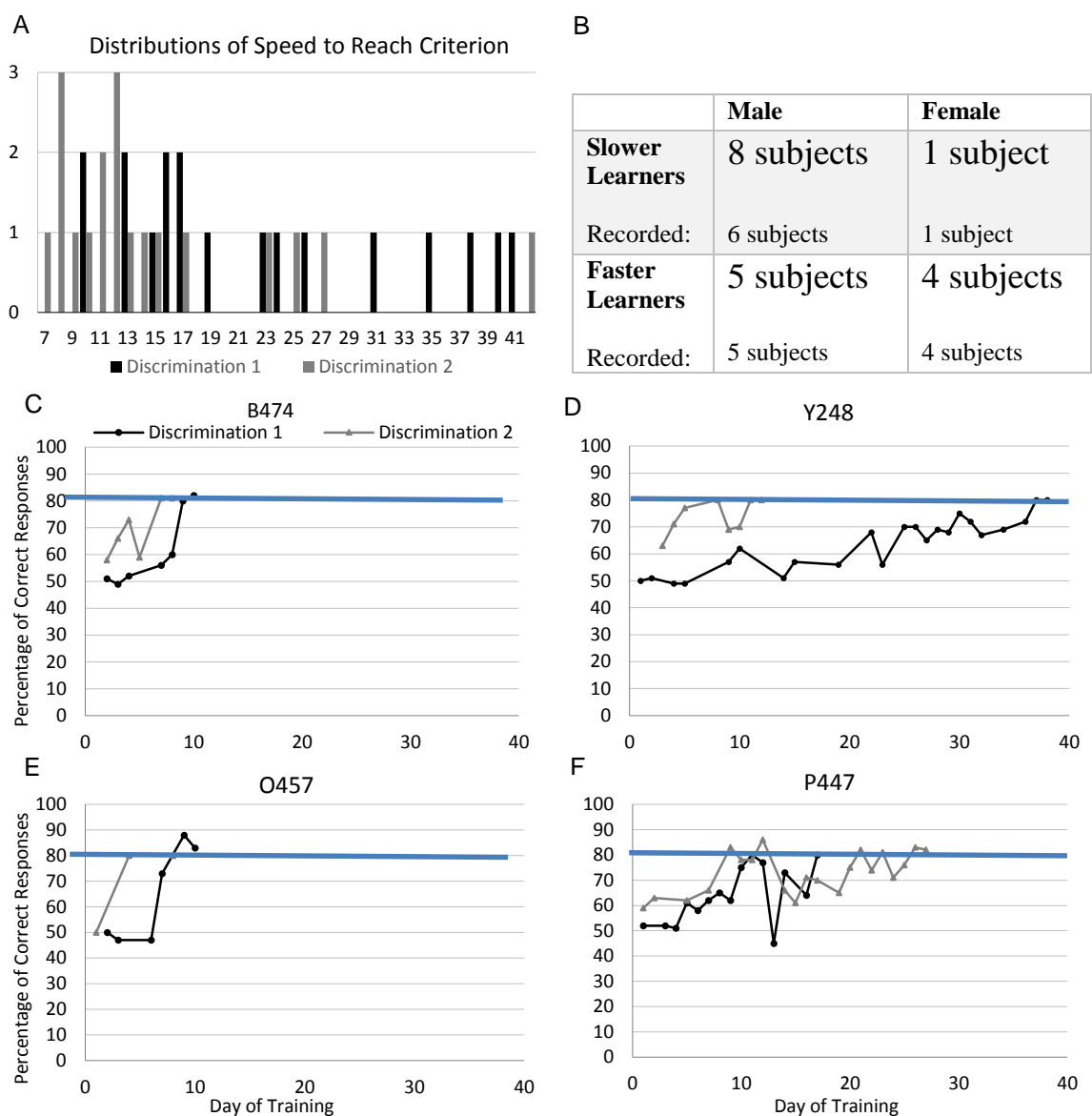


Figure 7. Speed of behavioral acquisition in males and females. Individuals vary in the speed with which they reach behavioral criterion (80% accuracy) on the operant GO/NoGO task. (A) The distributions of days to reach criterion for discriminations 1 and 2, for all subjects. (B) Table of subjects. The learning curves of the (C) fastest-learning male, (D) slowest-learning male, (E) fastest-learning female and (F) slowest-learning female subjects. Therefore, male and female subjects were median-split into two groups, (C&E) faster and (D&F) slower learners, by their average speed to acquire both auditory discriminations, as show in (B).

