ASSESSING PARENTAL EXPERIENCE IN A SOCIALLY MONOGAMOUS SONGBIRD: NEURAL PROCESSING OF AND BEHAVIORAL RESPONSES TO FLEGDLING BEGGING CALLS IN MALE AND FEMALE ZEBRA FINCHES

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ABSTRACT OF THE DISSERTATION

Assessing parental experience in a socially monogamous songbird: neural processing of and behavioral responses to fledgling begging calls in male and female zebra finches

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Animals invest heavily in pair bonds and offspring, and benefit if they are able to recognize their family when they are separated. This study investigates how experience modulates the neural memory for auditory signals that can be used in individual recognition, with a focus on parent-offspring interactions in the zebra finch (ZF), a socially-monogamous species with bi-parental care. Bi-parental care, which is rare in mammals, makes this a powerful system in which to study sex differences in neural processing and behavior. Furthermore, auditory brain regions have been identified that respond preferentially to conspecific vocal signals, including songs and various communication calls. This study will focus on the fledgling call (FC), a short, high-frequency call produced in juveniles. FCs signal to parents that offspring need to be fed, and elicit a direct behavioral response. Thus, FCs are a behaviorally-relevant category of vocalization for ZF parents of both sexes, but may be meaningless to adult ZFs that have not yet mated and produced offspring (virgins). Although adult ZFs show behavioral and

neuronal memories for the songs and calls of familiar individuals, the neural processing of and behavioral responses to FCs have not been thoroughly examined.

Neural processing of these socially-relevant stimuli was assessed in the avian auditory forebrain, in parents and virgin subjects. In addition, parental behaviors elicited by FCs were tested in a novel nest-entry paradigm. Finally, the behavioral and neural data collected from parents were used to determine whether ZF parents can discriminate between the FCs of their own vs. unfamiliar fledglings. Results show that neural responses to FCs are stronger in parents of both sexes than in virgins and that this effect is lateralized. Enhancement of FC responses may be due to differences in multi-unit and single-unit tuning properties that include higher best frequencies in parents, perhaps reflecting a shift toward the high frequencies of FCs. Parents also showed neuronal and behavioral recognition of the calls of their own fledglings, although there were some sex differences. In a nest-entry behavioral paradigm that assesses components of parental feeding responses, FC playback also elicited parental behaviors more frequently in parents than in virgins. However, results showed unexpected sex differences in the frequency of parental behaviors (nest-box entries, food-collected, etc) which support the possibility that male and female parents distribute parental duties in bi-parental species. To further investigate the various sex differences observed and potential neural mechanisms, neural and behavioral responses to FCs were assessed in virgin ZFs that had been treated with the avian analogs of the parental care mediating hormones, vasopressin and oxytocin. Results provide evidence for sex-specific functions of these hormones and establish the ZF as a valuable model for investigating how parental experience affects neural and behavioral processing, in both female and male parents.

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1. INTRODUCTION AND BACKGROUND

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). For example, it is known that birds recognize each other by their vocalizations (Miller, 1979; Clayton, 1988; Riebel, 2000; Vignal, Mathevon & Mottin, 2004; Vignal, Mathevon & Mottin, 2008), often used to communicate between individuals who hide to avoid predation. However, it is unknown how the familiarity of a unique complex signal (that identifies another individual) 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 that can be used in individual recognition, with a focus on parent-offspring interactions.

In addition to producing a memory for a particular sound signal, repeated experience with auditory objects can also contribute to the production of a neural memory for a 'category' of sound signals. During speech acquisition, children are constantly forming categories of their native-language syllables through their experience with those sounds. Their auditory experience with those syllables subserves their ability to learn their native language, by enhancing distinctions between syllable categories at the cost of diminishing their ability to discriminate sounds within a syllable category

(Kuhl, Williams, Lacerda, Stevens & Linbolm, 1992; Werker & Tees, 1984; Kuhl, 1998). There are various similarities between vocal learning in humans and vocal learning in songbirds (Doupe & Kuhl, 1999). One of those similarities is the predisposition to respond to own-species vocalizations. Songbirds are able to distinguish between conspecific and heterospecific songs, even at a young age (Dooling & Searcy, 1980; Nelson & Marler, 1993), and neurons in several brain areas have been shown to respond preferentially to conspecific songs (Mello, Vicario and Clayton, 1992; Chew, Mello, Nottebohm, Jarvis & Vicario, 1995; Chew, Vicario & Nottebohm, 1996a). Furthermore, neurons in some of these areas also undergo a process of stimulus- specific adaptation (SSA), a form of neural recognition memory for individual songs. This memory is long-lasting for conspecific songs but not for heterospecific songs or other sounds, suggesting that these areas may be specialized for the processing of socially important acoustic information (Mello et al, 1992; Mello, Nottebohm & Clayton, 1995; Chew et al, 1995; Chew et al, 1996a; Terpstra, Bolhuis & den Boer-Visser, 2004, Smulders & Jarvis, 2013).

Song is a socially important auditory signal, used in both reproductive and territorial communication between songbirds in their natural habitats (Zann, 1996). Songbirds learn their songs from adult tutors through a process of vocal imitation with many parallels to speech acquisition (Doupe & Kuhl, 1999). 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. In addition to songs, zebra finches produce a variety of different types of call signals as well. Males learn to

produce a structured “long call” in the same way that they learn their song. However, females do not learn their long call and produce a long call that is innate, but still has idiosyncratic features in each individual (Simpson & Vicario, 1990; Simpson & Vicario, 1991). These calls are used by families to locate one another, and it has been shown that both male and female adult birds recognize the call of their mate in behavioral studies (Vignal et al, 2004; Vignal, et al, 2008). Early in development, before these long calls are produced, zebra finches of both sexes produce a different sort of call, a short, high-frequency fledgling call (FC), during their juvenile period (Zann, 1996; Elie & Theunissen, 2016). These calls signal to their parents that they need to be fed, and elicit a direct behavioral response from the parents. As a result, zebra finch parents have extensive experience with FCs. Thus, FCs are a behaviorally-relevant category of vocalization for adult zebra finches that have been parents, but may be meaningless to adult zebra finches that have not yet mated and produced offspring (virgins/naive). Although zebra finches show behavioral and neuronal memories for the songs and long calls of familiar individuals (Clayton, 1988; Vignal et al., 2004; Vignal et al., 2008; Menardy et al., 2012), the neural processing of fledgling calls has not been tested in parentally-experienced adults and the behavioral responses of adults to those calls has not been thoroughly examined.

A. Zebra Finch Songs and Calls

I. Production of Songs and Calls

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 (Doupe & Kuhl, 1999). 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.

At the same time that a young male zebra finch is learning to produce a stereotyped song from his tutor, he is also learning to produce a 'long' call, a communicative signal used in a majority of zebra finch social interactions (besides mating and territory-guarding). Due to this learning process the calls of different male zebra finches are individually distinct, much like songs (Zann 1990; Simpson & Vicario 1990; Simpson & Vicario, 1991). Male long calls contain complex acoustic features such as: fast-

frequency modulation, short duration and an elevated fundamental frequency (Simpson & Vicario, 1990). Female zebra finches also produce long calls that are used in social interaction; however, females do not learn to produce these calls. The female long call is a harmonic stack with almost no frequency modulation at a fundamental frequency of 500-600 Hz that lasts 100-500ms (Simpson & Vicario, 1990). These calls are far simpler than those produced by males and therefore less individually distinct. Nevertheless, they do have individually-specific features and behavioral studies have shown that male zebra finches can recognize their female mate by her call (Vignal et al., 2004).

Before male and female zebra finches begin to produce long calls, they produce shorter, high-frequency, 'begging calls.' The zebra finch is an altricial species and young need to be fed by their parents to survive. Therefore, even at a very young age (3 days post-hatch) young birds begin to call to attract attention when they are hungry, so that they can be fed (Muller & Smith, 1978). These calls are produced at high intensities, so that they can be heard by parents from as far away as 100 meters (Levrero et al., 2009). Although young zebra finches first leave the nest at 17-22 days old, they still need to be fed by their parents for 13-18 days after fledging (Zann, 1996). Therefore fledglings frequently produce these calls up to day 35 post-hatch (Muller & Smith, 1978). Although these calls are unlearned and innate, recent studies have shown that individual fledgling's begging calls are complex enough to support identification (Levrero et al., 2009; Reers et al, 2011). Further, experimenters have recently shown, using a call-back behavioral paradigm, that a fledgling's parents and siblings are all able to recognize its call one day before fledge (~18 dph) and preferentially call back to their own kin (Levrero et al, 2009; Ligout et al., 2015). The finding that parents discriminate between own and novel

fledglings has proved contentious, though, as a later experiment (using a similar call-back paradigm) showed that zebra finches do not preferentially call back to the calls of their own fledglings only a few days later (~22 dph; Reers, Jacot & Forstmeier, 2011). However, as call-responses to own-fledgling calls are not a natural ethological response to begging calls, further examination of the ability for parents to recognize their offspring is necessary at both the behavioral (using parental feeding behaviors) and neural level.

II. Social Value of Songs and Calls in Songbird Ethology

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. Female zebra finches do not sing. The consistent and unique (due to imperfect copying) features of each male zebra finch's song and call makes it a likely candidate for individual recognition. A songbird's experience with conspecifics involves repeated exposure to both the songs and distance "long 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; Clayton, 1988; Riebel, 2000; Vignal et al., 2004; Vignal et al. 2008). 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 their tutor's song that males exhibit (Miller, 1979; Clayton, 1988).

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 and, through sexual imprinting, later use that template to make mate selection decisions in adulthood, showing a preference for songs that are similar to their tutor's (Riebel, 2000; Riebel, 2002). In addition, female zebra finches show a mating preference for 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 (Nowicki, Peters & Podos, 1998). 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 et al. 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 and call as they are 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 (Clayton, 1988; Lind, Dabelsteen & McGregor, 1996; Vignal et al., 2004; Vignal et al., 2008).

B. Auditory Plasticity in Mammals and Songbirds

I. Learning-induced Tuning Changes in Neurons of Mammalian Auditory Cortex

Although primary sensory areas were classically considered simple stimulus-analyzers, learning-induced plasticity has been shown in primary visual, primary somatosensory and primary auditory cortices through a multitude of behavioral and neurophysiological methods, in recent years (Durup & Fessard, 1935; Delacour et al., 1987; Galambos, 1956). In A1 of the mammalian brain, neurophysiological measures have detected changes due to learning for over 50 years (Galambos, 1956; Bakin & Weinberger, 1990; Recanzone, Schreiner & Merzenich, 1993). After learning (classical, discriminative, perceptual, sensitization), primary auditory cortex (A1) shows changes in tuning through: decreased threshold for firing, narrowing of bandwidth, and changes in tonotopic maps to increase representational area for an auditory conditioned stimulus associated with reward or punishment (CS+). Primary auditory cortex is organized in a

tonotopic map. Regions of the cortex contain neurons that are tuned to the same frequency, and as you move along the map, tuning of neurons changes such that neighboring neurons are tuned to nearby frequencies. Typically, after classical training, the frequency associated with a reward or punishment becomes over-represented in the tonotopic map (Recanzone et al, 1993; Rutkowski & Weinberger, 2005; Hui et al., 2009). This increase in the representational area for the CS+ frequency is called high-order associative representational plasticity (HARP, as defined by Bieszczad & Weinberger, 2010a). HARP is a consistent neural correlate of learning, and therefore a plausible candidate for a 'memory code.'

The idea that a 'memory code' or any memory content could be held in a primary sensory cortex (areas previously thought of as simple stimulus analyzers) has been highly debated throughout the past 50 years, since the first EEG correlates of memory were detected in primary sensory cortices (Weinberger, 2004; Galambos, 1956). Therefore, there have been various studies into A1 tuning changes and their relationship to behavioral memory throughout the years. There are five major characteristics of memory that have been shown to be related to A1 tuning plasticity after learning. The five characteristics of memory that A1 shows are: associativity, rapidity, specificity, consolidation and long-term retention. Learning-induced tuning changes develop through contingent association of a sound with a reward or punishment (associative; Morell, 1961), within as few as 5 trials of training (rapid; Edeline, Pham & Weinberger, 1993). Tuning changes are specific (within an octave) of the frequency associated with reward/punishment (specificity; Merzenich et al., 1996; Calford, 2002), and tuning changes increase between 1 hour and 3 days after training, without additional training

(consolidation; Edeline & Weinberger, 1993; Galvan & Weinberger, 2002). Further, these changes are maintained up to (and possibly further than) 8 weeks after training has ceased (long-term retention; Weinberger, Javid & Lepan, 1993).

Finally, the mechanism through which HARP develops has also been studied in great detail in recent years (Weinberger, Ashe, Metherate & Diamond, 1990; Suga & Ma, 2003). In a simple fear classical conditioning paradigm, for example, the CS (tone) is processed by the inferior colliculus, which projects that information to the ventral medial geniculate nucleus (vMGN) of the thalamus as well as medial MGN (mMGN), which then project to the auditory cortex. At the same time, the US (shock) is processed in mMGN and, from there, projects to the auditory cortex, as well as the amygdala. The amygdala uses information from the shock to produce a conditioned response (freezing) as well as to activate the nucleus basalis (NB) to release acetylcholine (ACh), which then projects to A1. When ACh processing of the shock converges with tone information in the auditory cortex, plasticity is induced (Weinberger, 2004). In this model, activation of NB and release of ACh is vital in the mechanism of HARP development in A1. Recent studies have demonstrated that ACh influx from NB into A1 is both sufficient for learning-induced tuning changes there, as well as possibly necessary for it (Ji, Gao & Suga, 2001; Ji & Suga, 2003; McLin, Maisnikov & Weinberger, 2002). There are two mechanisms through which acetylcholine can causes tuning changes in AC, in layers 5/6 through muscarinic g-coupled metabotropic receptors (perhaps slower-developing, long-term memory; Miasnikov, McLin & Weinberger, 2001), as well as in layer 1 through ionotropic nicotinic receptors (perhaps quick, short term memory; Letzkus et al., 2011).

Both of these mechanisms allow plasticity to occur through disinhibition of auditory cortex pyramidal cells.

II. Role of Excitation and Inhibition in Auditory Cortex Plasticity

Inhibitory mechanisms of sound processing in the auditory cortex are of specific interest because inhibitory kinetics in this region are faster than typical of primary sensory cortices (Hefti & Smith, 2002). This suggests that even primary auditory cortex is capable of performing high-level processing of sounds. In particular, the balance between excitation and inhibition, and how that balance relates to changes in tuning after learning, has been extensively studied in the primary auditory cortex. In sensory cortices, excitation is provided primarily to layer 4 auditory neurons, from thalamocortical transmission. A few milliseconds after excitation in adult auditory cortex, GABA inhibition is provided to auditory neurons in a feed-forward mechanism. The balance between excitation and inhibition, as well as the precise timing of those inputs, controls integration of input and produces very specifically-timed action potentials post-synaptically (Volkov & Galazjuk, 1991; Wehr & Zador, 2003).

Therefore, the structure of an auditory neuron's receptive field (RF) is controlled by both excitation (EPSPs) and inhibition (IPSPs). In auditory cortex, neighboring cells provide lateral inhibition to one another, which narrow the bandwidth of the RF. Without this locally-derived inhibitory influence, RFs widen to all frequencies that excite a neuron, called its extra-classical RF. Therefore, inhibition has a direct influence on the RF of the neuron, and when inhibitory drive changes, the RF of a pyramidal cell can fluctuate, without synaptic modifications or gene transcription (Carcea & Froemke, 2013). During development receptive fields of sensory cortices are highly plastic and this

is reflected in the uncorrelated activity of excitation and inhibition in the cortex at this time (show little E:I balance). The uncorrelated excitatory and inhibitory inputs allow RFs to change quickly during early critical periods, and those changes are maintained long-term. However, as an animal ages, excitatory tuning curves and inhibitory tuning curves become highly correlated, introducing stability into sensory neuron RFs, an attribute that is highly desirable for the 'sensory analyzer' roles of primary sensory cortices, to allow for stable perception (2013).

Due to the importance of E:I balance for the maintenance of stable receptive fields, one of the simplest mechanisms for neural plasticity in the auditory cortex is disinhibition. Froemke and colleagues have studied the role of inhibitory mechanisms in A1 plasticity extensively. They find that when a tone is followed by reinforcement, inhibition in the auditory cortex for the CS+ decreases, which allows excitatory changes to take place (Froemke et al., 2013). This decrease in inhibitory drive to pyramidal neurons is achieved through NB stimulation and ACh activity in the auditory cortex. Neuromodulators, such as ACh, work on sensory neurons to decrease their inhibitory drive, and put them back into a plastic, development-like state that enables RF changes. Acetylcholine works through both muscarinic receptors and nicotinic receptors in A1 to produce disinhibition in pyramidal cells of A1. There are g-protein coupled metabotropic muscarinic receptors in layers 5 and 6 which have a role in GABA transmission in that area, that likely disinhibit the pyramidal cells of layers 5 and 6, allowing them to make long-term changes to RFs (Froemke, Merzenich & Schreiner, 2007). This mechanism of change likely affects downstream processing in the sub-cortical structures and subsequently, behavior. In addition, in layer 1 of A1 (and V1) there are ionotropic

nicotinic ACh receptors, which function quickly to produce short-term changes in RFs and cause disinhibition of pyramidal cells in layers 2/3 of the cortex (Letzkus et al., 2011). When the RFs of these layer 2/3 pyramidal cells are placed into a plastic state, information about the tone that they are provided with (by the thalamus) can influence their tuning. In addition, as layer 2/3 pyramidal cells have cortico-cortical connections with their cortical neighbors, they can also influence the horizontal processing of the sound in other pyramidal cells, expanding representational area.

III. Learning-Induced Plasticity for Songs and Calls in Avian Forebrain

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 decrease over subsequent presentations to reach an asymptote (Chew et al., 1995; Smulders & Jarvis, 2013). 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 (> 24 hours) neuronal

memory for individual songs (Chew et al., 1996a).

The avian auditory forebrain also shows lateralized neural responses to conspecific vocalizations (Phan and Vicario, 2010; Moorman et al., 2012; as reviewed in: Moorman & Nicol, 2015). 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 (stronger in the right hemisphere, **Figure 3**; Phan and Vicario, 2010). In addition, the lab has shown that the direction of lateralization can be affected by environmental changes; experience in a novel acoustic environment reverses the typical direction of lateralization of auditory responses in zebra finches (Yang & Vicario, 2015). In addition, lateralization in this area may be related to the quality of a songbird's auditory learning such that birds with stronger left-lateralization of auditory responses learn more quickly in operant tasks (Bell, Phan & Vicario, 2015). 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). Left-hemisphere intact zebra finches perform better than right-hemisphere intact subjects at discriminating two songs while right-hemisphere intact birds are better at a harmonic-profile task. In addition, adult birds that produce songs most similar to their tutor's song show increased incorporation of new neurons in the left hemisphere NCM, when compared with the right (Tsoi et al., 2014). Therefore, successful auditory learning may depend more strongly on

one hemisphere of the avian forebrain (NCM/CMM) than the other.

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, Hulse & Ball, 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 (2004). In neurophysiological recordings after training, 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; Bell et al., 2015). 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. These results suggest that learning not only changes how the bird (and the brain) reacts to a given stimulus, but that these changes are also long-lasting. The mechanism through which neural memories for behaviorally-relevant songs develop in NCM and CMM is still being determined. However, the Gentner lab has shown that when inhibition is blocked (Thompson, Jeanne & Gentner, 2013) the effects of learning on auditory responses in these regions do attenuate, suggesting there is a role for inhibition in this plasticity.

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; Menardy 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 (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, Phan, Lu & Vicario, 2015). 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 (Phan et al, 2006). For males, NCM seems to be particularly important in holding and retrieving the tutor song memory as 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, Lu & Vicario, 2012; Terpstra, Bolhuis, Riebel, vad 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). In addition, the neurons in NCM respond differently to a

female's mate's call than they do to novel call stimuli (Menardy et al., 2012). Playback of mate's song to females causes also 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.

C. Parental Communication in Mammals

I. Parental Behavior in Mammals

In mice, parental care includes: nursing, licking/grooming and pup retrieval by the female parent. During pregnancy, the hormones oxytocin, prolactin and estrogen levels increase and fluctuate in the maternal brain, which causes various organizational effects in areas implicated in parental care: paraventricular nucleus (PVN), supraoptic nucleus (SON), medial preoptic area (MPOA), bed nucleus of the stria terminalis (BNST), the amygdala, lateral septum (LS), prefrontal cortex (PFC) and olfactory bulb (OB). Many of these areas increase their levels of prolactin and oxytocin receptors (PRL-R & OXT-R) throughout pregnancy (Grattan et al, 2001; Kokay et al., 2006; Zingg et al., 1996; Neumann et al., 1993). The increased receptor levels in three areas of particular interest, MPOA, BNST and olfactory bulb, allow for the rapid production of maternal behavior at parturition, when hormones such as OXT surge (Bridges, 1990; Rosenblatt, Mayer & Giordano, 1988). After pregnancy and parturition, when pups are present, the stimuli

associated with them (tactile, auditory, olfactory) serve to maintain the changes induced by pregnancy, and lead to further plastic effects on the maternal brain (Stern, 1989). Many of these changes occur throughout sensory systems (as reviewed in: Miranda & Liu, 2009).

Olfaction is particularly important in the development of parental behavior in female rodents. In virgin (and even pregnant) mammalian males and females, there is a large avoidance, and even fear response to the odor of young. This fear response is caused by responses in the olfactory bulb (to pup odors) which project to the medial amygdala (MeA) and then the periaqueductal grey (PAG) to induce fear and avoidance of offspring in non-parental virgins. This OB, MeA, PAG activity typically inhibits another circuit of pup odor processing, which goes through OB and MeA to the bed nucleus of stria terminalis (BNST) and medial preoptic area (MPOA) to induce maternal behaviors (Rosenblatt & Mayer, 1995). At the time when offspring are born, there is a decrease in the inhibitory action of the PAG which allows for the BNST and MPOA pathway to take over, so that maternal behaviors can be induced. The BNST and MPOA, at that point, also produce downstream activation of the dopamine reward system (nucleus accumbens, VTA, ventral pallidum) to release dopamine during interactions with pups, which rewards maternal behaviors and trains the animal to show more maternal care (Rosenblatt & Mayer, 1995). Therefore, sensory stimuli that are experienced by mothers during pup interactions (calls, odors, etc.) are also associated with dopamine release.

An additional component of mammalian parental care behavior is individual differences in the degree to which parents respond to their offspring, which is influenced by oxytocin and prolactin receptor levels in the nucleus accumbens. There are OXT-Rs

and PRL-Rs on NAcc dopamine neurons; therefore the amount of hormonal influx as well as the number of receptors in the NAcc can influence how rewarding individuals find parental care and the frequency with which they will exhibit parental care behaviors (Francis, Champagne & Meaney, 2000; Francis, Young, Meaney & Insel, 2002; Shieh & Pan, 1999). Many of the plastic changes in the parental brain that are induced in mothers by interaction with pups (through PRL influxes) are also present in virgins that are exposed to pups for an extended period. Therefore, virgin females can be induced to act parentally after interaction with pups; this process is called sensitization (Pedersen, Ascher, Monroe & Prange, 1982). Finally, these individual differences in oxytocin expression are associated with high levels of maternal care in a variety of mammalian species, from the simple rodent models described here to complex human behavior (rat: Francis et al., 2000; macaque: Maestripieri et al., 2009; human as reviewed in: Feldman, 2015).

II. Enhanced Neural and Behavioral Responses to Pup Calls in Maternal Mice

A common behavioral paradigm used to test maternal behavior in mice is the pup-retrieval paradigm. In this paradigm, an adult female mouse is placed in a cage with multiple mouse pups; there is typically a nest at one end of the cage, while the pups are isolated (outside of the nest) at the opposite end of the cage. Female dams will collect all isolated pups and bring them back to the nest. However, due to the virgin pup-avoidance behavior, female mice (of the C57 strain) that have not had experience with pups will not respond to the isolated pups. Further, when virgin females have been sensitized to pups through previous interaction with them, those sensitized virgins will collect isolated pups and return them to their nest. This behavior has been shown to be highly motivated by

ultrasonic (> 25 kHZ) isolation calls that the pups emit when separated from their nest (Noirot, 1966; Sewell, 1968; Sewell, 1970; Haack, Markl & Ehret, 1983). Mothers preferentially approach pup-like ultrasonic sounds, as compared to neutral sounds, while virgin female mice do not (Ehret, Koch Haack & Markl, 1987). This behavioral preference for pup calls demonstrates that mothers recognize the behavioral relevance of this category of calls, while virgins do not.

Auditory processing of pup isolation calls has also shown to be influenced by maternal experience in mammals. The behavioral differences between maternal and virgin mice was first extended to auditory cortex with a c-Fos immediate early gene (IEG) study which showed distinct auditory cortical fields in the two groups, after pup call sound exposure (Fitchel & Ehret, 1999). Electrophysiological examination of neural responses to isolation calls in mothers and virgins was further explored by Liu and colleagues; results show that A1 multi- and single-units show improved cortical representation in mothers as compared to virgins (Liu, Linder & Schreiner, 2006; Liu & Schreiner, 2007). Liu further showed that this improved cortical detection is due (at least in part) to changes in A1 lateral band suppression, which produces more stereotyped responses to pup calls in mothers and sensitized virgins than virgins (Galindo-Leon, Lin & Liu, 2009; Lin et al., 2013). Their lab suggests that enhancement in neural responses to ultrasonic pup-isolation calls is achieved through lateral-band suppression of responses in neurons that are tuned outside of the ultrasound range, which increases in strength over the course of maternal care thereby increasing the population level signal-to-noise ratio (Shephard, Chong & Liu, 2016). Although sensitized virgins also show plastic changes in A1 inhibition in response to pup isolation calls, they do so for only a short time after

experience with pups. Mothers with the same amount of pup exposure show longer-lasting changes, which suggests their physiological state serves a role in the development of long-term memories for pup calls (Lin et al., 2013).

III. Role of Oxytocin in Female Parental Auditory Cortex

Dams experience increases in oxytocin, prolactin and estrogen levels throughout pregnancy. In 1982, administration of exogenous oxytocin to female virgin rats was shown to facilitate pup-retrieval behaviors (Pedersen et al., 1982). This finding has been replicated in non-rodent mammals and more recent research by Froemke and colleagues has identified a possible neural mechanism of this phenomenon (sheep: Kendrick, Keverne & Baldwin, 1987; mouse: Marlin et al., 2015). To test how oxytocin may influence neural processing of pup isolation calls, Froemke's lab used a designer antibody to label oxytocin receptors in A1 of mother and virgin female mice (2015). Marlin et al. found that not only are there neurons with oxytocin receptors in AC, but also that the expression of those receptors is lateralized (more expression on the left than right). Further, mucimol inactivation in left AC was shown to impair pup-retrieval behavior in dams while inactivation of the right AC did not show an effect, a result which supported previous studies showing a left-side dominance for pup-call recognition in mothers (2015; Ehret, 1987). These results demonstrate that oxytocin is working through action in left auditory cortex to facilitate pup-retrieval in mothers.

Marlin et al. (2015) further found that 30-40% of the parvalbumin and somatostatin-positive neurons in A1 also show oxytocin receptors; suggesting that oxytocin asserts influences on inhibition in this region. Due to the fact that neural

responses are evoked by pup calls in mothers (but not virgins) because of differences in inhibitory responses to those calls in those two groups, Froemke and colleagues explored the influence of oxytocin on EPSPs and IPSPs using whole-cell voltage clamp recordings (Marlin et al., 2015). In maternal female mice there is balanced excitation (EPSPs) and inhibition (IPSPs) in response to pup calls that allows for temporally-precise spiking responses. Virgin females do not show correlated excitation and inhibition in response to pup calls. However, when auditory cortical neurons of virgin females are treated with exogenous oxytocin while being exposed to pup call stimuli, oxytocin causes rapid disinhibition of the A1 neurons (within 15 minutes). Thirty minutes after disinhibition, inhibition returns and becomes highly correlated to the timing of excitation, similar to what is seen in parental females (2015). Therefore, oxytocin causes experience-dependent plasticity in the responses of auditory cortical neurons via a similar mechanism as acetylcholine, through inhibitory processes.

D. Parental Communication in Zebra Finches

I. Parental Behavior in Zebra Finches

In birds, parents show nest-building, incubation, fledgling-feeding, and nest-guarding behaviors. Both nest-building and incubation occur before fledglings hatch, and are accomplished through a coordinated effort by both the male and female parent (Boucaud, Mariette, Villain & Vignal, 2015). After young birds hatch, however, the primary function parents must perform to ensure the survival of their young is fledgling-feeding. Young zebra finches are born altricial and cannot survive without constant care from their parents. At a young age, fledglings begin producing a vocal ‘begging call’ to

call attention to themselves and request food. The frequency with which a nestling produces these calls increases with their level of hunger and stimulates parental feeding (Redondo & Castro, 1992; Ottoson, Backman & Smith, 1997; Leonard & Horn, 2001).

Recorded begging calls are sufficient to produce feeding behavior in parents only when recorded from offspring 12-16 days or older (Muller & Smith, 1978). Although early feeding behavior is therefore likely not dependent on auditory cues, parents learn this category of sound and its behavioral relevance quickly. In fact, various studies have shown that parental birds need to hear these begging calls in order to properly care for their offspring (von Haartman 1953; Nottebohm & Nottebohm, 1971; Schleidt, Schleidt & Magg, 1960; Betts 1954; Betts, 1956). Deafened ring doves will not feed their offspring enough to sustain their lives and deafened turkeys will actually kill their offspring when they hatch (Nottebohm & Nottebohm, 1971; Betts 1954; Betts, 1956). Therefore, auditory communication is essential in avian parent-offspring interactions and the neural responses to this learned auditory signal (begging calls) are likely to show plasticity after experience.

Zebra finch parental behavior is strikingly different from that of mammalian model systems (rats, mice, etc.) because both male and female zebra finches perform parental behaviors. Only 6% of all species show biparental care; typically one parent performs all parental behaviors. In most mammals the female parent is the sole care-giver, but in 90% of avian species both the mother and father take care of offspring (Ketterson & Nolan, 1994). This is of particular interest because 40% of human societies show a moderate to high level of paternal care (Barry & Paxson, 1971). For this reason, the study of paternal care behavior in avian species may be of particular interest for its applications to human

fathers, as the data from paternal mammals is scarce. Data suggests, because the evolutionary distance between animals that do show paternal care is so large, that paternal care is under the control of a highly conserved, similar circuit in all species that exhibit it (as reviewed in: Dulac, O'Connell & Wu, 2014), although it could also be the result of convergent evolution. Therefore data on the neural mechanism of avian paternal care may be highly relevant to paternal care in humans.

II. Effects of Mesotocin and Vasotocin on Songbird Brain and Behavior

It is well known that the social hormone oxytocin serves many roles in mammalian pregnancy and maternal behavior. Oxytocin levels peak at parturition, inducing labor, and then remain high throughout lactation, allowing for bonding between moms and their pups during nursing. The avian analog of oxytocin is a hormone called mesotocin (MT). Social interactions and bonding are associated with oxytocin action in female mammals and with arginine-vasopressin in male mammals (Donaldson & Young, 2008). In biparental priairie voles arginine-vasopressin increases paternal behavior while antagonism of vasopressin receptors decreases the expression of these behaviors (Wang, De Vries & Ferris, 1994). Songbirds (and other birds) also express a hormone analogous to vasopressin in mammals, called vasotocin (VT). As MT and VT are closely related in amino acid sequence and structure they are able to bind to one another's receptors; VT receptors show a relatively high affinity for MT and vice versa. Receptors for both MT and VT are widely expressed throughout the zebra finch brain, strongly in areas of the social-behavioral network such as the hypothalamus and lateral septum (Leung et al., 2011).

The social function of these hormones in the avian brain has been examined by Goodson and colleagues. Females treated with mesotocin antagonists show reduced sociality: decreased time spent in a large group, decreased allopreening behaviors, increased latency to pair with a mate and less stable mate-pairing (Goodson et al., 2009; Klatt & Goodson, 2013a). When MT and VT synthesis are knocked down by RNA interference VT knockdown produces a reduction in gregariousness in both sexes and an increase in aggression toward other birds in males, whereas MT knockdown reduces side by side perching and affects stress coping in both sexes while also producing female-specific deficits in gregariousness, pair bonding and nest cup ownership (Kelly & Goodson, 2014).

These hormones are implicated in the control of parental behaviors in songbirds as well. Peripheral injections of MT antagonists cause a decrease in nesting behaviors exhibited by females and injections of VT antagonists produce smaller effects in both females and males (Klatt & Goodson, 2013b). In fact, Fos (IEG) expression increases in mesotocinergic neural populations in male and female zebra finches after nest-building (Hall, Healy & Meddle, 2015). Further, in males, the amount of Fos expression in vasotocinergic neural populations is related to the amount of material gathered for nest-building and the amount of time that a male spends in the nest with his mate (2015). Therefore there is evidence for sex differences in the functions of MT and VT, much like oxytocin and vasopressin in the mammalian system. Further, a recent study showed that these hormones play a role in social development of fledglings. When vasotocin is administered to nestlings on days 2-8 post-hatching (dph), fledglings show increased affiliative interest in parents throughout the juvenile period and males show increased

interest in females when sexually mature (Baran, Shlar & Adkins-Regan, 2016). However, when a vasotocin antagonist is administered during this time period instead, males showed decreased affiliative interest in the opposite sex at sexual maturity (2016). Thus, the roles of mesotocin and vasotocin in songbird mating, parenting, bonding and time spent in social contact with others, have been established and are analogous to those for oxytocin and vasotocin in mammals.

In addition to finding receptors for MT and VT in the social behavioral network, Leung et al. showed extensive labeling for MT and VT receptors throughout the zebra finch song control and auditory processing pathways, including NCM and CMM (2011). Experimenters showed receptor labeling for both VT and MT receptor subtypes in NCM, but observed receptor labelling for VT only in CMM. Therefore, it is possible not only that these social hormones have effects on neural processing of (social) auditory signals, but also that these social hormones affect neural processing of auditory stimuli differentially in NCM and CMM. Further, if parental experience does cause neural plasticity and enhanced neural responses to fledgling calls in adult zebra finches, then this may be accomplished or modulated by action of mesotocin or vasotocin in these structures through a mechanism similar to the one documented in mice.

E. Overview of Experiments:

In the interest of investigating how social interactions shape the sensory brain, the current experiment aimed to explore the common social behavior observed across the largest variety of species: parenting, and its influence on auditory processing in a bi-parental species. We specifically investigated the neural processing of socially-relevant

juvenile-produced auditory stimuli in the NCM and CMM regions of the avian auditory forebrain, in both parental and virgin adult zebra finches. When significant effects of parental experience were detected in the neural data, we went on to explore a mechanism by which they may be produced by examining the role of neuropeptide hormones involved in avian social behavior (mesotocin and vasotocin) in the neural differences found between parental and virgin subject groups. These hormones are of particular interest as they are the avian homologs to oxytocin and vasopressin, the hormones involved with pair-bonding and parental-care in mammals. Vasopressin is associated with male-typical social behaviors such as aggression, territoriality, paternal care and pair-bonding in males; while oxytocin is implicated in female-typical social behaviors including parturition, maternal attachment, and pair-bonding in females. In addition, the behavioral responses that FCs elicit in parents and virgin subjects were assessed using a novel behavioral paradigm. The role of vasotocin and mesotocin in parental behaviors in zebra finches was then studied by measuring the behavior of hormonally-treated naïve males and females, in the same behavioral paradigm. Finally, the behavioral and neural data collected from parental subjects was used to determine whether zebra finch parents can discriminate between their fledgling calls of their own offspring, as compared to unfamiliar fledglings.

The zebra finch provides a scientifically valuable model species for an investigation of how parental experience affects neural and behavioral processing of the acoustic signals produced by offspring because zebra finches are a socially-monogamous, bi-parental species. The role of oxytocin in the development of A1 neural plasticity has been demonstrated in mice (Marlin et al., 2015). However, most mammalian species

currently used in neuroscientific study do not show bi-parental care (with the exception of prairie voles and California mice) and these recent results only apply to female parents. Therefore the auditory processing results found in male zebra finches using the male-typical social hormone vasotocin are potentially valuable in their applications to the neurobiology of human male parental care, a topic which is unfortunately understudied due to model constraints.

In experiment 1 (Chapter 2), we quantified differences in the strengths of multi-unit neural responses to FCs between virgin and parental groups, with the prediction that parents have stronger responses to FCs than virgins in the avian auditory structure CMM (due to behavioral relevance). We also compared the neural responses of parents to calls of their own offspring to the neural responses they show to novel fledgling calls, to determine whether parents show a neural memory for the calls of their own juveniles, after those young birds have stopped producing fledgling begging calls. We hypothesized that parental subjects would show both multi- and single-unit neural discrimination between own and novel fledgling calls, through slower adaptation of the neural responses of the avian auditory forebrain to the familiar stimulus.

After finding effects of parental experience on neural responses to fledgling begging calls in experiment 1, experiment 2 (Chapter 3) then aimed to test a possible mechanism for those plastic changes: hormonal action within the avian auditory forebrain. In this experiment, we tested whether microinjections of the social hormones mesotocin and vasotocin could cause plastic changes in the neural responses of naïve animals. We hypothesized that hormones would cause plasticity in a similar manner as parental

experience, and further that mesotocin would show stronger effects in females than males, and vice versa for vasotocin.

Experiment 3 (Chapter 4) then aimed to show behavioral correlates of the neural effects of parental experience, by investigating parental behaviors of virgin and parentally-experienced zebra finches in a novel behavioral paradigm. We tested animals with a nest-entry paradigm, using FCs as stimuli, and hypothesized that both male and female zebra finches with parental experience would show more frequent parental behaviors (nest entries, call responses, food gathering) than virgins. This experiment also aimed to test whether the frequency of behaviors exhibited by parents, in response to FCs, differs when the auditory stimuli are calls of their own offspring, as opposed to novel fledglings. We predicted that subjects could make this behavioral discrimination, and that parents would be more responsive to the calls of their own offspring. However, results from this experiment were inconclusive. Therefore, in experiment 5 (Chapter 6) we tested the same hypothesis through more a more appropriate method, the "behavioral approach" assay. Finally, in experiment 4 (Chapter 5), nest-entry behavior was tested under the influence of the social hormones mesotocin and vasotocin. Peripheral injections of these hormones were predicted to increase parental behaviors in naïve subjects, in the same way oxytocin sensitizes virgin female mice/rats. As in experiment 2, we hypothesized that female parental behaviors would be more influenced by mesotocin injections and male parental behaviors would be affected more so by vasotocin injections.

2. Experiment 1: Neural Responses to Fledgling Begging Calls

RATIONALE

The aim of our first experiment was to broadly assess how parental experience affects the auditory processing of fledgling begging calls, using electrophysiological methods. Within this aim there were two experiments. In the first study, we tested male and female zebra finches for their neural responses to the 'category' of novel fledgling begging calls, aiming to look at how the two groups - parentally-experienced and virgin/naïve subjects - process this category differently. As learning-associated plasticity in the neural responses of adult songbirds has been demonstrated in both structures of interest here, NCM and CMM, we hypothesized that parents would have learned the behavioral relevance of fledgling begging calls and show a neuronal memory for the category that would not be seen in naïve subjects. Most commonly, the responses of CMM have been elevated in response to learning-associated stimuli in songbirds, and for this reason we expected to see an enhancement in responding to FCs in parents in relationship to naives, and for this result to occur in CMM (Gentner et al., 2004; Gentner & Margoliash, 2003; Bell et al., 2015). In addition, in this study the tuning of both parentally experienced and naïve subjects was measured to test whether any plasticity in the neural responses of parental subjects was achieved through differential tuning than what naïve subjects show. Fledgling begging calls have higher mean frequencies than other auditory cues that zebra finches produce, such as adult calls and songs (Elie & Theunissen, 2016). Therefore we hypothesized that we would find higher best frequency tuning in parental subjects than naives.

The second study in experiment 1 investigated whether parental subjects show neuronal memories for the calls of their own fledglings. Previous studies have shown that zebra finches show behavioral preference for and neuronal memories for the song/calls of other animals with which they have interacted with socially, such as a tutor or mate. Specifically, the neurons of NCM show slower adaptation of their neural responses for familiar stimuli, such as a tutor's song (Phan et al., 2006). For this reason, we expected to find that the neurons of NCM would adapt more slowly to the parent's own fledgling's call (OFC) than the calls of novel fledglings, in parents. As adaptation occurs in both multi-unit and single-unit neural responses of these structures, we assessed neural discrimination of own and novel calls in both multi-unit and single-unit data. In addition, absolute responses and spike-rates were assessed to determine whether the behaviorally-relevant OFC was responded to more strongly than novel FCs in our subjects. In this experiment, we hypothesized there would be a neural correlate for the behavioral relevance of the FC auditory category, in addition to a neural memory for OFC (specifically), in parents of both sexes.

