

Does operant conditioning alter the
neurogenomic response to song
presentation?

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Chapter 2: Dr Rob Lachlan helped with the programming and hardware development, and Dr Julia George helped with hardware development.

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Chapter 4: Joelle Clayton coded the videos.

Chapter 5: Dr Julia George helped with the sectioning.

Abstract

Many levels of biological activity contribute to the encoding of specific perceptual experiences. From the discovery that conspecific song playback induces gene expression in a part of the zebra finch brain now referred to as the auditory forebrain, researchers have found increasingly complex roles for the auditory forebrain in song representation. Here we test the idea that the same exact auditory stimulus, varying only in its previously learned association, can induce differential patterns of gene expression. We identify Go/No-Go operant conditioning as a method for generating different meanings associated with a stimulus and develop a suite of software and hardware to conduct this training. We then analyse both the learning of the Go/No-Go behaviour as well as the behavioural response to acute song playback of the stimuli learned during Go/No-Go conditioning. We also examine the expression of *ZENK*, an immediate early gene, in response to the acute playback of one of the trained stimuli. We find evidence to support the idea that Go/No-Go learning requires two separate psychological processes. We also find that acute song playback of a learned stimulus does not elicit discrete behavioural states, or indeed any behavioural response at all. Finally, we demonstrate that overall levels of *ZENK* expression in the auditory forebrain do not vary by condition, but that the auditory forebrain responds in a more coordinated way to Go songs than to No-Go, novel, or habituated songs. We conclude that, when active learning is minimised, *ZENK* expression in the auditory forebrain subtly reflects the previously learned association of stimuli.

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Chapter 1

Introduction

Many levels of biological activity of the brain are involved in the encoding of specific perceptual experiences. Though most work to date has focused on the role of synaptic activity (Dubnau, Chiang, & Tully, 2003), gene expression has been posited as a mechanism through which memories are stored (D. F. Clayton, 2000). Acute and salient experiences induce changes in fast-responding genes that have multiple downstream effects (immediate early genes; IEGs) (e.g. Jarvis, Schwabl, Ribeiro, & Mello, 1997; Wheeler et al., 2013) and neurons that express IEGs are involved in memory formation (Minatohara, Akiyoshi, & Okuno, 2016). Long-term memory formation is impaired for mice that do not have the IEG *Arc* (Plath et al., 2006), and juvenile zebra finch males do not learn tutor song accurately when a pathway associated with the IEG *ZENK* is disrupted (London & Clayton, 2008). Moreover, IEGs have been shown to be differentially neurally expressed in response to stimuli with varying levels of salience and novelty (Mello, Vicario, & Clayton, 1992; Terpstra, Bolhuis, Riebel, Burg, & Boer-Visser, 2006). What remains unclear is whether the presentation of an identical stimulus, which through experience has acquired a meaning, elicits differing neuroanatomical distributions of an IEG. If so, the neuroanatomical distribution of that IEG would reflect the meaning of that stimulus, and provide new evidence as to how the brain encodes memories.

For this thesis, I test this idea using a combination of behavioural and molecular techniques, and the auditory discrimination abilities of a songbird, the zebra finch (*Taeniopygia guttata*). In this introduction, I begin by reviewing the evidence for perceptual learning and discrimination of auditory patterns in songbirds. I then describe the use of a particular IEG (*ZENK*) as a tool for studying the anatomical distribution of plasticity-related neural activity. Finally, I explain

operant conditioning techniques used to shape the associations an animal has with a stimulus, and how this can be used to investigate the neural encoding of those associations.

1.1 Neural encoding of song stimuli in songbirds

1.1.1 Songbirds as a model species for auditory memory

Members of the songbird clade, especially the zebra finch, are frequently used as a model species for the investigation of auditory perception and auditory memory. Both male zebra finches and humans have a sensitive period for vocal learning as well as similar learning phases (i.e. a social phase followed by/overlapping with a sensorimotor phase that begins with babbling) (Doupe & Kuhl, 1999). Although female zebra finches do not learn to produce song, they do learn to recognise individuals by their songs, and their preference for song is shaped by early life experiences (N. S. Clayton, 1988; Holveck & Riebel, 2014; Lauay, Gerlach, Adkins-Regan, & Devoogd, 2004). Further, the neuroanatomy of the song system is fairly well understood in the songbird (Doupe, Perkel, Reiner, & Stern, 2005; Mello, Velho, & Pinaud, 2004).

In the songbird brain, for both males and females, auditory input arrives in the MLd (dorsal lateral nucleus of the mesencephalon) in the brainstem, where there is some preliminary tuning of neural responses (e.g. functional MRI evidence suggests greater activation in response to conspecific song than heterospecific song in the male zebra finch left MLd (Poirier, Boumans, Verhoye, Balthazart, & Van der Linden, 2009)). The MLd projects to the nucleus ovoidalis, which projects auditory information to be filtered through Field L, a collection of tonotopically organised regions similar to the mammalian auditory cortex (Gehr, Capsius, Gräbner, Gahr, & Leppelsack, 1999). Field L projects to CLM (caudolateral mesopallium) and NCM (caudomedial nidopallium), which in turn project to CMM (caudomedial mesopallium) (Moorman, Mello, & Bolhuis, 2011; Vates, Broome, Mello, & Nottebohm, 1996). The part of the brain that includes NCM, CMM, and Field L2 has been referred to as the auditory forebrain (Theunissen et al., 2004). Regions in the auditory forebrain then project to HVC and RA, which are part of the song production system in male songbirds.

Evidence from large-scale gene expression studies indicates that the auditory forebrain is involved in complex auditory processing. Within the auditory forebrain,

at least five miRNAs vary depending on whether a bird has been exposed to a song or to silence (Gunaratne et al., 2011). One of these, miR-2954, affects the expression of around 1000 downstream mRNAs, suggesting that this single miRNA might mediate a large network of neurogenomic changes that are involved in song perception (Lin, Balakrishnan, & Clayton, 2014). Further, many of the genes affected by miR-2954 are downregulated when birds are exposed to song to which they have habituated (Dong et al., 2009). Dong et al. (2009) found that detection of this habituation profile does not require presentation of the stimulus immediately before tissue collection; rather, simple exposure to repeated presentation of a single song can induce large-scale changes in gene expression the day before tissue collection. These broad dynamic shifts suggest that patterns of gene expression in the auditory forebrain contribute to, or at least reflect, recent exposure to song stimuli.

Connectivity between regions in the auditory forebrain is rich and complex (Vates et al., 1996). Field L2 has reciprocal projections to CLM, which has reciprocal projections to CMM, which has reciprocal projections to NCM. Despite the interconnectivity within the auditory forebrain, different regions, and even subregions, display vastly different responses to auditory stimuli. For example, some neurons in Field L and CMM selectively respond to conspecific songs, but other neurons selectively respond to white noise (Grace, Amin, Singh, & Theunissen, 2002). Further, medial parts of Field L and CMM exhibit greater selectivity for conspecific songs than lateral parts (Grace et al., 2002). In contrast to the similarity of electrophysiological responses in Field L and CMM in that particular study, Mello et al. (1992) found that in response to conspecific song playback, an immediate-early gene called *ZENK* is expressed in NCM and CMM, but not in Field L2. NCM and CMM also show differential responses to song playback; *ZENK* expression in female zebra finch NCM is higher in response to conspecific song than heterospecific song, but *ZENK* expression in CMM is similar in response to both conspecific and heterospecific song (D. J. Bailey, Rosebush, & Wade, 2002).

For some who study male song production, the auditory forebrain has been characterised as a secondary auditory processing centre that receives auditory input and feeds into the male songbird's song production system, and much work has been done to understand the processing of tutor song and bird's own song in the auditory forebrain (see Mooney, 2009; Theunissen et al., 2004). However, the role of the auditory forebrain is more likely that of a general song processor for both males and females. For example, for female zebra finches, who do not sing, temporary inactivation of NCM leads to females failing to show a preference for

males singing natural song (Tomaszycki & Blaine, 2014). NCM has been further implicated in the processing of sexually relevant stimuli for females; for female starlings, who prefer longer songs, Gentner, Hulse, Duffy, & Ball (2000) found that *ZENK* expression was higher in ventral NCM for females exposed to longer songs than females exposed to shorter songs, but that expression was uniform in response to both song lengths in CMM. In contrast, for female zebra finches, who prefer directed song to undirected song, *ZENK* expression in NCM is modulated by familiarity of songs, whereas *ZENK* expression in CMM is modulated by the directedness of the song as directed songs tend to be preferred over undirected songs (S. C. Woolley & Doupe, 2008). Similarly, *ZENK* expression in CMM is higher for female zebra finches who are exposed to their father's song than for birds exposed to novel songs (Terpstra et al., 2006), and *ZENK* expression in CMM is higher for female canaries who are exposed to sexy syllables compared to nonsexy syllables (Leitner, Voigt, Metzdorf, & Catchpole, 2005). In one surprising study of natural and time-distorted songs played to female zebra finches, both NCM and CMM responded with similar increases in *ZENK* expression in response to the time-distorted songs (Lampen, Jones, McAuley, Chang, & Wade, 2014). Therefore, for female songbirds, the auditory forebrain responds to birdsong in complex ways, with some aspects of preference, salience and familiarity leading to differential expression in NCM, and others leading to differential expression in CMM.