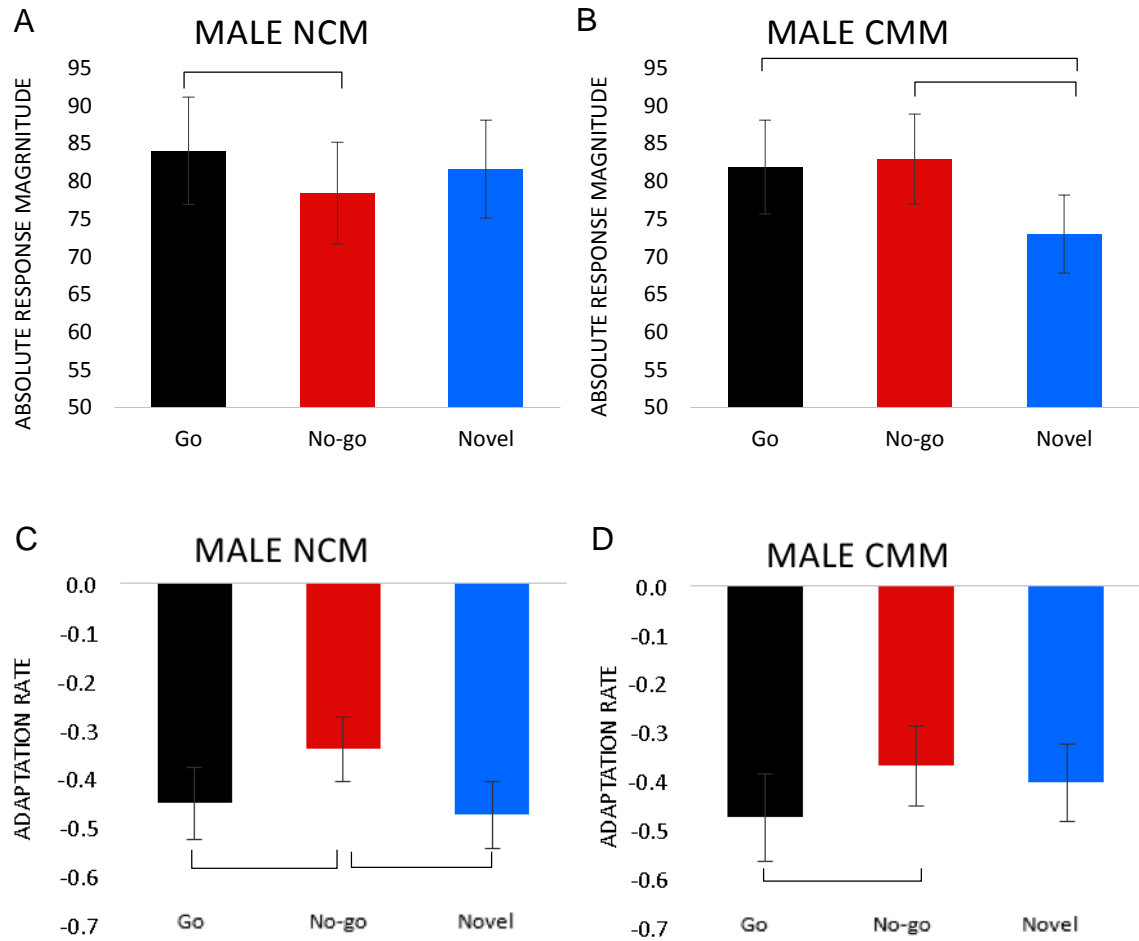


Figure 8. Effects of training on ARMs and multi-unit adaptation rates. The mean ARMs and adaptation rates of multi-unit recording sites in NCM and CMM for male subjects (n=11) in response to GO, NoGO and novel auditory stimuli. (A) There was a significant main effect of training on ARMs in NCM ($p < 0.05$, repeated-measures ANOVA) responses were stronger for GO stimuli than NoGO stimuli ($p < 0.05$, t-test). (B) There was also a significant main effect of training on ARMs in CMM ($p < 0.001$, repeated-measures ANOVA), here novel stimuli evoked lower ARMs than reward-predictive (GO and NoGO) stimuli did ($p < 0.001$ in both cases, t-test). In addition, multi-unit adaptation rates showed main effects of training in NCM ($p < 0.001$, repeated-measures ANOVA) and CMM ($p < 0.05$, repeated-measures ANOVA). (C) In NCM, NoGO stimuli were adapted to more slowly than GO ($p < 0.01$, t-test) and novel stimuli ($p < 0.01$, t-test). (D) In CMM, NoGO stimuli were adapted to more slowly than GO stimuli ($p < 0.05$, t-test).