METHODS

Subjects:

The subjects of the experiment were 12 male and 12 female adult zebra finches (**Table 1**). Subjects were reared in an aviary, maintained on a 12:12 light:dark cycle and had access to cuttlebone. Subjects had *ad libitum* access to food and water throughout the entire experiment. Half of the males and females were parentally experienced. Parentally-experienced subjects were assigned mating partners and allowed to cohabit with that partner while being provided with a nest, nesting-material, and the proper

nutrition to stimulate breeding. All parental subjects produced at least 1 surviving offspring with their mate and cohabited with both their partner and any offspring throughout development, until at least 60 dph (see **Figure 4**).

Families were isolated and recorded for auditory interactions throughout the development of subject's offspring, using an Audio-Technica microphone and power module. Continuous recordings of all vocalizations were collected between days 15 and 30 dph with Sound Analysis Pro V1.04 (Sound Analysis Pro, Tchernichovski et al., 2000). These recordings were used to create two stimuli for each clutch/subject, to be used in neurophysiological recordings and behavioral testing. One of these stimuli was a 2-3 second bout of calls from multiple offspring in the clutch (stimulus: fledglings) and the other stimulus was a 1-2 second bout of repeated calls from one fledgling (stimulus: fledgling). Both stimuli were recorded on the same day for each clutch, at 21.48 ± 0.3 dph. As young zebra finches only produce begging calls until 35 dph (at the latest), all behavioral and neural testing in parents was done 25 days or more after their offspring would have stopped producing the calls used in testing, at 60 dph or later. Therefore, any significant results indicate a long-term memory for the 'begging call' sound category.

To standardize the auditory stimuli, recordings were filtered, equated for loudness and 5 msec of silence was added to the start and end of each bout of calls (Signal; Engineering Design). Each subject heard 6 'fledgling' stimuli and 6 'fledglings' stimuli during neural recording. The same filtering procedure was used to produce the other vocalization stimuli used in neurophysiological experiments: (3) zebra finch songs, (3) canary songs, (6) adult male zebra finch calls and (6) adult female zebra finch calls. We then characterized the auditory parameters (duration (s), amplitude (dB) & mean

frequency (Hz)) of our single fledgling call stimuli using Sound Analysis Pro 2011 Software (Tchernichovski et al, 2001). Sound Analysis Pro was also used to calculate the mean frequencies for the novel zebra finch song and adult call stimuli, to determine how they compared to the frequencies found in fledgling begging calls (FCs). Finally, two t-tests were run to test whether the average mean frequency of the recorded FCs was higher than the average mean frequencies of the adult vocalization stimuli used in this experiment (adult call and song).

Electrophysiology:

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 pins are used 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 a variety of auditory stimuli. Stimulus sets included calls of individual fledglings (stimulus: fledgling), calls of an entire clutch of fledglings (stimulus: fledglings), adult male and female calls, novel zebra finch and canary songs as well as sine tuning sets. Parents were played the calls of one of their own fledglings, as well as the calls of their entire clutch to test for individual recognition. Stimulus sets were counter-balanced across subject groups.

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; stimuli were played through a speaker placed directly in front of subjects (0.5m). 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 amplified (x19,000, band-pass filtered: 0.5-5 kHz). Electrode data and the sound stimulus were collected at 25kHz per channel (Spike 2 software, CED, Cambridge, England). All 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 6s interval between stimuli.

Histology

Once all recordings were complete, 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 µA 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 µm slices using a vibratome (Series 1000), placed onto subbed slides, and stained with cresyl violet. Sections were visualized under a light

microscope to confirm electrode placement with respect to identified landmarks and cyto-archtectonic aras. 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, using Spike 2 analysis software (CED, Cambridge, England). Recordings from both NCM and CMM were visually inspected to set spike detection thresholds. 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 shorter than 2ms <=2% of the time were deemed to be true single-units. Only 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, following established procedures (Phan et al, 2006). In addition, the rate of adaptation of neural responses was 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. In addition, a 'fledgling response strength' (FRS) was calculated at each site, using the average response to fledgling calls and the average response to all stimuli at that site, to calculate a response to fledgling normalized by a site's response(formula below). This measure was used to eliminate any gross differences between subject groups in multi-unit activity, due to differential neural recruitment.

$$\text{FRS} = \frac{\left(ARM_{Fledglings} - ARM_{All\ Stimuli}\right)}{AVERAGE(ARM_{Fledglings}, ARM_{All\ Stimuli})}$$

Sites were excluded from further analyses if: 1) they were not verified histologically within NCM or CMM (above); or 2) more than half of the adult songs and calls played during testing showed responses at that site that 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 (see **Figure 6**). 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.

Statistical Analysis

Analyses were performed to test whether: parental subjects show stronger responses to fledgling calls than virgins (using FRS and single-unit firing rates), tuning best-frequencies are higher (close to the mean frequencies of fledgling calls) in parents than virgins and whether neural responses to own fledgling show slower adaptation than responses to novel fledglings in parental subjects (due to SSA). These analyses were performed using factorial ANOVAs, to fully explore all factors. For between subjects comparisons (FRS, single-unit firing rates, etc.), factorial ANOVAs included the following factors: sex of subject, parental experience of subject, side of recording (left/right), and area of auditory forebrain (NCM/CMM). Best frequency tuning was also analyzed as a between-subjects comparison, but for all tuning comparisons, ANCOVAs were used and depth was included as a covariate in addition to the factors of interest, as NCM shows tonotopic tuning. When significant factors were detected, non-parametric tests (Mann-Whitney U tests) were used for post-hoc analyses of the data. For the within-parent comparisons conducted to test whether parents neurally recognize the calls of their own young, experimenters used repeated-measures ANOVAs, with stimulus as the repeated measure, at each electrode. These ANOVAs included: stimulus, sex of subject, parental experience of subject, side of recording and area of recording as factors. In this way experimenters tested whether multi-unit ARMs, single-unit firing rates, or adaptation rates are different for own fledgling and novel fledgling calls. Tukey HSD and non-parametric Wilcoxon signed-rank tests were run as post-hoc analyses for any significant factors in the ANOVA.

RESULTS

For Experiment 1A, which focused on the between groups differences in the strength of response to fledgling begging call stimuli, data was collected from six male virgins, six female virgins, 6 male parents and 6 female parents. Between subjects comparisons were run using multi-unit electrophysiological data collected from a total of 339 responsive recording sites histologically verified to be in NCM (170 sites) or CMM (169 sites) in 24 adult zebra finches. Of those 339 sites, 161 were recorded in female subjects and 178 sites were collected in males. Finally, 166 of the units were isolated in parental subjects and 173 recorded in naïve subjects. Therefore, the samples recorded and analyzed were not strongly skewed to any one subject group, or structure.

A total of 326 single units were isolated offline from that multi-unit data set, to analyze single-unit tuning, 108 in NCM and 218 in CMM. CMM tended to show better unit separation. An exemplar raster plot for one NCM single-unit, in response to 2 novel and 1 familiar FC stimulus, is plotted in **Figure 6**. In addition, the average PSTHs of all units recorded from naïve and parental subjects, , to an exemplar fledgling call stimulus are plotted in **Figure 7A&B** for both NCM and CMM. In **Figure 7C, t**he average PSTH for units recorded in parents hearing their own FC stimulus, is then plotted against the average PSTH for that stimulus for units recorded in all other parents. Single-units recorded in parental subjects show significantly higher responses to the exemplar FC than those recorded in naïve subjects, at time points indicated with an asterisk (**see Figure 7A&B**). In addition, in parental subjects, single neurons respond more strongly to their own offspring's FC stimulus than the units recorded in parents for whom this FC is novel at time points indicated with an asterisk (see exemplar CMM data in **Figure 7C**).

Parental Subjects show Stronger Neural Responses to Fledgling Begging Calls

The strength of the multiunit neural responses to fledgling begging calls was compared across parental and naïve subjects, to test the effect of parental experience and behavioral relevance on the neural representation of that category. But first, the average neural response (ARMs) to all auditory stimuli was compared across these two groups, to ensure there were no gross differences in neural processing of sound in these two subject groups. However, when a factorial ANOVA was run on the average response to all stimuli in these two groups, there was a significant interaction between subject group and area of recording ($F(1,322) = 15.264$, $p < 0.001$; see **Figure 8**). Specifically, NCM sites of naïve subjects had significantly stronger neural responses to all stimuli (74.63 ± 4.16) than parental subjects (51.94 ± 4.21; Tukey HSD post hoc $p < 0.001$). At CMM sites, on the other hand, responses of naïve subjects were slightly lower (41.94 ± 4.16) than those of parents (51.97 ± 4.35), although not significantly different (Tukey HSD, ns). This general difference between naïve and parental subjects in auditory responses at NCM multiunit sites necessitated the use of a normalized measure of fledgling begging call responding, to focus on the response strength of a subject to that stimulus category relative to the strength of their auditory responsiveness in general. For this reason, the fledgling response strength (FRS) was used for the remainder of the between subjects comparisons in this experiment (1A). Because this is a difference measure relative to the average response, and FCs have low responses, FRS is typically a negative number; thus, relatively stronger responses show as less negative on this measure.

When a factorial ANOVA was run on FRS values using parental experience, sex of subject, area and side of recording as factors, parental experience emerged as the only

factor which had a main effect on fledgling response strengths ($F(1, 321)=10.17$, $p < 0.01$; see **Figure 9**). Parental subjects showed significantly stronger FRS values (-0.233 ± 0.04) than naïve subjects (-0.41 ± 0.039). This effect was true in both parentally experienced males and females (Female main effect of parental experience: $F(1, 153)=5.77$, $p< 0.05$; Male main effect of parental experience: $F(1, 168)=5.36$, $p< 0.05$; see **Figures 10A and 10B**). Parents of both sexes had higher FRS values (males: -0.209 ± 0.05; females: -0.257 ± 0.05) than their naïve counterparts (males: -0.419 ± 0.05; females: -0.402 ± 0.05). Sex did not have an effect on fledgling responses strengths in naïve subjects ($F(1, 164)=.0383$, ns; see **Figure 10C**) and sex did not interact with parental experience to influence FRS values ($F(1,321) = 0.341$, ns).

A fascinating effect emerged: there was a significant interaction between parental experience, sex and side of recording such that the effect of parental experience on FRS was seen primarily in the right hemisphere of females and the left hemisphere of males ($F(1, 321) = 4.47$, $p < 0.05$; see **Figure 11**). In males, parents had significantly lower FRS values at multiunit sites in the left hemisphere (-0.113 ± 0.076) than did naïve subjects (-0.454 ± 0.076; Tukey HSD $p < 0.05$) and did not show a difference in the right hemisphere (parent: -0.306 ± .076; naïve: -0.384 ± 0.075, Tukey HSD, ns). In females, the largest difference between parents and naïve subjects lay in the right hemisphere with parents showing higher FRS values (-0.211 ± .086) than naives (-0.460 ± 0.079), however this did not come through as significant in post-hoc tests (Tukey HSD, ns). The left hemisphere of females showed no difference between parents and naïve females (parents: -0.344 ± 0.079; naives: -0.303 ± 0.077; Tukey HSD, ns). This interaction suggests that there may be some lateralization in the mechanism through which parental experience

influences the auditory processing of fledgling begging calls, and further that this lateralized mechanism may differ in the two sexes.

The final significant result that emerged from the factorial ANOVA on FRS values was an interaction between parental experience and the structure in which neural responses were recorded ($F(1, 321)=4.1038$, $p< 0.05$; see **Figure 12**). Post-hoc Tukey HSD tests revealed that the significant difference between parental and naïve subjects was specific to CMM responses, where parents showed stronger FRS values (-0.176 ± 0.057) than naives (-0.466 ± 0.055; $p < 0.01$). NCM sites of parents also showed stronger FRS values (-0.291 ± 0.055) than naives (-0.355 ± 0.055) but this was not a significant result (ns). Therefore, the effect of parental experience on fledgling response strengths is driven by activity in CMM, an area whose neural responses are commonly associated with the behavioral relevance of auditory stimuli (Gentner et al., 2004; Gentner & Margoliash, 2003; Bell, Phan & Vicario, 2015)

Changes in Tuning are Associated with Parental Enhancements in FRS

Neural data collected during the presentation of a tuning set of sine tones was used to explore the possibility that a parental enhancement in fledgling begging call responding could reflect a change in tuning. We analyzed parameters of the stimuli used, and found that fledgling calls have fundamentally different auditory characteristics than other social zebra finch auditory stimuli, such as songs and calls. The average fledgling call is 143.01 ± 15.7 ms in duration and 43.63 ± 0.71 dB in amplitude. When the mean frequencies of fledgling begging call stimuli were compared to the mean frequencies of adult calls, there was a significant difference ($t(16) = -7.38$, $p < 0.001$; see **Figure 13A**).

Adult calls are composed of significantly lower frequency components (Hz) (3909.62 ± 98.7) than fledgling begging calls (6117.48 ± 385.04). In addition, when a t-test was run to compare the mean frequencies of song stimuli to those of a bout of fledgling calls, there was a significant difference ($t(7) = -11.23$, $p < 0.001$; see **Figure 13B**). Adult songs are also composed of significantly lower frequency components (Hz) (2946.8 ± 1.14) than fledgling begging calls (5904.93 ± 180.02).

When multi- and single-unit sites were characterized by the sine tone that caused their strongest response (best frequency) there were significant effects of parental experience on tuning in both types of units. ANCOVAs were run to test the effect of parental experience on best frequency tuning data, with depth as a numerical factor, because NCM shows a tonotopic structure along its ventral-dorsal axis. Results showed that multi-unit best frequencies were higher in parents (2706.08 ± 82.4) than naïve subjects (2475.24 ± 81.5; $F(1,333) = 3.93$, $p < 0.05$; see **Figure 13C**). Single-unit data showed the same effect, the units of parental subjects had significantly higher best frequency tuning (3011.68 ± 142.3) than units in naïve subjects (2541.18 ± 131.3; $F(1,160) = 5.89$, $p < 0.05$; see **Figure 13D**). To further analyze these group differences in tuning, we also looked at the depth at which BF tuning was recorded in both groups, to ensure this difference in tuning was not due to differential electrode placement along the dorsal-ventral tuning axis (as depth from the dorsal surface of NCM increases, BF tuning also increases; Terleph, Vicario & Mello, 2006). Our single-unit data showed no effect of electrode depth on tuning BFs in the ANCOVA ($F(1,160) = 1.73$, ns), and there was no difference in the depth of placement between parental and naïve subjects ($t_{(161)} = 1.08$, p = ns). On the other hand, our multi-unit data did show a significant effect of electrode

depth on BF tuning, such that more ventral sites showed higher BFs, as expected ($F(1,333) = 8.42$, $p < 0.01$). However, a t-test showed that electrode placement was actually deeper in naïve subjects (449.48 ± 12.47 μm) than in parents (404.33 ± 14.02 μm; $t(334) = 2.41$, $p < 0.05$). Therefore, the difference in BF tuning between parental and naïve subjects was not due to differences in placement along the dorsal-ventral tuning axis.

In experiment 1B, data recorded from the same parental subjects as used in 1A were analyzed to determine whether parents had a 'neural memory' for their own fledgling's begging call. As both NCM and CMM show stimulus specific adaptation (SSA) a stimulus that has an adaptation rate significantly slower than the adaptation rate to novel, at that site, is considered 'familiar' to the subject. Therefore, throughout these comparisons repeated measures ANOVAs were used to compare neural responses to one's own fledgling's call to those of novel fledglings. As only parental subjects could be used for this comparison, the data reported here are from 166 multiunit sites, 84 in NCM and 82 in CMM. From those sites we isolated 64 single units: 23 units in NCM and 41 units in CMM; 33 in males and 31 in females.

Parental Subjects show a 'Neural Memory' for their Own Fledgling's Call 25 Days Later

Data was recorded from parental subjects at the fledgling's post-hatch day 60, or later. Therefore, fledglings had not produced begging calls for at least 25 days. In fact, parental subjects were recorded from at day 98.6 ± 13.6 on average, meaning they had not heard their own fledglings produce this type of call in over 60 days. To assess

whether parents showed an elevated neural response to the call of their own fledgling (recognition of OFC), as they do for the category of fledgling calls, repeated measures ANOVAs were run on multiunit ARMs and single-unit spike rates in NCM and CMM (there was no need to use FRS values here, as comparisons were run within-group). Results showed that parents have an enhanced response to their OFC in both multi- and single-unit activity (multiunit: $F(1, 148)=4.65$, $p< 0.05$; single-unit: $F(1, 60)=4.87$, $p< 0.05$; see **Figure 14A&B**). Multiunit ARMs were significantly stronger in response to OFC (40.2 ± 0.79) than the calls of novel fledglings (36.8 ± 0.79), in both male and female subjects (stimulus/area interaction: $F(1,148) = 0.726$, ns). Single-unit spike rates were significantly faster in response to one' own fledgling (11.0 ± 0.39) than novels (9.31 ± 0.39; as illustrated in an exemplar unit in **Figure 7C**). These results were also further analyzed to determine in which structure the significant enhancement of OFC neural response lay. Repeated measure Wilcoxon non-parametric tests were run on ARMs in NCM and CMM and showed that the difference was primarily seen in NCM. In NCM, ARMs to one's OFC (42.9 ± 4.82) were significantly stronger than ARMs to novel calls (37.2 ± 3.40; $Z = 1.98$, $p < 0.05$; **Figure 15A**). On the other hand, ARMs to own (39.0 ± 3.30) and novel (37.1 ± 3.30) showed no difference in CMM ($Z = 1.41$, ns; **Figure 15C**). Finally, repeated measure Wilcoxon tests were run on NCM and CMM spike rates, to determine in which structure showed the significant difference between spiking to own and novel stimuli. In NCM, spike rates to own (8.08 ± 1.49) and novel stimuli (7.52 ± 1.13) did not differ ($Z = 0.243$, ns; see **Figure 16A**). However, in CMM spike rates were significantly faster to OFC (13.9 ± 2.20) than the calls of novels (11.4 ± 0.056; $Z = 2.64$, $p < 0.01$; see **Figure 16B**).

In addition, when multiunit adaptation rates were analyzed, they showed slower adaptation to their own fledgling's call than novels, indicating that there was a long-term neural memory (as assessed by SSA) for one's own fledgling's call ($F(1, 148)=11.35$, $p< 0.001$; see **Figure 14C**) in both sexes (stimulus/structure interaction: $F(1,148) = 1.37$, ns). Male and female parents adapted significantly more slowly (-0.191 ± 0.011) to the call of their own offspring than to the calls of novel fledglings (-0.265 ± 0.011). This result was further explored to assess which, if either, structure was more responsible for this result than the other. Adaptation rates to own and novel fledgling calls were analyzed for within-subjects differences in NCM and CMM separately, using repeated measure Wilcoxon non-parametric tests. In NCM, multiunit adaptation rates to parent's own fledgling's begging call were significantly slower (-0.134 ± 0.032) than adaptation to novel stimuli (-0.239 ± 0.026; $Z = 3.42$, $p < 0.001$; see **Figure 15B**). In CMM, however, adaptation rates to one's own fledgling (-0.274 ± 0.066) did not differ from those to novel fledgling calls (-0.0313 ± 0.053; $Z = 1.74$, ns; see **Figure 15D**). Therefore, familiarity with OFC had an effect on adaptation rates only in NCM, but familiarity with OFC caused an enhancement of neural responding in both NCM and CMM.

When the same comparison was run on the single-unit data, however, results did not show a significant difference in how quickly the units of parents adapted to the familiar stimulus, as compared to novels ($F(1, 60)=.608$, ns; see **Figure 14D**). Adaptation rates for own (-0.503 ± 0.016) and novel stimuli (-0.476 ± 0.016) were similar. When single-unit adaptation rates were analyzed separately for NCM and CMM, as done for multi-units, neither structure showed a significant effect of stimulus familiarity (NCM: $Z = 1.125$, ns; CMM: $Z = 0.888$, ns; see **Figure 16C&D**). This failure

to find a result in single-units that was present in the multi-unit data may be due, in part, to the much smaller sample size, or to the participation in multi-unit recordings of units with smaller spikes that were less likely to be isolated.

DISCUSSION:

The results of experiment 1 support the hypothesis that parental experience has an effect on the neural-processing of fledgling begging calls in the auditory forebrain structures (NCM and CMM) of adult zebra finches. Further, these experiments provide evidence that parental experience influences the auditory processing of these cues in two ways: (1) through increased neural processing of the 'category' of fledgling calls as well as (2) a distinct 'neural memory' for the calls of one's own offspring. Results indicate a memory for the 'category' of fledgling calls as a greater response to the calls of novel fledglings in parents than in virgin subjects, as measured by the fledgling response strength (FRS). Parental subjects of both sexes showed stronger FRS values than virgins in the avian auditory forebrain. However, a significant interaction between parental experience and area of neurophysiological recording indicated that this effect was strongest in the multi-unit sites of CMM, an auditory structure whose neural responses have been repeatedly associated with the behavioral relevance of stimuli (Gentner et al., 2004; Gentner & Margoliash, 2003; Bell, Phan & Vicario, 2015). Therefore, results are consistent with the hypothesis that parental experience with fledgling call stimuli teaches birds the social and behavioral relevance of this category of auditory cues, thereby enhancing neural responses to all stimuli of this category. This result suggests that parental experience is a learning process through which neural representations of behaviorally-relevant stimuli develop in NCM and CMM, just as occurs in songbirds for

more classical types of learning (e.g. operant, Bell et al., 2015).

In addition, results indicated a significant interaction between parental experience, sex of subject and side of neurophysiological recording, such that parents showed higher FRS values than virgins in the left hemisphere of the avian auditory forebrain of males and the right hemisphere of females. This result was not specifically hypothesized, however it does suggest that parental experience influences neural responses to fledgling begging calls in a lateralized manner, which is consistent with what has been observed in mammals (Ehret, 1987; Marlin et al., 2015). Female mice show enhanced single-unit responses to the isolation calls of young pups in A1, through an oxytocin-mediated mechanism that is specific to the left hemisphere where oxytocin receptors are expressed on auditory neurons (Marlin et al, 2015). In mammals, however, this lateralized effect has only been observed in females. Our results replicate this lateralized phenomenon and extend it to male parents as well, suggesting that both sexes show similar effects of parental experience, but that they do so perhaps through differently lateralized mechanisms. If parental experience exerts its effects on neural processing through hormonal mechanisms in zebra finches, as it does in mice, males and females may show these effects via different hormones, or through the same hormone if there are sex differences in how receptors for that hormone are expressed in the left and right hemispheres. Unfortunately, the important experiments conducted by Goodson and colleagues up to this point have not yet explored sex-differences and lateralization of MT and VT receptors (Leung et al., 2011). Therefore, to explore this effect, experiments that investigate the distribution of hormonal receptors in the avian auditory forebrain are necessary to learn whether males and females show different patterns of expression, and

how receptors are distributed across left and right hemispheres. Such a follow-up experiment could also shed light into the comparative difference in lateralization of this parental-experience effect, detected in the left hemisphere of female mammals and here in the right hemisphere of female birds. Although lateralization may similarly function to improve the efficiency of auditory-information processing in both these species, the mechanism of lateralization may have emerged differently in mammals and birds, due to their evolutionary distance. This explanation may also be true for the lateralization of auditory processing of vocal communication signals, which is left-hemisphere dominant in humans (mammals) and right-side dominant in songbirds (Phan & Vicario, 2010).

To further explore mechanisms by which the parental-experience enhancement of fledgling call processing occurs, we looked at neural responses to sine tones in virgins and parents. As the mean frequencies of fledgling begging calls are significantly higher than the mean frequencies in the other social vocal signals of adult zebra finches (shown for calls and songs here and the rest of the zebra finch vocal repertoire in Elie & Theunissen, 2016), we predicted higher best frequency tuning in the neural recording sites of parental subjects than in those of virgins. Results supported this hypothesis, in both multi-unit and single-unit sites the average 'best-frequency' was higher in parents than virgins. Neurons in parental subjects may undergo a change in tuning as they encode the behavioral relevance of these high-frequency fledgling begging calls, which then contributes to the greater neural responses to this category of cues seen in parents at testing. Further experiments are needed to determine how these plastic changes in auditory neuron tuning are accomplished, and whether social hormones like oxytocin are involved (see Chapter 3). There is extensive research on learning-associated tuning

changes in the neurons of the auditory cortex, and the role of acetylcholine-mediated disinhibition in the process by which this plasticity occurs (as reviewed in Weinberger, 2004). Oxytocin has also been shown to cause disinhibition in the neurons of the mammalian auditory cortex and therefore could be responsible for tuning plasticity in the same way (Marlin et al., 2015). In mammals, oxytocin causes a marked decrease in inhibition of A1 neurons within 15 minutes of hormone-treatment and E:I balance stabilizes within 30 minutes of exposure (2015). Therefore, the rapid changes in RFs of avian auditory neurons documented here (detected 30 minutes to a couple hours after drug-treatment) might occur through a similar mechanism, through changes in lateral inhibition that do not require synaptic or gene expression changes. In addition, previous research has shown inhibitory mechanisms are important in the development of learning-associated plasticity in the avian auditory forebrain structures studied here (Thompson et al., 2013).

Finally, the results of experiment 1 showed a 'neural memory' for the specific calls of a subject's own offspring, in addition to enhanced responding to the category of FCs. When the neural responses of parental subjects were assessed for whether there was a difference in how a subject responded to the call of his/her own fledgling's call (OFC) and the call of novel fledglings, results indicated that auditory neurons do discriminate between own and novel fledgling calls, as hypothesized. Familiarity to OFC was first assessed through neural adaptation rates to fledgling calls as both NCM and CMM show stimulus specific adaptation, a neuronal memory for the songs/calls with which a subject has been presented. The neural ARM to a song/call is robust at first and declines with repeated presentations of that stimulus, adapting rapidly at first and slowing as the

adaptation of responses reaches asymptote. This adaptation is then maintained for up to 96 hours from stimulus playback (Chew et al., 1996b). Therefore, familiar auditory stimuli are adapted to more slowly than novels. In the data collected from our parental subjects, multi-unit neural responses adapted more slowly for OFC than novel fledgling calls, as hypothesized. This effect, interestingly, occurred primarily in NCM rather than CMM. This observation fits with various previous findings that suggest that NCM's primary role in stimulus processing is encoding stimulus familiarity whereas CMMs encodes the behavioral relevance of stimuli (Gentner et al., 2004; Gentner & Margoliash, 2003; Thompson & Gentner, 2010; Bell, Phan & Vicario, 2015). Unexpectedly, the data from single-unit responses to OFC and novel calls failed to replicate this finding. However, this failure to reach significance in single-unit data may have been due to the fact that the sample size was much smaller for this comparison. In addition, both multi- and single-unit responses showed neural discrimination of own and novel fledgling calls through a greater response (ARMs, spike-rates) to OFC than the calls of novel fledglings. This enhanced response to the behaviorally-relevant and familiar stimulus, OFC, was not region-specific, occurring in NCM multi-unit responses and CMM single-unit responses. Nevertheless, the results show robust evidence (through adaptation rates, ARMs and spike-rates) that the auditory neurons of parents recognize and show differential responses to the calls of their own fledglings, as compared to novels. This is a novel finding that supports the claims from Levrero et al., 2009 and Ligout et al., 2015 that parents can behaviorally discriminate between the calls of their own and novel pups, as behavioral recognition would have to begin at the neural level. In addition, these results are powerful with regard to the strength of the neural memory for one's own offspring's

call, as this memory was detected in parents that had not heard these calls in 60 days on average (25 days at the minimum) while stimulus-specific adaptation lasts only a few days if stimuli are presented through a passive-playback procedure. In this case, the behavioral importance of OFC (and fledgling calls in general) may be responsible for the long-term effects observed in this experiment. Further experiments to test the time course of these effects are necessary to determine whether parental experience exerts a permanent effect on neural responses to OFC, fledgling calls and sine tones, or if instead, these effects are transient. In fact, the effects detected in this experiment may be weak in comparison to those we would observe in parents at an earlier stage when offspring are still producing FCs or soon after they cease to produce these begging calls.

3. Exp 2: Effects of Central Injections of Mesotocin and Vasotocin on Neural Responses

RATIONALE:

Experiment 2 aimed to describe NCM and CMM neural responses to fledgling calls in zebra finches that have not been exposed to mating and child-rearing (naïve/virgin), and determine whether hormone-treatment to these structures changes neural responses as has been seen in mammals (Marlin et al., 2015). Results in experiment 1 of this study showed that naïve subjects differ from parentally-experienced subjects on a couple of neurophysiological measures: parents show stronger multi-unit neural responses to FCs than virgins (in a lateralized manner), and the multi-unit/single-unit sites of parents show tuning toward higher frequencies than sites of naïve subjects. As the mean frequency of fledgling call stimuli is significantly higher than that of adult zebra finch songs and calls (see Chapter 2), the parental-experience associated changes in auditory neuron receptive fields may account for the stronger responses to FCs that were observed as well. Therefore, the current experiment was designed to investigate a possible mechanism through which parental experience may cause animals to show this change in auditory neuron RFs.

Learning-associated receptive field plasticity has been studied in the neurons of the mammalian auditory cortex for many years now. Throughout that time, many studies have implicated acetylcholine release and action in the auditory cortex as the mechanism through which learning induces RF changes in the neurons of A1 (as reviewed in Weinberger, 2004). More recent work has shown that ACh release is sufficient to produce these changes and that it works through disinhibition of auditory neurons to

induce plasticity (Ji, Gao & Suga, 2001; Ji & Suga, 2003; McLin et al., 2002; Froemke et al., 2007; Letzkus et al., 2011; Froemke et al., 2013). When neurons of the auditory cortex are released from lateral inhibition rapid RF plasticity is possible due to the extra-classical tuning of auditory neurons. Most recently, the Froemke lab showed that inhibitory neurons of the auditory cortex show receptors for oxytocin and that the hormone's release there also causes disinhibition and plasticity (Marlin et al., 2015). Therefore, as oxytocin levels are high throughout parenting in mammals and exogenous oxytocin induces pup-retrieval in virgin females, the probable cause of differences in how dams and virgins process pup-isolation calls in the auditory cortex is oxytocin.

The avian analog of the mammalian hormone oxytocin is mesotocin and the avian analogy of vasopressin is vasotocin. Both of these hormones have been implicated in parental behavior in mammals as well as zebra finches. In addition, the auditory forebrain of songbirds shows receptors for these hormones just as the auditory cortex of mammals does for oxytocin. Receptors for vasotocin can be found in both NCM and CMM while receptors for mesotocin can only be found in NCM (Leung et al., 2011). However, the role of these receptors in songbird auditory processing has not been elucidated. We hypothesized that these receptors perform similar functions in songbird auditory forebrain as oxytocin does in mammalian A1, and that microinjections of both hormones into the forebrain would induce plasticity into the activity of auditory neurons in NCM, and CMM to a lesser extent (as it does not show both receptor types). As these social hormones are important for the expression of parental care and we see specific changes in neural processing after parental experience, it was hypothesized that hormone-treatment would increase neural responses to FCs and change tuning properties of NCM

and CMM neurons. The mammalian analogs of vasotocin and mesotocin show sex differences in their functions and receptor expression, therefore we further predicted there would be sex differences in how these hormones affect the neural responses of male and female zebra finches. Specifically, we hypothesized there would be a greater influence of mesotocin in females and vasotocin in males.

METHODS:

Subjects:

The subjects of the experiment were an additional 19 male and 19 female adult zebra finches (**Table 1**). Subjects were reared in an aviary, maintained on a 12:12 light:dark cycle and had access to cuttlebone. Subjects had *ad libitum* access to food and water throughout the entire experiment. All subjects for hormonal manipulation experiments were naïve to breeding and parenting offspring. Ten naïve males and ten naïve females were centrally injected with mesotocin on one side of the avain auditory forebrain (NCM and CMM); 9 naïve subjects of each sex were centrally injected with vasotocin on one side of the avian auditory forebrain. The hemisphere of injection was counter-balanced across subjects.

Central Drug Manipulations and Electrophysiology:

Two days prior to experimentation, subjects ($n_{male} = 19$, $n_{female} = 19$) 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 a variety of auditory stimuli. Stimulus sets included calls of individual fledglings (fledgling), calls of an entire clutch of fledglings (fledglings), adult male and female calls, novel zebra finch and canary songs as well as sine tuning sets. Stimulus sets were counter-balanced across subject groups.

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 and dura were opened bilaterally over NCM and CMM. At this point, a stereotaxic arm was used to lower a micropipette (Drummond Wiretrol I, 5 uL, Drummond Scientific Company, Bromall, PA) filled with drug solution (50μMolar mesotocin in .9% saline, 50μMolar vasotocin in .9% saline) or saline vehicle into both structures at a 30 degree angle (concentrations based on Goodson et al. 2009). Drug was injected into one hemisphere, and saline into the other and this was counter-balanced across subjects. Both NCM and CMM received the same treatment in any one subject. NCM injections were made, at both 1000uM (~15 nL) and 1100uM (~34 nL) below the surface of the brain. CMM injections were made at both 800uM (~15 nL) and 900uM (~34nL) below the surface of the brain. This ensured a dispersion of drug/saline throughout a large portion of both structures.

After all injections were made, a Microdrive was used to place 16 tungsten electrodes bilaterally on the surface of the brain, near the bifurcation of the mid-sagittal

sinus and injection sites (**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 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 6s interval between stimuli.

Histology

Once all neural 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 µA 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 µm slices using a Vibratome (Series 1000), placed onto slides, and stained with cresyl violet. Sections were visualized under a light microscope to confirm electrode placement. 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). Recordings from both NCM and CMM were visually inspected to set spike detection thresholds. 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 shorter than 2ms <=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, following established procedures (Phan et al. 2006). In addition, the rate of adaptation of neural responses was 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. In addition, a 'fledgling response strength' (FRS) was

calculated at each site, using the average response to fledgling calls and the average response to all stimuli at that site to calculate a response to fledglings normalized by a site's response, as in experiment 1. Sites were excluded from further analyses if: 1) they were not verified histologically within NCM or CMM (above); or 2) more than half of the adult songs and calls played during testing showed responses at that site that 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.

Statistical Analysis

Neural data was analyzed using ANOVAs as described above (experiment 1) for neural effects of drug manipulations on neural responses (multi- and single-unit) to fledgling calls. Analyses were conducted across naïve untreated and vasotocin/mesotocin treated groups to assess group differences due to hormonal injections. In addition, secondary ANOVAs were used to assess within-group comparisons in the mesotocin- and vasotocin-treated groups, to assess whether responses to fledgling calls differ, within a bird, between saline and drug treated hemispheres. Further, experimenters will assess whether there is lateralization of the system by testing whether the effects of drug are influenced by into which hemisphere they are injected. Analyses were also performed to

assess whether hormonal injections influenced best frequency tuning in structures NCM or CMM. For all tuning comparisons, ANCOVAs were used and depth was included as a covariate, as NCM shows tonotopic tuning. Tukey HSD and non-parametric wilcoxon signed-rank tests were run as post-hoc analyses for any significant factors in the ANOVA.

RESULTS:

Neural responses to all auditory stimuli, fledgling calls and sine tones were assessed in untreated (from experiment 1), vasotocin-treated and mesotocin-treated subjects to investigate how social hormones influence neural processing of auditory stimuli in the avian auditory forebrain. Multi-unit responses of the two hormone-treated groups were first analyzed separately against responses in untreated naïve 'controls', followed by separate ANOVAs testing within the vasotocin and mesotocin-treated groups to fully analyze the responses at drug-treated and saline-treated sites in relationship to the other factors: side of recording, area of recording, and sex of subject. In these analyses, data from a total of 275 sites collected in subjects centrally-injected with mesotocin were analyzed, 123 from NCM and 152 from CMM; in addition, data from 262 sites of vasotocin-injected birds were included, 123 from NCM and 139 from CMM.

Hormones Show Variable Effects on FC and Tuning Responses across Naïve Groups

As in experiment 1, multi-unit data was first compared across subject-groups to test whether hormone-treatment had an effect on the average ARM responses to 'all stimuli' played during neurophysiological recordings. Across the naïve and centrally manipulated groups there were global group differences in multi-unit neural responses ($F(2,685) = 34.86$, $p < 0.0001$). There was a significant interaction between group and area of

recording on the average neural response ($F(2,685) = 8.44$, $p < 0.001$; see **Figure 17**). In NCM, responses in untreated naives (74.61 ± 3.72) were significantly higher than responses in both vasotocin (39.66 ± 3.12) and mesotocin-treated (35.70 ± 3.15) subjects (Tukey HSD, $p < 0.05$). In CMM, responses in untreated naives were also stronger (41.63 ± 3.71) than those in mesotocin treated subjects (25.71 ± 2.76; Tukey HSD, $p < 0.05$), although they did not differ from those in vasotocin-treated subjects (33.25 ± 2.92, ns). These strong global differences in how untreated and hormone-treated groups responded to auditory stimuli necessitated the use of normalized response measures to investigate any specific changes in neural responding to fledgling begging calls.

Fledgling response strengths of hormone-treated subjects were compared to those in untreated naives. Comparisons were conducted separately for male and female subjects to test the hypothesis that these hormones would have sex-specific effects. There was no effect of hormone-injection on the FRS responses of naïve male subjects (vasotocin v. untreated: $F(1,212) = 1.947$, ns; mesotocin v. untreated: $F(1,209) = 1.15$, ns; see **Figure 18A**). Male subjects also did not show any interaction between hormonal injection and area of recording on FRS values (vasotocin/area interaction: $F(1,212) = 0.532$, ns; mesotocin/area interaction: $F(1,209) = 0.134$, ns). In females, however, FRS responses of untreated naives were significantly higher (-0.402 ± 0.041) than responses in mesotocin-treated females (-0.591 ± 0.038; $F(1,222) = 8.78$, $p > 0.01$) and there was a trend in the same direction for vasotocin-treated females (-0.503 ± 0.032; $F(1,206) = 3.70$ $p = 0.06$; see **Figure 18B**). Once again, this result was equally true of both NCM and CMM (mesotocin/area interaction: $F(1,222) = 1.48$, ns; vasotocin/area interaction: $F(1,206) = 0.571$, ns). However, this result was unexpected and in direct conflict with our

experimental hypothesis.

Finally, we also compared best-frequency tuning of the multi-unit sites in these three subject groups in both avian auditory forebrain structures NCM and CMM. In NCM, both social hormone treated groups showed higher best frequency tuning than untreated naives (see **Figure 19A**). The main effect of drug-treatment trended toward significance in the ANCOVA comparing vasotocin-treated subjects (2666.94 ± 120.2) to untreated controls (2322.9 ± 141.4; $F(1,193) = 3.011$, $p = 0.08$). However, the average best frequency of multi-unit sites in untreated subjects (2397.16 ± 138.8) did not significantly differ from the best frequency of mesotocin-treated subjects (2731.7 ± 128.2; $F(1,180) = 2.748$, ns; see **Figure 19A**). Results in CMM were very different than those recorded in NCM. Rather than hormonal treatment increasing best frequency tuning, here vasotocin-treated animals showed significantly lower best frequencies (2213.82 ± 83.0) than untreated controls (2669.44 ± 104.7) at their multi-unit sites ($F(1,208) = 11.50$, $p < 0.001$; see **Figure 19B**). Mesotocin-treated (2608.03 ± 93.0) and untreated subjects (2653.26 ± 110.9), however, did not show a main effect of treatment on best-frequency tuning in CMM ($F(1,197) = 0.969$, ns; see **Figure 19B**). The effect of hormonal injections on NCM and CMM best frequency tuning was not affected by the sex of subject in either the vasotocin- (NCM sex/group interaction: $F(1,193) = 0.295$, ns; CMM group/sex interaction: $F(1,208) = 2.56$, ns) or mesotocin-treated group (NCM sex/group interaction: $F(1,180) = 0.166$, ns; CMM group/sex interaction: $F(1,197) = 3.44$, $p = 0.07$).

Results of Within-Group Comparisons Show Sex Differences in the Effects of Hormone Injections

When neural responses to all auditory stimuli were compared within groups, so that saline and hormone-treated hemispheres could be compared, there was little influence of either vasotocin or mesotocin on ARMs in the avian auditory forebrain. In the vasotocin-treated group there was no effect of hormone-treatment on ARMs to 'all stim' in either structure (drug main effect: $F(1,245) = 0.143$, ns; drug/region interaction: $F(1,245) = 0.195$, ns; see **Figure 20A**). Likewise, in the mesotocin-treated group there was no effect of hormone treatment on the neural responses to auditory stimuli, in general ($F(1,258) = 0.398$, ns; see **Figure 20B**). There was an interaction between the region of recording and the effect of mesotocin treatment on ARMs to 'all stim' ($F(1,258) = 4.41$, $p < 0.05$), but neither structure showed a significant effect of hormone treatment on neural responses in post-hoc Tukey tests (NCM: $p = 0.06$, CMM: ns; see **Figure 20B**). Therefore, absolute response magnitude measures were used for all within-group comparisons of multi-unit neural responses to fledgling calls at saline and hormone-treated recording sites.