One potential explanation for the range of effects seen in the auditory forebrain is that subregions, which are not clearly visible using common neuroanatomical staining techniques such as hematoxylin and eosin, respond differentially. For example, *ZENK* expression in response to song playback decreases from medial to lateral sections in NCM, but the same effect of laterality is not found for CMM (Gentner et al., 2000). Further, responses to auditory stimuli within the NCM vary on the dorsoventral axis; for canaries, dorsal NCM preferentially responds to low frequencies, and ventral NCM responds to high frequencies (Ribeiro, Cecchi, Magnasco, & Mello, 1998). For the mesopallium, despite discrete nomenclature for CMM and CLM, these two regions are generally separated by their distance from the midline, and they do not respond similarly to all stimuli (Jeanne, Thompson, Sharpee, & Gentner, 2011). Some reports collected data from CMM between 320-700 μm from the midline (S. C. Woolley & Doupe, 2008), and a frequently used zebra finch atlas shows CMM from 200-1700um from the midline (Oregon Health & Science University, 2013). Multiple studies provide no clear indication of laterality (e.g. Jarvis, Mello, & Nottebohm, 1995; Lampen et al., 2014). Given the complex multi-dimensional nature of responses to auditory stimuli in the auditory

forebrain, there is a clear need for high spatial resolution, which modern methods, such as RNASeq and fMRI of the entire auditory forebrain, cannot provide.

1.1.2 Probing the neurobiology of song recognition using the *ZENK* gene

In situ hybridisation is one method that provides extremely fine spatial resolution. In contrast to electrophysiology, which also provides high spatial resolution, *in situ* hybridisation supports the imaging of large areas of tissue. *In situ* hybridisation suffers from low temporal resolution, and when studying neural tissue, only one time-point per animal can be assessed. Despite this, IEGs, which are genes that respond rapidly to stimuli and have a broad range of downstream effects, have been widely used in neuroscience to measure activity and learning in the brain (D. F. Clayton, 2000; Minatohara et al., 2016). Within the avian neuroscience literature, the IEGs *ZENK* (and its protein product ZENK) (Lampen et al., 2014; Mello et al., 1992), *c-fos* (Z. J. Hall, Bertin, Bailey, Meddle, & Healy, 2014; Kimpo & Doupe, 1997), and *BDNF* (Li, Jarvis, Alvarez-Borda, Lim, & Nottebohm, 2000) have all been used. Of these, the most frequently measured is *ZENK*. *ZENK* is the avian homologue of and an acronym for *zif268*, *egr-1*, *NGFI-A*, and *Krox24* (Mello et al., 1992), and though NCBI has standardised the use of *egr-1*, I will refer to the gene here as *ZENK* due to its longstanding use in the avian literature. Within the auditory forebrain, expression of *ZENK* correlates strongly with electrophysiologically measured activity in response to songs (Chew, Mello, Nottebohm, Jarvis, & Vicario, 1995; Chew, Vicario, & Nottebohm, 1996; Stripling, Volman, & Clayton, 1997).

The last twenty-six years have been fruitful in characterising the *ZENK* gene response in the auditory forebrain; conspecific songs elicit strong *ZENK* expression in NCM and CMM and this peaks 30 minutes after acute playback of the song (Mello & Clayton, 1994; Mello et al., 1992). *ZENK* expression in NCM and CMM varies depending on the salience of features. For instance, *ZENK* expression is, on the whole, greater in response to conspecific songs than heterospecific songs, and greater in response to heterospecific songs than tones or silence (Mello et al., 1992). The *ZENK* response is also likely to occur in natural settings: only 10 repetitions of a conspecific song are necessary to induce the full *ZENK* response (Kruse, Stripling, & Clayton, 2000), it can be induced in wild birds listening to acute playbacks (Jarvis et al., 1997), and *ZENK* expression is higher across the auditory forebrain for birds in an aviary compared to birds isolated in a sound attenuation

chamber (George, Bell, & Clayton, 2016). Further, the *ZENK* response in NCM and CMM habituates to repeated song playback (Mello, Nottebohm, & Clayton, 1995). This effect is song-specific; if played a novel song, the *ZENK* response in the auditory forebrain recovers (Mello et al., 1995). Further, this recovery from habituation can occur with the same song played in a new context, such as from a different perceived spatial location (Kruse, Stripling, & Clayton, 2004). Whether the stimulus is conspecific or heterospecific, novelty, and spatial location are all examples of varying levels of inherent salience, and there is ample evidence that the *ZENK* response in the auditory forebrain encodes this.

1.1.3 Associative learning in the auditory forebrain

Evidence also demonstrates that the *ZENK* response reflects learned salience. Stimuli with no differences in inherent salience, but that have been associated with a stimulus with inherent salience, vary in the levels of *ZENK* they induce. Jarvis et al. (1995) found that, using a classical conditioning methodology, *ZENK* expression in NCM and CMM is greater when a song is paired with a shock than when songs and shocks are played/given at the same rate but unpaired. For starlings, Gentner et al. (2004) argue that novel songs elicit high levels of *ZENK* protein induction in NCM, whereas songs that birds have been trained to associate with a food reward or lights-out punishment elicit similarly low levels of *ZENK* in NCM. In contrast, in CMM, they found that the novel condition elicited the highest density of *ZENK*, followed by the trained songs, with silence significantly lower than novel and trained songs (Gentner et al., 2004). This study confounded stress and associative learning, and as the *ZENK* response can be altered by placing a bird under stress (Park & Clayton, 2002), the findings are difficult to interpret. However, it is clear that subregions of the auditory forebrain respond in complex ways to auditory stimuli with learned associations.

Electrophysiological studies have also aided our understanding of the role of the auditory forebrain in associative auditory learning. In Gentner & Margoliash (2003), electrophysiological recordings of anaesthetised starlings' CMM demonstrated that CMM neurons respond more to songs that have been associated with reward than to songs that have been associated with punishment. They also found a greater neural response to songs associated with punishment than to novel songs (NB: in direct contrast to Gentner et al., 2004). A more recent study has shown that after learning to associate one song with a reward and another song with a punishment, neurons in awake birds' NCM responded more to rewarded songs than punished songs, with novel songs eliciting middling responses (B. A. Bell, Phan,

& Vicario, 2015). In CMM, neurons were most responsive to rewarded songs, less responsive to punished songs, with novel songs eliciting a very low level of responding (B. A. Bell et al., 2015). Bell et al. (2015) therefore replicated Gentner & Margoliash's (2004) finding that CMM preferentially responds to stimuli that have been intensely trained to be associated with a reward or punishment.

Despite this wealth of both gene expression and electrophysiological investigations into associative learning in the auditory forebrain, no studies have yet controlled the song stimulus experience tightly enough to determine if gene expression is related to the stimulus' association. To do so, birds must be trained to associate two song stimuli with two different conditioned stimuli, and the presentation of the song stimulus, to which the *ZENK* response is measured, must come after confirmation that the birds have learned the associations. One simple way to achieve this is using Go/No-Go learning, an operant conditioning paradigm.

1.2 Use of operant conditioning to shape stimulus association

Operant conditioning is a form of learning whereby behaviour is altered by experience; effectively, it is the form of learning that leads to habit formation (Staddon & Cerutti, 2003). In contrast to classical conditioning, where a stimulus is associated with an outcome, operant conditioning creates an association between a behaviour and an outcome (Kirsch, Lynn, Vigorito, & Miller, 2004). Early studies demonstrated that random behaviours can be shaped by the regular presentation of rewarding feedback and highlighted that pattern-seeking is fundamental to learning processes (Skinner, 1948). The earliest work into operant conditioning focused on fundamental variables such as trial timing (Skinner, 1938) and the relationship between reward frequency and the conditioned behaviour (Herrnstein, 1961). However, more recently, the role of cognition, such as perceived choice (Washburn, Hopkins, & Rumbaugh, 1991) and motivation (Lawrence & Illius, 1989), in operant responding has also been investigated. Fortunately for avian researchers, much of the early work into operant conditioning was conducted on pigeons (Brown & Jenkins, 1968), and a wide array of operant conditioning paradigms, such as the 2-alternative forced choice task and Go/No-Go, have been developed and extended for use in understanding bird behaviour (Hulse, 1995).

Go/No-Go training is a form of conditioning whereby an animal learns to associate one stimulus with a positive reinforcement and a second stimulus with a positive

punishment. It does this by way of operant conditioning; i.e. it learns to associate a behaviour with a stimulus, which is associated with a reinforcement or punishment. More simply, the animal is presented with two stimuli (the Go stimulus and No-Go stimulus) and must learn that when it encounters the Go stimulus it must make the Go behaviour (e.g. pecking at a sensor). If it does so, the Go behaviour is reinforced (with, for example, a food reward). If it does not do so, the animal does not receive any reinforcement. However, when the animal encounters the No-Go stimulus it must make the No-Go behaviour (i.e. withholding the Go response, or simply not responding). If the animal makes the Go behaviour in response to the No-Go stimulus, the behaviour is punished (with, for example, the lights going out). If the animal makes the No-Go behaviour in response to the No-Go stimulus, the response is neither reinforced nor punished. In this way, the bird learns to associate the Go stimulus with a Go behaviour and a reinforcement, and also learns to associate the No-Go stimulus with a No-Go behaviour and a punishment. This learning of differential associations is often characterised as discrimination learning.