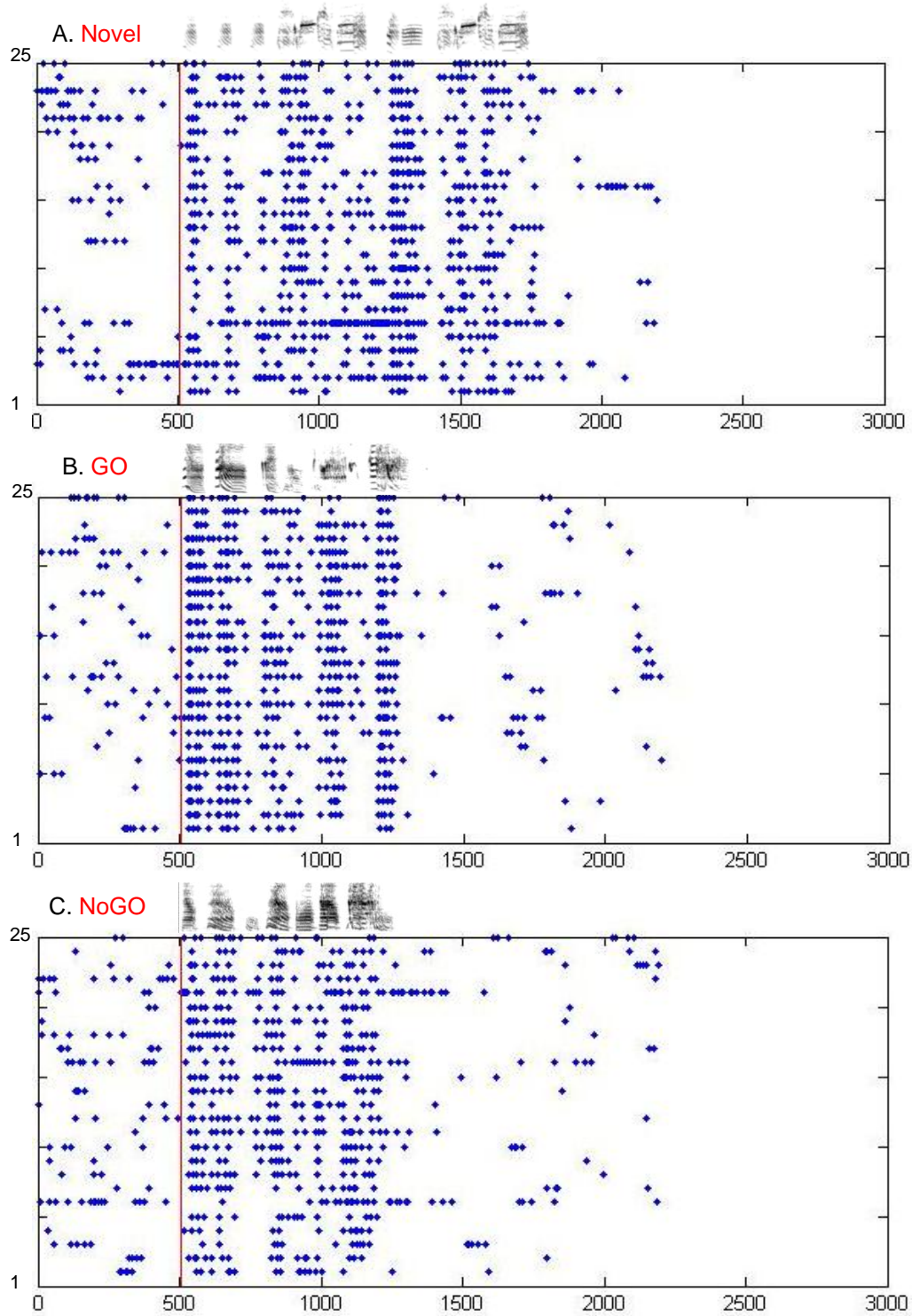


Figure 9. Single-unit Responses to GO, NoGO and Novel Stimuli. Raster plots show the responses to one of the NCM units to an example (A) novel, (B) GO and (C) NoGO stimulus. Responses from trials 1 to 25 (y-axis) are plotted for each stimulus from 500ms before stimulus onset (control period) to 2500ms after onset. The sonogram of the stimulus is represented above each plot along the x-axis (time).