Contrary to initial hypotheses, multi-unit responses to FC stimuli were not affected by either vasotocin or mesotocin treatment, matching the results seen for 'all stim.' When neural responses to FCs were compared across saline and vasotocin-treated sites, there was no main effect of hormone treatment on ARMs ($F(1,246) = 0.268$, ns) and no interaction between hormone-treatment and region of recording ($F(1,246) = 0.182$, ns; see **Figure 21A**). Results in the mesotocin-treated group were similar, showing no effect of hormone treatment on ARMs to FCs ($F(1,259) = 0.445$, ns) in either region ($F(1,259) = 1.860$, ns; see **Figure 21B**). However, results from the ANOVAs analyzing the ARMs to FCs in vasotocin and mesotocin-treated subjects did show unexpected main effects of the

sex of the subject on neural responses, in both groups. In the vasotocin-treated group, males showed significantly higher ARMs to FCs (33.53 ± 2.90) than females did (19.53 ± 2.89; $F(1,246) = 11.55$, $p < 0.05$; see **Figure 22A**). On the other hand, in the mesotocin-treated group, females showed a stronger response to FCs (26.08 ± 2.01) than males (15.16 ± 2.30; $F(1,259) = 12.76$, $p < 0.001$; see **Figure 22B**). These results, in conjunction with results from chapter 2 that showed no difference in how male and female subjects responded to FCs (see **Figure 10**), suggest that the social hormones mesotocin and vasotocin affect males and females differently, as hypothesized.

The sex-specific effects of social hormones on ARMs to FCs were also specific to the avian auditory area NCM. There were significant interactions between the sex of subject and the region of recording on neural responses to FCs in both mesotocin and vasotocin-treated groups (mesotocin: $F(1,258) = 6.29$, $p < 0.05$; vasotocin: $F(1,245) = 5.89$, $p < 0.05$; see **Figure 23**). Vasotocin-treated males showed stronger ARMs to FCs (42.63 ± 4.23) than vasotocin-treated females (18.49 ± 4.23) in NCM (Tukey HSD, $p < 0.05$), but not CMM (male: 24.42 ± 3.98; female: 20.58 ± 4.00; ns). Likewise, mesotocin-treated females showed stronger ARMs to FCs (34.73 ± 2.89) than vasotocin-treated males (16.11 ± 3.60) in NCM (Tukey HSD, $p < 0.05$), but not CMM (female: 17.42 ± 2.81; male: 14.20 ± 2.86; ns).

To further investigate whether the hormone-injections in NCM were working to increase ARMs to FCs in males treated with vasotocin and females treated with mesotocin, we also analyzed ARMs to FCs at hormone-treated sites as compared to saline-treated sites, in both sexes separately. In the NCM of female subjects, mesotocin-treated sites did show higher ARMs to FCs (41.02 ± 7.41) than saline-treated sites (28.90

± 3.97), but this effect was not significant (Z = -0.676, ns; see **Figure 24B**). NCM activity in mesotocin-treated males showed similar ARMs to FCs at mesotocin (18.28 ± 3.19) and saline-treated (15.85 ± 3.43) sites (Z = -0.325, ns; see **Figure 24A**). Similarly, NCM activity in vasotocin-treated females showed similar ARMs to FCs at vasotocin (19.46 ± 2.93) and saline-treated sites (17.35 ± 2.72; Z = -0.556, ns; see **Figure 24C**). Vasotocin-treated males did show stronger ARMs to FCs at vasotocin-treated NCM sites (44.21 ± 11.26) than saline-treated sites (38.95 ± 8.16), but this hormone-treatment effect was not significant (Z = -0.325, ns; see **Figure 24D**). Therefore, the stronger responses seen in the NCM of males under the influence of vasotocin and in the NCM of females under the influence of mesotocin were not specific to the sites that had been treated with the hormone. In fact, even saline-treated sites of vasotocin-treated males showed stronger responses to FCs than both vasotocin- and saline-treated sites of females in that group, and saline-treated sites in mesotocin-treated females showed stronger responses to FCs than both mesotocin- and saline-treated sites of males. Therefore the hormonal microinjections may be influencing (and in this case enhancing) neural responses in both the hormonally-injected sites but also at the contralateral saline-treated sites to a lesser extent, due to connected circuitry or perhaps hormonal feedback.

Finally, to determine whether these sex-specific effects of mesotocin and vasotocin were true for neural responses to fledgling calls specifically, or instead for all auditory stimuli, the main effects of sex on ARMs to 'all stim' were analyzed in the mesotocin- and vasotocin-treated groups. Results showed that there were main effects of sex on ARMs to 'all stim' in both the mesotocin- and vasotocin-treated groups (mesotocin: $F(1,258) = 21.23$, $p < 0.00001$; vasotocin: $F(1,245) = 9.33$, $p < 0.01$). As with the

responses to FCs, females showed higher ARMs to 'all stim' (38.64 ± 2.4) than males (22.04 ± 2.7), in the mesotocin-treated group. In addition, males showed higher ARMs to 'all stim' (44.89 ± 3.2) than females (28.36 ± 3.2), in the vasotocin-treated group. Therefore, this sex difference in the effect of social hormones on neural responses in the avian auditory forebrain was not specific to neural processing of FCs but rather was general to all auditory processing in our subjects.

Mesotocin and Vasotocin Injections Increase BF Tuning in Avian Auditory Structures

Multi-unit responses also showed sex-specific effects of hormonal treatment on best-frequency (BF) tuning. In the mesotocin-treated group, there was a strong trend for an interaction between the sex of the subject, the region in which responses were recorded and hormonal-treatment on BF tuning ($F(1,208) = 3.82$, $p = 0.05$). Once again, the effects of hormonal treatment on neural responses were sex-specific. In male subjects, mesotocin-treated multi-unit sites showed significantly higher BF tuning (3388.89 ± 247.7) than saline treated sites (2575.0 ± 257.2), in NCM ($Z = -1.99$, $p < 0.05$; see **Figure 25A**). There was no effect in male CMM ($Z = 0.471$, ns; see **Figure 25C**). In female subjects, however, there was a trend for an effect of hormone-treatment on BF tuning in CMM ($Z = -1.84$, $p = 0.07$; see **Figure 25D**). Here as well, hormone injections were associated with higher BF tuning (2957.14 ± 159.3) than was found at saline-treated sites (2529.41 ± 170.1). The NCM of females did not show an effect of treatment on BF tuning ($Z = 0.200$, ns; see **Figure 25B**). Contrary to the results in the mesotocin-treated group, there was no interaction between hormone-treatment, sex of subject and region of recording on BF tuning in the vasotocin-treated group ($F(1,232) = 1.45$, ns). The best-frequency tuning of multi-unit sites in CMM were unaffected by vasotocin-injections in

both male (Z = 0.109, ns; see **Figure 26C**) and female subjects (Z = 1.25, s; see **Figure 26D**). There was also no effect of mesotoin-treatment on NCM BF tuning, in males (Z = -0.300, ns; see **Figure 26A**). The NCM sites of female subjects, however, did show a trend for an effect of vasotocin-treatment on BF tuning (Z = -1.85, p = 0.07; see **Figure 26B**). Vasotocin-treated sites showed higher tuning (3017.86 ± 239.2) than saline-treated sites (2362.07 ± 252.9), in this structure. Therefore, both mesotocin and vasotocin injections affected the tuning of the neurons in the avian auditory forebrain.

In all ANOVAs, the side of the brain in which recordings were done was used as a factor, to assess lateralization. Although there was no significant interaction between sex of subject, area of recording and vasotocin-treatment on BF tuning (**see Figure 26**), there was a significant interaction between those three factors and the side of recording on BF tuning in the vasotocin-treated group ($F(1,232) = 4.84$, $p < 0.05$, see **Figure 27**). Post-hoc Tukey HSD tests showed that vasotocin-treatment had an effect on tuning in the NCM of males. The best-frequencies of male vasotocin-treated NCM sites (left: 3193.86 ± 256.2; right: 1930.5 ± 290.9) were: higher than saline treated sites in the left hemisphere (1750.25 ± 271.7) and lower than saline treated sites in the right hemisphere (3771.78 ± 327.8; $p < 0.05$ for both comparisons; see **Figure 27A**). This result is in accordance with results from experiment 1, which showed a left-lateralized enhancement of male neural responses to high-frequency FCs (as seen in **Figure 11**). In females, however, the effect of vasotocin-treatment on BF tuning was weaker, with neither hemisphere showing a significant effect (ns; see **Figure 27B**). Once again we see that vasotocin injections have a greater influence on neural responses in males than females. CMM tuning of both sexes was unaffected by vasotocin-treatment, in both hemispheres

(Tukey HSD, ns) and the mesotocin-treated group did not show this lateralized effect (interaction of sex, treatment, structure and side: $F(1,208) = 0.142$, ns). Nonetheless, these results do show that social hormones not only influence neural responses (including BF tuning) but also that they do so in a lateralized manner, supporting our earlier results which suggest that the mechanism through which parental experience causes plastic changes in auditory processing may be some form of lateralized hormonal action.

Single-Unit Spike Rates were Unaffected by Hormones

Single-unit spike rate responses to fledgling calls were analyzed to determine whether central injections of the social hormones vasotocin or mesotocin influenced the neural responses to these social cues. Spike rate responses at units of hormonally-treated subjects were investigated by first spike-sorting the multi-unit data and isolating single-units. In the group of mesotocin treated subjects (n = 20) 117 units were isolated in total, 37 in NCM and 80 in CMM. Units were further broken down by whether they had been injected with saline or drug, of the 37 NCM units 17 were drug-treated and of the 80 CMM units 45 were drug treated. In the group of vasotocin-treated subjects (n = 18) data was gathered from a total of 92 isolated units, 42 in NCM and 50 in CMM. When those units were broken down by drug-treatment group, 14 of the 42 NCM units and 28 CMM units were in drug-treated hemispheres. There was no effect of drug-treatment on single neuron isolation as a total of 104 of the single-units analyzed were in a hormone-treated hemisphere of the avian auditory forebrain while 105 were saline-treated. As in experiment 1, CMM tended to show better unit separation.

There was no effect of vasotocin-treatment on spike rates to stimuli, in general ($F(1,76) = 0.283$, ns; see **Figure 28A**). Drug-treatment also did not interact with the

region of recording of single-unit data to influence spike-rates; spike rate responses to auditory stimuli were similar in saline and vasotocin-treated neurons of NCM (saline: 18.09 ± 2.66; vasotocin: 17.77 ± 4.37) and CMM (saline: 15.93 ± 2.85; vasotocin: 22.87 ± 2.57; $F(1,76) = 1.40$, ns). Similarly, mesotocin-treatment did not affect the spike rate responses to 'all stim' (drug effect: $F(1,102) = 0.129$, ns; drug/area interaction: $F(1,102) = 0.0177$; see **Figure 28B**). Spike rate responses were no different in saline and mesotocin-treated single-units of NCM (saline: 14.58 ± 2.08; mesotocin: 13.18 ± 4.77) or CMM (saline: 12.06 ± 1.73; mesotocin: 11.44 ± 1.47). These results suggest saline and drug-treated single-units do not differ in baseline spiking, and therefore that comparisons made within a group of drug treated subjects need not be normalized to assess the effect of mesotocin or vasotocin on responses to fledgling calls. Therefore, spike-rate responses to fledgling calls were analyzed to determine whether social hormones enhanced neural responses to these social cues. In the group of subjects that were centrally-injected with vasotocin, there was no effect of the hormone on spike rate responses to FCs ($F(1,77) = 0.438$, ns; see **Figure 29A**). Vasotocin injections had no effect on spike rate responses to fledgling calls in NCM (saline: 12.79 ± 2.08; vasotocin: 11.03 ± 3.20) or CMM (saline: 9.85 ± 2.23; vasotocin: 14.86 ± 2.00; drug/area interaction: $F(1,77) = 1.92$, ns). Finally, results also showed that mesotocin-injections did not exert a significant effect on single-unit responses to fledgling call stimuli ($F(1,102) = 0.00038$, ns; see **Figure 29B**). This was true of both NCM (saline: 8.44 ± 1.56; vasotocin: 8.53 ± 3.57) and CMM neural responses (saline: 5.94 ± 1.30; vasotocin: 5.94 ± 1.10; $F(1,102) = 0.00045$, ns).

Vasotocin Increases Best Frequency Tuning in NCM Single Neurons

Single-unit spike rate responses to sine tones were analyzed to determine whether

central injections of the social hormones vasotocin or mesotocin influenced the tuning of NCM or CMM. Spike rate responses at units of hormonally-treated subjects were investigated by first spike-sorting the multi-unit data and isolating single-units. In the group of mesotocin treated subjects (n = 20) 60 units were isolated in total, 18 in NCM and 42 in CMM. Units were further broken down by whether they were recorded in saline or drug-treated hemispheres, of the 18 NCM units 10 were drug-treated and of the 42 CMM units 27 were drug treated. In the group of vasotocin-treated subjects (n = 18) data were gathered from a total of 69 isolated units, 18 in NCM and 51 in CMM. When those units were broken down by drug-treatment group, 5 of the NCM units were drug-treated and 29 of the CMM were drug-treated. There was no significant effect of drug-treatment on single neuron isolation as a total of 71 of the single-units analyzed were in a hormone-treated hemisphere of the avian auditory forebrain while 51 were saline-treated. As in previous experiments, CMM tended to show better unit separation.

Single-unit best frequency tuning was compared across untreated, vasotocin and mesotocin treated naïve groups. In NCM, best frequency tuning in vasotocin and mesotocin treated subjects did not significantly differ from the tuning found in untreated controls (vasotocin: $F(1, 51) = 1.022$, ns; mesotocin: $F(1,40) = 0.356$, ns; see **Figure 30A**). Results from single neurons in CMM also showed no difference between the tuning of untreated naives and virgin subjects treated with vasotocin ($F(1,96) = 0.447$, ns) or mesotocin ($F(1,95) = 0.0215$, ns; see **Figure 30B**). However, when single-unit best frequency tuning was compared within the hormone-treated groups, allowing for a comparison of drug treated and saline treated sites, there was an effect of hormonal manipulation on tuning. In vasotocin treated subjects, single unit's treated with vasotocin

showed significantly higher best frequencies (3035.58 ± 222.9) than those in the saline-treated hemisphere (2362.37 ± 207.7; $F(1,64) = 4.88$, $p < 0.05$). Further, that effect lay predominately in NCM as results showed a strong trend for an interaction between vasotocin-treatment and region of recording on single-unit best frequencies ($F(1, 64) = 3.63$, $p = 0.06$; see **Figure 31A**). NCM was tuned to higher frequencies at vasotocin-treated sites (3614.81 ± 368.0) than at saline-treated sites (2350.71 ± 314.7; Tukey HSD, $p = 0.06$). The tuning of CMM sites, however, was unaffected by vasotocin-treatment (vasotocin: 2456.35 ± 255.3; saline: 2374.02 ± 237.8; Tukey HSD, ns). In the mesotocin-treated group there was no effect of hormone-treatment on single-unit tuning ($F(1,52) = 0.227$, ns) in either structure (drug/area interaction: $F(1,52) = 0.515$, ns; see **Figure 31B**).

DISCUSSION:

In this experiment, male and female zebra finches (naïve to parental experience) were assessed for their neural responses to: all auditory stimuli, fledgling calls and tuning tones, at neural sites that were microinjected with either saline or one of two social hormones, prior to testing. For each dependent variable assessed in this experiment, results were analyzed separately for the two social hormones used, mesotocin and vasotocin. In addition, we investigated how neural responses were affected by hormone-injections in the two avian auditory forebrain structures, NCM and CMM. Results differed for those two structures, the two hormones used and the two sexes investigated. To some degree, this is what we expected to find in this experiment. However, some of the more specific hypotheses for this study did not match the results we found. The most obvious divergence between the results observed and our expectations for them, was that there was no effect of either hormone on the strength of neural responses (ARMs; spike-

rates) to fledgling begging calls. We found that multi-unit ARMs and spike-rate responses to FCs were similar at saline- and hormone-treated (mesotocin and vasotocin) sites. This result is in direct conflict with the hypothesis that hormonal action in auditory structures would increase neural responses to FCs, similar to what is seen in parentally-experienced zebra finches. In fact, our between-groups comparisons showed that multi-unit responses to FCs were lower in hormone-treated female subjects than naives. Thus these hormones do not simply mimic the effects of parental experience, at least at the short time scale at which we tested neurophysiology in these subjects. However, results did show that both mesotocin and vasotocin injections caused changes in neural responsiveness and BF tuning in the avian auditory forebrain, suggesting that these hormones are responsible for short-term plasticity in these structures, as oxytocin is in mammalian A1 (Marlin et al., 2015). Therefore, short-term action of these hormones may cause disinhibition and RF plasticity in the avian auditory forebrain, while more long-term action may have to be combined with learning about the behavioral relevance of the begging calls of young birds in order to produce changes in how neurons respond to FCs, specifically.

The most consistent effect of hormonal injections on neural responses was observed in BF tuning of multi- and single-unit sites. There were effects of hormonal injections on best frequencies in analyses of: single-unit activity, within-group multi-unit activity and across-group multi-unit activity (the three major analyses performed here). In all but one case (vasotocin v. untreated groups' CMM activity) the neurons treated with hormone (mesotocin or vasotocin) showed tuning toward higher frequencies than saline or untreated sites. Mesotocin increased multi-unit BF tuning in the NCM of male subjects

and the CMM of female subjects. Vasotocin increased single-unit BF tuning in NCM in addition to multi-unit BF tuning in female NCM, and vasotocin-treated subjects had higher multi-unit best frequencies than the untreated group of naives. The upward shift in tuning seen in hormone-treated sites fits well with results from experiment 1 which showed that parental subjects have higher BF tuning than naïve subjects. As FCs have higher mean frequencies than adult calls/songs, and social hormones vasotocin and mesotocin cause changes in tuning that shift BF tuning upward during parental experience (which is associated with the action of these hormones), then these upward shifts may have a role in the subsequent increase in responding to FCs.

The final tuning effect found during analysis of the hormonally-treated multi-unit neurophysiology data, was a significant interaction between the structure in which recordings were done (NCM/CMM), the sex of the subject (male/female), the side in which recordings were done (left/right) and vasotocin treatment. Results showed that in the vasotocin-treated group, males had greater BF tuning at vasotocin-treated sites than saline-treated sites in the left hemisphere of NCM while vasotocin-treated sites showed lower best frequencies than saline-treated sites in the right hemisphere of that same structure. Therefore, there were lateral effects of hormone treatment, in this experiment as in experiment 1. Furthermore, those lateral effects were in the direction in which one would expect from the results of experiment 1, which showed that parental male subjects had enhanced responses to FCs in the left hemisphere of the avian auditory forebrain, but not the right. The significantly stronger FRS value that parental male subjects show (as compared to virgins) in the left hemisphere of the avian auditory forebrain may be downstream of a upward shift in the RFs of neurons in the left hemisphere of males, due

to vasotocin release during parental experience. Females, on the other hand, did not show any significant differences in post-hocs for the interaction between these four factors, demonstrating the sex differences that abounded in the results of this experiment.

An additional hypothesis was that there would be sex differences in how mesotocin and vasotocin influenced neural responses in the auditory forebrain, due to the sex differences that the mammalian oxytocin and vasopressin hormones show in their functions, in addition to the indications that vasotocin and mesotocin show sex-specific functions in zebra finch parental behaviors as well (from the few studies that have investigated it: Klatt & Goodson, 2013b; Hall et al., 2015). Specifically, we hypothesized mesotocin would affect neural responses in females more than males and vasotocin would affect males more than females. Fitting this hypothesis, results showed that mesotocin and vasotocin have sex-specific effects on neural processing of auditory stimuli (FCs and all other stimuli), in the avian auditory forebrain. Neural responses to FCs and the other auditory stimuli used were stronger in females than males in the mesotocin-treated group while responses were stronger in males than females in the vasotocin-treated group. As there were no sex differences in neural responses to FCs in the untreated naïve birds, these enhanced responses were due to hormonal action in the avian auditory forebrain. Therefore, not only do we see plasticity in auditory responses under the influence of social hormones, but the effects of those hormones are sex-specific in the way that one would expect due to their homology to oxytocin and vasopressin. This supports our hypothesis that although both sexes care for offspring in this species, the neural mechanisms through which parental care influences auditory processing of FCs are different for males and female zebra finches.

Finally, results indicated that the enhancement of neural responses to auditory stimuli in mesotocin-treated females and vasotocin-treated males was specific to the forebrain structure NCM. In fact, the majority of significant effects identified in this experiment were specific to NCM, suggesting that social hormones have a greater influence on neural activity there. This may be true, due to the distribution of MT and VT receptor subtypes; as NCM shows receptors for both VT and MT while CMM shows receptors for VT only (Leung et al., 2011). However, studies of the distribution of MT and VT receptors in the avian auditory forebrain have not separately assessed male and female subjects, or discriminated between left and right hemispheres in their results (2011). Thus, the results of this set of microinjection experiments, which reveal lateralized and sex-specific effects of mesotocin and vasotocin in NCM and CMM, further demonstrate the need for further labeling studies for these receptor subtypes in the auditory forebrain of zebra finches to fully interpret these effects (as discussed in Chapter 2). In addition to these receptor-labeling studies, experiments using *in vitro* electrophysiology techniques, such as patch-clamp, would enable us to answer the question how mesotocin and vasotocin are exerting changes in neural activity, in these areas. This study should be followed up with patch clamp experiments that test whether mesotocin and/or vasotocin cause disinhibition in neurons of the avian auditory structures, as oxytocin does in mammalian A1.

4. Experiment 3: Nest Entry Behavior in Parent and Naïve Zebra Finches

RATIONALE:

The aim of experiment 3 was similar to that of experiment 1 in that the goal was to compare responses to the category of FC auditory stimuli in parent and virgin zebra finches. In the current experiment, however, the responses being investigated were subjects' behavioral responses (to FCs) that may be indicative of parental motivation/investment. To test if parentally experienced and virgin birds respond differently to the calls of young, in a similar manner as what has been demonstrated in the retrieval responses of mice females to pup-isolation calls, a novel behavioral paradigm was designed for assessing zebra finch parental behavior in this experiment. We know that young birds call when they are hungry and that zebra finch parents learn the meaning of those calls and begin to produce feeding behaviors in response to them throughout the juvenile period. Before offspring fledge, parents must enter the nest to feed them. After fledging, young birds move in and out of the nest. At that point, parents must orient to the location of the calls and feed the young birds wherever they may be, in or out of the nest. Consequently, very often a parent's feeding response begins with them entering the nest, where chicks are located until ~18 dph. For that reason, in this experiment, we play FCs from behind a nest and measure how often birds enter the nest in response. To further assess entry responses to FCs, we measured the amount of time spent in the nest by subjects as well as the amount of time spent in an area directly outside of the nest. Before an adult zebra finch can regurgitate food to a fledgling, they must gather seed into their crop; therefore, the amount of food collected by the adult bird subject was also measured in this behavioral test paradigm. Nest entries, the amount of

time spent in/in front of the nest and the amount of food collected by subjects were all hypothesized to be higher in parents than virgins.

One additional behavior measured in this experiment, and hypothesized to be related to parental motivation in zebra finch males and females, was call-back responses to fledgling calls. Zebra finches often call in response to the calls of others (as discussed in Chapter 1) and previous studies looking at parental recognition of one's own offspring in this species have used call responses to FCs as the measured variable (Reers et al., 2011; Levroro et al., 2009). Results of these studies, however, have shown conflicting results as far as whether parental songbirds behaviorally discriminate between their own offspring and novel fledgling calls. Therefore, in this experiment we also aimed to answer the question of whether songbird parents respond behaviorally more strongly to the calls of their own offspring, as compared to novels. To answer this question, we used the same measurements used previously (nest-entries, time spent in nest/area, food gathered (g) and call backs), and tested whether responses to fledgling calls were more robust on sessions of trials in which the stimuli were one's from a subject's own clutch than in sessions where novel stimuli were played.

METHODS:

Subjects:

The subjects of the experiment were 12 male and 12 female adult zebra finches (**Table 1**). Subjects were reared in an aviary, maintained on a 12:12 light:dark cycle and had access to cuttlebone. Subjects had *ad libitum* access to food and water throughout the entire experiment. Half of the males and females were parentally experienced in the same manner as subjects from the first experiment. Parentally-experienced subjects were

those that had been assigned mating partners and allowed to cohabit with that partner while being provided with a nest, nesting-material, and the proper nutrition to stimulate breeding. All subjects produced at least 1 surviving offspring with their mate and cohabited with both their partner and any offspring throughout development, until at least 60 dph of the chicks (see **Figure 4**).

Families were isolated and recorded for auditory interactions throughout the development of subject's offspring, using an Audio-Technica microphone and power module. Continuous recordings of all vocalizations were collected between days 15 and 30 dph with Sound Analysis Pro V1.04 (Sound Analysis Pro, Tchernichovski et al., 2000). Clips from these recordings were used to create two stimuli for each clutch/subject, to be used in neurophysiological recordings and behavioral testing. One of these stimuli was a 2-3 second bout of calls from multiple offspring in the clutch (stimulus: fledglings) and the other stimulus was a 1-2 second bout of repeated calls from one fledgling (stimulus: fledgling). Both stimuli were extracted from recordings on the same day for each clutch, at 21.48 ± 0.337 dph. To standardize the auditory stimuli, recordings were filtered, equated for loudness and 5 msec of silence was added to the start and end of each bout of calls (Signal Engineering Design). As young zebra finches only produce begging calls until 35 dph (at the latest), all behavioral and neural testing in parents was done 25 days or more after their offspring would have stopped producing the calls used in testing, at 60 dph or later. Therefore, any significant results indicate a long-term memory for the 'begging call' sound category.

Behavioral testing:

One day prior to behavioral testing with our novel paradigm (for testing parental

behaviors), subjects 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). Adjacent to this chamber was a nest box identical to the ones used in the aviary for breeding, with which parental subjects should have been familiar. Inside the nest box there was nesting material and a model young bird. An open doorway allowed for subjects to move between the chamber and the adjacent nest at any point during acclimation (see **Figure 32**).

The next morning, 30 minutes after lights turned on in isolation boxes, subjects were placed in the nest-box for 15 minutes to ensure they had experienced both chambers. Fifteen minutes after the nest box acclimation, behavioral testing began with session 1. During a session, subjects heard 2 auditory stimuli 20 times each, within 1 hour. These stimuli were fledgling calls; during a particular session the first stimulus was the call of one fledgling from a particular clutch, and the second stimulus would be the calls of all members of that same clutch. Therefore, during each session, the stimuli were all from the same clutch. Subjects were tested on four sessions with this paradigm and stimuli were changed between sessions such that each bird heard three different stimulus sets (1 set was repeated and this set was from a bird's own clutch if the subject was a parent). Two sessions were conducted on day 1 of testing, one in the morning and one in the afternoon, and two were conducted on day 2 of testing, in the same way.

Begging call playbacks were stimulated by the experimenter every ~1.5 minutes. After a stimulus was played, entries into the next-box were measured using an infrared beam mounted in the opening between the chambers and monitored through the ARTSy program and MATLAB (Gess *et al.* 2011; David Schneider, Columbia University, New

York, NY, U.S.A). All sessions were video recorded. Using video scoring, experimenters blind to the experimental conditions measured: call responses (within 30 seconds of stimulus), time spent in the nest-box, time spent in a defined area directly in front of the nest (nest area, **Figure 32**) and the amount of food collected by the subject between the beginning of the session and the end of the session (difference in grams). The infrared beam and ARTSy software were used to measure any nest entries that occurred between sessions.

Statistical Analysis

The effects of sex and parental experience were tested by measuring food collected during behavioral sessions, nest entries, time spent in nest and area adjacent, and call-responses. Factorial ANOVAs were used to test interactions between sex, parental experience, and session number on food collected, time spent in nest and area and call-responses. The frequency with which these groups (male/female and virgin/parent) entered the nest were then tested using chi-square analyses. Alpha values were Bonferroni corrected for multiple comparisons. Finally, to test whether parents behaved differently in response to their own and novel fledgling calls, a repeated measure ANOVA was run to test responses to sessions with novel calls as stimuli to those with own fledgling calls as stimuli, for each subject. Post-hoc Tukey HSD tests were used to identify the significant differences from these ANOVAs.

RESULTS:

The behavior of parents and naïve subjects was compared for a number of dependent variables, which were all hypothesized to relate to parental investment in a young zebra finch. Results are reported for all of the dependent variables measured: the number of

call-back responses to fledgling calls, the amount of food collected (grams) by a subject throughout a session of FC playbacks, the amount of time subjects spent (seconds) in and in front of the nest during FC playbacks, and the number of nest entries subjects performed (within and out of each playback session). If the value of the of one of these measurements, at any one trial session, exceeded the overall average for that behavior plus/minus 3 times the standard deviation around that average, that observation was considered an outlier for that behavior and was eliminated from analyses.

Female Parents Perform more Nest Entries

Chi-square analyses were used to assess the frequency of nest entries both in session periods (when FCs were played back for ~1 hour) as well as time between sessions, in male and female parents and virgins. When the frequency of nest entries during a playback session was analyzed, the chi-square was significant, showing an influence of parental experience and sex on entries made ($\chi^2 = 39.34$, $p < 0.001$; see **Figure 33 & Table 2**). Females showed significantly more box entries than males ($\chi^2 = 58.88$, $p < 0.001$; see **Table 2**). In addition, parents showed significantly more box entries than naïve subjects ($\chi^2 = 20.84$, $p < 0.001$; see **Table 2**). However, this parental effect was not equal for both sexes. When post-hoc chi squares were run comparing parental and naïve entry frequencies, for each sex separately, results showed that although females did significantly increase the frequency with which they entered the nest relative to virgin females ($\chi^2=38.7$, $p < 0.0001$; see **Table 2**), males actually decreased their entry behaviors as parents when compared to the behaviors of virgin males ($\chi^2= 11.08$, $p < 0.001$; see **Table 2**).

Chi-square analyses of nest entry behavior during the out of session period, when

no stimuli were played back, show strikingly similar results. The chi-square analyzing the influence of parental experience and sex on nest entry behavior showed an interaction between the two factors (χ^2=131.23, $p < 0.001$; see **Figure 33 & Table 2**). As in the in-session data, parents showed more frequent entries than virgins ($\chi^2 = 18.74$, $p < 0.001$; see **Table 2**). This effect, however, once again interacted with the sex of the subject; although female parents did enter more than their naïve counterparts ($\chi^2 = 119$, $p < 0.0001$), males entered less as parents than they did as virgins ($\chi^2 = 18.8$, $p < 0.0001$; see **Table 2**). Contrary to the results seen during the in-session period, out-of-session nest entries did not show a greater overall number of entries by females, as compared to males ($\chi^2 = 0.48$; $p = 0.49$, see **Table 2**). Rather, the influence of sex on the frequency of nest entries was parental experience specific. Parental females entered more than males ($\chi^2 = 38.52$, $p < 0.0001$) while naïve males entered more than females ($\chi^2 = 90.02$, $p < 0.001$, see **Table 2**). Therefore, both in and out-of-session results show a consistent interaction between sex and parental experience on nest entry behavior. Females perform more nest entry behaviors as parents than female virgins do while males perform more nest entry behaviors as virgins than they do as parents.

Females are Stimulated to Enter the Nest by FCs while Males are Inhibited

Chi-square analyses were also used to test the frequency of nest entries across the two time points, in-session periods and out-of-session periods. To determine whether nest entries were more frequent during the in-session period than the out-of-session period, and whether subjects were actually entering the nest at a rate that was influenced by the playback of FCs. To analyze this, we calculated the expected number of entries into the nest for each time point for each sex, using the total number of entries for males

and females, and multiplying those by the fraction of time animals spent in session (4/17 hours) and out of session (13/17 hours) throughout the entire behavioral paradigm (see **Table 3A**). This 'expected frequency' assumes that animals are entering equally across two time points. When chi-squares of the observed frequencies were run against those expected frequencies, results showed that both male and female subjects did not enter the nest equally between the in-session and out-of-session periods. Females showed significantly more nest entries during in-session periods when FCs were played back than they showed during the out-of-session period ($\chi^2 = 46.11$, $p < 0.001$; see **Table 3B**). Further, this effect was seen in both naïve and parental subjects (naïve: $\chi^2 = 47.84$, $p < 0.0001$; parent: 25.11, $p < 0.0001$; see **Table 3B**). Males, on the other hand showed significantly more entries into the nest during the out-of-session period of time, when no stimuli were played back, than was predicted by chance ($\chi^2 = 16.55$, $p < 0.001$; see **Table 3C**). The male effect was also true of both parental experience groups (naïve: $\chi^2 = 6.64$, $p = 0.01$; parent: 11.34, $p < 0.001$; see **Table 3C**), although it did not reach significance in naïve males under the Bonferroni corrected alpha. Nevertheless, results are consistent with the pattern that males and females show opposite patterns of nest entry behavior. Specifically, females are stimulated by the playback of fledgling call stimuli to enter the nest while males are actually inhibited by FCs from performing this behavior, an unexpected result.

Interactions between Sex and Parental Experience on Parental Behaviors

The interactions between sex and parental experience on the chi-square nest entry frequency data was paralleled by similar results in some of the other dependent variables used to assess parental motivation in the nest-entry behavioral paradigm. Most similar to

the results seen for nest entries was the amount of time spent (s) in the nest and area adjacent to it, a behavior which by definition is related to the number of nest entries performed. On an average trial, females spent significantly more time in the nest and area adjacent (106.54 ± 26.8) than males (9.70 ± 26.8; $F(1, 78) = 6.53$, $p < 0.05$; see **Figure 34A**). There was also a trend for parental subjects to spend more time in the nest and area (91.64 ± 27.8) than naives (24.60 ± 25.8; $F(1, 78) = 3.13$, $p = 0.08$; see **Figure 34B**). The failure of the latter result to reach significance was likely due to the influence of male behavior, and a strong trend for an interaction on time spent in the nest and area, of sex and parental experience ($F(1, 78)=3.71$, $p= 0.057$; see **Figure 35A**). Post hocs revealed that female parents spent more time in and near the nest as parents (176.52 ± 39.24) than as virgins (36.56 ± 36.47), a strong trend (Tukey HSD, $p = 0.051$). However, males spent more time in the nest and nest area as virgins (12.64 ± 36.47) than as parents (6.76 ± 39.24), paralleling the nest entry frequency data (Tukey HSD, ns).

The amount of food that subjects collected during trials of fledgling call playback also showed a significant interaction between sex of subject and parental experience, in the same pattern ($F(1, 88) = 11.90$, $p< 0.001$; see **Figure 35B**). Post hoc tests show that males collected significantly more grams of food (1.19 ± 0.19) as virgins than as parents (0.573 ± 0.19; Tukey HSD, $p < 0.05$). Females behaved oppositely, collecting significantly fewer grams of food as virgins (0.378 ± 0.19) than as parents (1.07 ± 0.19; Tukey HSD, $p < 0.05$). There were no main effects of sex or parental experience on the amount of food collected (sex: $F(1,88) = 0.675$, $p = 0.41$; parental experience: $F(1,88) = 0.042$, $p = 0.84$). These results, taken with the previous, suggest that there is a pattern by which parental experience influences behaviors underlying parental motivation; in which

females increase the frequency with which they perform parental behaviors (which they show low motivation to perform before breeding) after parental experience, and males decrease the frequency with which they perform these same parental behaviors (which they do perform as virgins) with parental experience.

The final variable measured during the nest-entry behavioral paradigm was the number of calling responses within 30 seconds of a fledgling call playback. The number of call back responses performed by subjects was not influenced by parental experience through either a main effect ($F(1,81) = 0.575$, $p = 0.45$) or an interaction with the sex of the subject ($F(1,81) = 0.179$, $p = 0.67$; see **Figure 35C**). There was a weak trend for male subjects to call back (86.92 ± 13.1) more frequently than females (55.88 ± 13.3; $F(1, 81) = 2.75$, $p = 0.10$).

Nest Entry Behavior Does not Discriminate Own and Novel Fledgling Calls

All subjects were tested and observed for their behavioral responses to four sessions of fledgling call playbacks. In two of these four sessions a set of novel fledgling calls were used as stimuli; for the other two sessions one set of fledgling calls was repeated. If subjects were parents, the repeated stimuli were the calls of the subject's own fledglings. Therefore, each parental subject could be assessed for their average response to a session of OFC playback as compared to their behavioral response to a session of novel fledgling call playback, on each of the previously reported dependent variables. Surprisingly, none of the variables measured showed an effect of familiarity on the behavior of parents (food collected: $F(1,10) = .999$, ns; nest entries: $F(1, 10)=.938$, ns; time spent in nest & area: $F(1, 9)= 1.998$, ns; call responses: $F(1, 10)= .0833$, ns; see **Figure 36**). The failure to find a behavioral recognition of one's own offspring's call was not consistent with results

from neurophysiology, suggesting that the lack of an effect may have been due to an experimental design failure.

DISCUSSION:

Parental motivation was assessed in experiment 3, through a novel nest-entry behavioral paradigm in which multiple behaviors hypothesized to be more frequently produced in parents than virgins in response to fledgling call playback were quantified. The behaviors assessed were: nest-entries, time spent in/adjacent to nest, the amount of food subjects collected and call-back responses produced within 30s of a FC playback. We predicted that the subjects with parental experience (who have experience with hearing and responding to FCs) would respond to them more frequently than virgins, as in mice dams who respond to isolation calls with pup-retrieval more often than virgins. The behaviors observed in this experiment showed a highly conserved pattern of results for three of the four dependent variables; nest entries, time spent in nest and the area adjacent as well as the amount of food collected all showed an interaction between parental experience and sex of the subject. The hypotheses for this experiment had been that parental subjects would show more of these behaviors than naïve subjects, but results were not that simple. Female parents did show more of all three of these behaviors during the behavioral paradigm than female virgins, however, males showed the exact opposite effect of parental experience on the frequency with which they performed these behaviors. Male virgins entered the nest more, spent more time in the nest/area and collected more food than male parents, an effect that was unexpected but consistent. Therefore, nest-entries, time spent in the nest/area and grams of food collected, are behavioral responses to fledgling calls that are influenced by parental experience, as

hypothesized. However, sex of parent has a far greater influence on parental investment and behavior than originally expected in this bi-parental monogamous species, such that females act more parentally after experience with fledglings while males act more "parentally" before becoming parents than after.

Specifically, the number of times subjects entered the nest was significantly greater in female parents than female virgins and in male virgins than male parents, both during the in-session (FC playback) period as well as the out-of-session (no playback) period. In fact, when a chi-square was conducted to test whether animals entered the nest equally across the in and out-of-session time periods or instead entered more when FCs were being played back, results were once again sex-specific. Female subjects entered the nest significantly more frequently during the in-session time period than was predicted by chance, indicating a stimulatory effect of FCs on this behavior. Males, on the other hand, were not stimulated by the calls of young birds to enter the nest as hypothesized. Instead, males entered the nest more frequently during the out-of-session period than was predicted by chance, indicating that they were actually inhibited from entering after playback of fledgling calls, a thoroughly unanticipated and thought-provoking effect. These results match those from food collected and time spent (in nest/area) behaviors, which show a significant and strong trend (respectively) interaction between parental experience and sex of subject. The sex difference in how parental-experience influences the parental behaviors of male and female zebra finches was unanticipated, but does not fully deviate from what has been documented in the literature on parental behavior in this bi-parental species. As early as 1966, when El-Wailley & Jasin investigated the amount of time male and female zebra finches invest into incubating their eggs, data have shown

that females spend significantly more time incubating than males. Those results were replicated by Delesalle in 1986, who also found that females spend significantly more time feeding their young than their male partners. Therefore, there are various pieces of historical evidence showing that females provide more of the care-giving to offspring than males during these periods of juvenile-development. As the current behavioral paradigm was designed to explore feeding behavior, our results showing greater parental motivation in females fit well with previous studies (Delesalle, 1986). However, as virgin males acted more parentally than virgin females, we further hypothesize that these results may be consistent with a distribution of parental investment of the two sexes across time during juvenile development, such that males act more parentally early in nest-building and incubation – although we did not study that period -, while females may perform more of the later behaviors, such as feeding.