1.2.1 Operant conditioning in songbird research

Operant conditioning has been used as a tool in many bird studies to assess perceptual abilities. For example, just-noticeable differences in harmonics and the effect of duration on similarity comparisons can be derived simply from patterns of responses (Beckers, Goossens, & Ten Cate, 2003; Lohr & Dooling, 1998). One common assay is to test how birds generalise to novel stimuli once learning the discrimination of the initial training stimuli. For example, recent investigations have highlighted that starlings do not generalise tonal sequences when the pitch and timbre are altered (Bregman, Patel, & Gentner, 2016) and that individual zebra finches use different tactics to learn artificial grammar rules (Heijningen, Chen, Laatum, Hulst, & Cate, 2013). Though these reports use different forms of operant conditioning (e.g. 2-AFC, Go/No-Go), what they have in common is the quantification of responses to novel/unconditioned stimuli, and an assumption that those responses reflect whether the subjects perceive the unconditioned stimuli as more like one or the other of the conditioned stimuli. These studies often fail to recognise that an inherent bias in responding (such as an overall likelihood to make the Go behaviour in response to all stimuli) can affect the statistical outcomes, especially when reporting response probabilities. Evidence that Bengalese finches employ multiple cognitive tactics to learn the Go/No-Go discrimination further complicates these analyses (Morisaka & Okanoya, 2009)

In contrast, we will use Go/No-Go operant conditioning solely as a tool to train the birds to associate one song with reward and a pecking behaviour and the other with punishment and no behavioural response. Although evidence suggests that some songbirds may preferentially learn to recognise the No-Go stimulus (Morisaka & Okanoya, 2009), and a range of human-based studies suggest that the Go and the No-Go responses are inherently unbalanced and require different cognitive processes (but see Criaud & Boulinguez, 2013; Simmonds, Pekar, & Mostofsky, 2008), we suggest that the clear differential responses to Go and No-Go stimuli (i.e. the Go and No-Go behaviour) indicate that some form of associative learning has occurred. For the purposes of understanding whether gene expression varies in response to stimuli learned through operant conditioning, we therefore need not be concerned with issues of learning strategies and motivation, though we recognise that the specific patterns of gene expression may rely on these variables.

1.3 Aims and objectives

We will combine molecular neurobiology with behavioural psychology to determine if differential IEG expression reflects memories of perceptual experiences. Go/No-Go operant conditioning provides us with a powerful method for forming associative memories. *In situ* hybridisation of the IEG *ZENK* allows us to assess neural gene expression with high spatial resolution. We will first train a bird to discriminate between one song (Go stimulus) and a second song (No-Go stimulus). Then we will play one of those two songs immediately before collecting tissue for *ZENK* *in situ* hybridisation. Finally, we will use the pattern of *ZENK* induction to assess which brain regions are involved in the perception of previously learned stimuli.

First, I present Operanter, a new suite of hardware and software that allows us to inexpensively conduct avian auditory operant conditioning. We successfully developed, from the ground up, Java-based software and non-proprietary hardware that has enabled us to train around 40 female zebra finches thus far.

Second, I use a fine-grained analysis of operant conditioning learning and maintenance to characterise individual differences in Go/No-Go learning, and to better understand the processes underlying the Go and No-Go responses. We find that birds' responses to two conspecific songs initially have a Go bias, and that birds are slower to learn the correct response to No-Go stimuli. We also argue that response latencies to Go and No-Go stimuli suggest separate cognitive processes for these responses. Finally, we show that the time of day a bird is preferentially

active relates to the bird's learning rate.

Third, I demonstrate that despite the profound differences in response to Go and No-Go stimuli during operant conditioning, avian responses to acute playbacks of the trained stimuli do not differ. Using an array of analytical techniques, we find that patterns of behaviour do not vary during playback of the trained stimuli, and we argue that this means that differences in neural gene expression cannot be caused simply by differences in physical activity levels.

Fourth, I show that overall levels of *ZENK* gene expression do not vary across the auditory forebrain in response to Go, No-Go, novel and habituated songs. Responses to novel and habituated songs do not elicit the same strikingly differential patterns of expression as found in previous experiments. However, using graph theory approaches, I demonstrate that the auditory forebrain responds in a more coordinated way in response to Go songs than in response to No-Go, novel, or habituated songs.

Finally, I summarise my findings and conclude that operant conditioning experiments, in conjunction with *in situ* hybridisation, provide rich anatomical detail that allows us to investigate the neurogenetic basis for encoding associative memories.

Chapter 2

Operanter: open source hardware and software for avian operant conditioning

Operant conditioning is a form of learning often used to study psychological and neural processes. Despite the widespread use of operant conditioning, commercially available setups (i.e. Skinner boxes) rely on proprietary software and/or hardware, and free/open source setups incorporate expensive components or are underdeveloped and inflexible. Here we introduce Operanter — free open source software for controlling operant conditioning. Originally designed for avian auditory Go/No-Go training, Operanter runs on a Raspberry Pi computer and is easy to modify using simple XML scripts. It supports other forms of operant conditioning, such as ABX and two-alternative forced choice designs, and can be used to run sound playback experiments. It controls day/night light cycles, reward/punishment procedures, and backup safety mechanisms. It also supports direct control of the operant conditioning hardware for auto- and hand-shaping training stages. Operanter provides summarised information about the training activity to enable animal care staff and researchers to easily determine an animal's progress. Daily logs are automatically exported to a .csv file for later processing. An associated wiki describes how to build an Operanter-compatible operant conditioning setup based on a Raspberry Pi 2 Model B with all the necessary hardware/electronics. A single setup with a dedicated computer, touchscreen display and reward/punishment hardware can cost as little as £250. The affordability and flexibility of Operanter-based setups allows researchers with small budgets or specific needs to carry out operant conditioning experiments.

2.1 Introduction

In order to investigate psychological and neural processes, many researchers use operant conditioning. This form of learning occurs when a behaviour is modified by a consequence, which can be either a reinforcement or punishment (Staddon & Cerutti, 2003). Operant conditioning is frequently used to investigate learning processes; this research usually involves rats or mice (e.g. Saar, Grossman, & Barkai, 1998; Sclafani & Ackroff, 2016). Moreover, by training animals using operant conditioning, researchers can investigate perceptual and cognitive abilities (e.g. Kwak, Lim, & Kaang, 2016; Miletto Petrazzini, Agrillo, Izard, & Bisazza, 2015; Toal, Radziwon, Holfoth, Xu-Friedman, & Dent, 2016). In linguistics and perceptual psychology, this type of research has frequently used songbirds, whose vocal learning shares similarities with human language development (e.g. Holveck & Riebel, 2007; Spierings & Cate, 2014). Operant conditioning is also used to study reward, addiction and drug mechanisms, mostly in rodents (Groeber Travis, Altman, & Genovese, 2015; e.g. Sclafani & Ackroff, 2016), but also in zebrafish and crustaceans (e.g. Bhimani & Huber, 2016; Parker, Millington, Combe, & Brennan, 2012).

Despite the commonness and utility of operant conditioning, most setups are expensive and require proprietary software and hardware. Few companies publish costs online (e.g Lafayette Neuroscience, Bioseb, Med Associates Inc, Harvard Apparatus), but the average cost of a single operant conditioning chamber has been estimated to be over USD 6000 (Pineno, 2014). Further, while these companies offer many modular features, they are designed only for rats and mice. Some universities have chambers designed specifically for their needs by an intra-university department, (e.g. Leiden University), but costs tend to remain high and altering boxes at a later date can prove expensive.

Open source solutions are currently underdeveloped or require expensive components. In avian operant conditioning, Sound Analysis Pro is often used (Tchernichovski, Nottebohm, Ho, Pesaran, & Mitra, 2000). However, it requires a National Instruments I/O card (£156) and only runs on the Windows operating system. Sound Analysis Pro is also difficult to customise for training regimens such as Go/No-Go or ABX. A new piece of free software from the Tchernichovsky lab, BirdPuffer, uses social interaction as reinforcement and a puff of air as a punishment (Tokarev, 2014). However, there are some instances for which this setup might not be ideal, such as when testing female preference for male song. Other open source solutions include ArduiPod Box, which is limited to rats inter-

acting with a touchscreen on an iPod touch (Pineno, 2014). Despite incorporating an Arduino computer, the ArduiPod Box software primarily runs on the iPod touch. Despite the author hoping the ArduiPod Box will be extended by users, there is currently no mechanism for doing so. Another open source solution, OpenBehavior, has many of the same aims as our system but appears to only support fixed-ratio reinforcement and is still in early development (H. Chen & Wang, n.d.).

This chapter describes Operanter; flexible and intuitive operant conditioning software. Originally built for a specific Go/No-Go auditory task with zebra finches, Operanter was designed to be easily extendable for all operant conditioning paradigms, including ABX/AXB and two-alternative forced choice tasks. It was also designed to facilitate inexpensive operant conditioning setups based on a Raspberry Pi computer module and customisable hardware. To this end, Operanter was written in Java but will require only simple XML files to edit the training schemes. Instructions on how to install Operanter and build an operant conditioning setup are provided on the Operanter GitHub Wiki pages (<https://github.com/rflachlan/Operanter/wiki>).

2.2 Methods

2.2.1 Hardware

The Operanter software controls multiple peripheral components using a Raspberry Pi running Raspbian.

2.2.1.1 Raspberry Pi

Operanter was developed on a Raspberry Pi B+ running Raspbian Jessie (Figure 2.1). It has been tested only on Raspbian Jessie 4.1 but should work on earlier versions, albeit without touchscreen support. The Operanter software is designed to be as lightweight as possible and currently requires less than 8MB for both installation and for activity log saving. Operanter uses less than 5% CPU of the Raspberry Pi B+, which allows for multiple other processes to run in parallel on the Raspberry Pi unit, such as sound or video recording/monitoring.

2.2.1.2 Peripheral components

Operanter is designed to work with the DIY hardware on our GitHub Wiki. We describe how to use inexpensive and manufacturer-independent parts to build three types of components: solid state relay-controlled lights, infrared sensors/LED devices for the interactive component, and a linear servo motor to power the food hatch covers for controlling access to food. These components are connected to the Raspberry Pi by ethernet cables, which help to minimise the number of cables necessary to control each chamber. An auditory operant conditioning setup with light deprivation as the punishment and access to food as the reward can cost as little as £250.