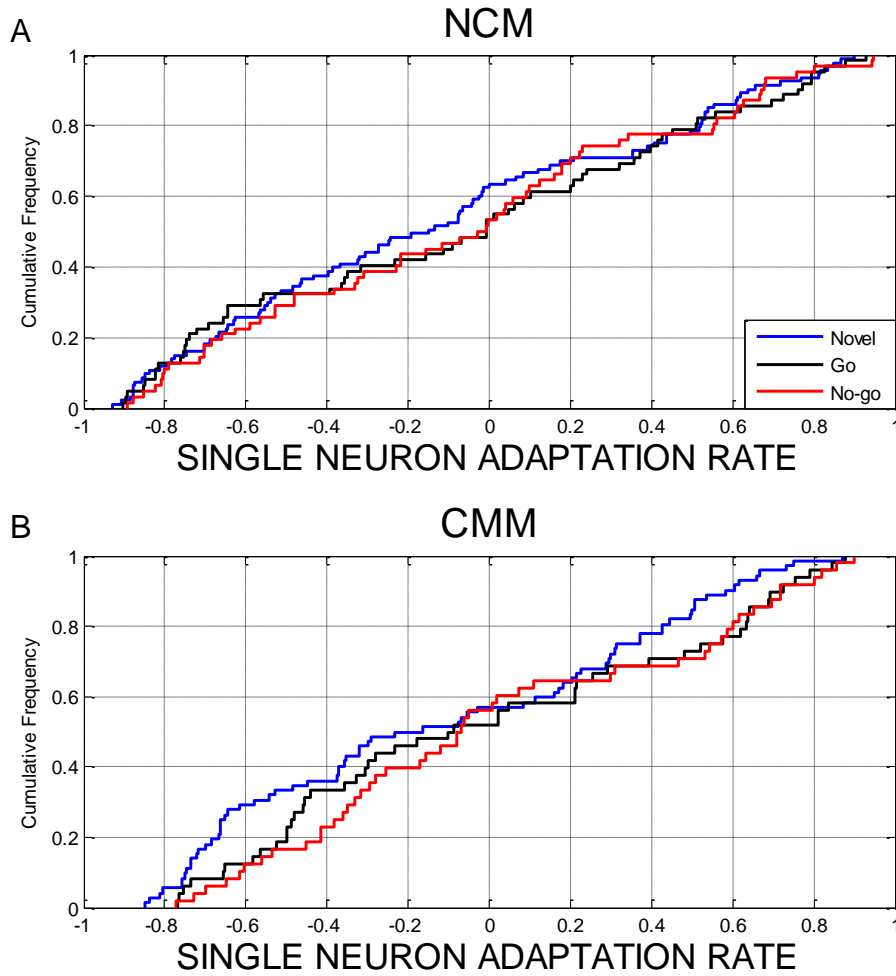


Figure 10. Effect of training on single-unit adaptation rates. The single-units isolated in both NCM and CMM were heterogeneous populations of both adapting (negative rates) and anti-adapting (positive rates) neurons. (A) In NCM, there was no effect of training on single-unit adaptation rates (n.s., Friedman's within subjects test). (B) In CMM, there was an effect of training on single neuron adaptation rate ($p < 0.05$, Friedman's within subjects test). Novel stimuli were adapted to faster than GO stimuli ($p < 0.05$ & $p < 0.01$, respectively, Wilcoxon sign-rank test).

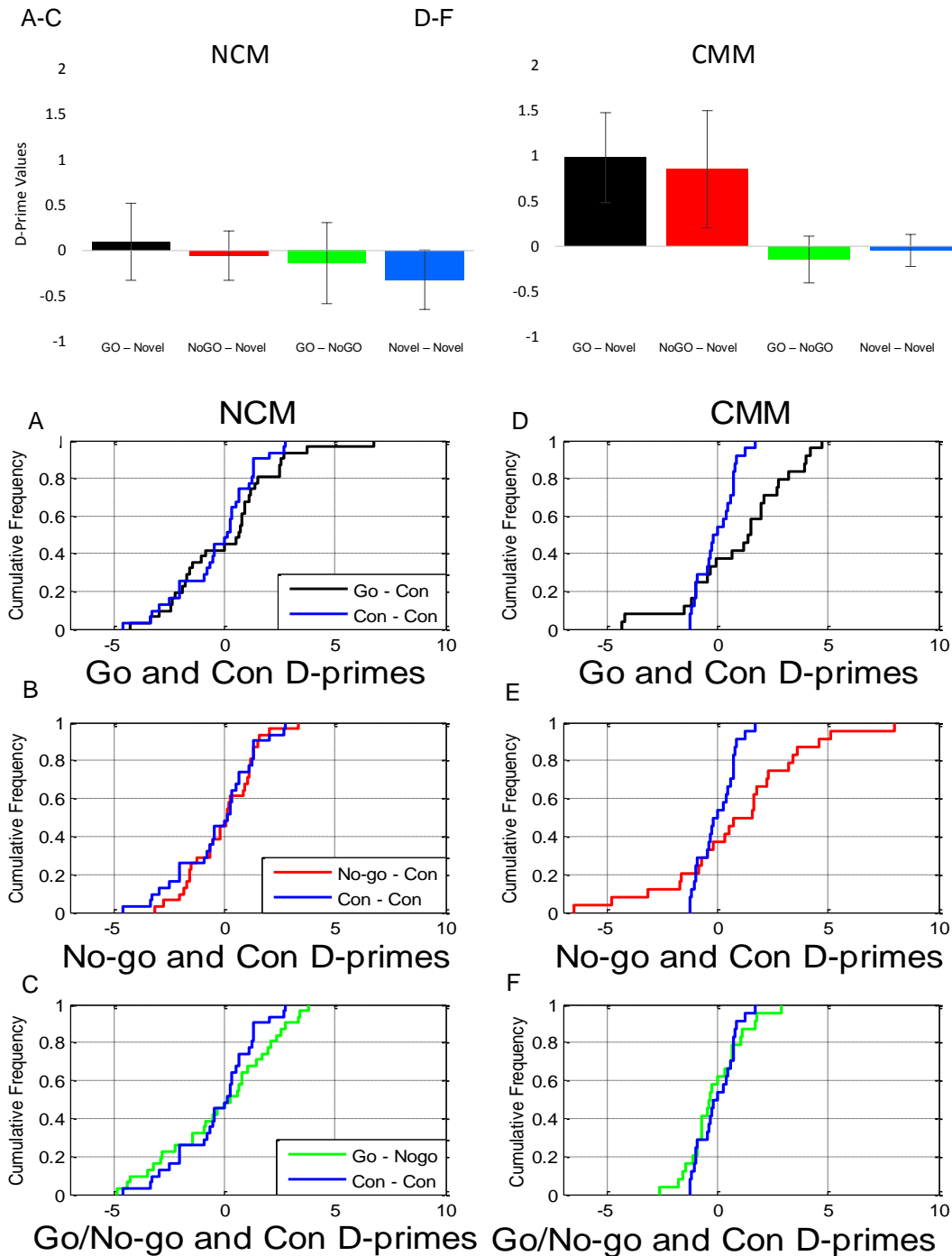


Figure 11. D-primes of single-unit spiking responses to trained and novel stimuli. Plotted here are the cumulative distribution frequencies of d-prime values in NCM and CMM comparing single-unit spike rate responses to: (A, B, D & E) trained stimuli (GO/NoGO) to those of novel songs, (C & F) GO stimuli to those of NoGO stimuli. D-prime values calculated by comparing single unit spiking responses to two novel conspecific songs (Con-Con) are also plotted for each area, as a baseline measure of discriminability of arbitrary song stimuli in that structure. (A, B & C) In NCM, d-prime values were no different for reward-predictive stimuli than they were for novel stimuli ($p = ns$, ks-test). (D, E & F) In CMM, however, responses to reward-predictive stimuli were significantly more discriminable from those to novel conspecific stimuli than baseline (D: $p < 0.01$; E: $p < 0.01$, ks-test).