The final behavior analyzed, call-back responses to fledgling call stimuli, was the only dependent variable of the nest-entry paradigm that was unaffected by parental experience. There was no effect of parental experience, sex or the interaction between the two on call-back responses in this experiment. Call responses, therefore, may not be as indicative of parental motivation as the other behaviors observed. If this is so, the earlier behavioral studies that assessed whether parents could recognize their own offspring through call-back response behaviors, may not be using the appropriate behavior to test whether parents have this ability. This may be why experimenters have made conflicting conclusions from such studies (Levrero et al., 2009; Ligout et al., 2015; Reers et al., 2011).

In our behavioral paradigm, we also attempted to test behavioral recognition of

OFC versus novel FCs, but found no difference. Nest entries, time spent in nest/area, food collected and call-back responses were no different for trials in which OFCs were played back as compared to trials in which novel fledgling calls were played back. This result was not consistent with results from experiment 1 which showed a neural memory for OFC in parental subjects. However, there were experimental design flaws that may have confounded results. Two of the four trial sessions parental subjects experienced in the nest-entry behavioral paradigm used OFCs as stimuli, whereas the other two trials used two different novel fledgling begging calls as stimuli. Therefore, the effect of behavioral adaptation to a (repeated) stimulus may have occluded any tendency for parents to respond with parental behaviors more after playback of OFC, in these results. It is also possible that the behaviors measured in this paradigm are not performed differently in response to own and novel FC stimuli, by parental subjects, although they do discriminate. If this is the case, further testing of alternative parental behaviors may better demonstrate recognition. For instance, there is evidence that parents call back in response to OFC and novel FCs equally, in one of the two studies of this behavior and parental recognition (Reers et al., 2011). Our experimental results further indicate that call-back responses to FC stimuli may not be a meaningful or consistent parental response. Therefore, the investigation of additional behavioral responses to FCs may be necessary to demonstrate behavioral recognition of OFC. For this reason, this question was further studied in Chapter 6, by means of a different method, i.e. the behavioral approach assay.

5. Exp 4: Effects of Peripheral Injections of Mesotocin and Vasotocin on Behavior

RATIONALE:

The fourth experiment in this study aimed to test whether inexperienced male and female zebra finches can be induced to act parentally (in a similar manner as parents did in Chapter 4) by treatment with social hormones vasotocin and mesotocin, as virgin rats do when treated with exogenous oxytocin (Pedersen et al., 1982). Although the behavioral response of female dams to pup-isolation calls differs significantly from that of virgin mice/rats, virgins can be prompted to retrieve pups through experience with them, or by treatment with oxytocin (1982). Peripheral injections of oxytocin produce increased brain levels of hormone, through either hormonal feedback mechanisms, active transport across the blood-brain barrier (BBB) or leakage across capillary-rich areas of the BBB (Neumann et al, 2013). In the current experiment, it was hypothesized that peripheral injections of mesotocin and vasotocin hormones into naïve zebra finch subjects would increase the number of parental behaviors they subsequently exhibit in the nest-entry behavioral paradigm used in Chapter 4. In this experiment only naïve subjects were used (as in experiment 2) to test whether virgin zebra finches can be 'sensitized' as virgin female mammals can.

Results from experiment 3 showed a high degree of sex-specificity in how parental experience affected the frequency with which animals performed the parental behaviors measured in the nest-entry paradigm. These results, in conjunction with the sex-specific roles that the mammalian analogs of mesotocin and vasotocin have in behavior, caused us to develop different hypotheses for the effects of these hormones in the two sexes. Previous results showed that parental females perform more nest entries, spend more time

in the nest/area and collect more food during nest-entry testing than naïve female zebra finches. Therefore we predicted that mesotocin injections increase these behaviors as well, as mesotocin/oxytocin is typically associated with maternal care. In males, however, previous results were quite the opposite of our behavioral hypotheses. Parental males showed fewer nest entries, less time in nest/area and less food collected, in the nest-entry behavioral paradigm, than naïve males do. If parental males have elevated vasopressin (evidence for this has been shown in (1) human plasma studies: Gray, Parkin & Samns-Vaughan, 2007; Atzil et al, 2012; Apter-Levi, Zagoory-Sharon & Feldman, 2014; and (2) prairie-vole gene expression: Wang, Liu, Young & Insel, 2000), and parental males show fewer of the parental behaviors that we measured (our data show this), then it may be that vasopressin actually acts to reduce these parental behaviors. Following this logic, we hypothesized that injections of the hormone associated with paternal care, vasopressin/vasotocin, would cause naïve males to behave as the parental males had in the previous experiment, decreasing the frequency with which they performed nest-entry and food-collection behaviors.

METHODS:

Subjects:

The subjects of the experiment were an additional 18 male and 18 female adult zebra finches (**Table 1**). Subjects were reared in an aviary, maintained on a 12:12 light:dark cycle and had access to cuttlebone. Subjects had *ad libitum* access to food and water throughout the entire experiment. All subjects for hormonal manipulation experiments were naïve to breeding and parenting offspring. Subjects were equally split into three treatment groups: vasotocin, mesotocin and saline-treated males and females.

Saline-treated naïve subjects were added as a supplemental control group after original testing, and therefore have not yet been video-scored for all nest-entry behaviors.

Peripheral Manipulations and Behavioral Testing:

One day prior to behavioral testing, virgin subjects (n_{male} = 18, n_{female} = 18) 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). Adjacent to this chamber was a nest box identical to the ones used in the aviary for breeding, with which parental subjects should have been familiar. Inside the nest box there was nesting material and a model young bird. An open doorway allowed for subjects to move between the chamber and the adjacent nest at any point during acclimation.

The next morning, 30 minutes after lights turned on in isolation boxes, subjects received 0.05 mL intramuscular injections of drug or saline (10μMolar mesotocin in .9% saline, 10μMolar vasotocin in .9% saline, .9% saline), after which they were placed into the nest-box for 15 minutes to ensure they had experienced both chambers (concentrations based on Castagna et al. 1998 & Goodson et al. 2009). Fifteen minutes after the nest box acclimation, behavioral testing began with session 1. During a session, subjects would be played 2 auditory stimuli 20 times each, within 1 hour. These stimuli were fledgling calls; during a particular session the first stimulus would be the call of one fledgling from a particular clutch, and the second stimulus would be the calls of all members of that same clutch. Therefore, during each session, the stimuli were all from the same clutch. Subjects were tested on four sessions with this paradigm and stimuli were changed between sessions such that each bird heard three different stimulus sets.

Two sessions were conducted on day 1 of testing, one in the morning and one in the afternoon, and two were conducted on day 2 of testing, in the same way. Before each subsequent session, birds were once again injected with drug/saline 30 minutes prior to the behavioral testing.

Begging call playbacks were stimulated by the experimenter every minute and a half (on average). After a stimulus was played, entries into the next-box were measured using an infrared beam mounted in the opening between the chambers and monitored through the ARTSy program and MATLAB (Gess *et al.* 2011; David Schneider, Columbia University, New York, NY, U.S.A). All sessions were video recorded. Using video scoring, experimenters blind to experimental conditions also measured: call responses (within 30 seconds of stimulus), time spent in the nest-box, time spent in a box directly in front of the nest and the amount of food consumed by the subject between the beginning of the session and the end of the session (difference in grams). The infrared beam and ARTSy software were used to measure any nest entries that occurred between sessions. The procedures for the nest-entry behavior paradigm and scoring were conducted exactly as they had been done in experiment 3 (besides the additional inclusion of peripheral injections in this experiment).

Statistical Analysis

Behavioral data was analyzed using factorial ANOVAs and chi-square analyses as described above (Chapter 4), to test whether control groups assessed in experiment 3 (virgin males, parental males, virgin females, parental females) behave differently than drug manipulated groups in the novel behavioral paradigm. For chi-square analyses, alpha values were Bonferroni corrected for multiple comparisons. Tukey HSD tests were

used as post-hoc tests for significant differences in the factorial ANOVAs.

RESULTS:

Mesotocin and Vasotocin Injections Influence Nest-Entry Behavior in Naïve Subjects

Nest-entry frequency data was analyzed to compare the behavior of naïve subjects that have been injected with social hormones (vasotocin and mesotocin) to the behavior of same-sex (untreated) naives. To do so, chi-square analyses were performed on both in-session and out-of-session frequency data for all groups (n=6 each). Alpha levels were Bonferroni-corrected for multiple comparisons. During the in-session period, injections of hormones exerted an inhibitory influence on behaviors, but hormonal manipulations did increase parental behaviors (via nest entries) during the out-of-session period.

During the in-session period, naïve subjects treated with mesotocin performed significantly fewer nest-entry behaviors than untreated naives ($\chi^2 = 13.82$, $p = 0.002$; see **Table 4A**). Post-hoc analyses showed that this effect was due to a decrease in nest entries in mesotocin treated males, as compared to untreated males ($\chi^2 = 11.08$, $p = 0.009$) and that female behavior was not affected by treatment ($\chi^2 = 3.68$, ns). This inhibitory effect on the responsiveness of males to FC playback, however, was specific to mesotocin treatment. Vasotocin injected naives did not show any difference in the frequency of their nest-entries from untreated naives (overall vasotocin v. naive: $\chi^2 = 6.24$, ns; male: $\chi^2 = 6.66$, ns; female: $\chi^2 = 0.96$, ns; see **Table 4C**). When hormone-manipulated subjects nest-entry behavior was compared to that of parental subjects during the same (in-session) period, results showed that female subjects injected with either vasotocin or mesotocin showed significantly fewer nest entries than female parents (mesotocin: $\chi^2 = 59.04$, $p < .0001$; vasotocin: $\chi^2 = 50.06$, $p < 0.001$; see **Table 4 B&D**).

Male behavior, however, was not different than that of male parents (mesotocin: $\chi^2 = 0$, ns; vasotocin: $\chi^2 = 0.5$, ns; see **Table 4 B&D**).

Results on out-of-session nest entry data showed very different patterns from those seen in-session. Both hormonal manipulations exerted a stimulatory effect on female nest-entries, as compared to untreated virgin behavior, during this time period. Vasotocin treated subjects showed significantly more nest entries than their untreated naïve controls during the out-of-session period ($\chi^2 = 45.32$, $p < 0.0001$; see **Table 5C**). However, post-hoc tests showed that this effect was specific to females, and that naïve male behavior was unaffected by vasotocin injections (female $\chi^2 = 121$, $p < 0.0001$; male $\chi^2 = 0.08$, ns). Similarly, in mesotocin treated subjects, nest entries were more frequent in treated females than untreated naives ($\chi^2 = 28.04$, $p < 0.0001$) and less frequent in treated males than untreated males ($\chi^2 = 71.28$, $p < 0.0001$; see **Table 5A**). This interaction between sex and hormonal treatment on nest entry frequency paralleled the sex difference we see on how parental experience influence nest entries in normal males and females. When hormonally-treated virgin nest-entry behavior was then further compared to the nest-entry behavior of parental subjects during the out-of-session period, mesotocin treated subjects showed fewer responses than the same-sex parents (male: $\chi^2 = 22.68$, $p < 0.0001$; female: $\chi^2 = 53.64$, $p < 0.0001$; see **Table 5B**). However, vasotocin-treated males showed more frequent responding than male parents ($\chi^2 = 15.82$, $p < 0.0001$) and vasotocin-treated females did not significantly differ, in the number of nest-entries they produced out-of-session, from parental females ($\chi^2 = 0$, ns; see **Table 5D**).

The Inhibitory Effect of Hormonal Injections on In-Session Behaviors

Factorial ANOVAs were run on the three dependent variables measured in the nest-entry behavioral paradigm (time in nest and area, call-back behaviors and the amount of food collected) to compare naïve performance to the performance of animals under vasotocin and mesotocin treatments, separately. The sex of the subject and the drug treatment group were the two factors used in these analyses. The amount of time (seconds) subjects spent in the nest and adjacent area, a behavioral measure that is tied to the number of nest-entries performed by subjects in the nest-entry behavioral paradigm, was not increased by peripheral hormonal manipulations. As results showed for the nest-entry frequency data, vasotocin treated and untreated naives did not differ in the amount of time they spent in the nest and area ($F(1,86) = 0.162$, ns; see **Figure 37A**). In addition, in keeping with the results from above chi-square analyses, subjects treated with mesotocin showed less time (6.39 ± 10.9) in the nest and area than untreated naives (23.78 ± 11.0) , although that effect was not significant ($F(1, 85) = 3.05$, $p = 0.08$; see **Figure 37A**). Neither of these behaviors were significantly influenced by the sex of the subject (vasotocin: sex*drug interaction: $F(1,86) = 0.00003$, ns, sex main effect: $F(1,86) = 1.50$, ns; mesotocin: sex*drug interaction: $F(1,85) = 0.228$, ns), although there was a trend for females to spend more time in the nest and area (23.9 ± 7.07) than males (6.32 ± 7.00; mesotocin: $F(1,85) = 3.10$, $p = 0.08$).

The final dependent variables for the nest-entry behavioral paradigm, call-back responses and the amount of food gathered by the subject throughout the trial session, both showed effects consistent with a general injection-induced inhibition of behavior. The number of call responses produced by subjects in a session of FC playbacks was greater in untreated naives (64.3 ± 8.42) than in both the vasotocin treated (31.2 ± 8.42)

and mesotocin treated (3.66 ± 8.53) naives (vasotocin: $F(1,88) = 5.25$, $p < 0.05$; mesotocin: $F(1,87) = 28.03$, $p < 0.00001$; see **Figure 37B**). This effect was also unaffected by the sex of the subject (vasotocin: sex/drug interaction: $F(1,88) = 2.63$, ns, sex main effect: $F(1,88) = 1.15$, ns; mesotocin: sex/drug interaction: $F(1,87) = 2.29$, ns) although there was a trend for males to call back more (44.9 ± 7.96) than females (23.1 ± 8.24; mesotocin: $F(1,87) = 3.64$, $p = 0.06$). Finally, the amount of food gathered by subjects during a session (g) was also greater in untreated naives (0.784 ± 0.077) than in naïve subjects treated with vasotocin (0.254 ± 0.081) or mesotocin (0.249 ± 0.076; vasotocin: $F(1,84) = 16.72$, $p < 0.0001$; mesotocin: $F(1,90) = 6.73$, $p < 0.0001$; see **Figure 37C**). However, this effect was specific to males for both drug treatments (vasotocin sex/drug interaction: $F(1,84) = 7.26$, $p < 0.01$; mesotocin sex/drug interaction: $F(1,90) = 10.45$, $p < 0.01$; see **Figure 38**). Naïve untreated males gathered significantly more grams of food (1.19 ± 0.126) than naïve males treated with vasotocin (0.311 ± 0.126) and mesotocin (0.273 ± 0.117; Tukey HSDs, $p < 0.001$) while untreated, mesotocin-treated and vasotocin-treated females did not show a significant difference in the amount of food they gathered throughout the session (Tukey HSD, ns). Males gathered significantly more food (vasotocin: 0.751 ± 0.089, mesotocin: 0.732 ± 0.084) than females (vasotocin: 0.287 ± 0.094, mesotocin: 0.301 ± 0.084; vasotocin: $F(1,84) = 12.77$, $p < 0.001$, mesotocin: $F(1,90) = 13.32$, $p < 0.001$).

To further elucidate the inhibitory effect of peripheral hormone injections on nest-entry behaviors, a saline-treated control group was added after original testing. We tested whether these subjects showed hypolocomotion in the behavioral paradigm, as compared to untreated naives (as was seen in the hormonally-treated subjects). Due to the late

addition of this group, not all behaviors have as of yet been scored for saline-treated subjects. However food-gathering and nest-entry behaviors have been analyzed in the same way as had been done previously for hormone-treated groups. Results show evidence for injection-induced hypolocomotion during in-session periods: saline-treated male and females did not enter the nest during this time point whatsoever. Therefore, the frequency with which saline-treated naives entered the nest during the in-session period was significantly lower than that of untreated naïve males and females (all subjects: $\chi^2 = 27.07$, $p < 0.0001$; males: $\chi^2 = 11.08$, $p < 0.001$; females: $\chi^2 = 24.04$, $p < 0.0001$; see **Table 6A**). In addition, the saline-treated group entered the nest significantly less frequently than the vasotocin-treated naives ($\chi^2 = 10.08$, $p = 0.002$), an effect that was driven by female behavior (females: $\chi^2 = 8.1$, $p = 0.004$, males: $\chi^2 = 0.5$, ns; see **Table 6C**). Saline and mesotocin-treated subjects did not differ in the frequency with which they entered the nest, in session (all subjects: $\chi^2 = 4.16$, ns; males: $\chi^2 = 0$, ns; females: $\chi^2 = 4.16$, ns; see **Table 6B**). The amount of food gathering during the in-session behavioral period showed similar results, the amount of food (in grams) gathered by naïve subjects (0.784 ± 0.087) was greater than the amount gathered by saline-treated subjects (0.344 ± 0.087; see **Figure 39**). As shown previously for both vasotocin and mesotocin-treated groups, this saline injection induced decrease in food consumption was specific to male subjects (treatment/sex interaction: $F(1,84) = 10.49$, $p = 0.002$) such that naïve males gathered significantly more food (1.191 ± 0.12) than saline-treated males (0.329 ± 0.13; Tukey HSD post hoc, $p < 0.0001$, see **Figure 39**).

The hypolocomotive effect of IM injections was less apparent during the out-of-session period, leading to less consistent results of saline-treatment during that period.

Out-of-session nest entries were similar in naïve untreated and saline-treated males (χ^2 = 3.62, p = n.s.; see **Table 6D**), however females treated with saline actually entered more frequently than untreated naives (females: $\chi^2 = 36.2$, $p < 0.0001$; males: $\chi^2 = 3.62$, ns). Vasotocin increased nest entries in treated naives as compared to saline controls (χ^2 = 34.34, $p < 0.0001$), and this result was driven by female behavior ($\chi^2 = 43.82$, $p < 0.0001$; males: $\chi^2 = 2.34$, p = n.s.; see **Table 6F**). On the other hand, although saline and mesotocin treated naïve females entered similarly during this time ($\chi^2 = 0.72$; p = n.s.), mesotocin treated males unexpectedly entered less frequently than those treated with saline (male: $\chi^2 = 47.04$. $p < 0.001$; all: $\chi^2 = 31.62$, $p < 0.0001$; see **Table 6E**).

DISCUSSION

Generally, there was an inhibitory effect of peripheral injections on the behavior of naïve subjects of both sexes in the nest-entry behavioral paradigm. Subjects were given intramuscular injections of vasotocin or mesotocin thirty minutes before trial sessions and after those injections behaviors were produced less frequently than they had been in untreated naïves (controls). Both call-back responses and food collected were greater in untreated naives than mesotocin and vasotocin treated naives. Time spent in nest/area was also greater in untreated naives than in mesotocin-treated subjects, while vasotocin treated subjects were no different than controls. In session nest-entries showed a similar result, subjects treated with mesotocin showed fewer nest entries than untreated controls. However this effect was seen in males only, as untreated female naives already showed floor levels of nest entries (0). Vasotocin treated subjects once again did not significantly different from the untreated naives. In every case, during the in-session period, (when nest entries, time in nest/area, call back responses, and food collected were

measured), any significant effect of the hormonal injections was seen as a decrease in behavior, as compared to untreated naives. Typically the inhibitory effect of hormone on behavior was stronger for mesotocin than vasotocin. These inhibitory results do not fit the hypothesis that mesotocin injections would increase parental behaviors in naïve female subjects, making them act more like parentally experienced individuals. One reason for this conflicting result may be due to the fact that during behavioral testing in session, subjects were quiet and moved very little, possibly due to the stress of the intramuscular injections.

During the out-of-session period, however, the hormonal manipulations exerted some stimulatory effects on nest-entries. Both mesotocin- and vasotocin-treated females showed more entries than the untreated naïve females, appearing more like the parental females than virgins. In fact, naïve females injected with vasotocin did not significantly differ from parental females in their frequency of nest entries. In males, results showed that mesotocin injections decreased the number of nest entries in naïves, while vasotocin did not have any effect. Mesotocin injections thus produced sex-specific effects on out-of-session nest-entry behaviors in the same direction as parental experience had in experiment 3; causing an increase in female behavior and a decrease in male behavior.

Results for the out-of-session period, therefore, fit the hypothesis that subjects would behave more like parents under the influence of social hormones than untreated naives had. This is of particular interest as out-of-session testing was relatively far from the time of injection when compared to in-session periods; all hormonal injections occurred 30 minutes prior to in-session testing (1 hr), while out-of-session testing began 1.5 hours from that time (and lasted for hours). If the generally inhibitory effects

measured for the in-session behavior were due to the lingering stress of IM injections, the behavioral results detected during the out-of-session period may be less occluded by stress effects, due to the increased time elapsed since subjects had been injected. To further test this hypothesis, a new control group of saline-injected naïve subjects were added to the experiment, to better assess the behaviors in hormone-injected subjects while controlling for injection-stress. Saline-treated naïve males and females showed hypolocomotion in the nest-entry behavioral paradigm, like the hormonally-treated groups. The saline treated subjects gathered significantly less food during behavioral testing than untreated controls and entered the nest significantly less during in-session testing than untreated controls. Therefore, results support the hypothesis that the stress of the peripheral injections was a cause of the inhibitory effects in our hormonally-treated groups, as measured in-session. In addition, the nest-entry behaviors exhibited by the saline-treated control group during the out-of-session time period were variable, in some cases being more frequent than those in untreated naives and in other cases being less frequent than the untreated group. Therefore, these results also support the hypothesis that hypolocomotion due to injection stress does not affect, or affects to a lesser extent, behavior during the out-of-session period. However, complete video-scoring of the saline-treated control group was not possible due to time constraints and the late addition of this group. The call-back behaviors and time spent in nest/area of this group should be scored to fully compare control behavior to the behavior of the hormone-treated groups, without the added confound of injection stress. In addition, the influence of mesotocin and vasotocin on parental behaviors could be further studied by completing the same experiment using male and female parents as subjects, and treating those zebra finches

with antagonists for the two hormones to test whether parental subjects act more like naives when hormonal action in the avian auditory forebrain is reduced pharmacologically.

6. Experiment 5: Behavioral Recognition of One's Own Offspring's Call

RATIONALE:

After finding no behavioral discrimination between parent's OFC and novel FCs in the nest-entry behavioral paradigm (see Chapter 4), experiment 5 aimed to test parent's recognition of their own offspring's call using a behavioral approach assay (as used in Miller, 1979; Clayton, 1988; Woolley & Doupe, 2008). In songbird research, simple tests for behavioral recognition of a salient stimulus have most commonly used either call-back or approach assays (Miller, 1979; Clayton, 1988; Clayton & Prove, 1989; Lind et al., 1996; Vignal et al., 2004; Vignal et al., 2008; Woolley & Doupe, 2008; Levrero et al., 2009; Reers et al., 2011). Call-back responses to own and novel fledgling calls have been studied by the Mathevon group (Levrero et al., 2009), Reers and colleagues (Reers et al., 2011) and again by us in experiment 3 (Chapter 4), and yet results have not yet definitively shown a behavioral recognition of one's own offspring's call. The question of whether parents recognize the calls of their own offspring and respond preferentially to them has not yet been assessed using a behavioral approach assay, and therefore we aim to do so here.

Behavioral approach assays have been used in adult zebra finch subjects to demonstrate an approach bias for the bird's original tutor song, as compared to novel and other familiar stimuli, in both males and females (Miller, 1979; Clayton, 1988). In addition, the same behavioral procedure has been used to demonstrate female preference for 'directed' song over 'undirected' male song; females prefer the stereotyped 'performance' songs males sing in social contexts over the songs they produce when alone and practicing (Woolley & Doupe, 2008). We therefore hypothesized that parental

subjects would similarly show a preference for the calls of their own offspring over those of novels, measurable as an approach bias for OFC in this experiment.

METHODS:

Subjects:

The subjects of the experiment were 11 male and 10 female adult zebra finches (**Table 1**). Subjects were reared in an aviary, maintained on a 12:12 light:dark cycle and had access to cuttlebone. Subjects had *ad libitum* access to food and water throughout the entire experiment. All subjects were parentally experienced in the same manner as subjects from the first experiment. Parentally-experienced subjects were assigned mating partners and allowed to cohabit with that partner while being provided with a nest, nesting-material, and the proper nutrition to stimulate breeding. All subjects produced at least 1 surviving offspring with their mate and cohabited with both their partner and any offspring throughout development, until at least 60 dph (see **Figure 4**).

Families were isolated and recorded for auditory interactions throughout the development of subject's offspring, using an Audio-Technica microphone and power module. Continuous recordings of all vocalizations were collected between days 15 and 30 dph with Sound Analysis Pro V1.04 (Sound Analysis Pro, Tchernichovski et al., 2000). These recordings were used to create stimuli for each clutch/subject, to be used in neurophysiological recordings and behavioral testing. Stimuli were produced from recordings of day 21.48 ± 0.337 post-hatch. One of the stimuli produced was a 1-2 second bout of repeated calls from one fledgling (stimulus: fledgling). To standardize the auditory stimuli, recordings were: filtered, equated for loudness and 5 msec of silence was added to the start and end of each bout of calls (Signal Engineering Design). The

'fledgling' stimulus was then further altered to prepare it for behavioral recognition testing using a behavioral approach assay. The 1-2 s fledgling stimulus was edited to play once, followed by a three second interval of silence, and then play again, to more closely resemble the way in which young birds beg for food (GoldWave software, Goldwave Inc., St. Johns, Newfoundland, Canada). In addition, experimenters edited the fledgling call stimuli so that they would only play out of one speaker (left or right) when played in stereo.

Behavioral testing:

Recognition testing was designed to assess whether parents show a long-term memory for their own offspring. As young zebra finches only produce begging calls until 35 dph (at the latest), all behavioral recognition testing was therefore conducted in parents 25 days or more after their offspring would have stopped producing the calls used in testing, at 60 dph or later. Testing procedures were adapted from those used in Clayton, 1988 and Woolley and Doupe, 2008.

One hour prior to behavioral approach testing, subjects were isolated and acclimated to a 18" x 8.5" x 10.25" wire bird cage inside of a 25" x 21.5" x 22" sound-attenuated box. The 18" length of the cage was divided into three areas: the left zone (5" x 8.5"), the center 'neutral zone' (8" x 8.5"), and the right zone (5" x 8.5") using yellow tape to visualize these zones (see **Figure 40**). In the neutral zone, food and water were provided ad libitum, in addition to a perch on which to sit. This encouraged subjects to spend their time in the neutral zone, rather than staying in one of the call-associated side zones.

After the one hour cage acclimation period was complete, behavioral testing began

with a six-trial acclimation set of stimulus presentations (three of OFC and three of the novel FC stimulus). Throughout acclimation trials, as well as the experimental trials, birds were played a stimulus only when in the neutral zone. This acclimation set was not recorded or analyzed for behavioral responding, but rather allowed animals to acclimate to the playback of auditory stimuli in the isolation box, because, in preliminary testing, we found they tended to respond with alarm and hyperlocomotion. After all acclimation procedures were complete, trial 1 of testing began. Subjects were played one of the stimuli, randomly selected and played back from either the left or right speaker by the same ARTSy program used earlier in our nest-entry behavioral paradigm (Gess et al. 2011; David Schneider, Columbia University, New York, NY, U.S.A). Although experimenters initiated stimulus playback when the bird was in the neutral zone, they were unaware of the stimulus being played back during testing. Half of subjects were played their OFC from a speaker on the left and half were played their OFC on the right. After each trial there was a 40s response period/inter-stimulus interval before the next trial began. Therefore, the minimum latency between two trials was 40s; however, the inter-stimulus interval could be longer, as the next trial was not started until subjects returned to the neutral zone (if they had moved in response to the stimulus). A session of 14 trials was completed in this way; for half of those trials OFC was played and for the other half the same novel stimulus was played. After one set of trials was complete, subjects were given a 15 minute break, and then another set of trials was completed in exactly the same way. For this second set, however, the stimuli were switched such that OFC came out of the speaker from which the novel stimulus was played before, and vice versa. All sessions were video recorded for subsequent behavioral scoring, and records

of which stimulus was played at which time was recorded by ARTsY.

The following behaviors were scored from subject's behavior on the recognition-test: approach behaviors defined as movement into the call-associated zone when that call was played (within 40s), avoidance behaviors defined as movement into the opposite zone when a call was played (within 40s), and the amount of time spent (s) in each call-associated zone across the entire test session. For each trial, the number of approach and avoidance behaviors were added to assess general 'response' behaviors and compare them across the two stimulus types, as well. Any animal that took over 1 hour to return to the neutral zone from one of the call-associated side zones was excluded from behavioral analyses as experimenters were not able to complete the test paradigm for those individuals (4 males, 0 females). In addition, any subject that did not leave the neutral zone throughout the entire experimental paradigm was excluded from analysis, as a non-responder (4 males, 2 females).

Statistical Analysis

To test whether parents showed discrimination between the novel stimulus and the call of their own fledgling, experimenters ran within-subjects paired t-tests on the number of: approach responses, avoidance responses and overall movement responses to the two stimulus types over the full 14-trial session. This was done separately for sessions 1 and 2. Finally, the amount of time spent in the OFC-associated zone was compared to the amount of time spent in the novel call-associated zone, for each session. Paired t-tests were run for one sex at a time, as various parental behaviors had previously been shown to function differently in males and females.

RESULTS:

All results are based on the behavior of 11 subjects in total, 10 of the subjects were eliminated as non-responders or were not able to complete the entire experimental paradigm. Of the 11 subjects that engaged in the behavioral approach assay, 3 were males and 8 were females. A greater proportion of female subjects were engaged by the fledgling call recognition test (80%) than male subjects (27%).

Females Discriminate between Own and Novel FCs on the First Session of Trials

In the first session, females showed discriminating responses on the behavioral approach assay. Paired t-test results showed that across the 14 trials, females approached in response to the novel call (3.86 ± 1.14) significantly more than in response to their own offspring's call (0.571 ± 0.286; $t_{(7)} = -2.875$, $p < 0.05$; see **Figure 41A**). In addition, females produced more avoidance responses after the playback of their own fledgling's call (3.71 ± 1.10) than they did in response to trials in which the novel fledgling call was played (0.429 ± 0.401; $t_{(7)} = 2.486$, $p < 0.05$; see **Figure 41B**). These two results counteracted such that females ended up responding (by avoidance or approach) equally to own and novel fledgling calls ($t_{(7)} = 0$, ns; see **Figure 41C**). There was also a trend for subjects to spend more time (s) in the novel zone (369.76 ± 166.3) than the zone associated with OFC (18.42 ± 12.55; $t_{(7)} = -2.07$, $p = 0.08$; see **Figure 41D**). The male subjects, however, showed no difference in their responding to their own fledgling's calls and their responding to the calls of a novel fledgling (approach: $t_{(2)} = 0.210$, ns, avoidance: $t_{(2)} = -0.574$, ns, movement response: $t_{(2)} = -1.732$, ns) and spent equal amounts of time (s) in both call-associated zones ($t_{(2)} = -0.34$, ns; see **Figure 41**).

During the second session of recognition testing, subjects did not show greater

approach for either stimulus. Unlike the earlier trial, females behaved similarly for OFC and novel FCs (approach: $t_{(7)} = 0.129$, ns; avoidance: $t_{(7)} = 0.267$, ns; time spent: $t_{(2)} = -1.16$, ns; movement response: $t_{(7)} = -1.24$, ns; see **Figure 42**). The male data showed the same result, (approach: $t_{(2)} = -1.11$, ns; avoidance: $t_{(2)} = -0.480$, ns; time spent: $t_{(2)} = -0.676$, ns; movement response: $t_{(2)} = -0.819$, ns; see **Figure 42**).

DISCUSSION:

Results of the OFC behavioral recognition test supported hypotheses and were consistent with the neurophysiological results of experiment 1. Parental subjects discriminated between the calls of their own offspring and novels, in a behavioral approach assay. However, this behavioral discrimination was only found in female parents, as the majority of male subjects did not engage in the behavioral paradigm, limiting the male results to a small sample size of n = 3. Females moved in response to fledgling call playback and showed significantly more approach responses to the novel stimulus than OFC as well as more avoidance responses to OFC than novel stimuli. There was also a trend for female subjects to spend more time (s) in the novel zone than the OFC zone, during testing. Results indicate that female subjects could discriminate between the novel and familiar stimulus, and further than they had a tendency to approach the novel stimulus (novelty preference). Preference for the novel fledgling call may be explained by the communicative value of FCs in parental subjects. Parents typically respond to FCs with a behavior that causes fledglings to cease producing them, feeding. In this context, FCs may be an aberrant sound to parents; one that they are motivated to silence when they hear it. Further, the call of one's own fledgling may be particularly aberrant when there is no behavioral response that will end it. Therefore, our

preference for the novel stimulus may be better understood as an ‘anti-preference’ for OFC.

The significant discrimination between OFC and novels was only present on trial 1 of OFC recognition testing. The failure of the second session to find discrimination in avoidance or approach behaviors, as well as the time spent in the two zones, was likely due to the fact that the novel fledgling call was no longer novel. If the behavioral discrimination observed here was, in fact, due to a preference for novelty, then habituation to repeated presentations of this stimulus could account for the absence of a preference for either stimulus, in the second session. An additional explanation for the failure to find behavioral discrimination in the later session may have been that subjects were no longer naïve to the apparatus on the second session of testing. If the subject’s preference for the novel stimulus in the early session caused them to form a preference for that side of the cage, that preference likely confounded results from the later session as animals would now have to move into the former OFC-zone (not-preferred) to approach the preferred stimulus. Therefore, the results of the second session are not meaningful.

The results of this experiment are not only consistent with the results of our earlier experiment that found a memory for OFC in the neural responses of the avian auditory forebrain, but also with the results of Levero et al., 2009 and Ligout et al., 2015 which showed behavioral recognition for OFC through call-back responses to FC stimuli collected at 18 dph. The stimuli used in the current experiment were collected from young birds at ~21 dph, and are therefore most comparable to the calls used in the Reers et al., 2011 study (~22 dph) in which experiments did not find a difference in how parents

responded to OFC and novel fledgling calls, through call-back behavior. However, as call-responses are not a ethological response to begging calls in zebra finch parental behavior and results from experiment 3 showed this behavior to be the least influenced by parental experience, the results of the current experiment may be a more accurate description of whether parents are able to discriminate between OFC and novel stimuli at this point in development. Future studies should test male recognition on a more engaging task, and assess whether recognition of one's own offspring can be affected by hormonal manipulations. Oxytocin has been shown to have a role in recognition of socially-familiar conspecifics, and as we now know that neural recognition of OFC occurs as early as the avian auditory forebrain structures and mesotocin influences neural responses there, it is possible that antagonism of this system may inhibit behavioral recognition of one's own offspring's call.

7. General Discussion and Conclusions:

This set of experiments aimed to test the role of experience-dependent auditory cortex plasticity in zebra finch parental behavior, and the possible hormonal mechanisms by which such plasticity is induced. Our results demonstrate that parental experience induces neural plasticity in areas analogous to the auditory cortex in a species evolutionarily distant from mammals, and further that hormonal mechanisms contribute to the changes in auditory responses associated with parental experience. Results of experiment 1 clearly show that parental subjects have stronger neural responses to the behaviorally-relevant category of fledgling begging calls and higher best-frequency tuning in auditory neurons than virgins do, weeks after offspring have ceased producing begging calls. However, our results are significant well beyond replicating a phenomenon that has been documented in mammals. The choice to conduct this experiment in the socially monogamous species, the zebra finch, is of particular interest because of the bi-parental care that zebra finches show. Bi-parental care is uncommon in mammalian species, including the rodents most commonly used as model systems. Therefore investigation into the neural basis of mammalian male parental care has been limited to experimentation performed in voles and California mice (Bamshad, Novak & de Vries, 1994; Parker & Lee, 2001; Lee & Brown, 2007; de Jong, Chauke, Harris, Saltzman, 2009). Our results are the first to show the effect of parental experience on neural responses in auditory structures of the brain, in males.

We have shown that experience with fledglings causes changes in neural responses to offspring vocalizations in auditory structures of the male brain as well as the female brain, in this species. However, we have also shown that there are qualitative

differences in how parental experience influences the neural and the behavioral responses to FCs in males and females. Results from experiment 1 showed that both male and female parents show increased response strength to FCs (over that of virgins), but also that this enhancement was found in the left hemisphere of male subjects and the right of females. Therefore, we have replicated the result from Marlin et al. (2015), which indicated that parental experience-dependent auditory cortex plasticity is lateralized, while extending those results to male subjects and showing that the direction of lateralization is dependent on sex. These results suggest that the mechanism through which parental experience affects auditory processing may differ for maternal and paternal care. Marlin et al. (2015) found that the effect of parental experience on neural responses to pup-isolation calls was lateralized because the distribution of oxytocin receptors in the auditory cortex are lateralized (specific to the left). Therefore, the results found here may be explained by a differently lateralized distribution of hormonal receptors in male and female zebra finches, or produced through the action of two different hormones (one male-specific and one female-specific).

Experiment two of this study explored the hypothesis that paternal and maternal experience influence neural responses in the avian auditory forebrain through the action of two different hormones: mesotocin for maternal care and vasotocin for paternal care. The results of this experiment revealed that mesotocin did increase neural responses to auditory stimuli in females, but not males, while vasotocin caused males to show more elevated responses to auditory stimuli than females did, in NCM. Results further indicated that microinjections of these social hormones caused upward shifts in tuning receptive fields such that mesotocin and vasotocin-treated multi- and single-unit sites had

higher average best frequencies than saline and untreated sites. In fact, in the NCM of male subjects, there was a vasotocin-dependent increase in BF tuning in the left hemisphere specifically, a result which matched the lateralized result identified in experiment 1 (paternal enhancement of FRS in the left hemisphere). Therefore, central administration of mesotocin and vasotocin mimicked the effect that parental experience produced on tuning in the avian auditory brain structures NCM and CMM, supporting the hypothesis that these hormones may be important in the neural mechanism of this plasticity.

These changes may occur through disinhibition of the auditory neurons' extra-classical RF, as has been repeatedly demonstrated for acetylcholine in mammalian A1 (as reviewed in Weinberger, 2004). Marlin et al. (2015) recently revealed that oxytocin acts to disinhibit neurons in the auditory cortex to affect neural responding in a similar manner as Ach. Therefore, this hypothesis is consistent with the mechanism that has been developed for the mammalian homologs of these hormones (2015). Although our data did not show any changes in neural responding to FCs, after microinjection with either social hormone, it is possible that these early changes in tuning work in combination with social learning about the behavioral relevance of FCs (throughout parental experience) to develop the enhanced FRS response that parental subjects ultimately show. Ultimately, the complex results found in this experiment support the hypothesis that the social hormones vasotocin and mesotocin may be involved in the neural mechanism of plasticity in the parental avian brain; while also demonstrating that further investigation into the functions of these hormones and their receptors is necessary

to better comprehend how male and female brains function differently to produce parental behaviors.

The latter experiments in our study investigated the behavioral responses adult zebra finches show to fledgling calls, how those are affected by parental experience, and whether peripheral injections of mesotocin and vasotocin can mimic parental experience as they had for neural responses. Experiment 3 investigated how parental and virgin subjects differ in their responses to fledgling calls, using the following behaviors: call-responses, food-gathering, time spent in nest/area and nest-entries. Results showed that parental females gathered more food, spent more time in the nest/area adjacent and entered the nest more than virgin females. On the other hand, virgin males gathered more food and entered the nest more than parentally-experienced males, an unexpected result. Once again, these outcomes indicated a noteworthy sex-difference in how subjects respond to fledgling call stimuli. However, in this case, we had not anticipated these sex-specific effects. We had hypothesized that parental behaviors would be increased in parental subjects, in both sexes, as the zebra finch is a bi-parental songbird species. However, there is historical evidence that female zebra finches perform more of the feeding behaviors than male zebra finches during juvenile development (Delesalle, 1986), as well as incubating more than their male counterparts (El-Wailley & Jasin, 1966). Interestingly, we also found a high level of parental care in male zebra finches that had not experienced mating or parenting (virgin subjects) that far exceeded the parental care exhibited by female virgins. These results may be explained by a distribution of labor in parental care, such that female zebra finches perform a majority of the incubating and food-provisioning behaviors while male zebra finches focus on nest-building early in the

mating process and territory defense in later child-rearing (assuming that the feeding-related variables we measured here index other early parental behaviors in males that we didn't measure). In fact, division of parental duties may be a vital component of parental care in bi-parental species. Animal species that evolve monogamous bi-parental mating systems may do so to facilitate cooperative parental care in particularly challenging environments, ones in which one parent cannot physically perform all the roles that offspring need for survival (food provisioning, incubating, foraging, defense). The 'harsh environment hypothesis' predicts that species that live in environments where food is scarce, there is high competition for resources, there is a high level of predation or extremely harsh weather conditions will show bi-parental care (Wilson, 2000; Amat & Masero, 2004; Wells, 2010; AlRashidi et al, 2011; Vincze et al, 2013). Therefore, further study into how model systems with bi-parental care (such as birds) divide parental labor may be important for understanding how cooperative care developed in humans as well.