Operanter interacts with the peripheral components via a Java class for each component. The exact control of the components can be modified with basic programming, but the creation of new classes for new component designs might require some knowledge of Java. However, with the inclusion of radio-controlled on/off outlets in our preliminary design, any component that can be turned on or off by an outlet can be controlled by Operanter without modification to the source code.

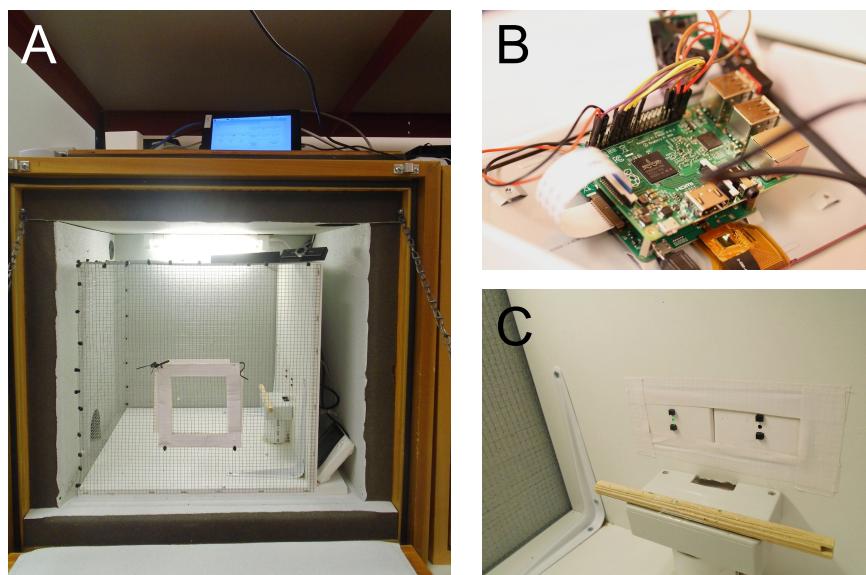


Figure 2.1: Operanter hardware, Raspberry Pi and electronics. A) Back of Raspberry Pi with GPIO connections to peripheral components. B) Infrared sensors and food hatch inside the cage. C) The chamber with individual Raspberry Pi on top.

2.2.2 Software

Operanter is written in the Java language; the Java Runtime Environment included in the Raspbian Jessie operating system distribution is sufficient to run Operanter. Operanter also uses the H2 database engine to record and analyse activity and the Pi4J library to communicate with the Raspberry Pi. These are included in the Operanter file and do not require separate installation. Operanter is distributed as a .jar file that runs by double clicking with administrative permissions and does not need installation. It can be downloaded from the Operanter GitHub website.

2.3 Results

2.3.1 What Operanter provides

The Operanter graphical user interface (GUI) comprises a single window with five tabs: Schedule, Operant Experiment, Log, Direct Control, and Stats (Figure 2.2). The Schedule tab allows the user to set a daily schedule for when the lights are on and when the experiment runs. This panel can be used to set a safety mechanism (maximum duration that the food hatch can remain closed) and to also set the time, or times, that Operanter automatically exports data. The Operant Experiment tab is where new schemes are created and saved. It provides an interface for setting some of the frequently modified defaults for the peripheral components. For example, the duration of the rewarder can be set here, but the rules that trigger the rewarder remain controlled by the programmed scheme and cannot be modified using the GUI. The Log tab shows a table of all activity and contains a button that forces Operanter to save the log file on demand, either as a comma-separated value or Excel file. Data can also be deleted from this table. The Direct Control tab is where the user can force peripheral component activity, which is especially useful during hand-shaping. For example, the LEDs can be flashed and the food hatch can be opened and closed on demand. The Stats panel shows a summary of how many times each action has been performed by each peripheral component since a user-input time, allowing researchers and animal caretakers to quickly determine the level of an animal’s activity and success.

Changes to the operant experiment design must be made at multiple levels. The GUI Operant Experiment tab is useful for changing a few simple settings for the peripheral components, but changes to the relationship between peripheral components must currently be made by editing a scheme in the Java code. We

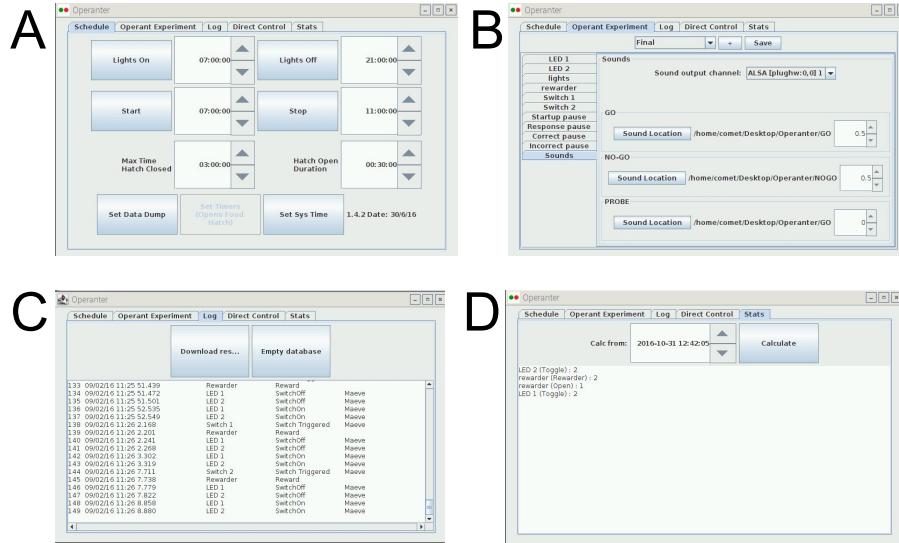


Figure 2.2: Operanter software. A) Schedule tab. B) Operant Experiment tab. C) Log tab. D) Stats tab.

plan to extend the software so schemes can be added by importing syntactically simple XML files with the logic for the new scheme. Only two schemes (Go/No-Go and a preliminary shaping phase) are currently available, but we plan to add schemes for ABX, AXB, 2-alternative forced choice, and preference test designs. Finally, for any users who need to make significant changes to Operanter, such as adding a new peripheral component, the source code is available on the Operanter Github website.

Operanter is robust software that has functioned as intended for the training of ~40 birds. It controls the daily light schedule and operant conditioning experimental design without fail, and allows a user to directly control the peripheral components with precision. Audio playback is of a high quality with no perceptible “clicks” or distortion. Anecdotally, the noise of the food hatch mechanism distrubs the birds to a similar degree as other setups (e.g. Leiden University).

2.3.2 Ease of use

Operanter has been designed to be as easy to use and as intuitive as possible. The GUI comprises five tabs with straightforward functionality. Instructions on the GitHub Wiki assume little knowledge of Raspberry Pi programming and electronics, and will soon be updated to be more thorough.

2.3.3 Mechanisms of support

Operanter is available through a GitHub repository; both the executable .jar file containing software and the source code can be downloaded. Operanter will continue to be updated with new bug fixes whenever they are discovered. The Wiki on the GitHub repository can be edited by users who would like to contribute documentation. The source code can be forked and modified by anyone who would like to extend the code.

2.3.4 Successful work completed with Operanter

Roughly 40 female zebra finches have successfully learned to discriminate two stimuli using Operanter. Female zebra finches can achieve a standard experimental criterion of discrimination of two conspecific songs within 400 trials. Zebra finches trained using the Operanter software and hardware achieve, on average, a lower asymptotic performance compared to zebra finches trained using proprietary software and hardware at the University of Leiden (Figure 2.3), but we suggest that this is likely due to significant differences in the conditioning protocol. Specifically, Operanter birds are only trained until the experimenter goes home, and receive food *ad libitum* after this time, to comply with UK Home Office regulations; in contrast, birds at the University of Leiden never receive food *ad libitum* and are engaged with the operant apparatus throughout the entire photoperiod.

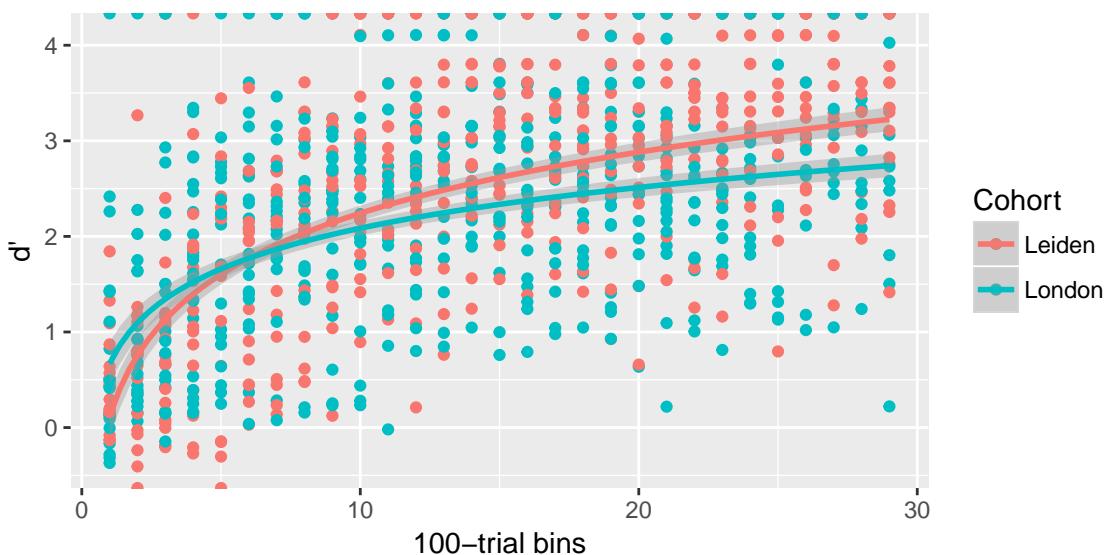


Figure 2.3: Learning curves for Leiden birds (proprietary) and London birds (Operanter).