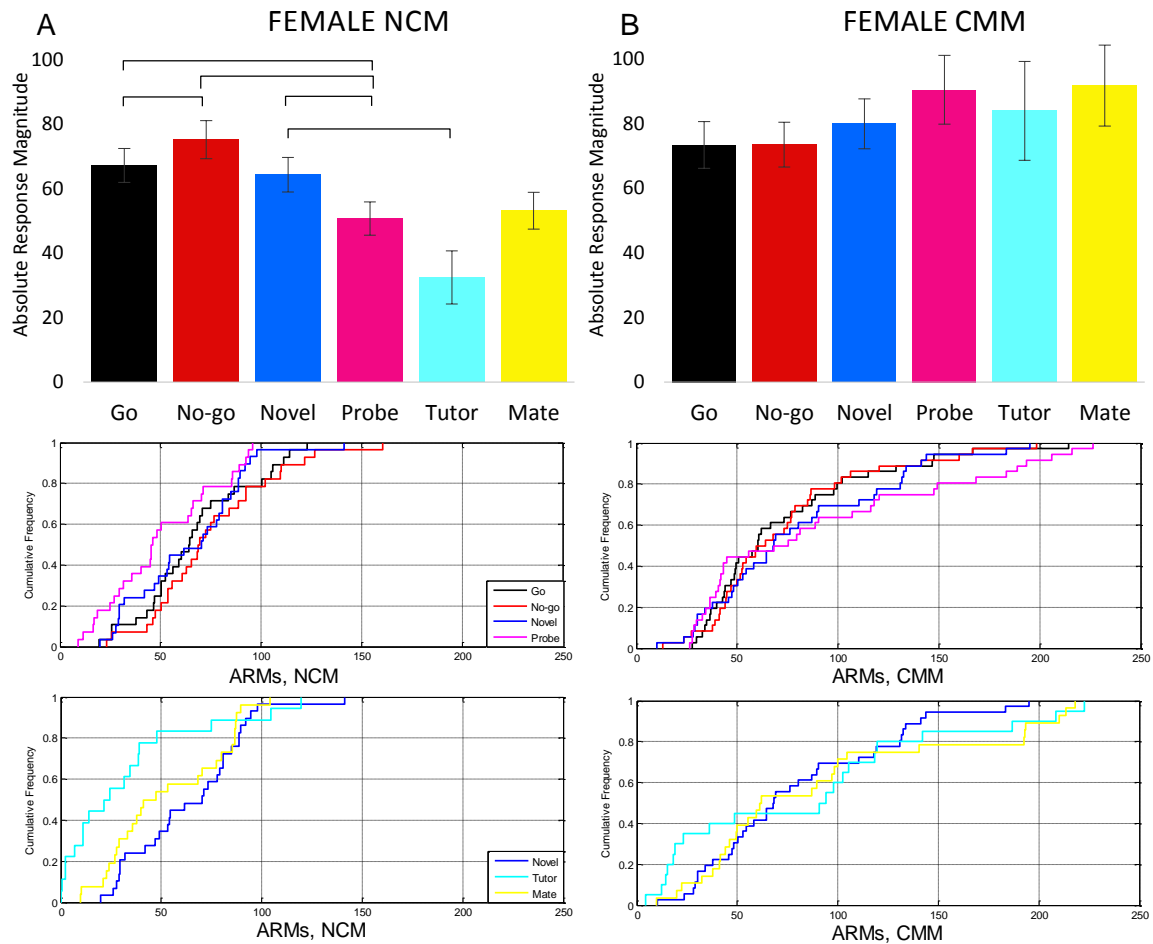


Figure 12. Female ARMs to socially-relevant and operantly-trained auditory stimuli. (A) Both socially-relevant and operantly-trained stimuli evoked responses significantly different from those evoked by novel stimuli in NCM ($p < .00001$, Friedman's within-subjects test). ARMs were stronger in response to novel stimuli than tutor stimuli ($p < 0.01$, Wilcoxon sign-rank test). Probe stimuli evoked weaker responses than GO, NoGO and novel stimuli ($p < 0.01$, $p < 0.001$ & $p < 0.001$, respectively, Wilcoxon sign-rank test). ARMs to NoGO stimuli were also stronger than ARMs to GO stimuli in this area ($p < 0.01$, Wilcoxon sign-rank test). (B) There were no significant differences between ARMs to novel stimuli and those to familiar (socially or operantly reinforced) stimuli in CMM (n.s., Friedman's within-subjects test).