When the effects of peripheral injections of the social hormones MT and VT were then tested for their ability to mimic parental behavior in naïve animals, there were sex-specific effects as well. Peripheral injections of both social hormones (mesotocin and vasotocin) increased the frequency with which naïve female subjects entered the nest during the out-of-session period and peripheral injections of mesotocin (only) decreased the frequency with which naïve male subjects entered the nest, during this period. So, after hormonal injections: naïve females perform more parental behaviors and naïve males perform less parental behaviors. These sex-specific effects of hormonal treatment corresponded well with the pattern of behavior that had been observed in parentally-experienced animals on this task, further supporting the role of these hormones in the

expression of parental behavior in zebra finches. Finally, these results confirm the hypothesis that the avian homologs of oxytocin and vasopressin affect behavioral responses to socially-relevant auditory stimuli. This evidence, in concordance with similar results found in rodents and most recently in the non-human primate, the marmoset (Taylor & French, 2015), suggests that the role of these hormones in auditory processing is conserved across species.

The choice to conduct this study in the zebra finch was made, in a large part, because of the individual differences zebra finch fledglings show in their fledgling begging call structures. Studies in rodents have not yet shown a distinction in the behavioral or neural responses to the calls of a dam's own pups as compared to her responses to the calls of novel pups. However, this failure to demonstrate individual-recognition is likely due to the simple structure of mouse pup calls. On the other hand, recent studies of the zebra finch have shown that fledgling calls are statistically discriminable at days 18-22 post-hatching (Levrero et al., 2009; Reers et al., 2011). Further, there has been some suggestion that parents can discriminate between the calls of their own and novel chicks in call-back studies (Levrero et al., 2009; Ligout et al., 2015) although this has evidence has been hard to replicate (Reers et al., 2011). Consequently, in both the neural and behavioral experiments of this study we aimed to answer this question, definitively, for fledgling calls produced at ~22 dph. This time point was chosen because it is shortly after birds fledge, when they may be in the nest or outside of the nest at the time of calling (Zann, 1996). Therefore, parents should use auditory cues to locate and identify the offspring to whom they will invest care.

As zebra finches live in large colonial nesting groups, if parents do not discriminate between their own and novel offspring, there is a high likelihood they will invest parental care into fledglings that do not carry their genes, a behavior that is incompatible with theories of natural selection. Results from our neurophysiology experiment fit our hypothesis; both female and male parents exhibited a neural memory for the calls of their own offspring in the avian auditory forebrain. This memory was robust; neural discrimination was replicated in both multi-unit and single-unit data, through adaptation rates and ARMs/spike-rates. For this reason, we hypothesized that there would be a strong behavioral discrimination between own fledgling's call (OFC) and novel fledgling calls, in male and female parents. When subjects were tested for behavioral recognition of OFC on a behavioral approach assay, we found that female subjects showed recognition of OFC, as expected. However, the majority of male subjects were unresponsive to both OFC and novel FC stimuli, and did not engage in the behavioral approach task. For this reason, many male subjects had to be excluded from analysis, leaving such a small sample that it was not possible to draw a conclusion for whether they could perform this behavioral discrimination or not. To further address the question of whether males show a behavioral recognition that matches their neural recognition of OFC, males should be tested again using a different task, one in which they are more motivated to respond.

Nevertheless, our results support the notion that recognition of offspring based on vocal signals has been selected for in this species, and provide evidence for neural mechanisms that contribute to this behavioral adaptation. In addition, we have shown here evidence for a fairly long-term memory (minimum: 25 days, average: 60 days) for

the vocal cues of a socially-familiar conspecific, as evidenced by both neural and behavioral discrimination for OFC. Heretofore there have been no published data showing a socially-learned auditory memory (for song or call) that lasts this long in zebra finches, besides the one for tutor song that is established through developmental imprinting. Although behavioral and neural memories for mate's song/call have been shown, the longest duration between exposure to that stimulus and test for memory in those studies has been a couple of days (Vignal et al, 2004; Vignal et al, 2008; Lind et al, 1996; Menardy et al, 2012). Therefore, our study establishes the zebra finch as a model system that not only shows plasticity for socially-relevant stimuli in the sensory structures of adult subjects, but also as one in which these neural changes are maintained such that they affect behavior for weeks.

Conclusions and Implications:

Parental care is one of the most common and highly-conserved social behaviors in the animal kingdom. However, although we have only begun to understand the neural mechanisms of complex social interactions such as maternal care, our understanding of paternal care still pales in comparison. We have learned a good deal about the importance and influence of parental care on outcomes in offspring throughout the last two decades. We now know that the degree to which parents nurture their offspring influences both the social and brain development of juveniles in a variety of species, and that oxytocin is one of the mechanisms through which parental behavior influences those outcomes (rodents: Francis et al., 2000; primates: Maestripieri et al., 2009). Oxytocin levels in the brains of rodent parents are associated with the level of care they provide to

their offspring, which has been shown to set up the oxytocin system of offspring and, in turn, influence the level of care they give their children as adults (Francis et al., 2000). These findings support the evidence in humans which has shown that parental oxytocin levels during the first few months of juvenile development predict: social engagement, friendships and empathy in their children (Feldman, 2007). Human babies are born highly immature, and due to the lengthy period of dependence on their parents, the social interactions between parent and baby have a greater influence on the social development of children than is seen in most other species (as reviewed in: Feldman, 2015). Therefore, the neural mechanisms that enable the behavioral expression of not only maternal, but also paternal care, have implications for the social functioning of the human race as a whole; and oxytocin has remained our biggest clue into that neural mechanism.

Oxytocin also has a role in the social recognition of familiar individuals. Mice with knockouts for either social hormone (oxytocin or vasopressin) show a reduced ability to recognize conspecifics as familiar after social experience with them (Ferguson et al., 2000; Bielsky et al., 2004). In fact, these social hormones may influence the neural processing of the visual, olfactory or auditory cues of socially familiar conspecifics from as early in the bottom-up processing of those stimuli as the sensory structures. However unlikely we would have judged this hypothesis years ago, primary sensory cortices have been shown to be much more than simple stimulus analyzers as neural responses in these structures have been shown to be influenced by the behavioral relevance of sensory stimuli again and again. Furthermore, receptors for oxytocin have been identified in the visual cortex of non-human primates as well as the olfactory bulb and auditory cortex of rodent model organisms (as reviewed in: Freeman & Young, 2016; Marlin et al., 2015).

Hormonal action at these receptors has also been shown to influence neural processing of sensory stimuli (2015). The localization of oxytocin receptors in the primary auditory cortex of mammals in conjunction with the identification of receptors for its avian analog (mesotocin) in the avian auditory structure analogous to superficial layers of mammalian A1 (NCM), suggest that the role of social hormones in auditory processing is conserved across species (2015; Leung et al., 2011; Wang et al., 2010). As auditory structures of the songbird also show receptors for the avian analog of vasopressin (vasotocin) and birds show biparental care, the songbird is a uniquely powerful model in which we can study the influence of male and female parenting hormones, on neural responses to natural auditory signals (Leung et al., 2011). Therefore, the results our of experiments which show sex-specific effects of vasotocin and mesotocin on neural and behavioral responses may be the first step towards identifying the mechanism by which the male and female brain coordinate to allow for efficient co-parenting through a distribution of duties. In fact, our behavioral results, which show a very high level of parental motivation in virgin males and parental females, but not parental males and virgin females, might be best understood as a distribution of parental responsibilities, across time.

Finally, the results found here for the influence of vasotocin and mesotocin injections on neural responses support findings from other species showing that these hormones are a possible mechanism through which parental experience causes brain plasticity. In addition, we have found evidence that parental experience may change the brain in a lateralized way in the zebra finch. The enhancement of neural responses to fledgling calls in parents was specific to the left hemisphere in males and the right

hemisphere in females. This result was not specifically hypothesized and is of particular interest, in the context of how cortical lateralization may improve neural processing. The production and perception of speech is cortically lateralized in humans; speech is processed primarily in the left hemisphere of most subjects (Friederici, 2011). Many other animals, including the zebra finch, also show lateralization in the production and perception of vocalizations (Ocklenburg, Ströckens, & Güntürkün, 2013; Moorman & Nicol, 2015). Although this characteristic of the perception of vocalizations has been shown to be evolutionarily widespread, the advantages of lateralized processing are still up for debate. One potential explanation is that lateralization may enhance the brain's momentary capacity for neural processing by reducing repetitive information (Vallortigara & Rogers, 2005).

In the NCM of zebra finches, perception of conspecific vocalizations has been shown to be right-lateralized (stronger response) for novel stimuli and left-lateralized for songs that are remembered by a subject (BOS, tutor song; as reviewed in: Moorman & Nicol, 2015). In fact, neuro-estrogen action in the left hemisphere of NCM, specifically, is necessary for zebra finch males to show behavioral preference for their own song (BOS; Remage-Healey, Coleman, Oyama & Schlinger, 2010). Therefore, our results are not the first to show left-lateralized NCM processing of behaviorally-relevant stimuli in male zebra finches. In fact, there is prior evidence showing that hormonal action on one hemisphere of NCM is particularly important for the expression of a behavioral response to a learned vocalization (Remage-Healey et al, 2010. Therefore, it is possible that mesotocin or vasotocin may act specifically in one hemisphere of the avian auditory forebrain to enhance neural responses to FCs.

In contrast, our results are the first to show differential lateralization of auditory processing in the two sexes. However, it is important to note that, of the eight studies reviewed in Moorman & Nicol, 2015 that focus on lateralized auditory processing in the zebra finch, only two studies included female zebra finches as subjects (along with their male counterparts). A majority of the research focused on auditory processing in the zebra finch has been conducted in male subjects, exclusively. For this reason, sex differences, such as those identified throughout these experiments, likely remain undiscovered. Auditory responses may be lateralized differently in male and female zebra finches, and this may be due to: differential distribution of receptors for neuromodulators such as vasotocin or mesotocin in males and females, or differences in the concentrations of those neuromodulators in the brains of males and females (due to their sex-specific functions). Further investigation into these two potential mechanisms, through which the origin of these lateralized effects could be explained, is necessary. Experiments aimed to address these questions will allow us to determine whether the lateralization of auditory responses to FCs emerges in NCM/CMM, or if the ascending auditory pathway is lateralized before reaching these structures. In addition, the function of this lateralization in auditory perception should be further explored through inactivation experiments, which may reveal differential effects on behavior in males and females. Ultimately, our understanding of the lateralized neural processing of this behaviorally-important vocalization in zebra finches may develop our understanding of the advantage(s) of lateralized neural processing of vocalizations, throughout the animal kingdom.

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Appendices

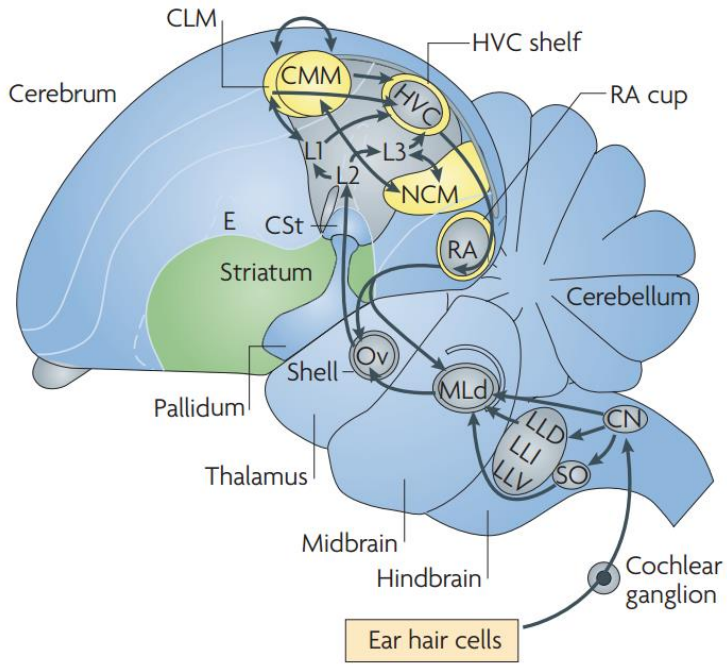


Figure 1. Anatomy of the bird song auditory system. Ascending auditory information projects from the midbrain area MLd (homologous to inferior colliculus) to nucleus ovidalis (Ov) which is homologous to mammalian medial geniculate nucleus of the thalamus. From there, information goes to the primary auditory area Field L2 (probable homolog of A1, layer 4). Field L2 projects to L3 and L1 which in turn project to higher auditory areas caudomedial nidopallium (NCM) and caudal mesopallium (CMM), shown in yellow. NCM and CMM may be analogous to mammalian secondary auditory cortex, or superficial layers of mammalian A1. Diagram also shows projections into vocal motor areas not studied here. Figure from Bolhuis et al., 2010.

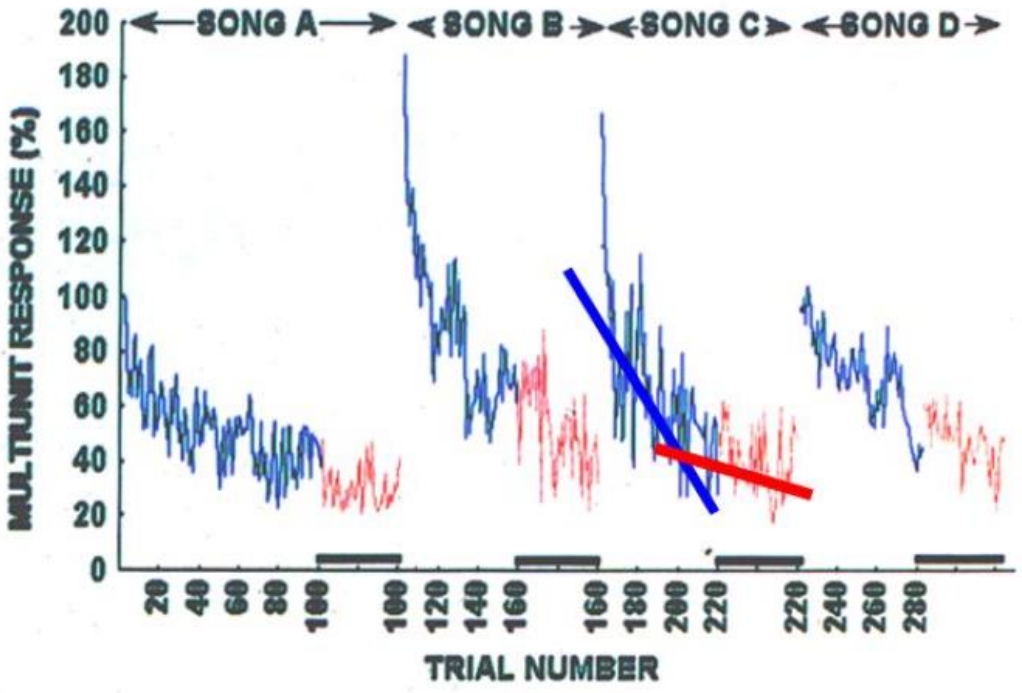


Figure 2. Stimulus specific adaptation (SSA). Stimulus-evoked multi-unit absolute response magnitudes decline over repeated presentations of an auditory stimulus. The reduction in neural responses to one stimulus does not cause an overall decrease in auditory responses to other stimuli, and is therefore 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 adapted from Chew et al., 1995.

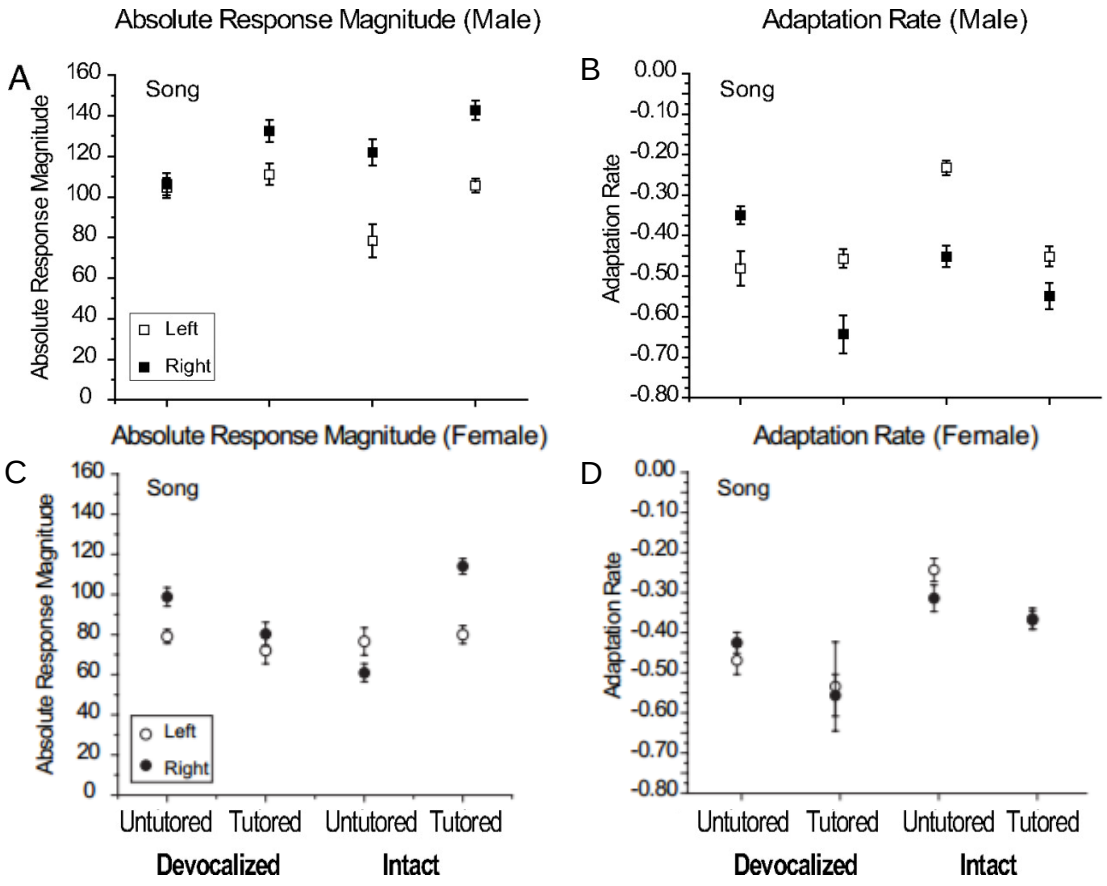


Figure 3. Lateralized auditory responses in NCM. ARMs and adaptation rates of multiunit responses to song in NCM of male and female zebra finches are lateralized. Males that have experienced song, either through tutoring or by hearing themselves vocalize, have: **(A)** stronger absolute responses to 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. **(C)** Females also show right hemisphere dominance for auditory-processing of auditory stimuli, in absolute response magnitudes. Figure from Phan and Vicario, 2010.

	Group	Exps 1A & 1B: Ephys. N = 24	Exp 2: Central Manipulation and Ephys. N = 38	Exp 3: Nest Entry Behavior N = 24	Exp 4: Peripheral Manipulation and Behavior N = 36	Exp 5: Recognition Test Behavior N = 21
Females N = 71	Naïve N = 49	6 subjects	Mesotocin: 10 subjects	6 subjects	Mesotocin: 6 subjects	-
			Vasotocin: 9 subjects		Vasotocin: 6 subjects	
			-		Pilot Saline: 6 subjects	
	Parent N = 22	6 subjects	-	6 subjects	-	10 subjects
Males N = 72	Naïve N = 49	6 subjects	Mesotocin: 10 subjects		Mesotocin: 6 subjects	-
			Vasotocin: 9 subjects		Vasotocin: 6 subjects	
			-		Pilot Saline: 6 subjects	
	Parent N = 23	6 subjects	-			11 subjects

Table 1: Experimental subjects (N = 143). The sample size for all experiments was 142 subjects, 72 male and 71 female. In experiment 1, electrophysiological recordings were conducted in 24 subjects, half male and half female. Experiment 1A investigated neural responses to fledgling call stimuli naïve and parental subjects. Experiment 1B assessed neural recognition of one's own offspring's call, using the same parental subjects that were used for Experiment 1A. Experiment 2 focused on the neural responses of naive subjects that had been centrally injected with the social hormones mesotocin and vasotocin. Ten males and females were used the mesotocin experiment and 9 males and females were used in the vasotocin experiment. Experiment 3 assessed behavioral responses to FCs in parental and naïve subjects, using 24 subjects, half male and half female. Experiment 4 then investigated the behavioral responses of naïve males and females to fledgling call stimuli, after injections of social hormones mesotocin and vasotocin. Six males and females were used in the mesotocin experiment and 6 males and females were used in the vasotocin experiment. An additional group of naïve subjects (n = 6 of each sex) was saline-injected and tested for hypolocomotion; these animals were included as a pilot group as not all data have been scored and analyzed at this time. Finally, in experiment 5, we assessed parental subjects for their ability to behaviorally recognize their own offspring's call using 10 female and 11 male subjects.

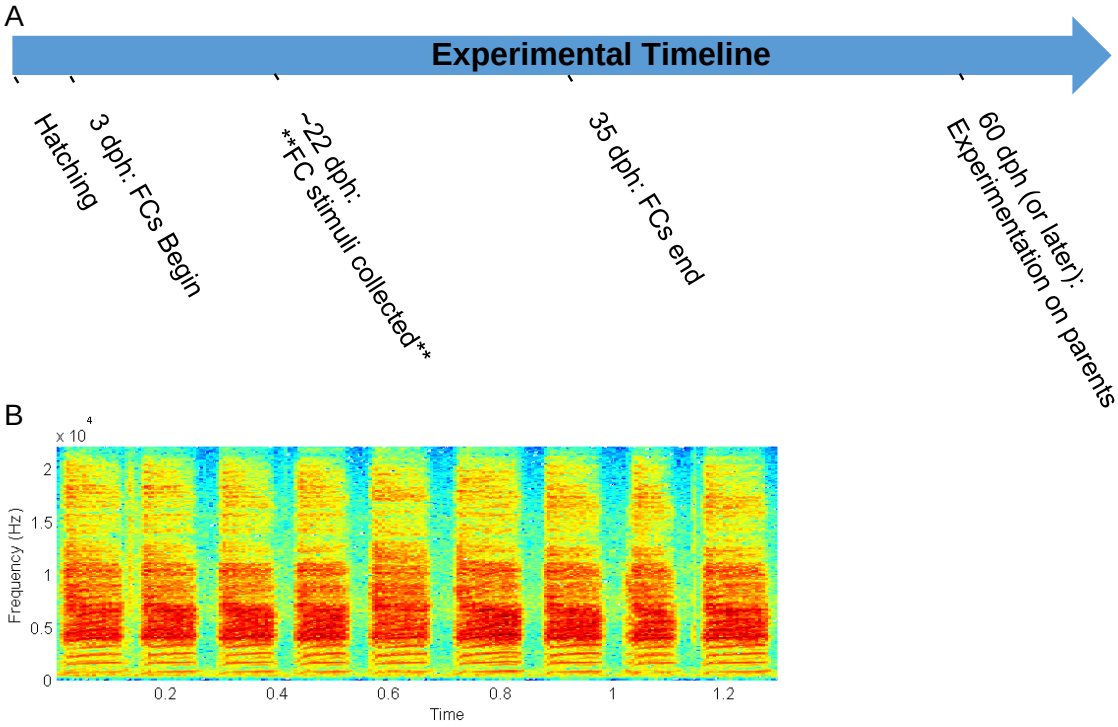


Figure 4. General experimental timeline and fledgling call spectrogram. For all experiments, subjects were either parents or aged matched controls, naïve to mating. **(A)** All parents experienced this experimental timeline before experimentation: they cohabitated with the opposite sex, mated, produced nests and layed eggs, incubated those eggs to hatching and then lived in single-family housing until 60 dph. Offspring produce FCs from 3 dhp to 35 dph, at the latest. Therefore all experiments tested long term memory for FCs (> 25 days from hearing the calls of their own offspring). The stimuli used in all experiments were collected at 21.48 ± 0.337 dph. **(B)** A spectrogram of an example bout of fledgling calls from one fledgling (all stimuli used were 1-2s).

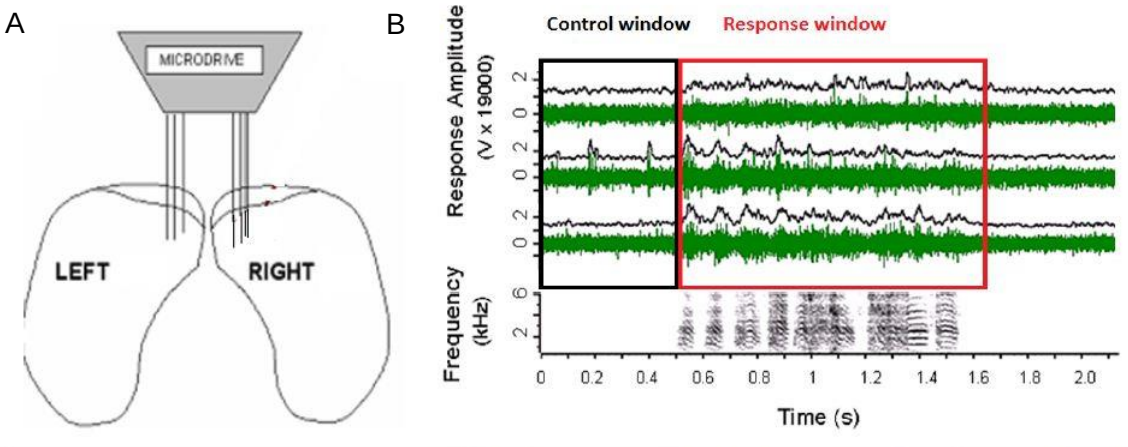


Figure 5. Electrophysiological apparatus and multi-unit recording. (A) Sixteen micro-electrodes are placed bilaterally in the avian auditory structures NCM and CMM (4 in each structure in each hemisphere). **(B)** Multi-unit recording of neural activity in NCM shown for 3 channels. Multi-unit recordings 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.

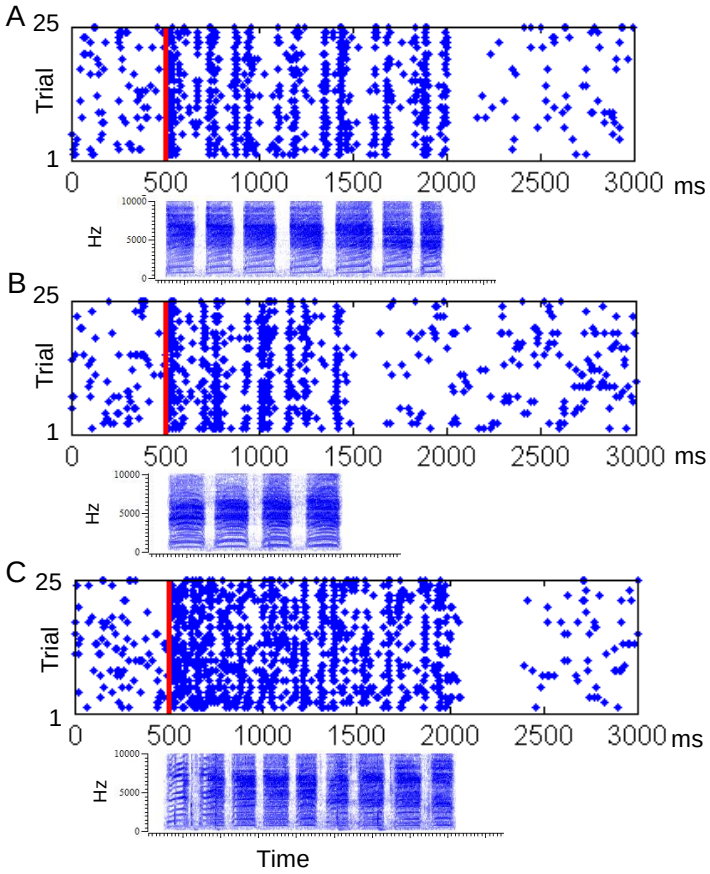


Figure 6. Exemplar single-unit raster responses to fledgling begging calls. The spiking responses of a parental subject's NCM unit to **(A&B)** two bouts of a novel fledgling's call and **(C)** a bout of own fledgling's call are shown in these rasters. All three rasters depict firing of the same neuron, from 500 ms before stimulus onset to 2.5 seconds after the stimulus began. Responses are depicted for each stimulus presentation, going from trial 1 on the bottom of the y-axis to trial 25 at the top. Beneath each raster plot, the spectrogram of the auditory stimulus that evoked those responses is depicted. Spike-rates were faster for OFC than novel fledgling calls.

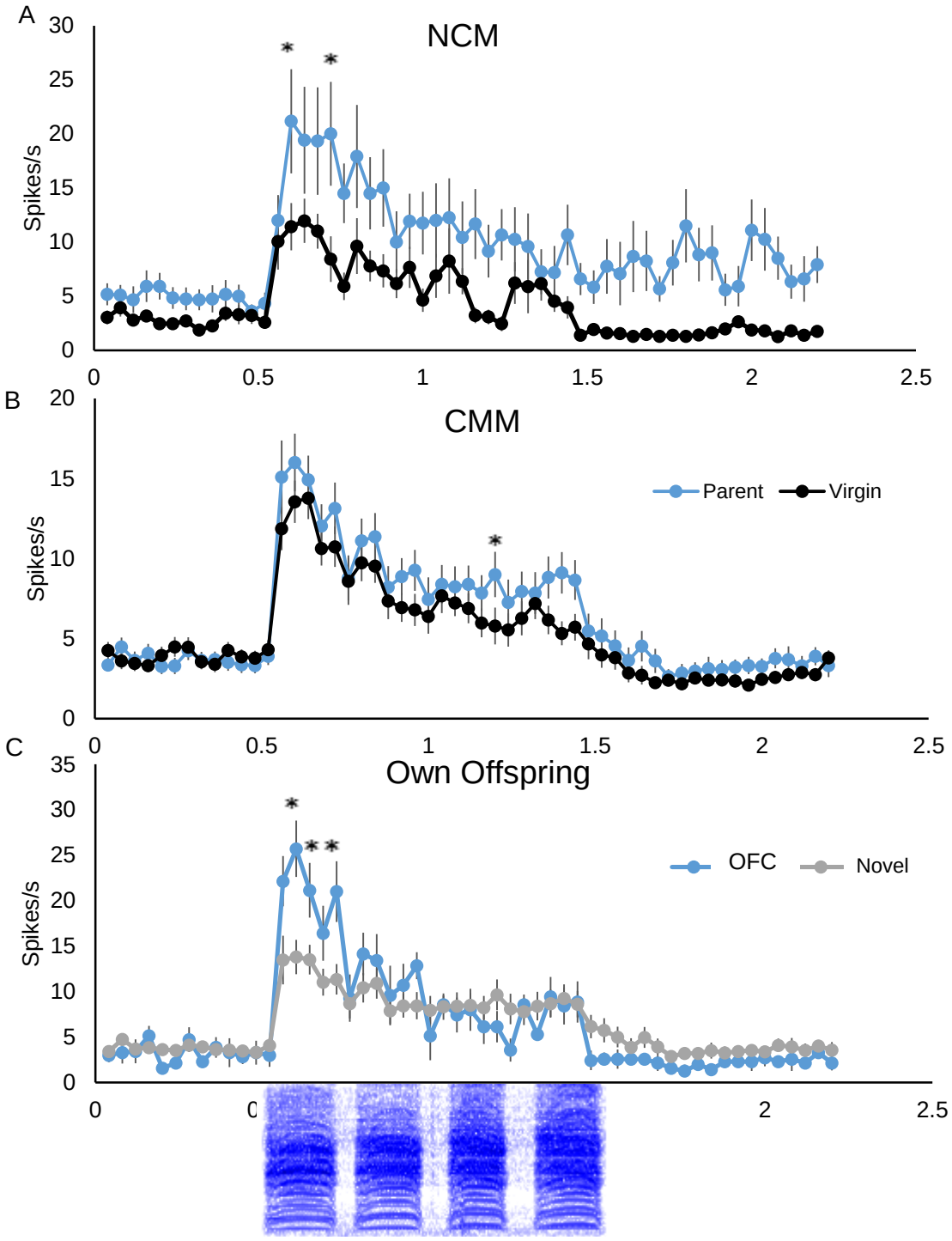


Figure 7. Average PSTH for fledgling call stimulus, in virgins and parents. (A&B) The average PSTH for single-unit NCM **(A)** and CMM **(B)** data is shown for one exemplar fledgling call stimulus. In NCM, parents show higher responses throughout the stimulus, the post-stimulus response period and at baseline. In CMM, the single-unit response to the stimulus is elevated in parents over virgins, however the magnitude of that enhancement is weaker. For this stimulus, the CMM response of parents (only) is depicted here in **(C)**, to compare responses of parents to novel fledgling calls as compared to their own fledgling's call. The elevated response to one's OFC occurs rapidly, within the first 40 ms and decreases over time.

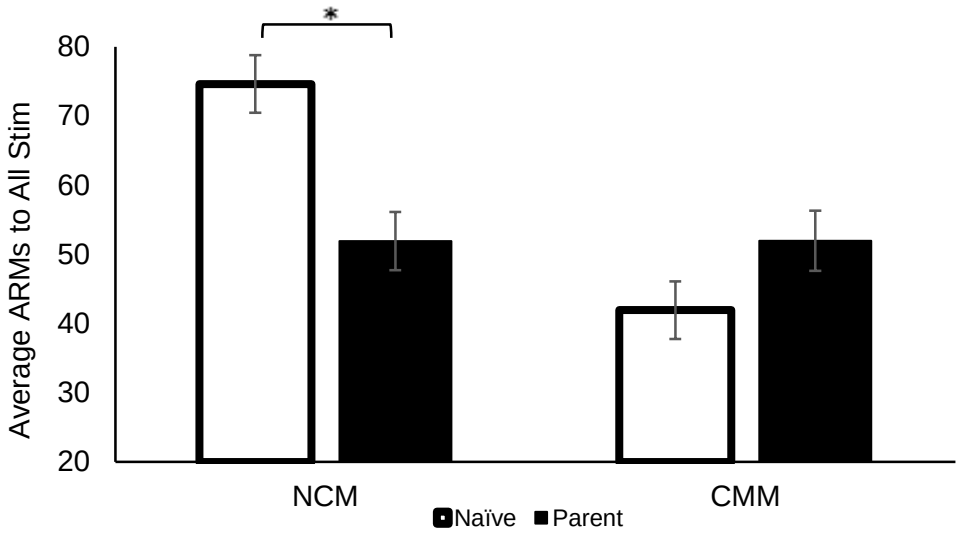


Figure 8. Naïve subjects show stronger responses to all auditory stimuli in NCM. There were global differences in neural responding between the parental and naïve groups. When the average response to all stimuli was analyzed with a factorial ANOVA, results showed a significant interaction between the area of recording and the subject group ($F(1,322) = 15.264$, $p < 0.001$). Post-hoc tests showed that naïve subjects had significantly higher neural responding to auditory stimuli at their multi-unit NCM sites than the parental subjects ($p < 0.001$). For this reason, normalized FRS values were calculated at each site to look specifically at the neural response to fledgling calls, as compared to the average response of that site, thereby eliminating these global group differences to focus on the comparison of interest: how parents and virgins differ in their responses to fledgling begging calls.

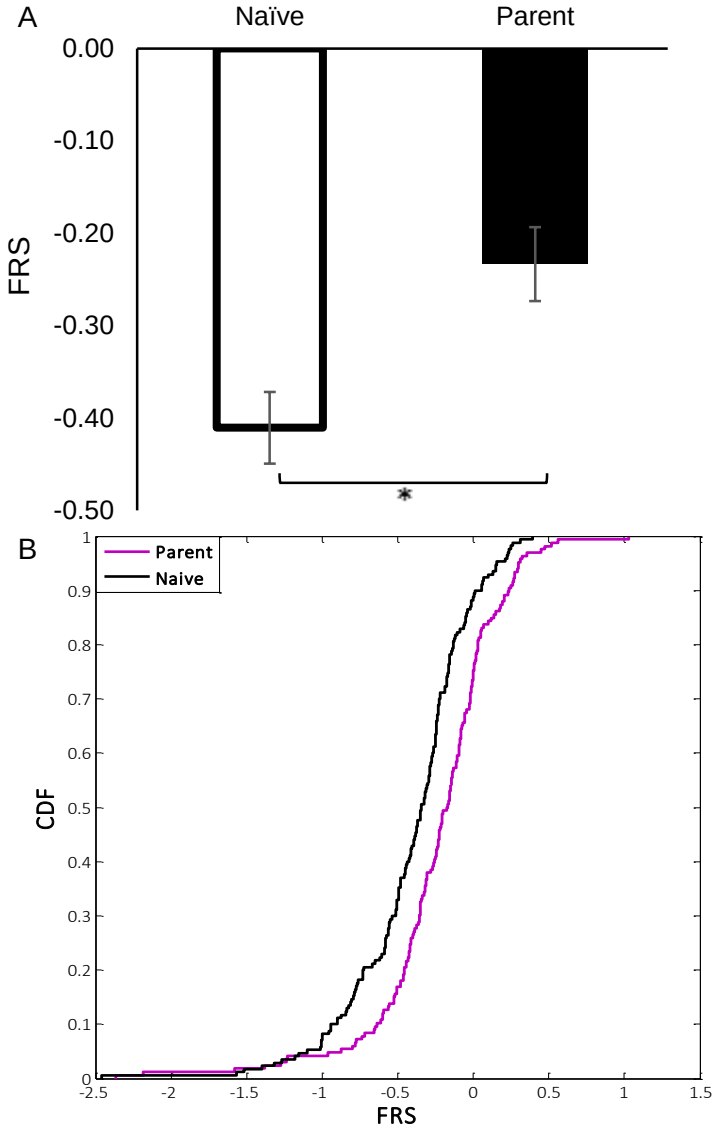


Figure 9. Parents show stronger responses to fledgling call stimuli than naïve subjects. Parents show stronger responses to fledgling calls (FRS) than virgins across all multi-unit recording sites (ANOVA main effect: $F(1, 321)=10.17$, $p < 0.01$; Mann-Whitney U Test: $Z = 4.71$, $p < 0.0001$). This comparison is represented here with both **(A)** traditional bar graphs and **(B)** a plot of the cumulative frequency of FRS values across the distribution of sites. Throughout figures, results will be visualized with traditional bar graphs for comparisons analyzed with factorial ANOVAs, for analyses in which multiple factors and interactions needed to be assessed simultaneously. In addition, CDF plots will be used when visualization of the entire distribution of results is necessary and when non-parametric tests are used. Asterisks indicate significant effects, with alpha set at 0.05.

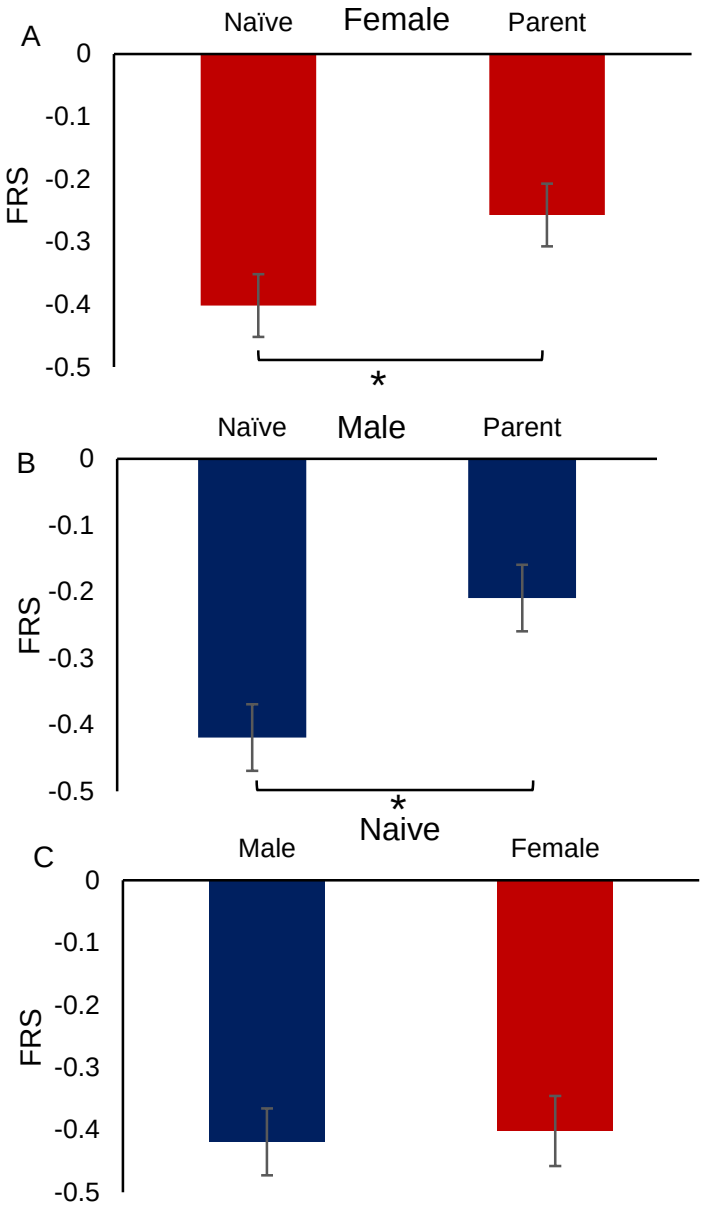


Figure 10. Fledgling call response strength is not significantly influenced by sex of subject. (A&B) Both males and female subjects show an influence of parental experience on FRS values, with stronger responses in parental subjects than naives (Female main effect of parental experience: $F(1, 153)=5.77$, $p< 0.05$; Male main effect of parental experience: $F(1, 168)=5.36$, $p< 0.05$). In addition, **(C)** naive male and female subjects do not show a difference in the strength of their neural responding to fledgling begging calls ($F(1, 164)=.03833$, $p=.84$).