2.4 Discussion

Our software will soon have the flexibility to implement any operant conditioning experimental design by importing an XML file. Further, though we do not provide any schemes for classical conditioning, these experimental designs can be written and implemented by Operanter with minor modifications to the source code that controls the peripheral components. As demonstrated by its success with controlling operant conditioning training for multiple experiments, Operanter should be considered robust. Because it is open source, it can be developed by any user who wishes to extend or improve the software.

We hope that Operanter will be used and developed by users in other labs who wish to conduct operant conditioning experiments. We make this as simple as possible by releasing Operanter through GitHub. We plan to start a library on the GitHub Wiki of XML schemes, and we hope that users will upload their own schemes to aid others. Further developments will include updates to the Stats tab to make preliminary data analysis easier. We plan to release R code that can parse the activity logs and provide simple statistics, such as d' . We also hope to provide some basic functionality for real-time auditory stimulus generation.

In conclusion, Operanter provides a much-needed open source alternative to commercial and proprietary operant conditioning setups. A comprehensive Wiki will make it possible for anyone, regardless of prior electronic or programming knowledge, to build a setup from inexpensive components. Operanter enables researchers with small budgets or specific requirements, such as non-rodent subjects, to build operant conditioning setups. These setups can be enhanced and maintained by end users without the need to rely on proprietary solutions that do not release source code.

Chapter 3

Characterising Go/No-Go learning and maintenance behaviour in the zebra finch

Go/No-Go operant conditioning is regularly used by ethologists to investigate perception in zebra finches. Despite this rich literature, little work has been done to investigate how the zebra finches learn this task. Here we avail of a large dataset of simple Go/No-Go discrimination learning of a conspecific song, and long-term maintenance of this discrimination behaviour. We find that the rate of learning the correct responses to Go and No-Go stimuli varies, with birds taking longer to learn to inhibit the No-Go response. Response latencies, or the interval from stimulus onset to pecking response, also vary between Go and No-Go stimuli, with incorrect responses to No-Go stimuli having longer latencies than correct responses to Go stimuli. We also highlight large individual differences in daily patterns of activity, and demonstrate a relationship between learning rate and when birds prefer to be active. These results have numerous implications for experimenters using Go/No-Go operant conditioning.

3.1 Introduction

The Go/No-Go paradigm is a form of operant conditioning where a subject is trained to associate the Go stimulus with a reward and the No-Go stimulus with a punishment. It does this by learning to produce the Go behaviour in response to the Go stimulus, which results in the presentation of a reinforcement; it must also learn to make the No-Go behaviour in response to the No-Go stimulus, as the Go behaviour results in the presentation of a punishment. Go/No-Go conditioning is frequently used for investigations of animal perception due to the ability of researchers to extract information about perceptual abilities from simple, easily measured behavioural responses (e.g. Chen, Rossum, & Cate, 2015; M. Long, Jiang, Liu, & Yao, 2015). But despite a long history of investigation of fundamental operant conditioning variables (e.g. Herrnstein, 1961; Skinner, 1938) and more recent attempts to understand specific cognitive aspects of Go/No-Go learning (e.g. Kalenscher et al., 2005; Thomas, Gonsalvez, & Johnstone, 2009; Yechiam et al., 2006), we still do not understand what facets of perception and decision making are captured by the binomial measure of response accuracy to presentations of Go and No-Go stimuli.

Classical conditioning, or the learning of stimulus-outcome associations, is often contrasted with operant conditioning, or the learning of response-outcome associations. But Go/No-Go operant conditioning goes beyond the simple response-outcome association, and, in fact, creates a stimulus-response-outcome association. That is, Go/No-Go creates “expectancies of particular outcomes when certain responses are emitted in the presence of an occasion setting (discriminative) stimulus” (Kirsch et al., 2004, p 378). Therefore, in contrast to simple operant conditioning paradigms, such as shaping, a thorough characterisation of Go/No-Go learning could benefit from our understanding of both classical and operant conditioning. Moreover, as Go/No-Go learning involves discrimination, the use of analytical methodologies derived from signal detection theory has enhanced researchers’ ability to use behavioural outputs to understand animal behaviour and perception (B. Kim & Basso, 2008; M. Long et al., 2015; Nevin, 1969).

Responses to Go/No-Go-trained stimuli have occasionally been compared to responses to alternative operant conditioning paradigms. 2-alternative forced choice (2-AFC) and Go/No-Go behavioural responses are both subject to bias (e.g. subjects can have a left or right bias for 2-AFC (Riebel & Slater, 1998), and a Go or No-Go bias for Go/No-Go (Carandini & Churchland, 2013)) and can be assessed with signal detection theory in order to quantify those biases.

However, the responses are not always equivalent: adaptation to probe stimuli (i.e. novel/untrained stimuli to which subjects respond with a Go or No-Go behaviour, embedded in a stream of trained stimuli) can change the bias of making the Go response, but this does not occur in (2-AFC) (M. Long et al., 2015). Additionally, the Go/No-Go bias can be altered by a wider range of factors, such as motivation, than the 2-AFC bias.

Motivation plays multiple roles in operant conditioning. For example, the valence of and preference for the reinforcement can alter the motivation of subjects to engage in the operant behaviour (Holveck & Riebel, 2014; Sclafani & Ackroff, 2016). For experiments where the operant stimulus is, itself, a reinforcement (e.g. Go/No-Go experiments on female birds where the stimuli are conspecific songs), subjects might initiate trials to receive the inherently rewarding stimulus, with no motivation to produce the reinforced behaviour. Within a Go/No-Go experimental design, this, of course, could lead to a No-Go bias. Further, the choice of the reinforcement and punishment can affect the ease with which subjects learn operantly-trained associations (Scheiner, Erber, & Page, 1999), and the discriminability of the two stimuli also affects the learning rate (Frontali & Bignami, 1974; Hagmann & Cook, 2010). As some subjects appear to become frustrated with the operant conditioning apparatus when regularly unsuccessful, the relative valence of reinforcement/punishment and stimulus discriminability may affect the subjects' motivation to produce responses (McMahon, pers. obs.).

Additionally, in standard avian perceptual operant conditioning, birds choose when to initiate the trials. Hunger, desire to hear the stimulus, or desire for enrichment could all affect the motivation of the bird to intiate a trial. Zebra finch operant conditioning generally lasts through the entire photoperiod (e.g. Spierings & Cate, 2014), but our laboratory recently reduced the operant conditioning period to morning and afternoon (but not evening) in an effort to improve animal welfare. Therefore, a characterisation of trial initiation times could enhance our understanding of response behaviour during training and maintenance, and also aid in the improvement of our experimental procedure.

The response behaviours themselves also require further characterisation. Unlike 2-AFC, where both stimuli require a similar motor behaviour for reinforcement, Go/No-Go requires a motor behaviour in response to one stimulus and a withholding of that behaviour in response to another stimulus. As such, Go/No-Go tasks have often been used to investigate inhibition of behaviours, and much work has been done on understanding whether the Go and No-Go responses are fundamentally different (Simmonds et al., 2008). Specifically, there is evidence

that the production of the No-Go behaviour is more effortful than production of the Go behaviour (Gao & Mingming, 2017; Shenoy & Yu, 2002). One meta-analysis suggests that electrophysiological signals measured in human Go/No-Go task performance primarily reflect differences in attentional resources, and not differences in motor responses or inhibition processes (Criaud & Boulinguez, 2013). Of critical importance is that human studies of Go/No-Go tasks do not require operant conditioning, and certainly do not involve the long-term acquisition and storage of associative memories that are involved in animal Go/No-Go operant conditioning tasks. In contrast, human Go/No-Go task discriminations are held in working memory and subjects respond without reference to long-term memory. Therefore, it is unclear to what extent we might expect to see similar patterns of effortfulness in avian Go/No-Go operant conditioning, but provisional support for these patterns could be found by measuring bias during learning.

Response latency has been used in many non-operant conditioning studies as a proxy for memory (e.g. Klein & Arbuckle, 1970). In contrast, almost all animal operant conditioning experiments use response accuracy to assess learning (e.g. Beckers et al., 2003; Bregman et al., 2016; Brodigan & Peterson, 1976). Response accuracy is simple to measure and intuitive, but provides far less resolution per trial than response latency. As some subjects learn to produce the No-Go response to No-Go stimuli very slowly, the development of response latency as a variable for assessing learning in animal operant conditioning might provide higher resolution to experimenters. Further, after learning, when error rates are negligible (i.e. correct responses are subject to a ceiling effect), response latencies may provide more information on subject performance (Kahana & Loftus, 1999). However, response latency and response accuracy do not necessarily measure the same aspect of memory: speed-accuracy tradeoffs exist (Reed, 1973), and response latency and accuracy have been suggested to measure two separate aspects of memory retrieval (MacLeod & Nelson, 1984). Therefore, the characterisation of response latencies during avian Go/No-Go conditioning could be of value to researchers who use this methodology.