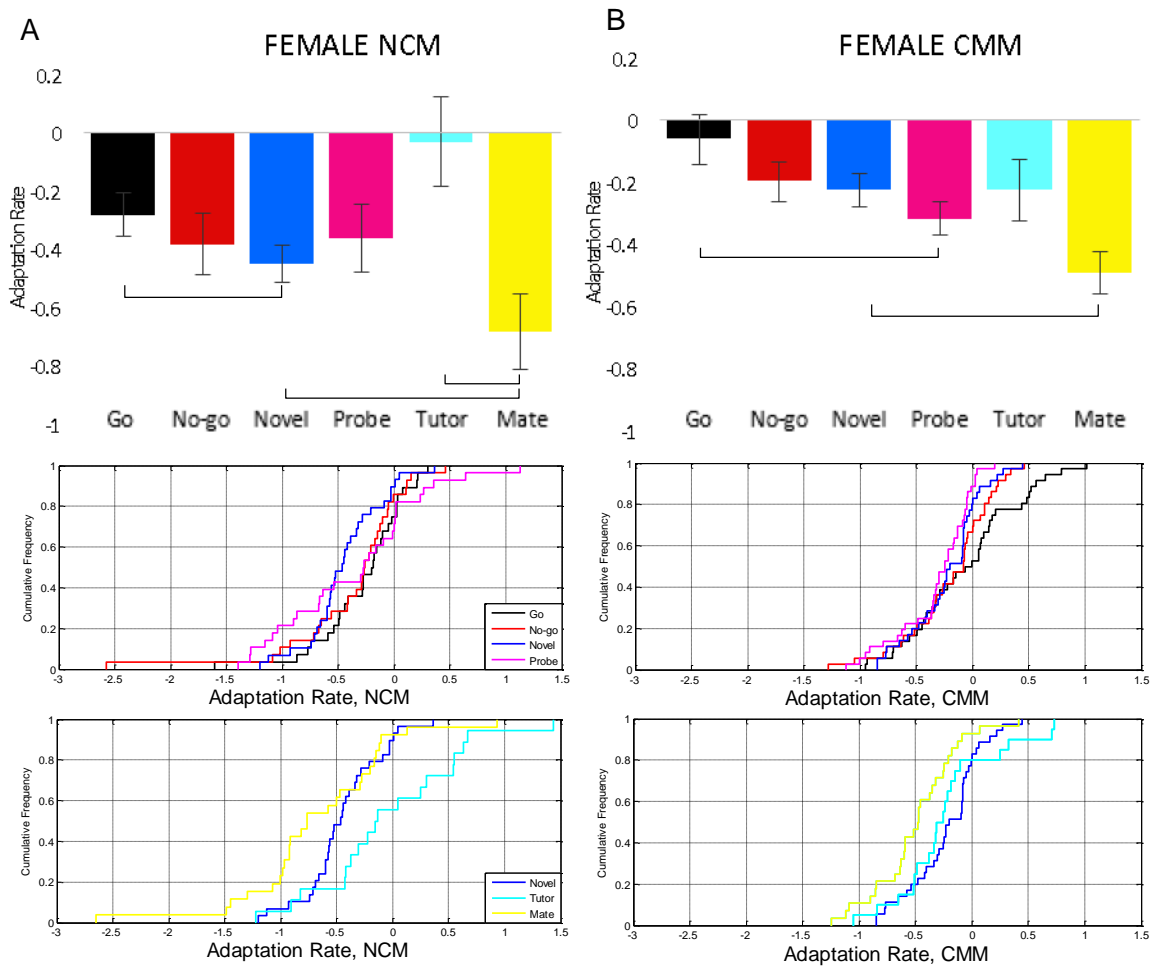


Figure 13. Female multi-unit adaptation rates to socially-relevant and operantly-trained auditory stimuli. (A) Socially-relevant and operantly-trained stimuli evoked multi-unit adaptations rates significantly different from those evoked by novel stimuli in NCM ($p < .05$, Friedman's within-subjects test). Multi-unit sites adapted to novel stimuli significantly faster than GO stimuli ($p < 0.05$, ks-test). A female's mate's song was adapted to faster than novel stimuli and tutor stimuli ($p < 0.01$ & $p < 0.01$, respectively, ks-test). (B) In CMM, there were significant differences in multi-unit adaptation rates based on social-relevance and operant-training category ($p < 0.01$, Kruskal-Wallis ANOVA). Mate's song was adapted to faster than novel stimuli ($p < 0.01$, ks-test) and GO stimuli were adapted to faster than probe stimuli ($p < 0.01$, ks test).