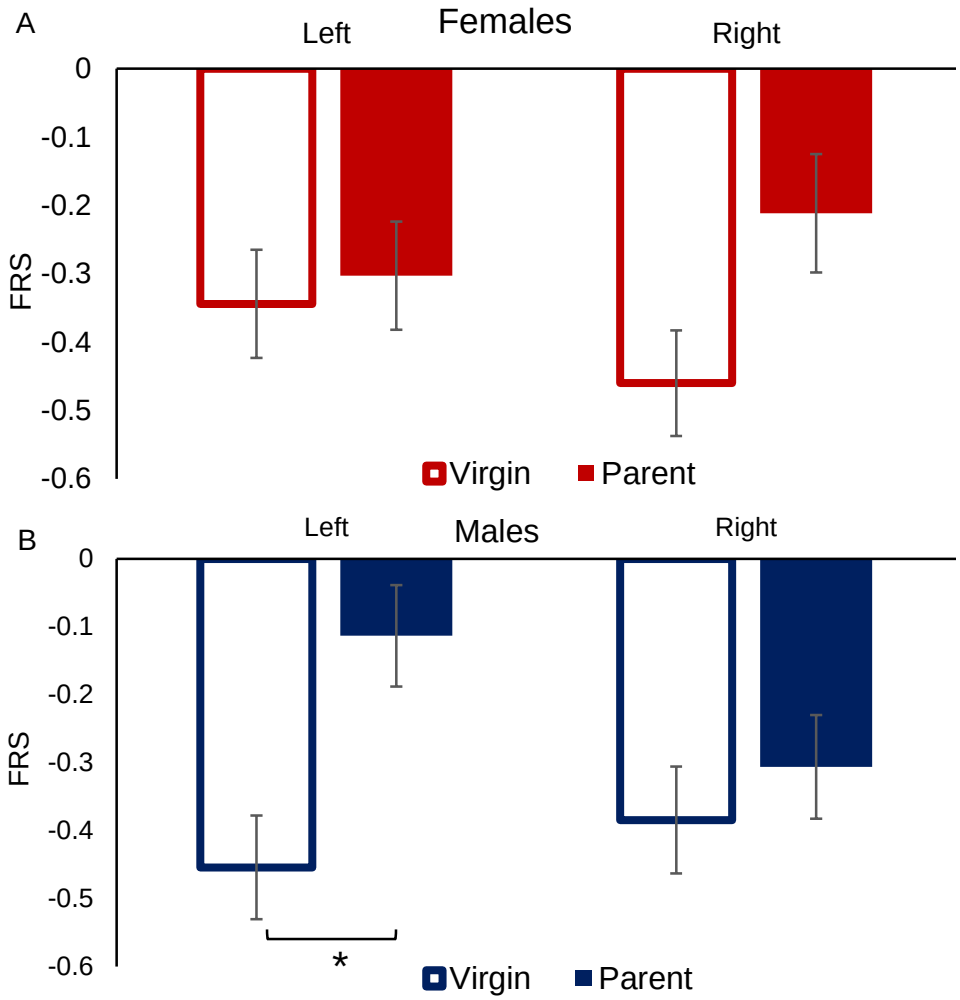


Figure 11. FRS is influenced by parental experience, sex and side of recording. Parents of both sexes showed stronger responses to fledgling calls (FRS) than their naïve controls (Female main effect of parental experience: $F(1, 153)=5.77$, $p < 0.05$; Male main effect of parental experience: $F(1, 168)=5.36$, $p < 0.05$). However, there was an interaction between parental experience, sex of subject and side of recording such that the effect of parental experience was seen primarily in **(A)** right auditory forebrain for female subjects while it was detected in the **B)** left hemisphere of male subjects (Interaction between sex, parental experience and side of recording: $F(1, 321) = 4.47$, $p < 0.05$). Post-hoc tests showed that males show a significant differences in responses to fledgling calls between virgins and parents in the left hemisphere ($p < 0.05$).

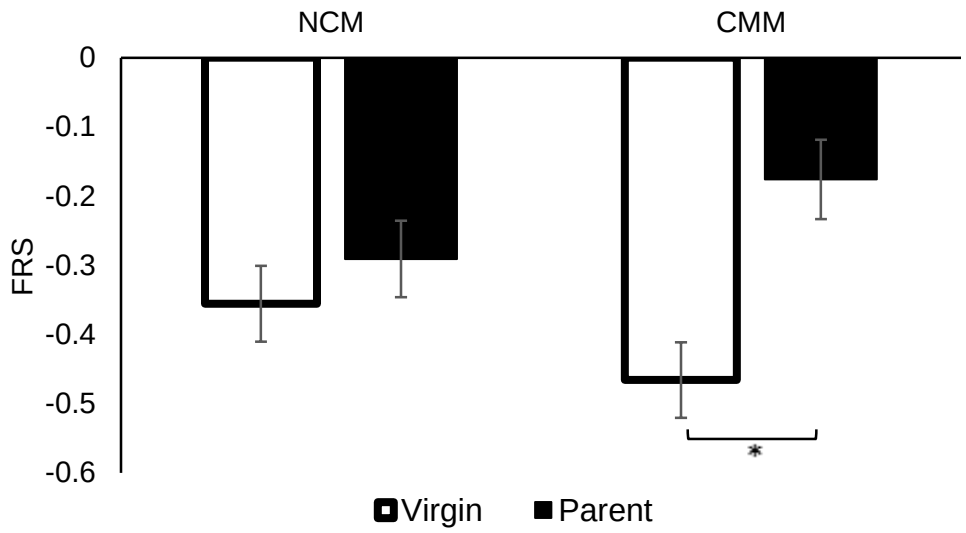


Figure 12. The effect of parental experience on enhanced FRS values occurs in CMM. Parents show stronger responses to fledgling calls (FRS) than virgins when NCM and CMM sites are collapsed (Main effect: $F(1, 321)= 10.17$, $p < 0.01$). However, this effect is primarily in CMM, an avian auditory forebrain area shown to be influenced by behavioral relevance. The results of the factorial ANOVA showed a significant interaction between parental experience and the area of recording ($F(1, 321)= 4.1038$, $p < 0.05$). Post-hoc tests showed that the significant difference between naïve and parental subjects occurred in CMM ($p < 0.01$).

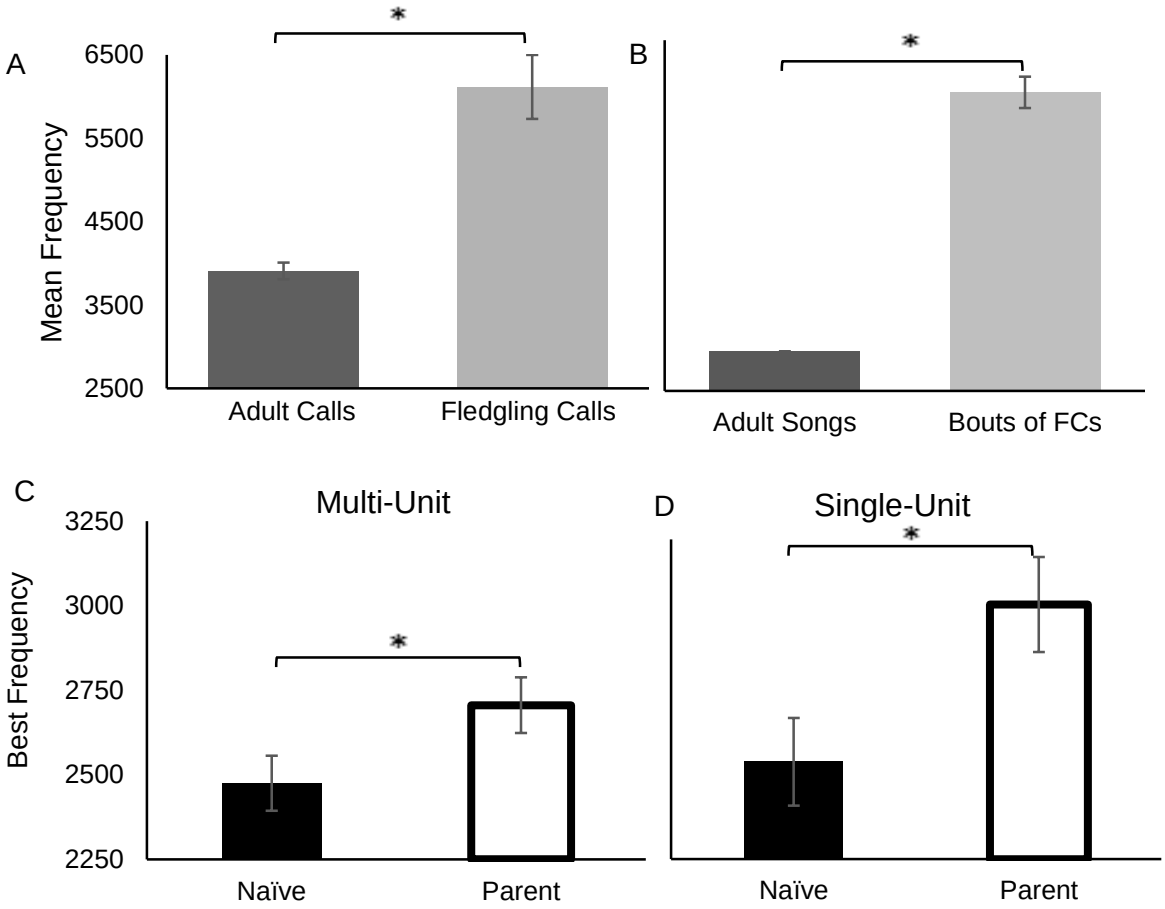


Figure 13. Auditory responses are tuned more closely to the frequencies found in FCs in the multi-unit and single-unit sites of parents. Fledgling calls show significantly higher mean frequencies than **(A)** adult calls (for our stim: $t(16) = -7.38$, $p < 0.001$) and **(B)** adult songs ($t(7) = -11.23$, $p < 0.001$). In addition, both **(C)** multi-unit and **(D)** single-unit responses showed higher best frequencies when subjects were parents (Multi-unit tuning: $F(1, 333) = 3.93$ $p < 0.05$; Single-unit tuning: $F(1, 160) = 5.89$, $p < 0.05$).

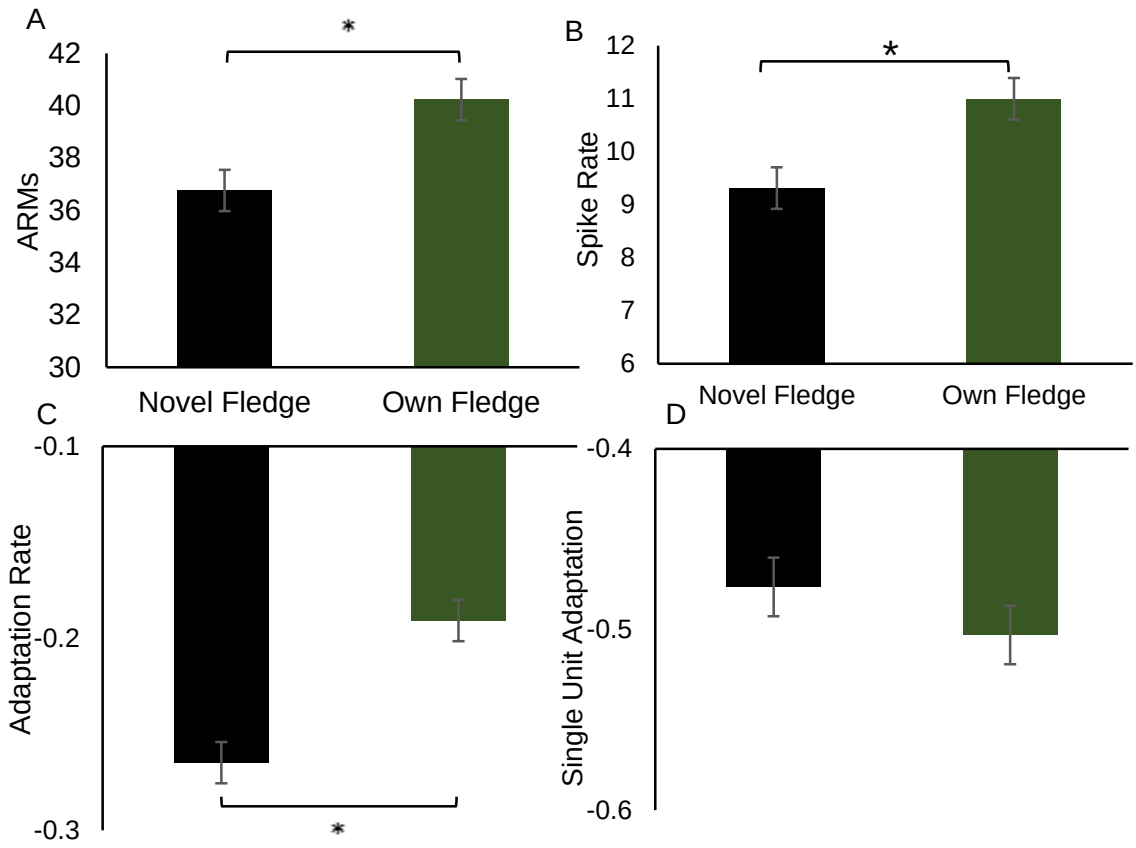


Figure 14. Parents show neural recognition for the calls of their own fledgling. (A) Parents show stronger ARMs to the calls of their own fledgling than novel fledglings ($F(1, 148)= 4.65$, $p < 0.05$). **(C)** In addition, they adapt to the calls of their own fledgling more slowly than the calls of novel fledglings, reflecting familiarity due to SSA ($F(1, 148)= 11.35$, $p < 0.001$). Single unit responses show a similar neural memory for the calls of one's own fledglings. **(B)** Single unit spike rates are faster in response to the calls of one's own offspring than novel fledglings ($F(1, 60)= 4.87$, $p < 0.05$). **(D)** Single unit adaptation rates, however, do not show an effect of stimulus familiarity ($F(1, 60)= 0.608$, ns).

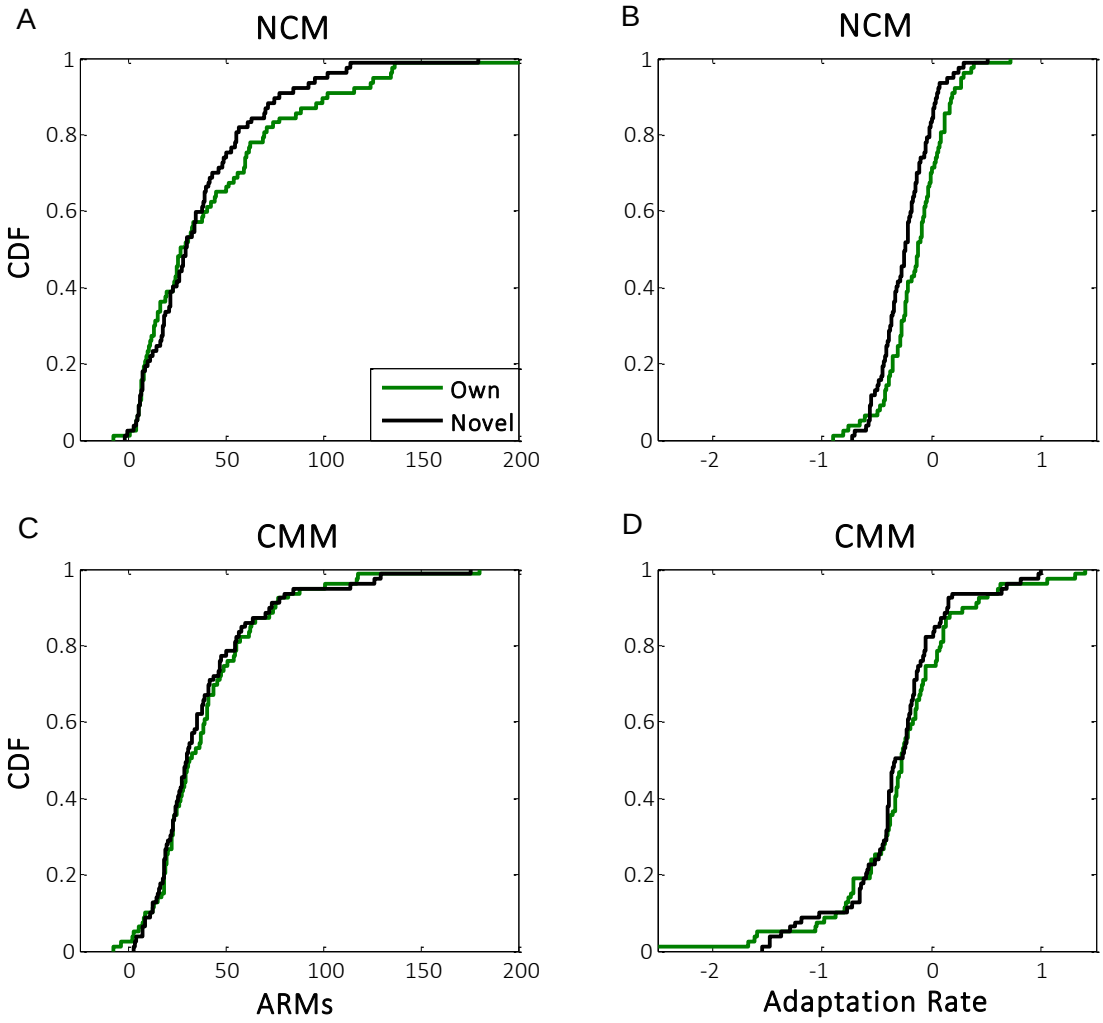


Figure 15. The neural recognition of one's OFC occurs in multi-unit NCM sites. When comparisons were run separately for the two structures studied, NCM multi-unit sites showed neural recognition of one's own offspring but CMM sites did not. **(A&B)** In NCM, non-parametric Wilcoxon signed-rank tests were run on multi-unit ARMs and adaptation rates, revealing that subjects showed stronger responses to the calls of their own fledgling than novels ($Z = 1.98$, $p < 0.05$) and slower adaptation rates to their own fledgling's call than novels ($Z = 3.42$, $p < 0.001$). **(C&D)** In CMM, however, neither ARMs nor adaptation rates discriminated between own and novel fledgling calls (ARMs: $Z = 1.41$, ns; adaptation rates: $Z = 1.74$, $p = 0.08$).

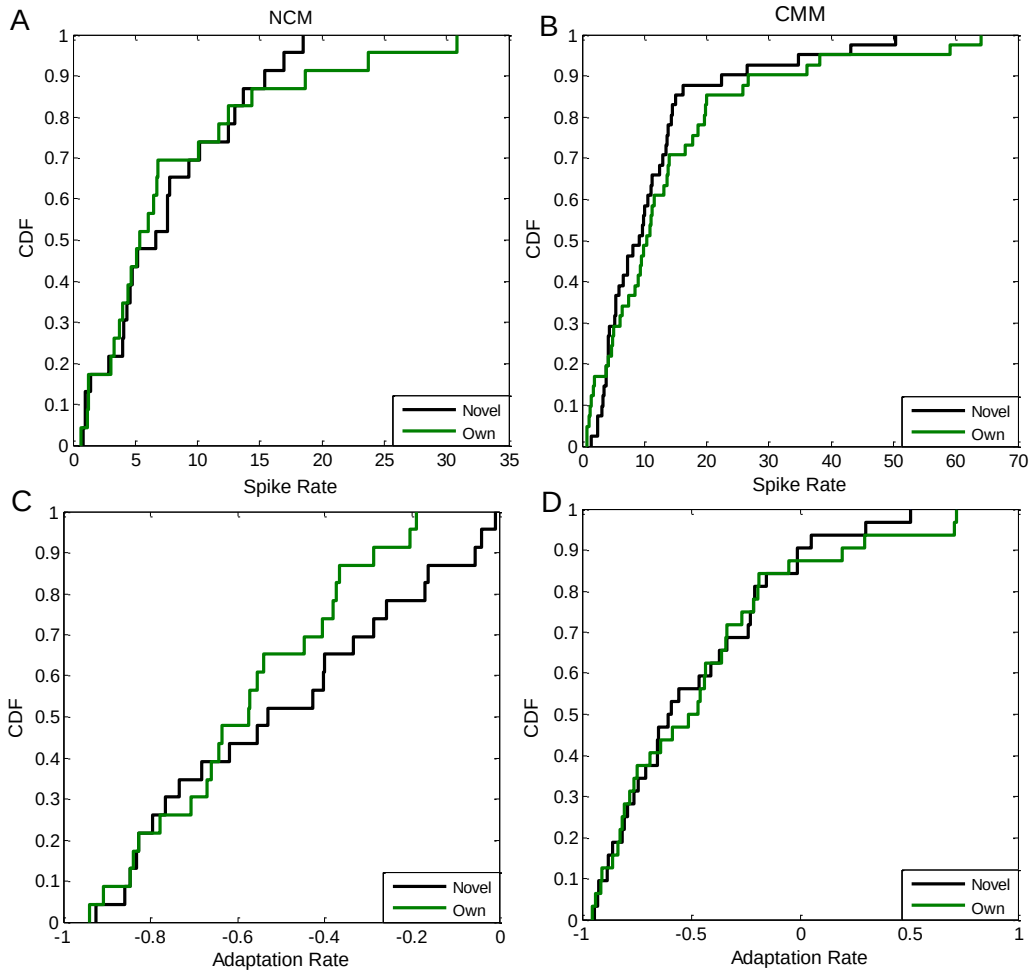


Figure 16. The neural recognition of one's OFC occurs in single-unit CMM sites. Single-unit spike-rate and adaptation responses to OFC and novel stimuli were analyzed separately for NCM and CMM, to determine where the spike-rate enhancement to OFC occurred. **(A)** In NCM, spike-rates to own and novel stimuli did not differ ($Z = 0.243$, $p = 0.81$). **(B)** In CMM, spike-rates to OFC were significantly faster than to the calls of novels ($Z = 2.64$, $p < 0.01$). **(C&D)** Single-unit adaptation rates did not discriminate between OFC and novel across the two structures, in NCM alone or in CMM alone ($F(1,60) = 0.608$, ns; NCM: $Z = 1.125$, $p = 0.26$; CMM: $Z = 0.888$, $p = 0.37$).

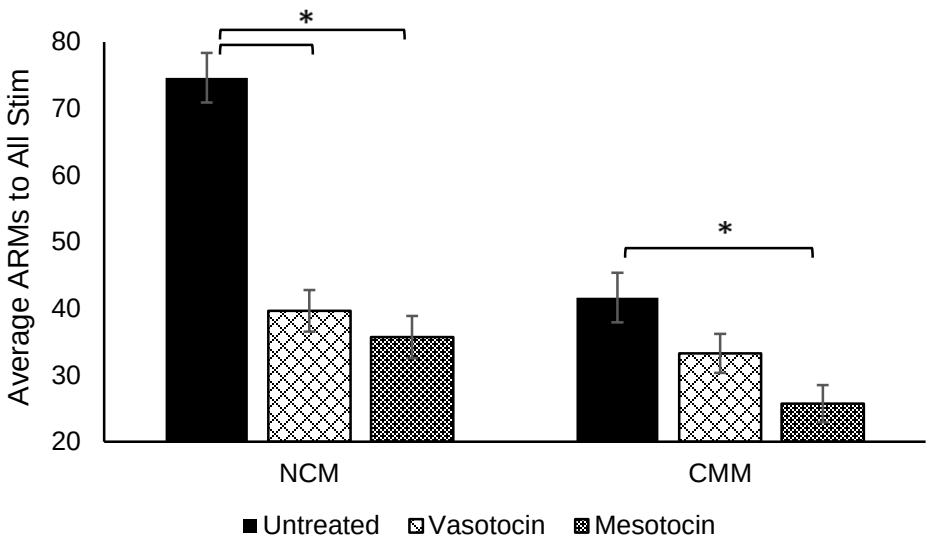


Figure 17. Multi-unit responses to all stimuli differ across naïve and hormone-treated groups. Across the naïve and (centrally) drug manipulated groups there were global group differences in neural responding. When responses to all stimuli were compared in the naïve, vasotocin and mesotocin injected groups, there was a significant interaction between group and area of multiunit recording on the average neural response ($F(2,685) = 8.43$, $p < 0.001$). Neural responses at the NCM and CMM multi-unit recording sites of centrally-manipulated subjects were lower than those of naives ($p < 0.05$). This was true across vasotocin, mesotocin and saline treated sites, possibly due to neural damage from either the saline injection, drug injection or micropipette placement. This suggests that any across group comparisons of fledgling call responding should be done using normalized FRS values.

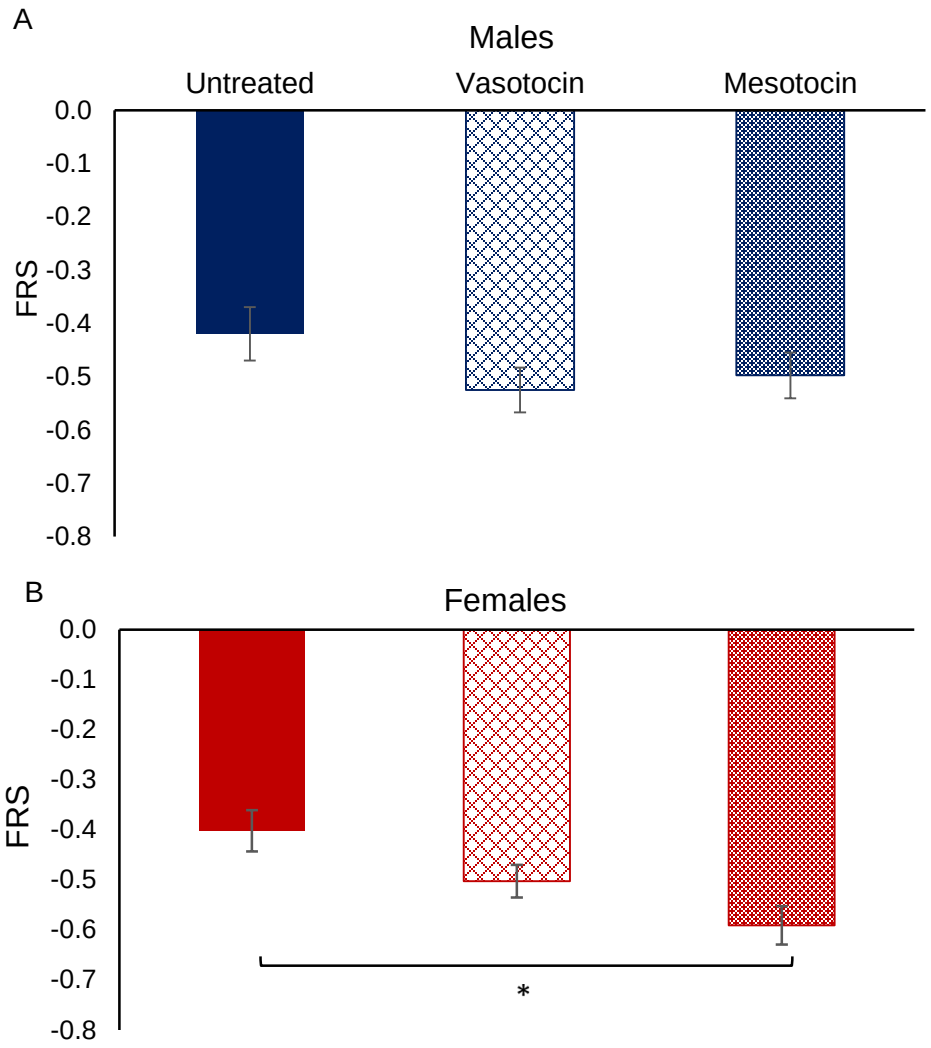


Figure 18. Central injections of mesotocin and vasotocin do not increase FRS values in naïve males and females. (A) Naïve male subjects centrally injected with social hormones show no difference in their FRS values, as compared to untreated naïve males (vasotocin: $F(1, 212)=1.9469$, ns; mesotocin: $F(1, 209)=1.1514$, ns). **(B)** In females, however, naïve untreated subjects unexpectedly showed higher FRS values than the mesotocin treated females ($F(1, 222)=8.78$, $p < 0.01$) and a trend for the same in vasotocin treated females ($F(1, 206)=3.70$, $p = 0.06$).

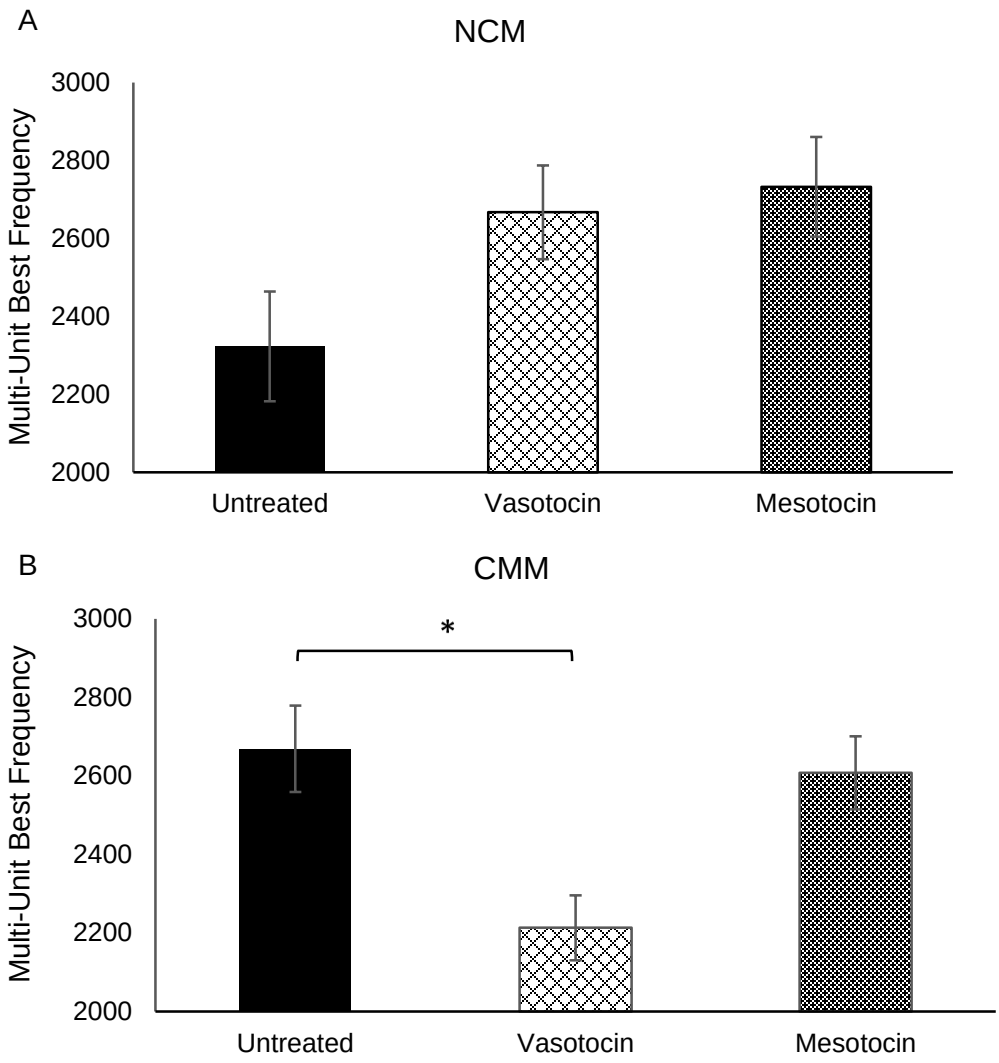


Figure 19. Vasotocin injections influence best frequency tuning in NCM and CMM. (A) In NCM, naïve subjects injected with both social hormones mesotocin and vasotocin showed higher best-frequency tuning than untreated controls. In vasotocin-treated subjects there was a trend toward significance in this effect ($F(1,193) = 3.011$, $p = 0.08$). In mesotocin-treated subjects the effect was not significant ($F(1,180) = 2.748$, ns). **(B)** Results were quite different in CMM. In this structure, mesotocin-treated subjects were tuned to similar frequencies as untreated naives ($F(1,197) = 0.0969$, ns). Contrary to hypotheses, vasotocin-treated subjects actually showed multi-unit tuning toward significantly lower best-frequencies than untreated naïves in this area ($F(1,208) = 11.50$, $p < 0.001$).

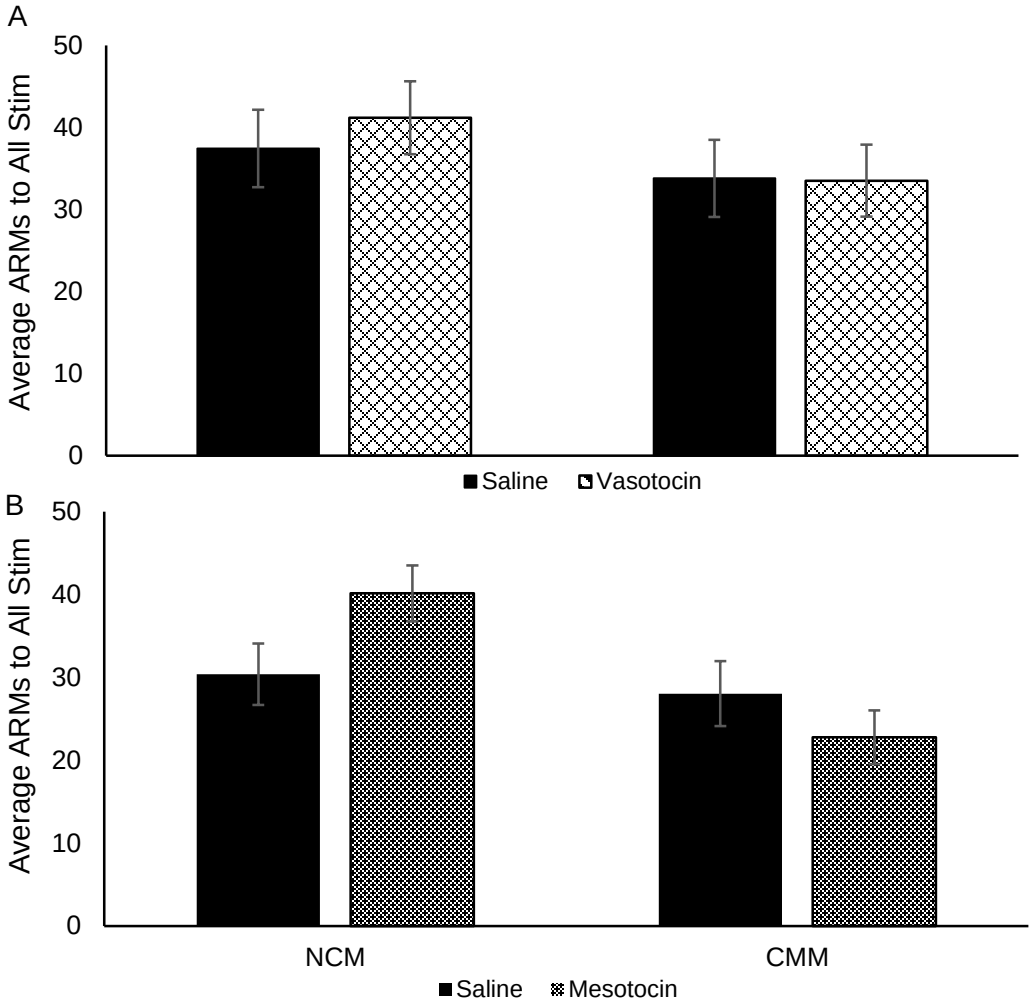


Figure 20. Central injections of mesotocin and vasotocin do not affect multi-unit responses to all auditory stimuli in NCM and CMM. When neural data was analyzed within a subject group, whether it be mesotocin or vasotocin microinjected subjects, there was not a significant effect of mesotocin or vasotocin on overall responding. **(A)** Responses to "All Stim" did not differ between saline and vasotocin treated sites ($F(1,245) = 0.143$, p = n.s.) in either structure (drug/area interaction: $F(1,245) = 0.195$, ns). **(B)** In mesotocin treated subjects, there was also no significant effect of drug treatment on ARMs to 'All Stim' ($F(1,258) = 0.398$, ns). There was an interaction between the direction of effects in NCM and CMM ($F(1,258) = 4.41$, $p < 0.05$) but neither structure showed a significant effect in post-hoc Tukey tests (NCM: $p = 0.06$, CMM: ns). Results suggest that comparions made within a group of drug treated subjects need not be normalized to assess the effect of mesotocin or vasotocin on responses to fledgling calls.

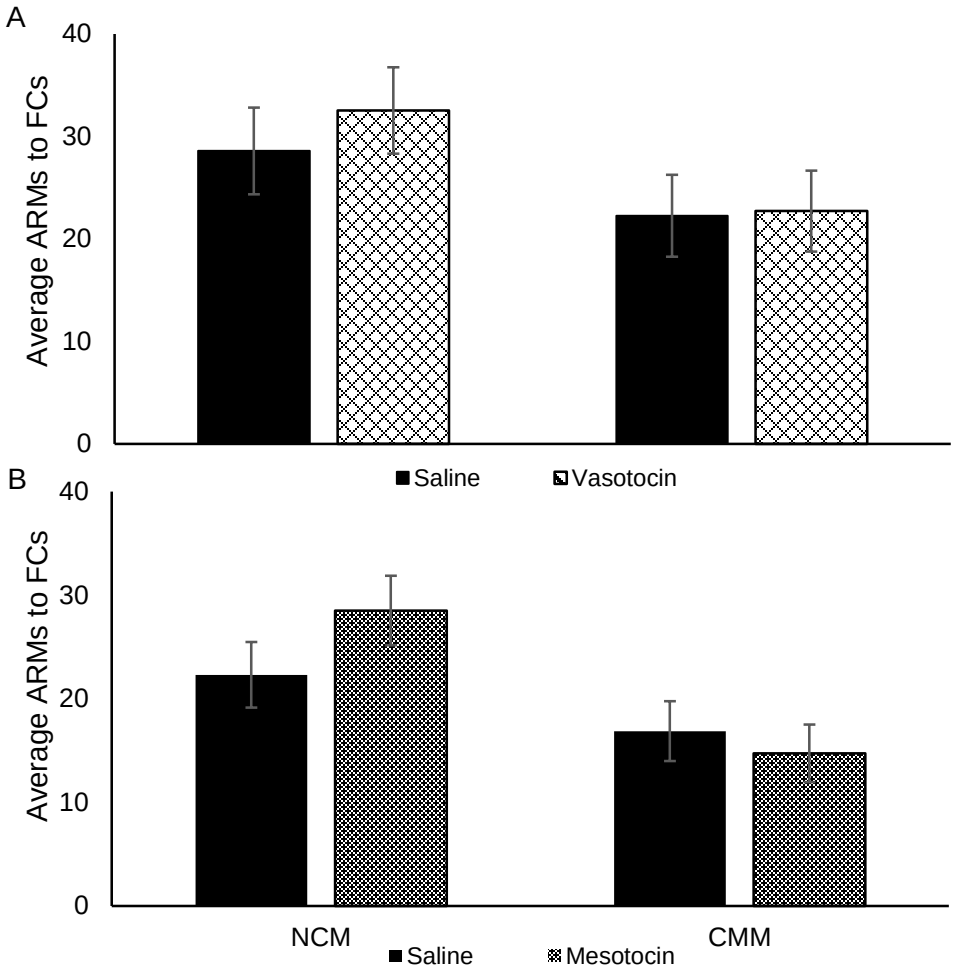


Figure 21. Central injections of mesotocin and vasotocin do not affect ARMs to FCs. When neural data was analyzed, within a drug-treated subject group, to test whether microinjections of social hormones had an effect on responses to fledgling calls, there were no differences between saline and drug-treated sites. **(A)** Vasotocin injections did not significantly affect the ARM response to FC stimuli in the avian auditory forebrain (drug v. saline main effect: $F(1,246) = 0.268$, ns) for either structure (drug/region interaction: $F(1,246) = 0.182$, ns). **(B)** Similarly, there was no effect of mesotocin treatment on ARMs to FCs (main effect: $F(1,259) = 0.445$, ns) in either NCM or CMM (drug/region interaction: $F(1,259) = 1.860$, ns).