3.1.1 Aims

In order to characterise the Go/No-Go discrimination of conspecific song stimuli, we utilised a large dataset of straightforward single conspecific song discrimination learning and maintenance. We hypothesised that motivation to hear male song would interact with hunger levels, and that this would be seen as a change in response bias throughout the day. We also predicted that birds would more rapidly

reach criterion for the Go stimuli than for the No-Go stimuli. As previously seen in Chapter 2, Leiden birds were on average faster to reach our discrimination criterion than London birds, despite using the same training methodology. We hypothesised that this could be caused by the longer time window during each day that London birds did not engage in training, and sought evidence to support this hypothesis. Finally, we aimed to characterise response latencies to No-Go stimuli to determine if they can be used as a finely tuned continuous indicator of learning performance.

3.2 Methods

3.2.1 Animals

24 female zebra finches (*Taeniopygia guttata*) bred at Queen Mary University of London were housed in a single sex aviary for at least a week before being placed singly into a sound attenuation chamber with an operant conditioning setup. The birds ranged in age from 332 to 909 days post hatch (mean = 558.8, sd = 200.2). The birds were kept on a 16:8 light cycle (7:00 to 23:00). Birds were given free access to food from 7:00 until 7:10, at which time the operant conditioning apparatus automatically initiated. Operant conditioning then continued until the experimenter left the premises, between 14:00 and 20:00. Animal housing and welfare were in compliance with the European directives for the protection of animals used for scientific purposes (2010/63/EU) under Procedures Project License PPL70-8183.

3.2.2 Apparatus

The birds were housed in a sound attenuation chamber fitted with an operant conditioning cage (43 cm w x 46 cm d x 42 cm h). The cage had a solid floor and back, with mesh on the remaining four faces. The back of the cage contained the operant conditioning peripheral equipment: a motorised food hopper and two LED/peck detectors. A Jawbone Mini Jambox speaker was placed on top of the chamber. A Raspberry Pi automatically controlled the operant conditioning, including the food hopper, LED/peck detectors, speaker, and the chamber light (as described in Chapter 2).

3.2.3 Stimuli

For all birds, the early training stages used a novel male zebra finch song and sine wave tone. For the final training stage, each bird received two novel songs in a counterbalanced design: one as the Go stimulus and another as the No-Go stimulus. These songs were matched for duration. All songs were from the population of zebra finches at the University of Leiden, and were therefore novel to the birds in this study. The song recordings were edited in Praat to include a 10ms on and off ramp (Boersma & Weenink, 2018).

Final song playbacks were created using Audacity, and consisted of one of the stimuli (either Go or No-Go) repeated once every 10 seconds for 10 minutes, for a total number of 60 song playbacks. All stimuli were played at a SPL of 70 dB, measured using a Realistic sound level meter (Cat. No. 33-2050, RadioShack) on the fast setting at the location of the bird's head after pecking a sensor. Each bird received a final playback of either their Go or No-Go stimulus.

3.2.4 Operant conditioning

The birds were allowed to acclimatise overnight to the sound attenuation chamber with *ad libitum* access to food and water. Four hours after the lights came on, the food hopper closed and the birds began the first stage of training. Birds retained *ad libitum* access to water and cuttlebone throughout the experiment.

The first stage of training involved the birds learning to associate a peck to either sensor and the subsequent opening of the food hopper for 10 seconds. Once the birds had pecked either sensor ~200 times, the birds progressed to stage two, when they had to learn to peck the sensors in sequence. During stage two, the birds were only rewarded with access to food if they first pecked the left sensor followed by the right sensor within 30 seconds of the first peck. This time was reduced to 6 seconds once the birds learned the pecking sequence. At this point, a song, which was not used for the final training, was played when the birds pecked the left sensor.

The third stage of training introduced the Go/No-Go procedure. The birds were taught that if they pecked the left sensor and heard the song, they could peck the right sensor (Go response) and receive a food reward, as in the latter parts of stage two. However, punished trials were introduced at a rate of 80% rewarded to 20% punished. For these trials, a sine wave tone (440 Hz) was played when

the bird pecked the left sensor; the bird had to learn not to peck the right sensor (No-Go response). If they did peck the right sensor, the chamber light would go out for 10 seconds and the bird would not receive a food reward. During stage four, the ratio of rewarded to punished trials was altered to 50% each.

Following training, the birds were swapped to two novel songs as the Go and the No-Go stimuli. Once they learned this discrimination to a criterion of 0.80 discrimination ratio (defined as the proportion of correct responses to Go stimuli divided by the summed proportion of correct responses to Go stimuli and the proportion of incorrect responses to No-Go stimuli), they had to maintain their performance for 4-5 days before initiation of the final playback.

3.2.5 Final playback

The afternoon before final playback, the operant conditioning apparatus was disabled and birds were again allowed *ad libitum* access to food. The following morning, between three and five hours after the lights came on, the final 10 minute playback was initiated. 20 minutes after the end of the playback, the bird was decapitated for an RNA-Seq experiment.

3.2.6 Statistics

All statistics were carried out using the base stats package in R v3.3.3 unless otherwise noted.

3.3 Results

3.3.1 Go and No-Go stimuli are learned at different rates

In order to characterise differential learning of the Go and the No-Go stimuli, an analysis of the learning curves was undertaken. From the first presentation of the two song stimuli, birds took longer to achieve 80% correct responses to No-Go stimuli (median < 400 trials) than they did to achieve 80% correct responses to Go stimuli (median < 100 trials) ($W = 50, p = 0.0001$; two-sample Wilcoxon rank-sum test) The averaged learning curves for all individuals show that the Go and the No-Go stimuli are not learned at the same rate (Figure 3.1. Panel A of

Figure 3.1 shows the proportion of correct responses to Go and No-Go stimuli, fitted with a loess regression (R packages: ggplot2). This figure also illustrates that birds, on average, reached asymptotic performance after the presentation of around 1000 trials (i.e. bin 10). Further, after 3000 trials (i.e. bin 30), many birds had completed the training. For this reason, all time-of-day analyses presented below are based on data from trials 1000-3000, which should be considered the average maintenance stage. Bins after 3000 trials are less frequent, due to fewer birds remaining in the experiment, and the visible decline in correct responses to No-Go stimuli after this point is likely an artefact due to small sample sizes. Panel B of Figure 3.1 shows the proportion of Go responses to Go and No-Go stimuli, with bin fraction (100-trial bin number divided by the maximum bin number for each bird) on the x-axis. Therefore, these curves have been normalised to remove learning rate (line of best fit modelled with a loess regression using ggplot2). This further illustrates that birds were slower to learn the correct response to No-Go stimuli than the response to Go stimuli.

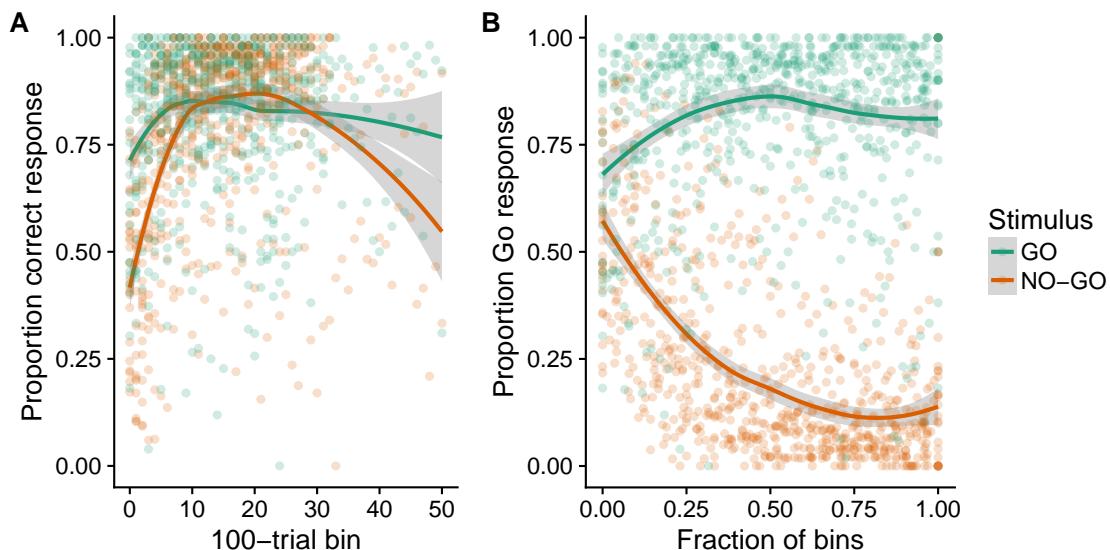


Figure 3.1: Averaged learning curves for all birds. A) Proportion of correct trials for 100-trial bins. B) Proportion of Go responses, normalised for each bird, where bin fraction is the bin number divided by the maximum number of bins for each bird. Lines of best fit are modelled with loess regression, with standard error shading.

3.3.2 Birds have a Go response bias during early training

In order to further characterise the learning process, an assessment of response bias during learning was conducted. Response bias (c ; mean of the sum of the z-score of the hit rate and z-score of the false alarm rate, multiplied by -1) is roughly independent of accuracy and provides a good indication of bias when performance

is at or near chance; it therefore provides an indication of whether the bird had a tendency to Go or to No-Go during learning, regardless of the stimulus (Macmillan & Creelman, 1990). A series of one-sample Wilcoxon rank-sum tests was carried out on the first 10 100-trial bins (with Bonferroni correction for multiple testing). Figure 3.2 shows that for the first 400 trials, birds had a slight bias towards a Go response, regardless of whether the stimulus presented was a Go or a No-Go song. This bias does not reliably continue throughout late learning and maintenance.