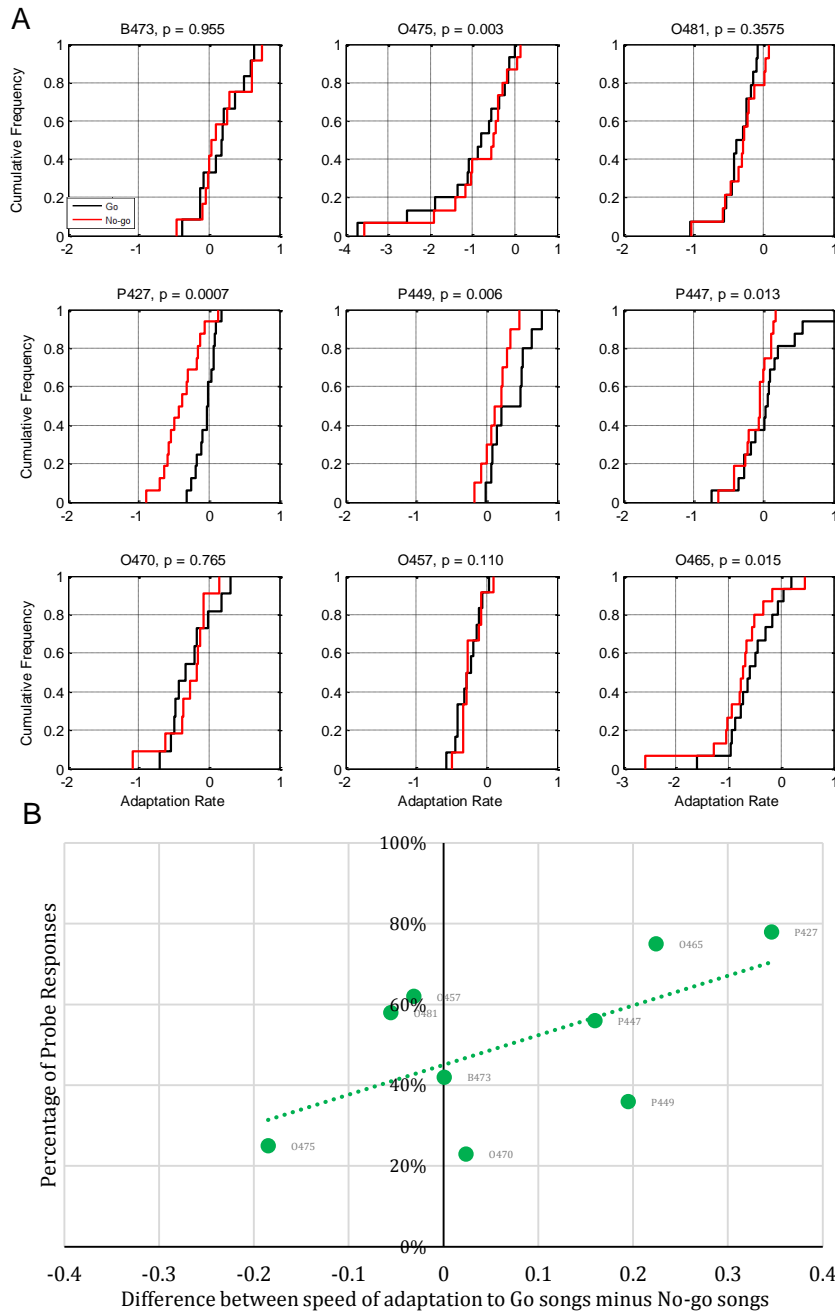


Figure 14. Familiarity of trained stimuli correlates with performance on probe trials. (A) Five of the nine subjects that participated in probe trials during behavioral training showed significant differences in speed of response adaptation to GO as compared to NoGO stimuli ($p < 0.05$, Wilcoxon-sign rank test). (B) The difference between speed of multi-unit adaptation to GO and NoGO stimuli (difference in familiarity) was correlated with behavioral responses to probe trials. Animals that were more familiar with (adapted to more slowly) GO stimuli responded more often to unreinforced probe stimuli as GO stimuli, and vice versa for NoGO stimuli ($r = 0.606$, $p = 0.08$).