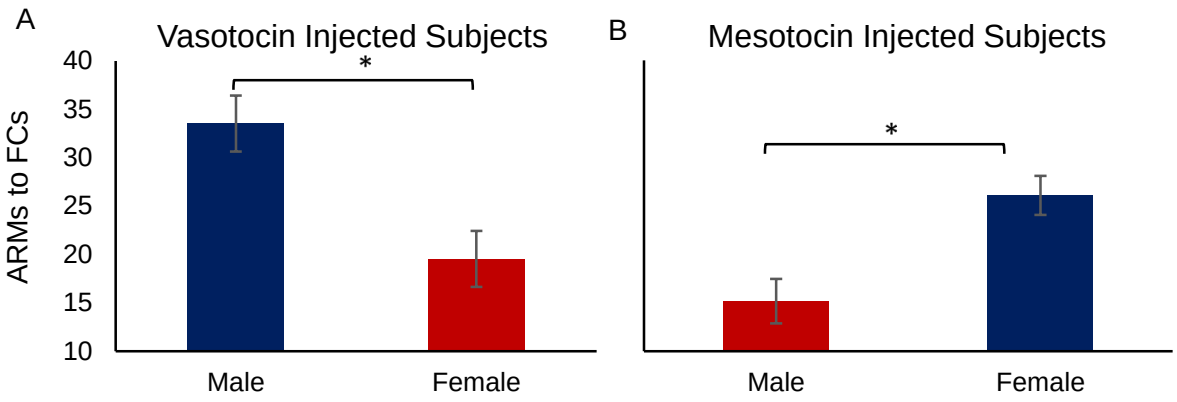


Figure 22. The effect of hormonal injections on FC responses differed in male and female subjects. Although there was not a main effect of hormone-treatment on ARMs to FCs in either hormonally-treated group, both groups showed main effects of sex on FC responses. This effect was unexpected as male and female naïve subjects did not differ in their strength of response to fledgling call stimuli in experiment 1 **(Figure 9A)**. **(A)** Vasotocin treated males showed significantly higher neural responses to fledgling begging calls than vasotocin treated females ($F(1, 246) = 11.65$, $p < 0.001$) while **(B)** mesotocin treated females showed significantly stronger neural responses than vasotocin treated males ($F(1, 259) = 12.76$, $p < 0.001$). Therefore, the social hormones mesotocin and vasotocin do affect males and females differently.

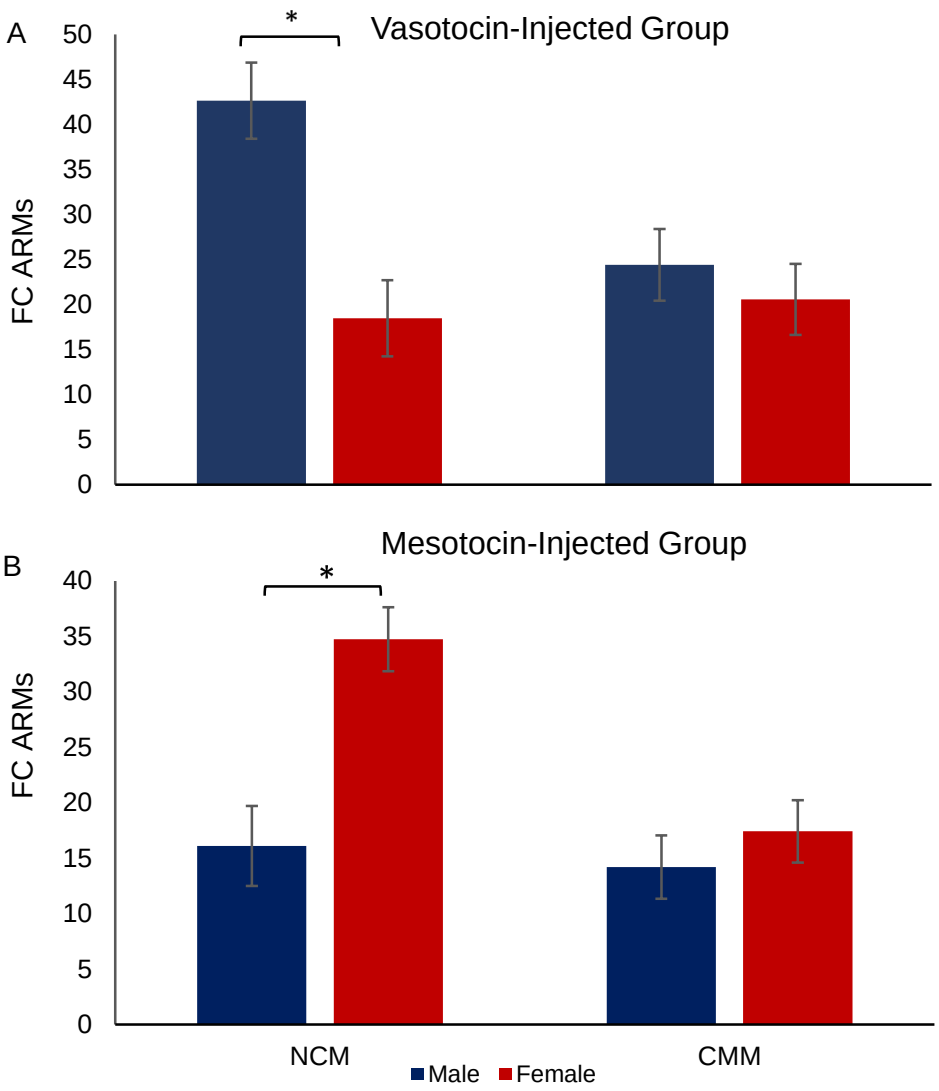


Figure 23. The sex-specific effects of mesotocin and vasotocin injections on ARMs to FCs occur in NCM. (A) In the vasotocin injected group, NCM responses to fledgling calls are stronger in males than females. There was a significant interaction between sex and structure of recording on multi-unit ARMs to fledgling calls in vasotocin treated subjects ($F(1,245) = 5.89$, $p < 0.05$). Although male and female responses were similar to FCs in the CMM of vasotocin treated subjects, males showed stronger responses in NCM than females (Tukey HSD, $p < 0.05$). **(B)** In the mesotocin injected group, NCM responses to fledgling calls are stronger in females than males. There was a significant interaction between sex and structure of recording on multi-unit ARMs to fledgling calls in vasotocin treated subjects ($F(1,258) = 6.29$, $p < 0.05$). Although male and female responses were similar to FCs in the CMM of vasotocin treated subjects, females showed stronger responses in NCM than males (Tukey HSD, $p < 0.05$).

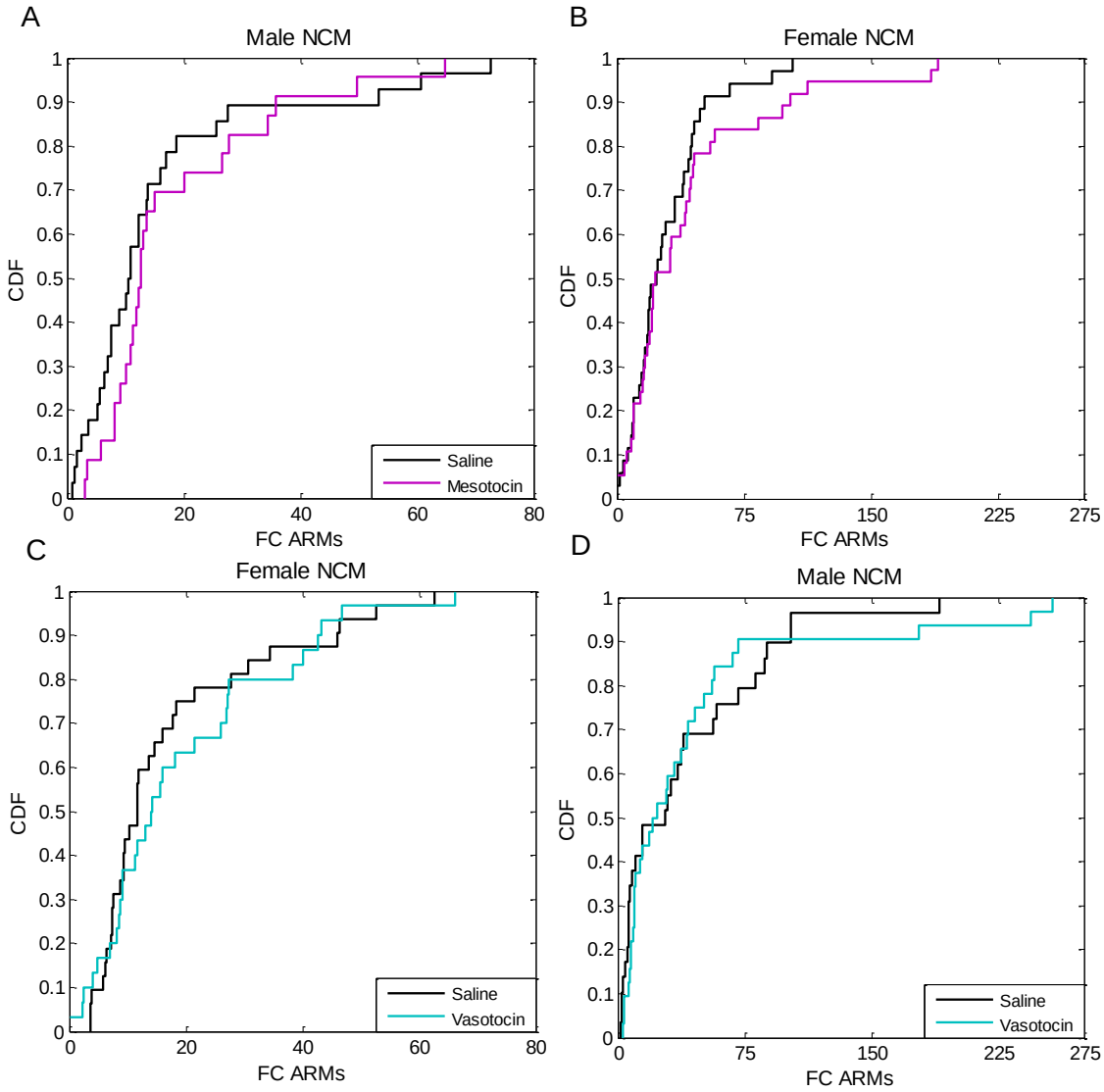


Figure 24. Saline and hormone-treated sites showed similar responses to FCs in the NCM of male and female ZFs. Previous results showed hormone-treatment increased ARMs to FCs in NCM, in males specifically under vasotocin-treatment and in females specifically under mesotocin-treatment. However, within NCM, **(D)** males showed no stronger responses to FCs at vasotocin treated sites as compared to saline-treated sites (Z = -0.325, ns), **(C)** similar to females (Z = -0.556, ns). **(B)** Females also did not show any difference in ARMs to FCs at saline and mesotocin treated sites (Z = -0.676, ns) in NCM. **(A)** Results were similar for mesotocin treated males (Z = -1.316, ns).

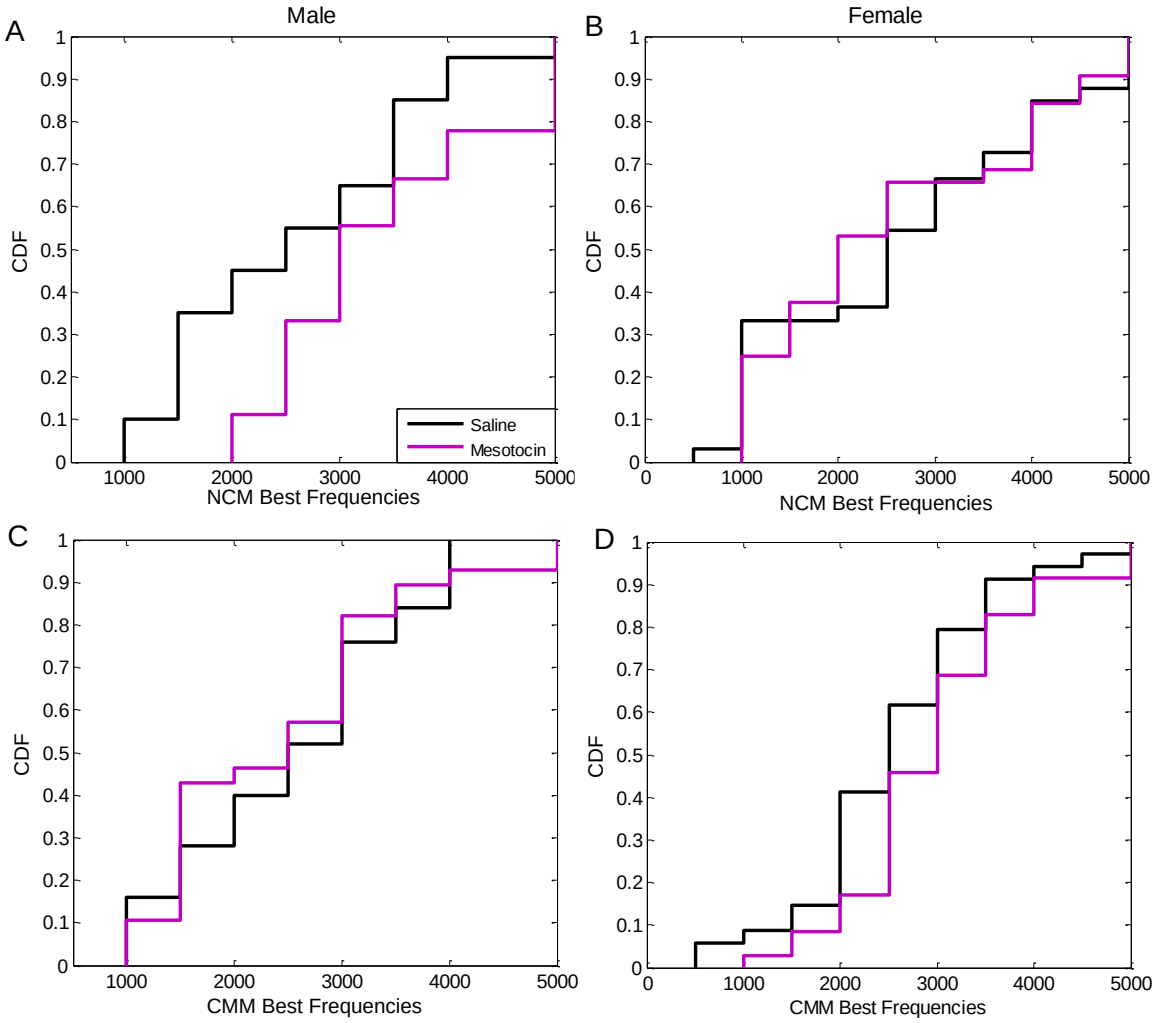


Figure 25. Central injections of mesotocin increase BF tuning in male NCM and female CMM. There was a strong trend for an interaction between the sex of subject, area of recording and hormonal-treatment of a site, on best-frequency tuning of multi-unit sites in the mesotocin-treated group ($F(1,208) = 3.82$, $p = 0.05$). Due to the complexity of the interaction, experimenters used non-parametric tests to assess tuning at saline and mesotocin-treated sites in each structure, separately for males and females. **(A)** Mesotocin-treated sites showed significantly higher best-frequency tuning than saline treated sites, in the NCM of male subjects ($Z = -1.99$, $p < 0.05$). **(B)** There was no effect of hormone-treatment on NCM tuning in females ($Z = 0.200$, ns). **(D)** In CMM, however, there was a trend toward a significant effect of hormone-treatment on best-frequency tuning in females ($Z = -1.84$, $p = 0.07$). **(C)** The CMM of male subjects did not show an effect of mesotocin-treatment on multi-unit BF tuning ($Z = 0.471$, ns).

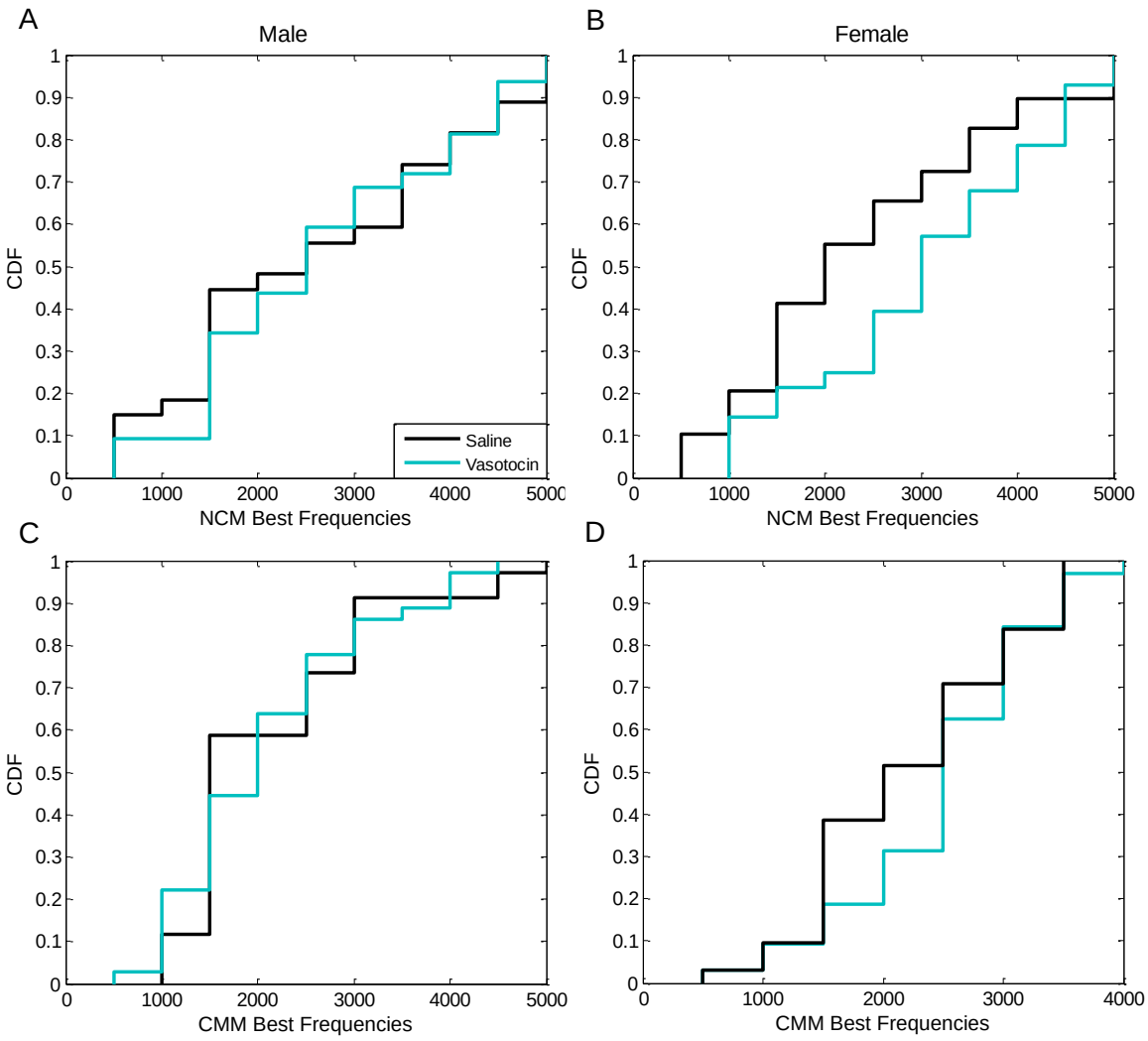


Figure 26. There is no interaction between sex of subject, area of recording and vasotocin-treatment on multi-unit BF tuning. There was no interaction between hormone-treatment, sex of subject and the region of recording on BF tuning in the vasotocin-treated group ($F(1,232) = 1.45$, ns) **(C&D)** The best-frequency tuning of multi-unit sites in CMM were unaffected by vasotocin-injections, in both male ($Z = 0.109$, ns) and female ($Z = 1.25$, ns) subjects. **(A)** In NCM as well, male subjects did not show an effect of hormonal-treatment on BF tuning ($Z = -0.300$, ns). **(B)** In the NCM of female subjects, however, there was a trend for vasotocin-treatment to increase BF tuning, as vasotocin had previously ($Z = -1.85$, $p = 0.07$).

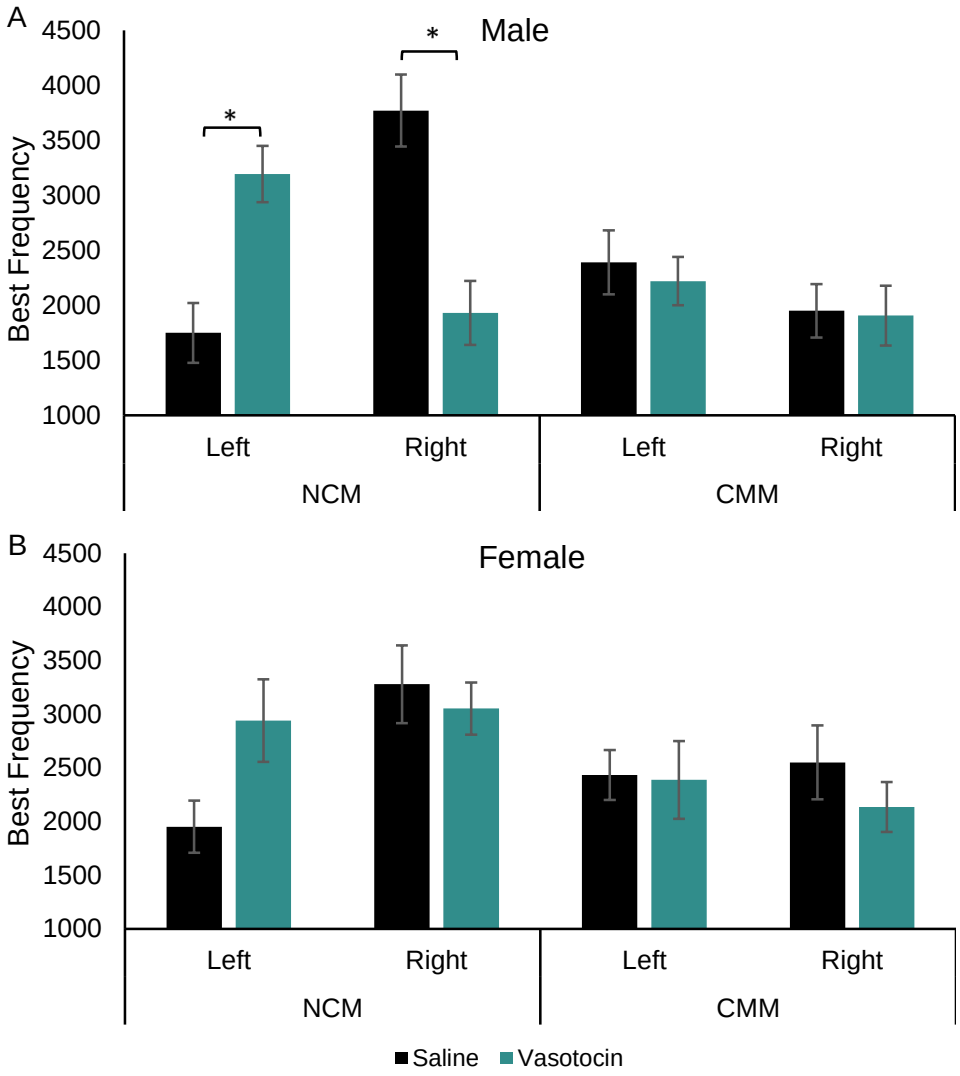


Figure 27. Vasotocin has lateralized effects on multi-unit BF tuning in male NCM. There was a significant interaction between sex of subject, area of recording, vasotocin treatment and the side of recording on BF tuning in the vasotocin-treated group ($F(1,232) = 4.84$, $p < 0.05$). Post-hoc Tukey HSD tests showed that **(A)** in male subjects, there was an effect of vasotocin-treatment on tuning in NCM, in left NCM BFs were higher in hormone-treated than saline sites, and in the right hemisphere of NCM BFs were lower at vasotocin-treated sites, as compared to saline ($p < 0.05$ for both comparisons). **(B)** In females, however, the effect of vasotocin-treatment on BF tuning was weaker, with neither hemisphere of NCM showing a significant effect of hormonal-treatment (Tukey HSD, ns). Tuning in the left and right hemispheres of CMM was unaffected by vasotocin-treatment, in both sexes (Tukey HSD, ns).

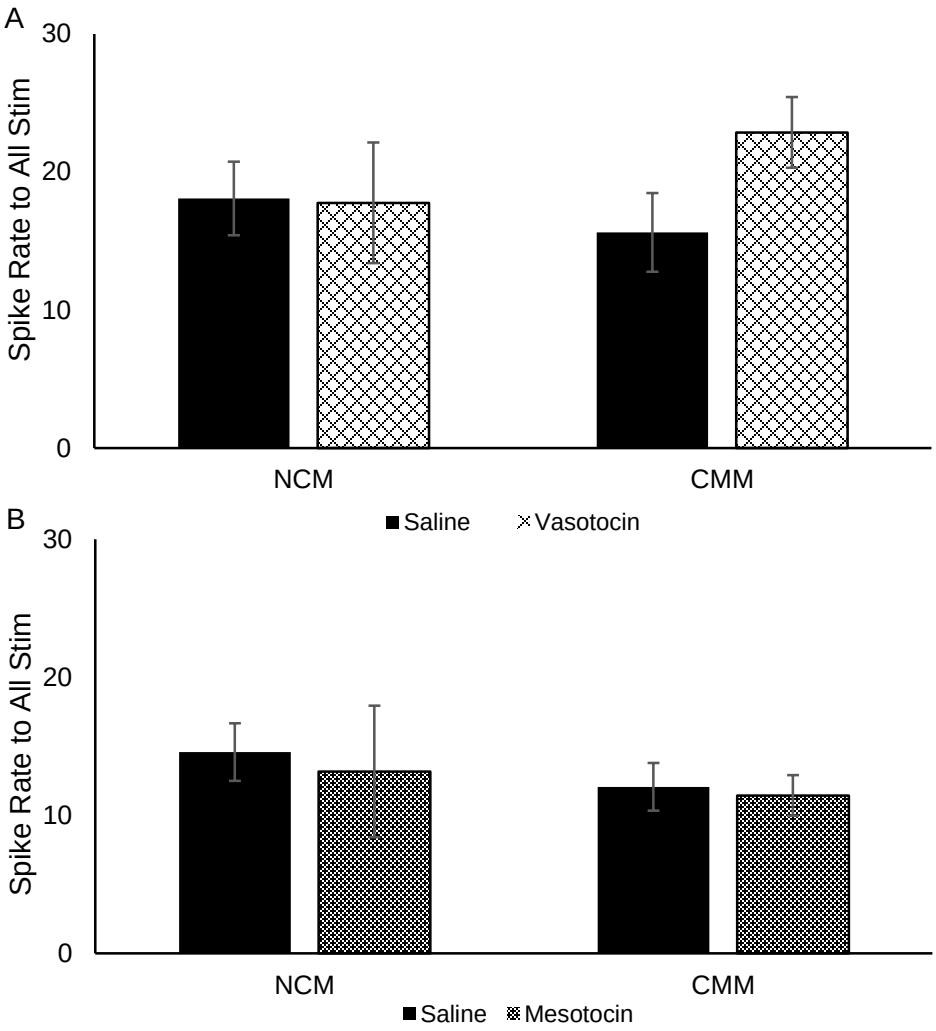


Figure 28. Single-unit spike-rates to all auditory stimuli are unaffected by hormone treatment. (A) There was no effect of vasotocin injection on spike-rates to all stim in single units of NCM of CMM (main effect of drug: $F(1,76) = 0.283$, ns, drug/region interaction: $F(1,76) = 1.40$, ns). **(B)** Single-unit spike-rates were also unaffected by mesotocin drug treatment in NCM and CMM (main effect of drug: $F(1,102) = 0.129$, ns, drug/region interaction: $F(1,102) = 0.0177$, ns). Results suggest that comparisons made within a group of drug treated subjects need not be normalized to assess the effect of mesotocin or vasotocin on responses to fledgling calls.

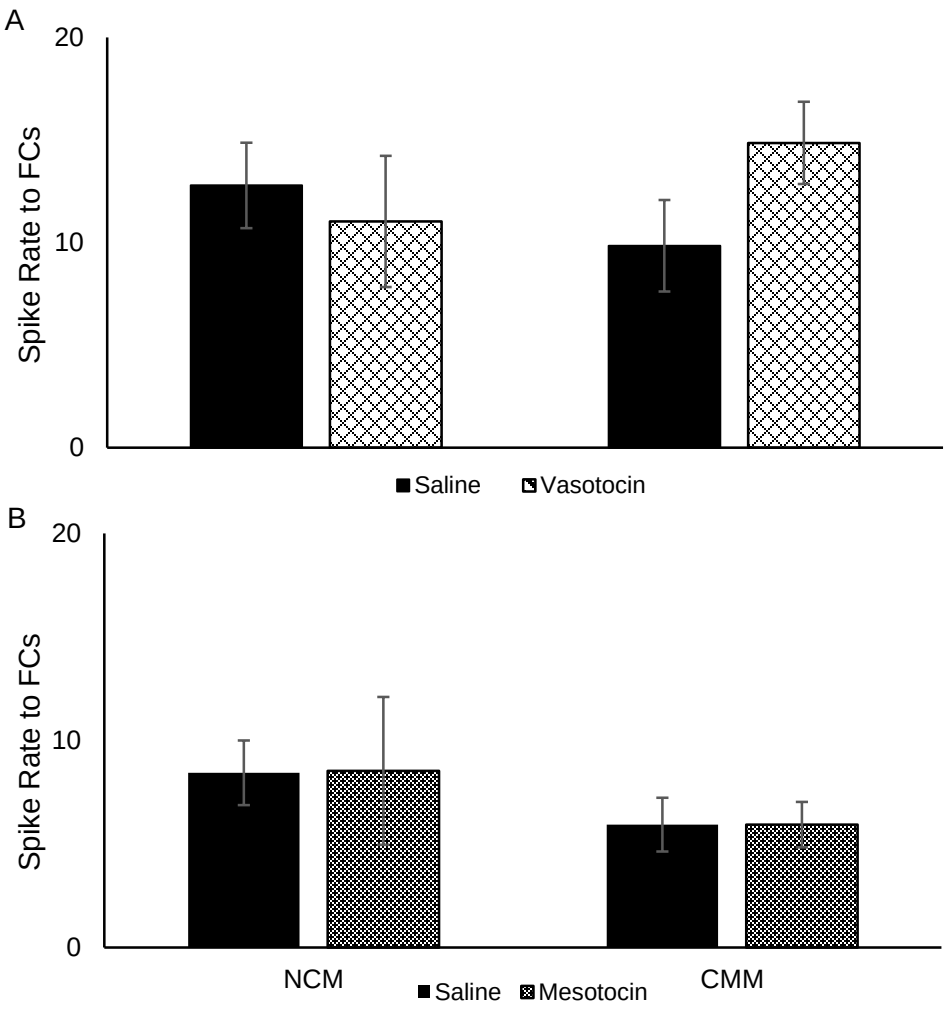


Figure 29. Single-unit responses to FCs are unaffected by hormone treatment. (A) There was no effect of vasotocin injection on best frequency tuning in single units of NCM of CMM (main effect of drug: $F(1,77) = 0.438$, ns, drug/region interaction: $F(1,77) = 1.92$, ns). **(B)** Single-unit tuning was also unaffected by mesotocin drug treatment in NCM and CMM (main effect of drug: $F(1,102) = 0.00038$, ns, drug/region interaction: $F(1,102) = 0.00045$, ns).

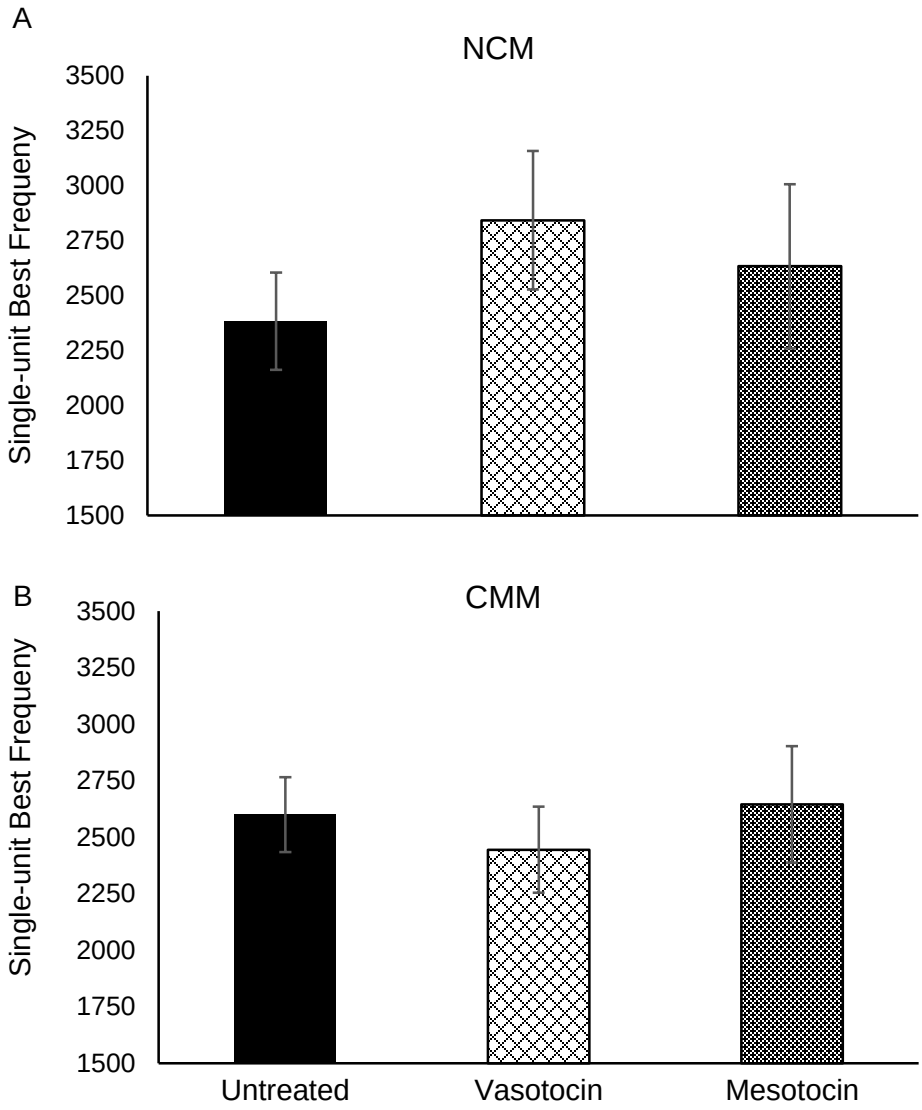


Figure 30. Single-unit tuning best frequencies did not differ across untreated and hormone-treated groups. (A) In NCM, untreated naïve subjects did not significantly differ from vasotocin ($F(1,51) = 1.022$, ns) or mesotocin treated ($F(1,40) = 0.356$, ns) groups in their single-unit best tuning frequencies. **(B)** In CMM, results also showed no significant difference in single-unit tuning between untreated naives and vasotocin ($F(1,96) = 0.447$, ns) or mesotocin-treated ($F(1,95) = 0.0215$, ns) groups.

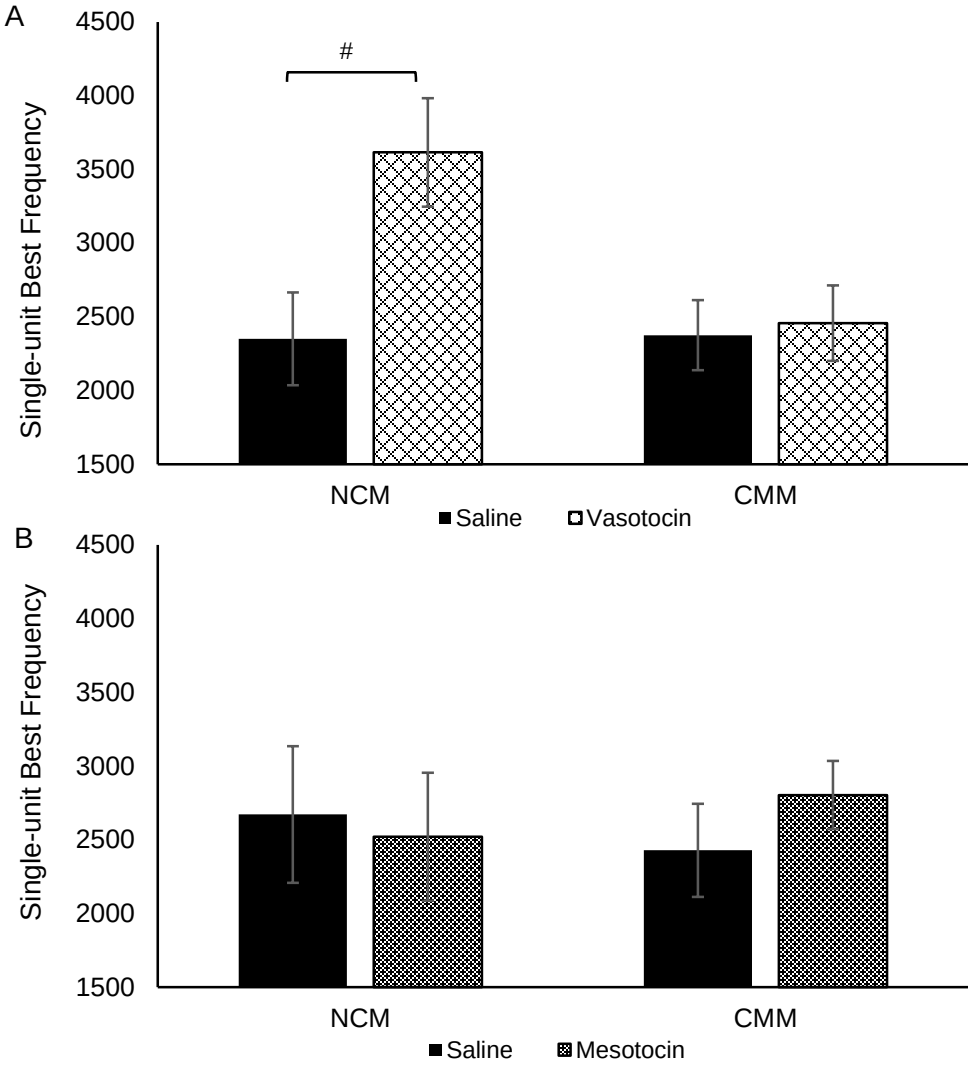


Figure 31. Hormone-treated single-units show higher tuning than saline-treated units in the NCM of vasotocin-treated animals. (A) In vasotocin treated subjects, single-unit's treated with vasotocin showed significantly higher best frequencies than saline treated sites ($F(1,64) = 4.88$, $p < 0.05$). There was a strong trend for the drug treatment to interact with the structure in which neurons were recorded ($F(1,64) = 3.63$, $p = 0.06$), and for the difference between vasotocin and saline tuning to lie in NCM (Tukey HSD $p = 0.06$, indicated with a #). **(B)** There was no difference between saline and drug treated single-unit tuning in mesotocin treated subjects ($F(1,52) = 0.0927$, ns) in either structure recorded (drug/area interaction: $F(1,52) = 0.515$, ns).