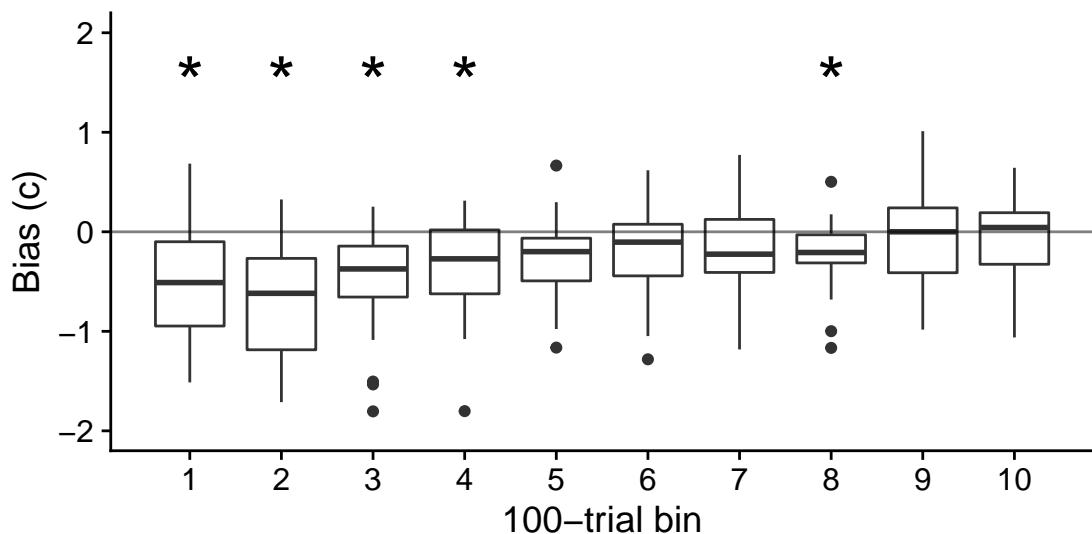


Figure 3.2: Bias (c) for first 10 100-trial bins, where scores > 1 indicate a No-Go bias and scores < 1 indicate a Go bias. Asterisks indicate significance at the 0.05 level (with Bonferroni correction).

3.3.3 Response latencies during learning and maintenance

To further characterise the patterns of responses to Go and No-Go stimuli, response latencies throughout learning and maintenance were compared. Response latencies to Go and No-Go stimuli appear qualitatively different, with longer latencies for incorrect responses to No-Go stimuli throughout learning and maintenance (Figure 3.3). Response latencies also appear to subtly vary between learning and maintenance for Go stimuli, with fewer long latencies during the maintenance stage than during learning (Figure 3.3; Panels A & B). In contrast, for No-Go stimuli, response latencies appear to diverge into a bimodal distribution during maintenance (Figure 3.3; Panels C & D).

In order to further characterise these differences, response latencies during learning (trials 1-1000) were explicitly compared to response latencies during maintenance (trials 1001-2000) for all non-outlier birds (i.e. the birds represented in Panels B and D in Figure 3.3). Response latencies during learning were from a significantly

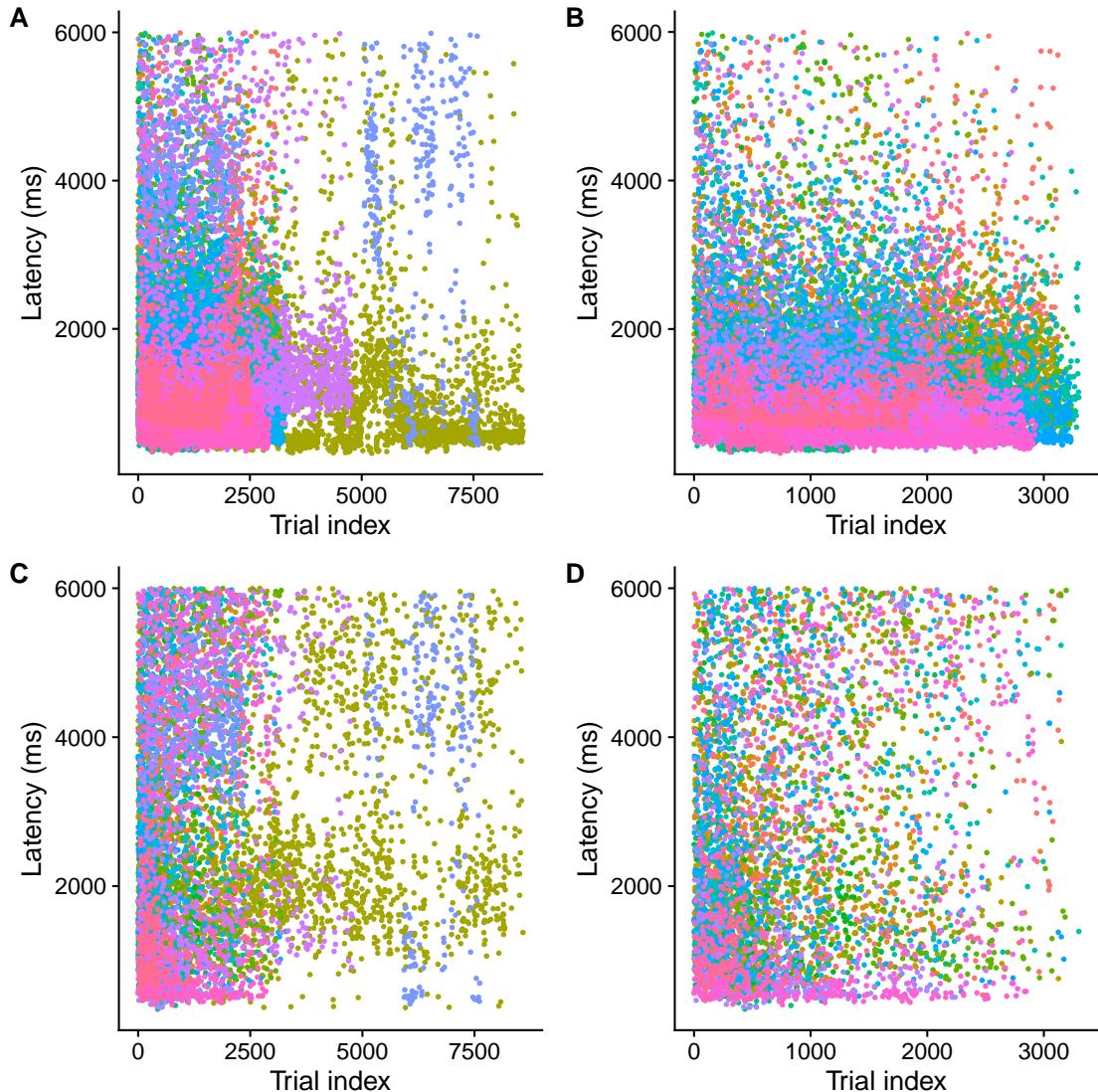


Figure 3.3: Response latencies (in milliseconds) to stimuli throughout learning and maintenance. Panels A and B are correct responses to Go stimuli, whereas Panels C and D are incorrect responses to No-Go stimuli. Panels A and C include all birds, including three outliers who learned extremely slowly. Those three outliers have been removed for Panels B and D. Colours represent individual birds.

different distribution than response latencies during maintenance for No-Go stimuli (two sample Kolmogorov-Smirnov test, $D = 0.15$, $p < 0.0001$), and for Go stimuli (two sample Kolmogorov-Smirnov test, $D = 0.051$, $p < 0.0001$). Though both Kolmogorov-Smirnov tests show significant differences due to the large sample sizes, the difference in response latencies appears to be much stronger and more qualitatively distinctive for the No-Go stimuli than for the Go stimuli (Figure 3.4). For the Go stimuli, response latencies shorten, with frequencies on the long right-hand tail diminishing during maintenance (t -test on log-transformed latencies, $t(17082) = 3.71$, $p = 0.0002$; Figure 3.4; Panels A & B). In contrast, for the No-Go stimuli, response latencies diverge during maintenance into a bimodal distribution, with a relatively increasing frequency of long-latency responses (Figure 3.4; Panels C & D).