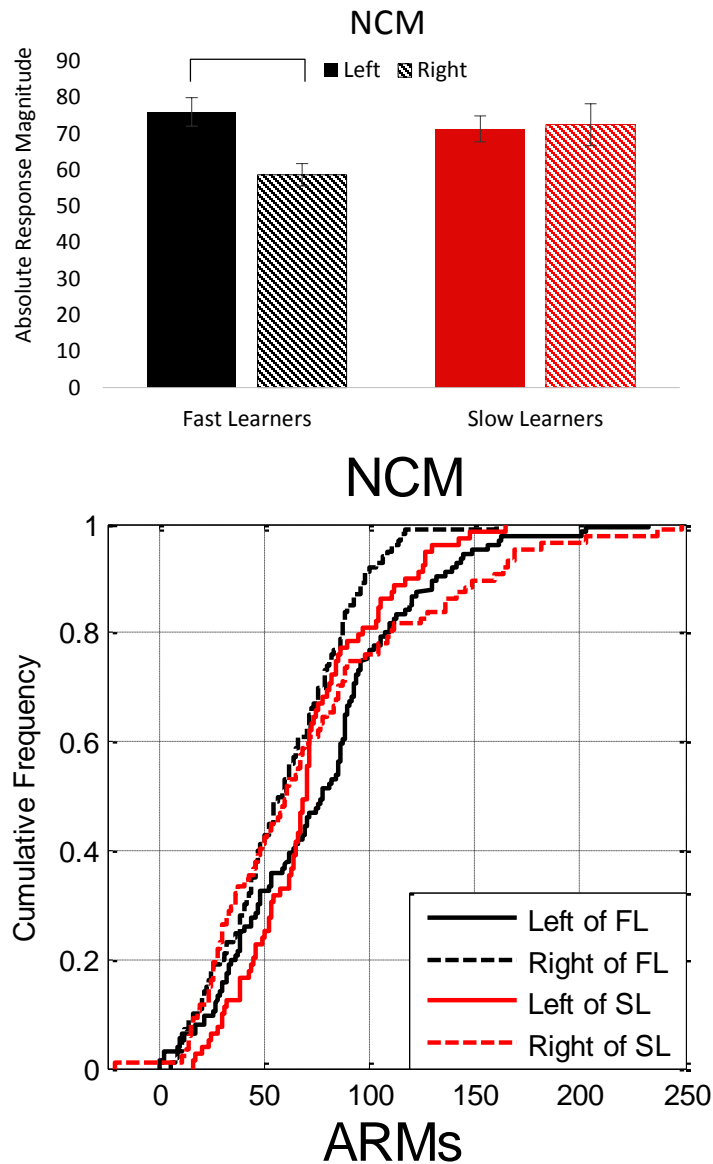


Figure 15. Speed of acquisition and Lateralization of NCM. When subjects (combined males and females) were median-split into two groups, according to their speed to reach criterion on the operant task in NCM, faster learners showed stronger ARMs in the left hemisphere as compared to the right ($p < 0.001$, ks-test). There was a trend for slower learners to show stronger responses in the right hemisphere as compared to the left ($p = 0.11$, ks-test).

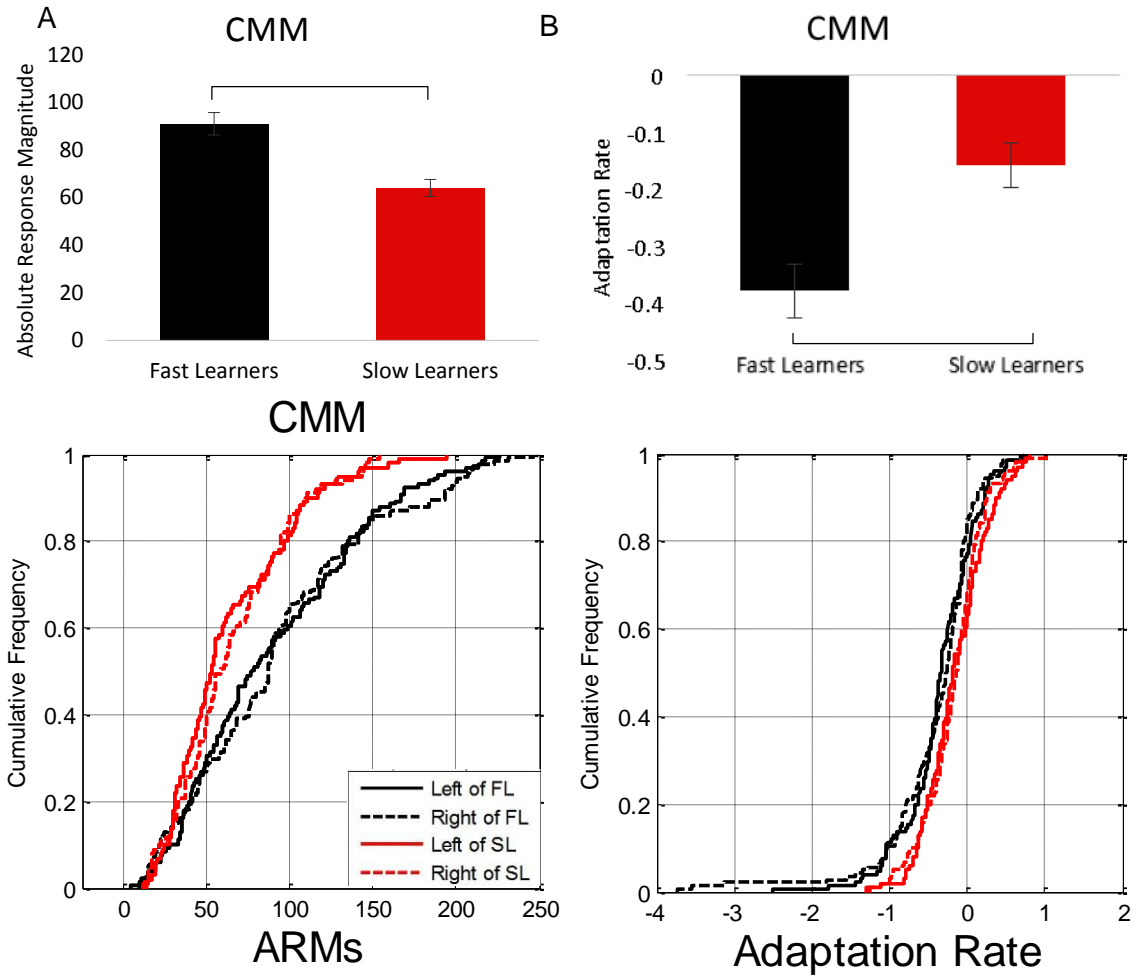


Figure 16. Speed of acquisition and CMM Auditory Responses. When male and female subjects were median-split into two groups, according to their speed to reach criterion on the operant task in CMM, faster learners (FL) showed (A) higher ARMs and (B) faster adaptation to all stimuli than slower learners (SL) ($p < 0.0000001$ & $p < 0.05$, respectively, ks-test).

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