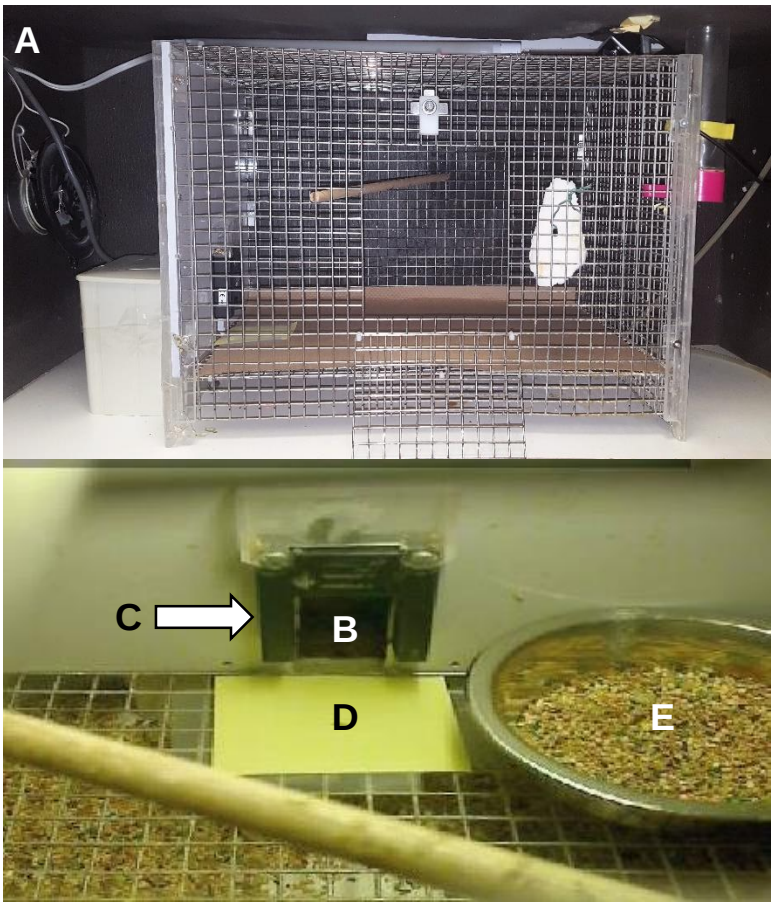
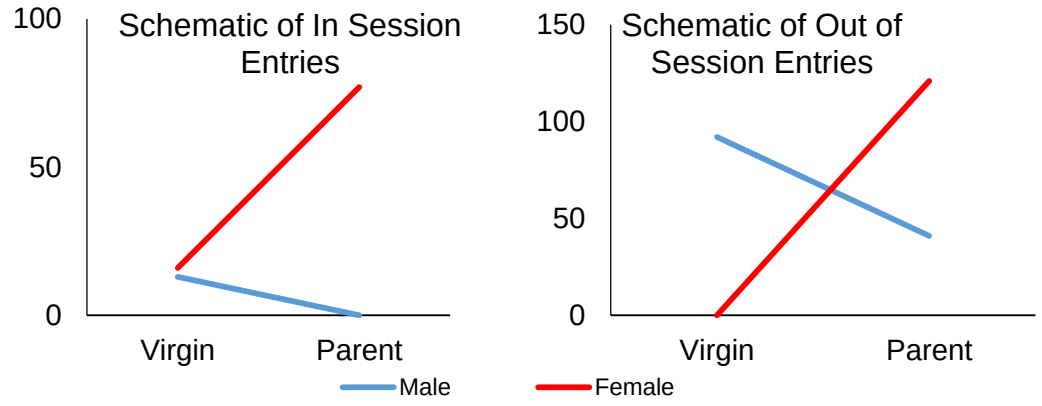


Figure 32. Nest-entry paradigm for assessing parental behaviors in ZFs. Subjects acclimated to **(A)** this box for a day before testing. During testing, fledgling calls came from a speaker placed outside the box, from the direction of the nestbox **(B)**. When subjects entered the nestbox an infrared beam **(C)** was broken, and ARTSy recorded the entry, as well as the exit (used in conjunction with stop-watched video monitoring) to calculate box spent in nest. The amount of food taken from the food bowl **(E)** was also recorded for each session. Blind video-scoring was used to assess the amount of time subjects spent in the nest area **(D)** as well as call responses to fledgling stimuli (within 30 seconds of stimulus).



In Session	Parent	Naïve		Out of Session	Parent	Naïve
Male	0	13		**Male**	41	92
Female	77	16		**Female**	121	0

Figure 33 & Table 2. Frequency of nest entries influenced by sex and parental experience. During behavioral sessions there was a significant effect of subject group on the number of entries into the box that subjects made ($\chi^2 = 39.34$, $p < 0.0001$). Females show significantly more box entries than males, and parents show more than virgins (sex: $\chi^2 = 58.88$, $p < 0.0001$, parental experience: $\chi^2 = 20.84$, $p < 0.0001$). These behavioral data match previous results which show an interaction between sex and parental experience, males showed more entries when they were virgins than when they were parents ($\chi^2 = 11.08$, $p < 0.001$) while females showed more entries after parental experience ($\chi^2 = 38.7$, $p < 0.0001$). Out of session box entries show a similar effect. There was a significant effect of subject group on the number of out of session box entries ($\chi^2 = 131.23$, $p < 0.0001$). Parents showed more box entries than virgins ($\chi^2 = 18.74$, $p < 0.0001$). However this effect was sex specific, virgin males enter the nest boxes significantly more than parental males ($\chi^2 = 18.8$, $p < 0.0001$) while parental experience stimulates box entries in females ($\chi^2 = 119$, $p < 0.0001$). Males and females did not enter at different frequencies across parental experience groups ($\chi^2 = 0.48$, $p = 0.49$). However, within the parental subjects, females did enter significantly more than males ($\chi^2 = 38.52$, $p < 0.0001$) and within the virgin group, males entered more than females ($\chi^2 = 90.02$, $p < 0.001$).

A Expected Nest Entries, Equally Across Time

	Parents tested for 17 hours	Naïve tested for 17 hours	Total hours: 34
In session: 4 hours	4/17 of parental entries	4/17 of naïve entries	4/17 of total entries
Out of session: 13 hours	13/17 of parental entries	13/17 of naïve entries	13/17 of total entries

B Female Nest Entries, Expected and Observed

	Parents: 198	Naïve: 16	Total: 214
In Session	Expected: 46.59 Observed: 77	Expected: 3.76 Observed: 16	Expected: 50.36 Observed: 93
Out of Session	Expected: 151.41 Observed: 121	Expected: 12.24 Observed: 0	Expected: 163.64 Observed: 121

C Male Nest Entries, Expected and Observed

Total entries	Parents: 41	Naïve: 105	Total: 146
In Session	Expected: 9.65 Observed: 0	Expected: 24.71 Observed: 13	Expected: 34.36 Observed: 13
Out of Session	Expected: 31.55 Observed: 41	Expected: 80.29 Observed: 92	Expected: 111.64 Observed: 133

Table 3. Entries into the nest are distributed across in-session and out-of-session time periods differently in the two sexes. **(A)** If subjects entered the nest without regard to the playback of fledgling begging calls, they would do so equally across the two time periods (in-session and out-of-session). **(B)** However, data show that females enter the nest more during stimulus playback trials than during periods of no-playback ($\chi^2 = 46.11$, $p < 0.001$). This was true in both the naïve and parental subjects (naïve: $\chi^2 = 47.84$, $p < 0.0001$; parent: 25.11, $p < 0.0001$). **(C)** Furthermore, males unexpectedly show an inhibition from responding (by entering the nest), entering significantly more during the out-of-session period than the in-session period ($\chi^2 = 16.55$, $p < 0.001$). This effect was also seen in both naïve and parental subjects when chi-squares were performed for the two groups in isolation (naïve: $\chi^2 = 6.64$, $p = 0.01$; parent: 11.34, $p < 0.001$).

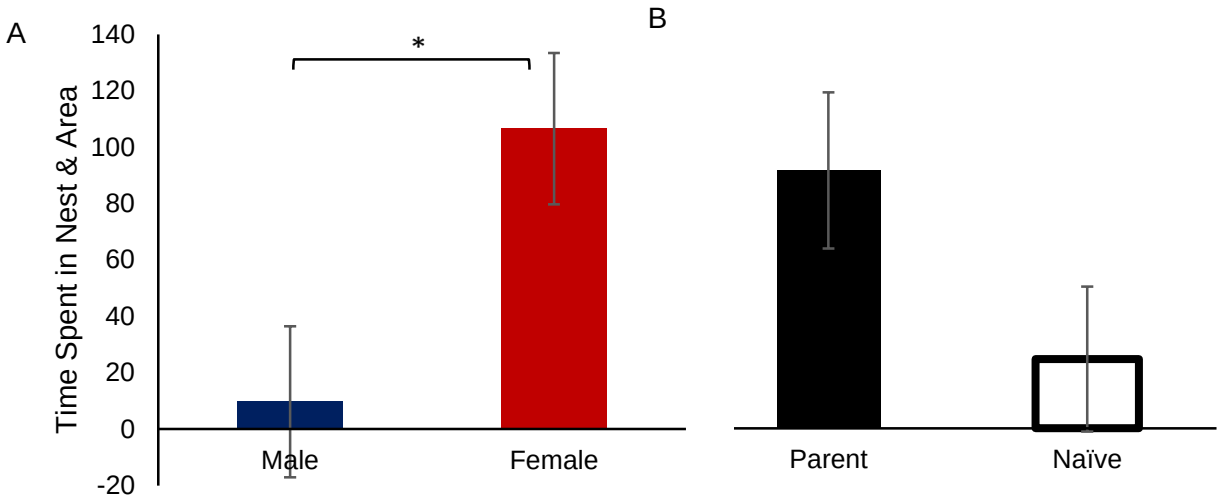


Figure 34. Time spent in nest/area showed greater parental motivation in females and parents. Similar to the results found with the frequency of nest entry data, ANOVA's analyzing average time spent in and around the nest, on a trial, showed effects of sex and parental experience. **(A)** Females spent significantly more time in the nest and area adjacent to the nest than did male subjects ($F(1, 78) = 6.53, p = 0.0125$). **(B)** In addition, parents showed a trend to spend more time in and around the nest than naïve subjects ($F(1, 78) = 3.13, p = 0.08$).

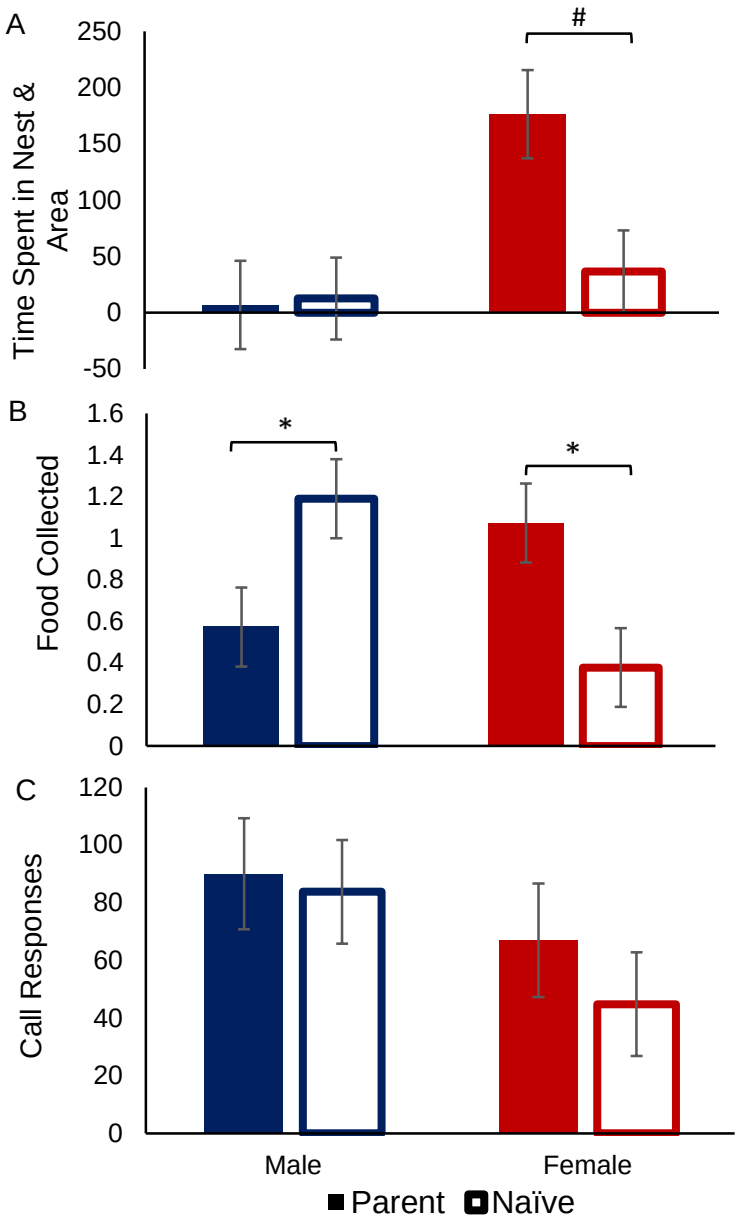


Figure 35. Behavioral responses to FCs show an interaction between parental experience and sex of subject. (A) There is a strong trend for an interaction between sex of subject and parental experience on the amount of time subjects spent in or near the nest during behavioral testing ($F(1, 78)=3.71$, $p= 0.057$). This result was pulled by a strong trend for female subjects to spend more time adjacent to the nest when they are parents than when they are virgins (Tukey HSD post hoc: $p = 0.051$, indicated with #). **(B)** There is also a significant interaction between sex of subject and parental experience on the behavioral measure of food collected during the trail (to eat, put in the nest, or near the nest) ($F(1, 88) = 11.90$, $p< 0.001$). Female parents collect significantly more food during sessions than virgins, while male parents collect significantly less than virgins (post hocs: $p < 0.05$). **(C)** Call back behavior, however, did not show an interaction between sex and parental experience ($F(1,81) = 0.179$, ns)

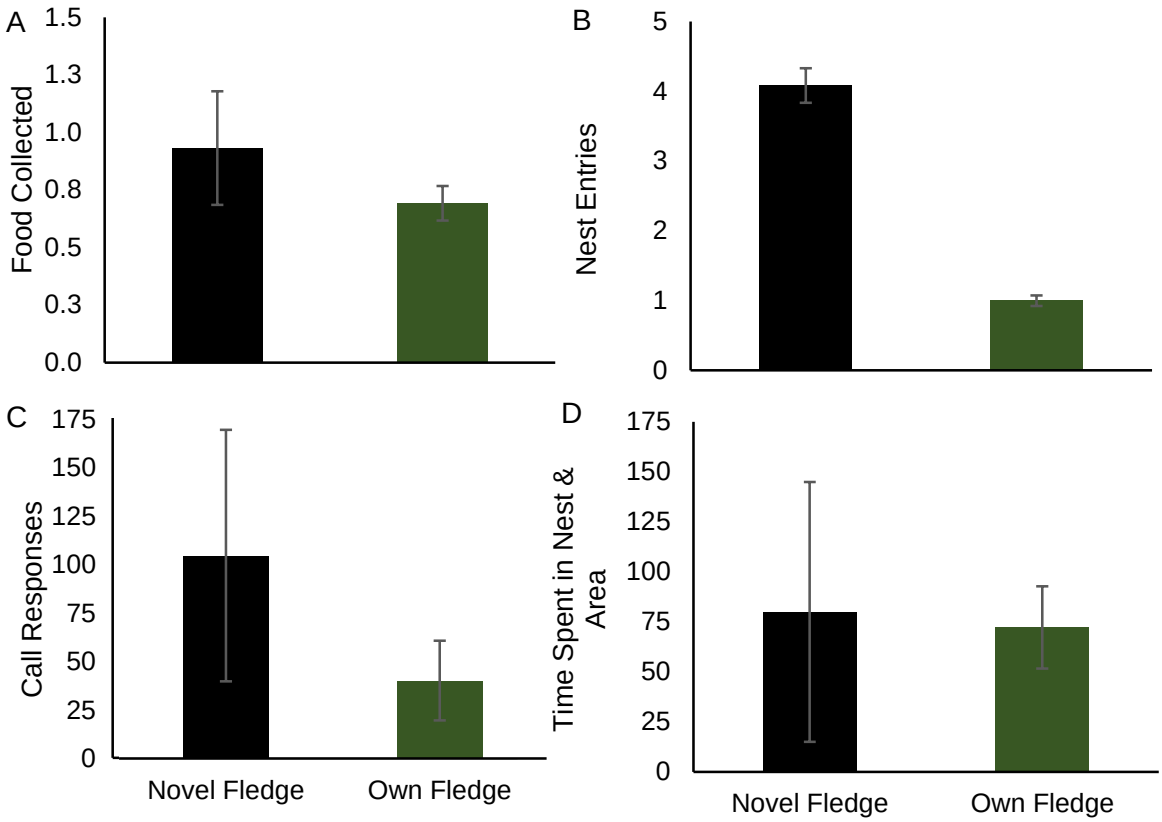


Figure 36. Parents do not behaviorally discriminate between OFC and novel FCs in the nest-entry behavioral experiment. When behavioral measures were analyzed using repeated measures ANOVAs to test whether stimulus type had an effect on behavioral responses to fledgling calls, all measures showed non-significant results (stimulus type main effect: **A.** food collected: $F(1,10) = .999$, $p = 0.341$ **B**. nest entries: $F(1, 10)=.938$, $p=0.356$; **C**. time spent in nest & area: $F(1, 9)= 1.998$, $p= 0.191$; **D**. call responses: $F(1, 10)= .0833=4$, $p= 0.779$).

In Session Entries: Drug v. Naïve

A

	Mesotocin	Naïve
Male	0	13
Female	6	16

C

	Vasotocin	Naïve
Male	2	13
Female	10	16

In Session Entries: Drug v. Parent

B

	Mesotocin	Parent
Male	0	0
Female	6	77

D

	Vasotocin	Parent
Male	2	0
Female	10	77

Table 4. Peripheral injections of mesotocin cause decreased nest entries in-session. (A) During the in-session period, naïve subjects injected with mesotocin performed significantly fewer nest entry behaviors than untreated naives ($\chi^2 = 13.82$, $p = 0.002$). This effect was due to a decrease in nest entries in mesotocin treated males (males: $\chi^2 = 11.08$, $p = 0.009$); females showed no difference in their frequency of nest entries (females: $\chi^2 = 3.68$, ns). **(C)** Naïve subjects treated with vasotocin, however, did not differ in nest entry behavior, as compared to untreated naives (overall vasotocin v. virgin: $\chi^2 = 6.24$, ns; male: $\chi^2 = 6.66$, ns; female: $\chi^2 = 0.96$, ns). **(B&D)** In addition, female subjects injected with both mesotocin and vasotocin showed significantly fewer nest entries than parental females (mesotocin: $\chi^2 = 59.04$, $p < .0001$; vasotocin: $\chi^2 = 50.06$, $p < 0.001$). Alpha Bonferroni-corrected for multiple comparisons.

Out-of-Session Entries: Drug v. Naïve

A

	Mesotocin	Naïve
Male	7	92
Female	30	0

C

	Vasotocin	Naïve
Male	87	92
Female	123	0

Out-of-Session Entries: Drug v. Parent

B

	Mesotocin	Parent
Male	7	41
Female	30	121

D

	Vasotocin	Parent
Male	87	41
Female	123	121

Table 5. Out-of-session peripheral injections of mesotocin and vasotocin cause increased nest entries in naïve females while mesotocin causes decreased entries in males. When the number of out-of-session nest entries of mesotocin and vasotocin treated males and females were compared to behaviors exhibited by naïve subjects, we found an increase in parental behaviors exhibited by naïve females when treated with social hormones. **(C)** Vasotocin treated naïve subjects showed a greater number of nest entries than untreated naives ($\chi^2 = 45.32$, $p < 0.0001$). However, when post hoc chi squares were calculated to determine whether this increase in nest entries occurred for both sexes, when treated with vasotocin, we found this was only true in females (female $\chi^2 = 121$, $p < 0.0001$; male $\chi^2 = 0.08$, ns). **(A)** Similarly, mesotocin treated females showed more nest entries than naïve untreated females ($\chi^2 = 28.04$, $p < 0.0001$) while mesotocin treated males showed fewer nest entries than naïve untreated females ($\chi^2 = 71.28$, $p < 0.0001$). When compared to the behavior of parents, **(B)** mesotocin treated males and females showed fewer responses than their same sex controls (male: $\chi^2 = 22.68$, $p < 0.0001$; female: $\chi^2 = 53.64$, $p < 0.0001$). **(D)** Vasotocin treated subjects, however, showed more frequent responding in treated males than male parents ($\chi^2 = 15.82$, $p < 0.0001$) and equal responding to parents in females treated with vasotocin ($\chi^2 = 0$, ns). Alpha Bonferroni-corrected for multiple comparisons.

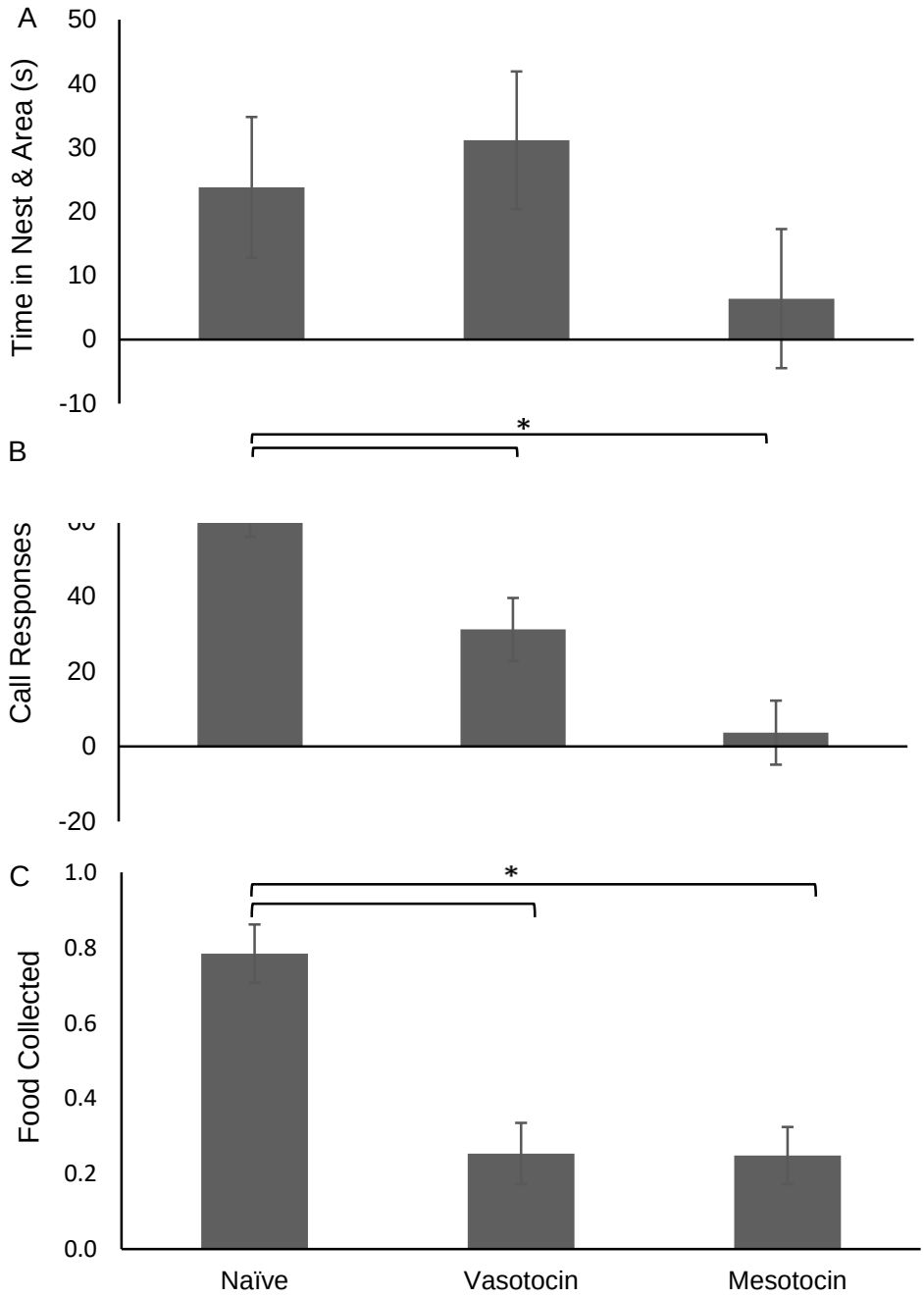


Figure 37. Peripheral injections of social hormones exerted an inhibitory effect on behavioral responses to FCs in the nest-entry paradigm. (A) When the average amount of time (s) spent in and near the nest during a behavioral session was analyzed to test whether untreated and hormone-treated subjects differed in their behavioral responses to FCs, there was no difference between untreated and vasotocin treated subjects ($F(1,86) = 0.162$, ns) and a trend for mesotocin treated subjects to spend less time in the nest/area than untreated naives ($F(1, 85) = 3.05$, $p = 0.08$). **(B)** Call responses to FCs were affected by peripheral injections of social hormones; untreated naïve subjects showed significantly more call back behaviors to FCs than both vasotocin treated ($F(1,88) = 5.25$, $p < 0.05$) and mesotocin treated naives ($F(1,87) = 28.03$, $p < 0.00001$). **(C)** The amount food that subjects collected throughout a session of trials also decreased in naïve subjects injected with social hormones; untreated naives gathered significantly more food than vasotocin treated ($F(1,84) = 16.72$, $p < 0.0001$) and mesotocin treated naives ($F(1,90) = 6.73$, $p < 0.0001$).

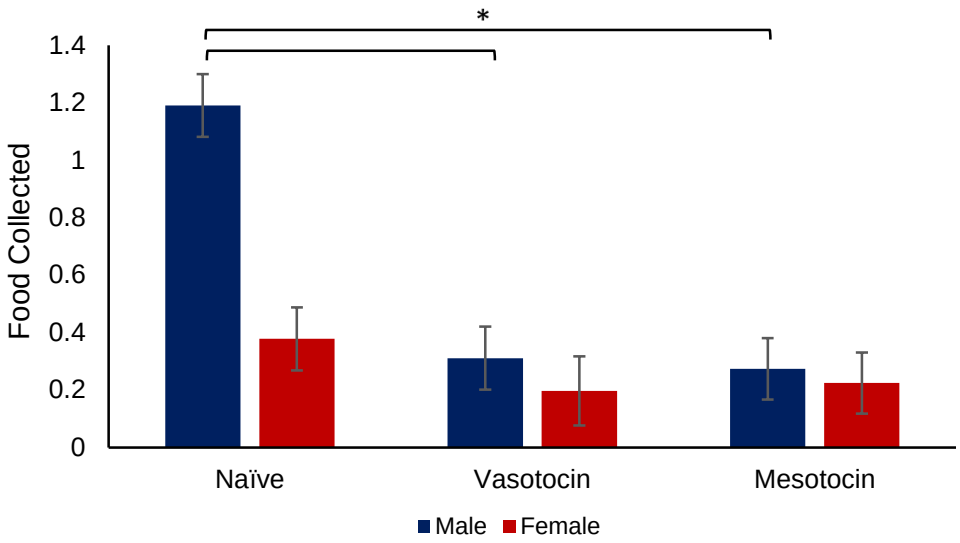
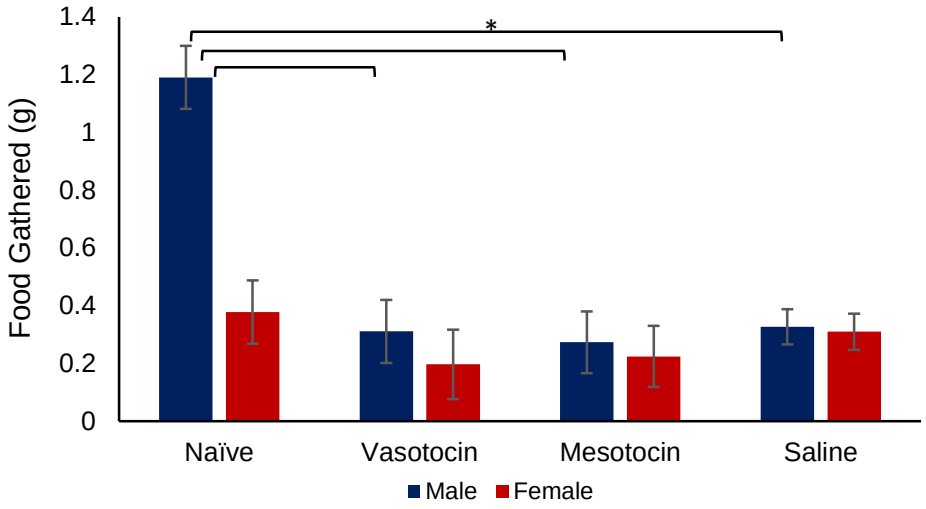


Figure 38. The hormone-induced decrease in food collection during behavioral testing is specific to male subjects. Both ANOVA comparisons of food collected during the behavioral paradigm for mesotocin and vasotocin showed a significant interaction between drug treatment and sex of the subject (vasotocin sex/drug interaction: $F(1,84) = 7.26$, $p < 0.01$; mesotocin sex/drug interaction: $F(1,90) = 10.45$, $p < 0.01$). The inhibitory effect of hormones on the amount of food naïve subjects gathered was specific to males (Tukey HSDs, $p < 0.001$), possibly due to the fact that they gather significantly more food than females (vasotocin: $F(1,84) = 12.77$, $p < 0.001$, mesotocin: $F(1,90) = 13.32$, $p < 0.001$). Females did not show an effect of drug treatment on food collected (Tukey HSDs, ns).



In-Session Entries:

A

	Saline	Naïve
Male	0	13
Female	0	16

B

	Saline	Mesotocin
Male	0	0
Female	0	6

C

	Saline	Vasotocin
Male	0	2
Female	0	10

Out-of-Session Entries

D

	Saline	Naive
Male	67	92
Female	38	0

E

	Saline	Mesotocin
Male	67	7
Female	38	30

F

	Saline	Vasotocin
Male	67	87
Female	38	123

Figure 39 & Table 6. Injection stress causes hypolocomotion in a saline-treated control group. The amount of good gathered by male subjects was greater in untreated naives than saline-injected naives (treatment/sex interaction: $F(1,84) = 10.49$, $p = 0.002$, Tukey post-hoc, $p < 0.001$). **(A)** Saline-treated naïve animals entered the nest significantly less than untreated naives during this period ($\chi^2 = 27.07$, $p < 0.0001$); this was true for both male ($\chi^2 = 11.08$, $p < 0.001$) and female groups ($\chi^2 = 24.04$, $p < 0.0001$). **(C)** Saline-treated naives also entered the nest significantly less frequently than the vasotocin-treated group ($\chi^2 = 10.08$, $p = 0.002$), this result was driven by a difference in female behavior ($\chi^2 = 8.1$, $p = 0.004$). **(B)** Saline and mesotocin-treated naives did not differ in the frequency with which they entered the nest during the in session period, in either sex (all subjects: $\chi^2 = 4.16$, p = n.s.; males: $\chi^2 = 0$, p = n.s.; females: $\chi^2 = 4.16$, p = n.s.). **(D)** Out-of-session nest entries were similar in naïve untreated and saline-treated males ($\chi^2 = 3.62$, p = n.s.), however females treated with saline actually entered more frequently than untreated naives ($\chi^2 = 36.2$, $p < 0.0001$). **(E)** Saline and mesotocin treated naïve females entered similarly ($\chi^2 = 0.72$; p = n.s.) during this time, but males treated with mesotocin actually entered less frequently than those treated with saline ($\chi^2 = 47.04$. $p < 0.001$). **(F)** Finally, vasotocin increased nest entries in treated naives over that of saline controls ($\chi^2 = 34.34$, $p < 0.0001$), and this result was driven by female behavior ($\chi^2 = 43.82$, $p < 0.0001$; males: $\chi^2 = 2.34$, p = n.s.). Alpha values were Bonferroni corrected.

Figure 40. Behavioral approach assay apparatus. For behavioral approach testing, subjects were isolated and acclimated to a wire bird cage inside of a sound-attenuated box. The 18" length of the cage was divided into three areas: the left zone, central neutral zone, and the right zone, using yellow tape. In the neutral zone, food and water were provided ad libitum, in addition to a perch on which to sit. This encouraged subjects to spend their time in the neutral zone, rather than staying in one of the call-associated side zones. Stimuli were played from speakers on either side of the sound-attentuated box when subjects were in the neutral zone and behavioral responses into the left or right zones were recorded.

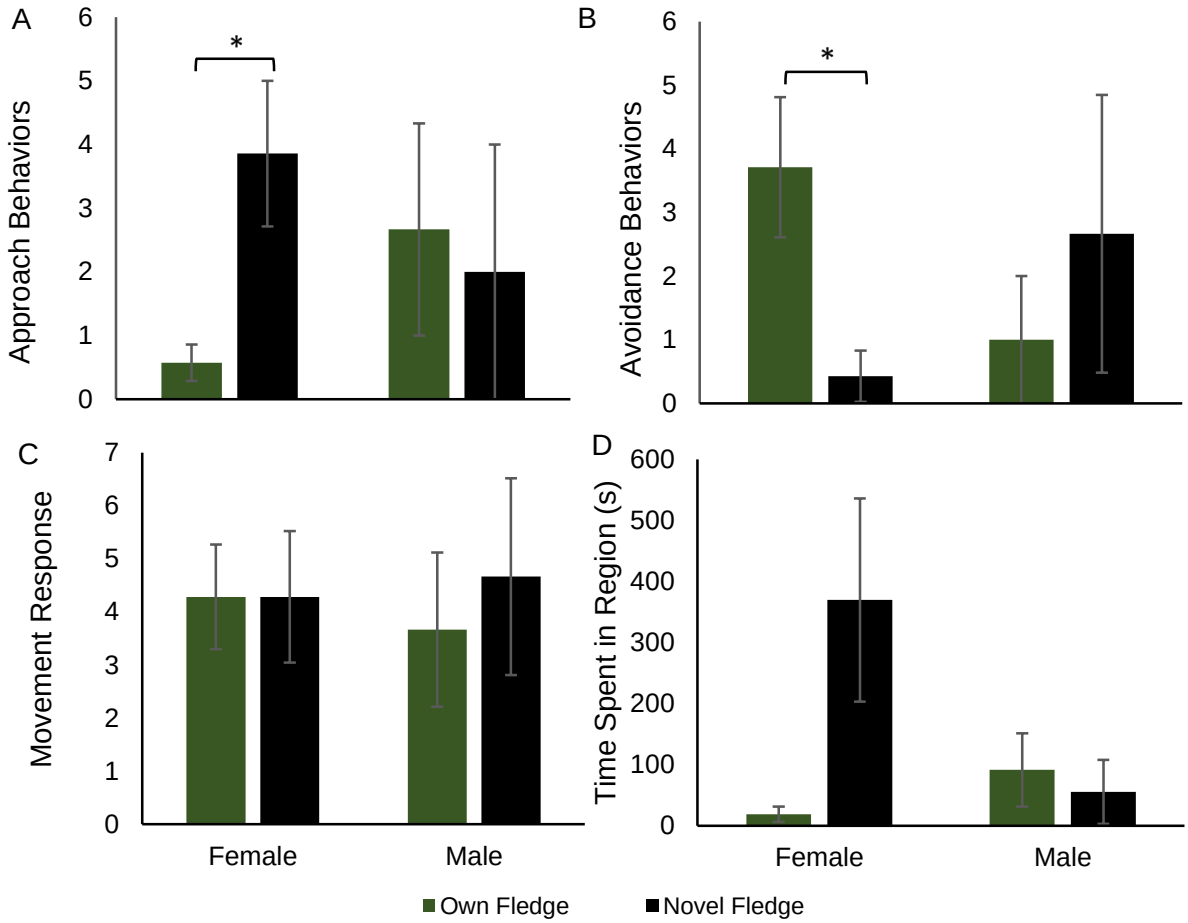


Figure 41. Female parents behaviorally discriminate between OFC and novel FCs in behavioral approach assay. In the recognition test, female parents behaviorally recognized their own fledgling's calls. **(A)** Females approached the novel call significantly more than their offspring's ($t_{(7)}$ = -2.875, $p < 0.05$) and **(B)** showed significantly more avoidance responses to their own offspring's calls than novels ($t_{(7)}$ = 2.486, $p < 0.05$), however **(C)** movement in response to either stimulus was equal ($t_{(7)}$ = 0, ns). **(D)** There was also a trend for female subjects to spend more time on the side from which the novel stimulus was played ($t_{(7)}$ = -2.07, p = 0.077). **(A, B, C & D)** Males showed no difference in their responses to own and novel fledgling calls for any of the measured behaviors (approach: $t_{(2)}$ = 0.210, p = 0.853, avoidance: $t_{(2)}$ = -0.574, p = 0.624, time spent in regions: $t_{(2)}$ = -0.34, p = 0.766, movement response: $t_{(2)}$ = -1.732, p = 0.225).

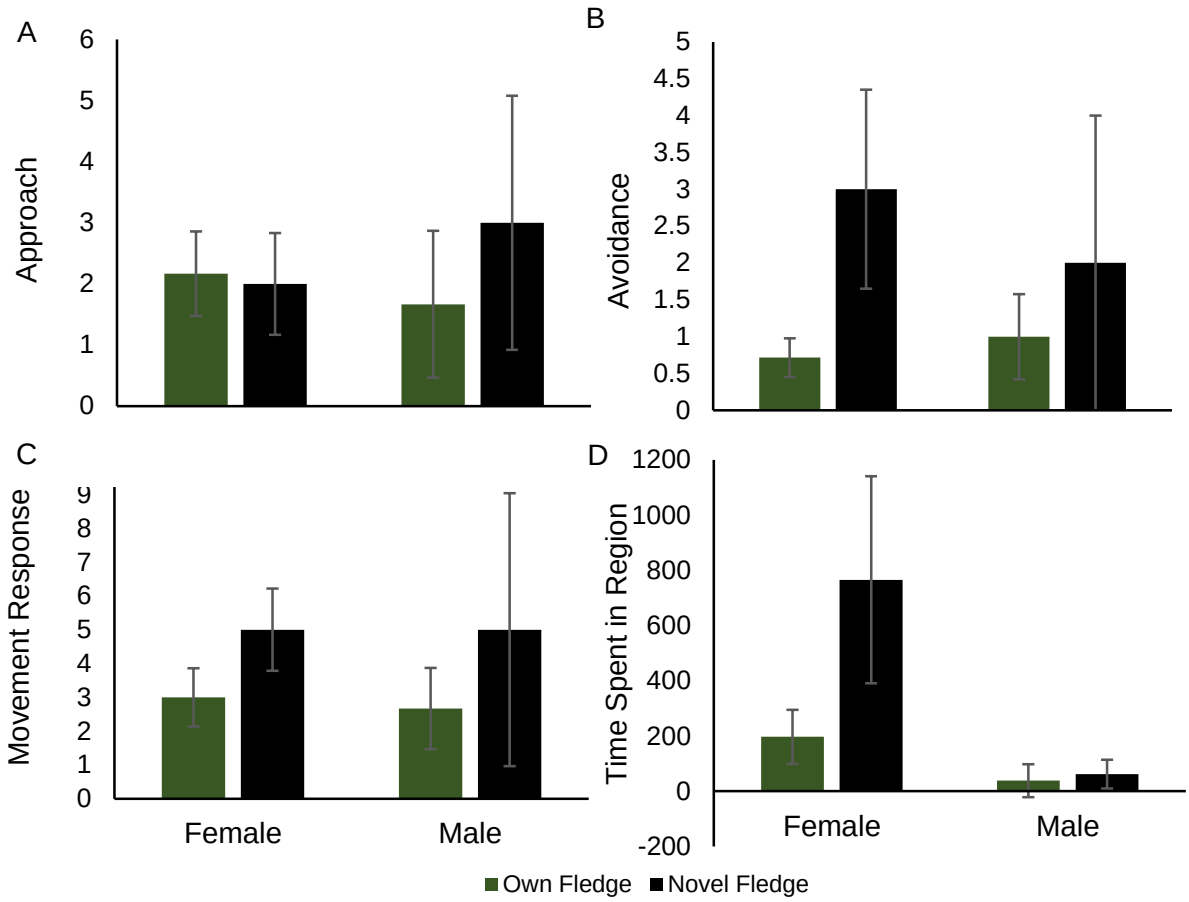


Figure 42. Behavioral approach assay showed no preference in session two. In the second session of the behavioral recognition test the recognition females showed in session one was not seen. Unlike the earlier trial, females behaved similarly for OFC and novel FCs (**A:** approach: $t_{(7)} = 0.129$, ns; **B:** avoidance: $t_{(7)} = 0.267$, ns; **C:** time spent: $t_{(2)} = -1.16$, ns; **D:** movement response: $t_{(7)} = -1.24$, ns). The male data showed the same result (**A:** approach: $t_{(2)} = -1.11$, ns; **B:** avoidance: $t_{(2)} = -0.480$, ns; **C:** movement response: $t_{(2)} = -0.819$, ns; **D:** time spent: $t_{(2)} = -0.676$, ns).