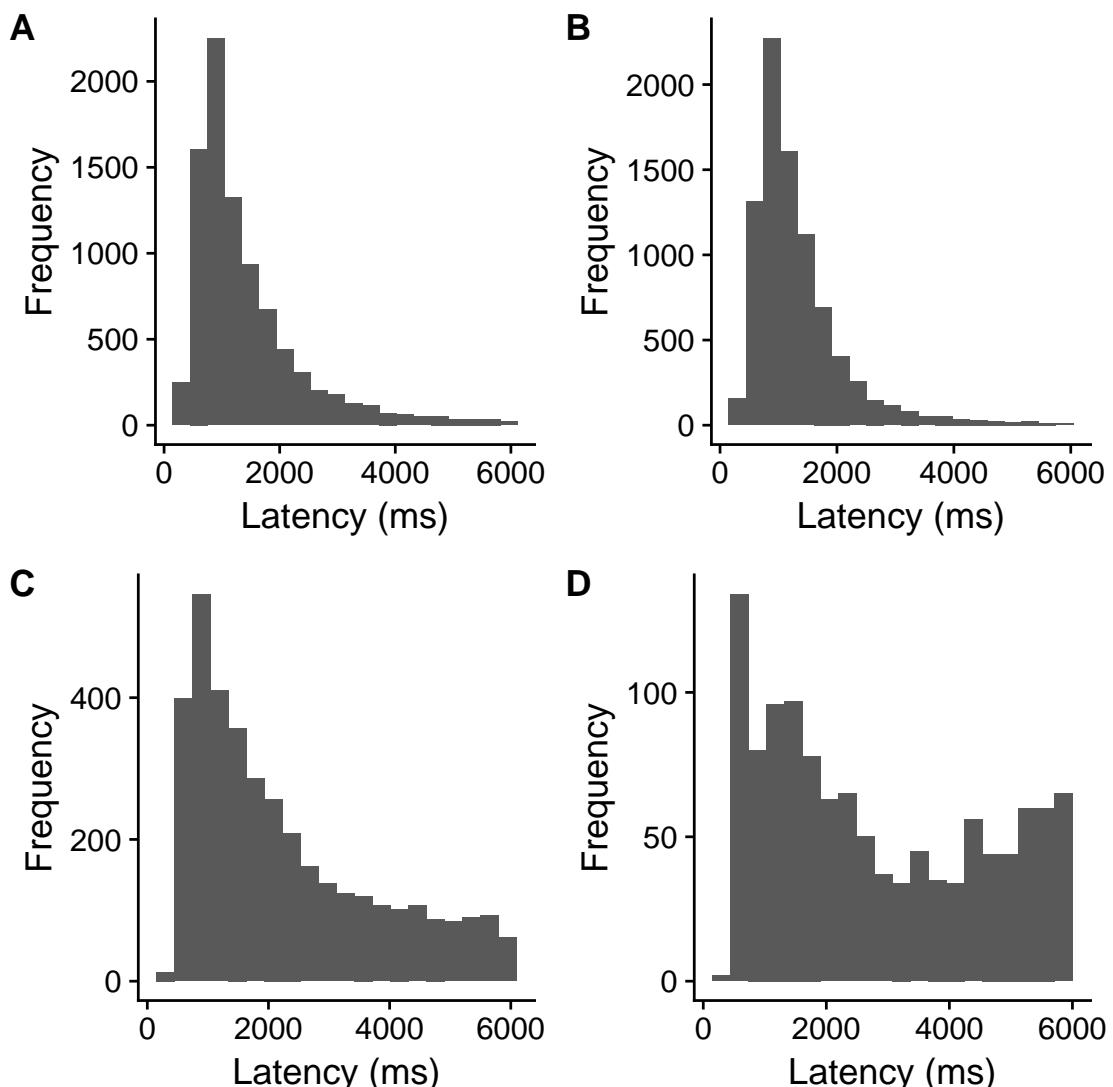


Figure 3.4: Response latencies in milliseconds. A & B) Correct responses to Go stimuli. C & D) Incorrect responses to No-Go stimuli. A & C) During learning (trials 1-1000). B & D) During maintenance (trials 1001-2000).

The difference between Go and No-Go response latencies during the maintenance stage can be described by plotting both on the same histogram. Specifically, a randomly generated normal distribution based on the mean and standard deviation of log-transformed Go response latencies was plotted alongside raw No-Go latencies; the length of the Go response latency normal distribution vector was determined by manually aligning the peak of the Go and No-Go response latency distributions (Figure 3.5). The No-Go latencies tend to be longer and do not follow a normal distribution after log transformation. Further, the maintenance stage Go and No-Go response latencies are not from the same distributions (Kolmogorov-Smirnov test; $D = 0.43$, $p < 0.0001$).

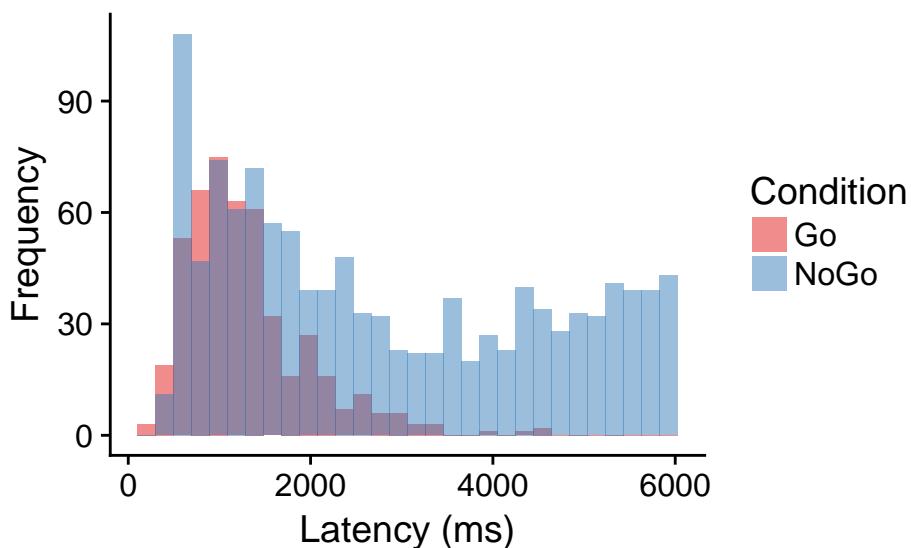


Figure 3.5: Histogram of Go and No-Go response latencies during maintenance. Red bars indicate a generated normal distribution that describes Go response latencies. Blue bars indicate raw No-Go latencies. The purple region is where Go and No-Go response latencies overlap.

3.3.4 Activity levels, but not accuracy or bias, vary according to the time of day

Half hour time bins (e.g. 7:00 to 7:30, 7:30 to 8:00) were calculated to assess behavioural changes through the day. Activity levels peaked around 8:30 (one and a half hours after the lights came on) and steadily decreased throughout the remainder of the day (Figure 3.6). Despite a group-level peak at 8:30, marked individual differences in patterns of activity can be seen, with a number of birds showing a peak in activity during afternoon hours. The time of day during which individual birds reached their median number of trials ranged from 9:00 to 14:00 (median = 11:00; inter-quartile range = 10:45 - 12:15).

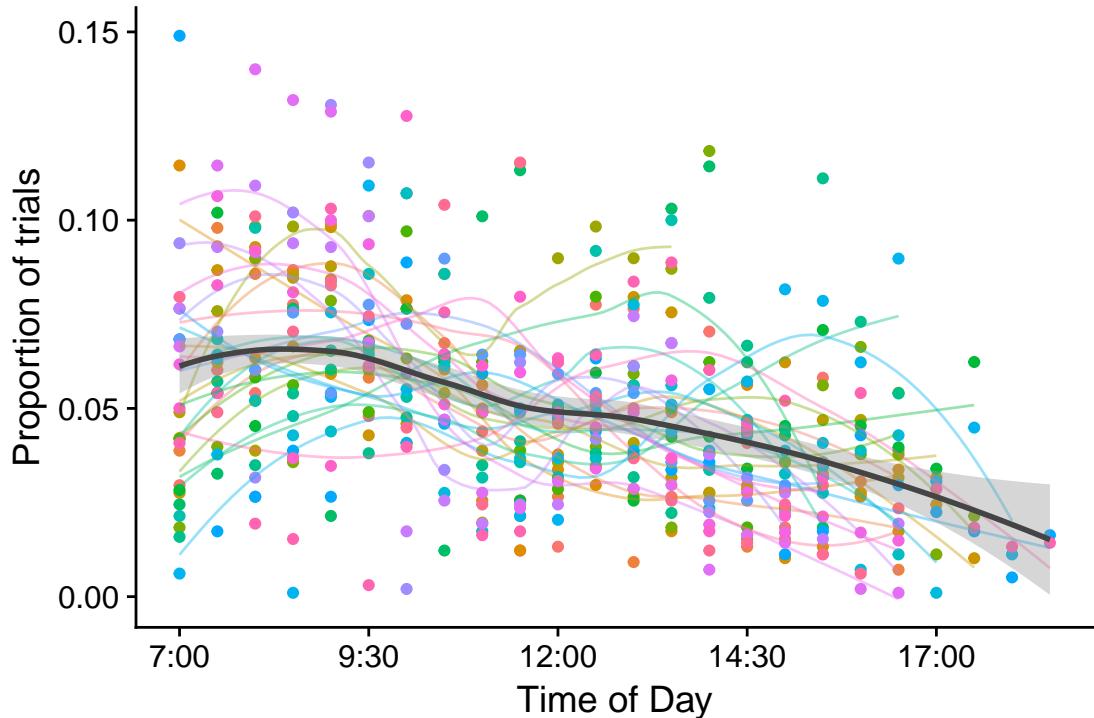


Figure 3.6: Activity levels for individual birds throughout the day, in half hour bins, during the maintenance stage. Each point indicates the mean proportion of trials during that half hour bin for each individual bird, across all days of maintenance. Lines of best fit are loess regression lines.

To determine if birds' motivation varied through the day, a number of metrics were calculated for each bird during the maintenance phase. Figure 3.7 shows four of these metrics: response latencies, d' (a measure of sensitivity/accuracy), discrimination ratio (a measure of accuracy more affected by bias than d'), and c (a measure of bias). To test for a relationship between time of day and the behavioural metrics, Spearman's correlations were conducted. There was no significant relationship between time of day and response latency (Go: $\rho = -0.018$, $p = 0.70$; No-Go: $\rho = -0.051$, $p = 0.31$). There was also no significant relationship between time of day and d' ($\rho = 0.068$, $p = 0.14$) or between time of day and discrimination ratio ($\rho = -0.052$, $p = 0.27$). However, there was a small but significant negative correlation between time of day and bias ($\rho = -0.10$, $p = 0.032$), with the tendency for birds to have a No-Go bias in the morning reducing throughout the day.

3.3.5 Early birds are slow learners

To understand whether the daily reduction in No-Go bias or activity changes throughout the day might be related to learning rate, learning rates were calculated

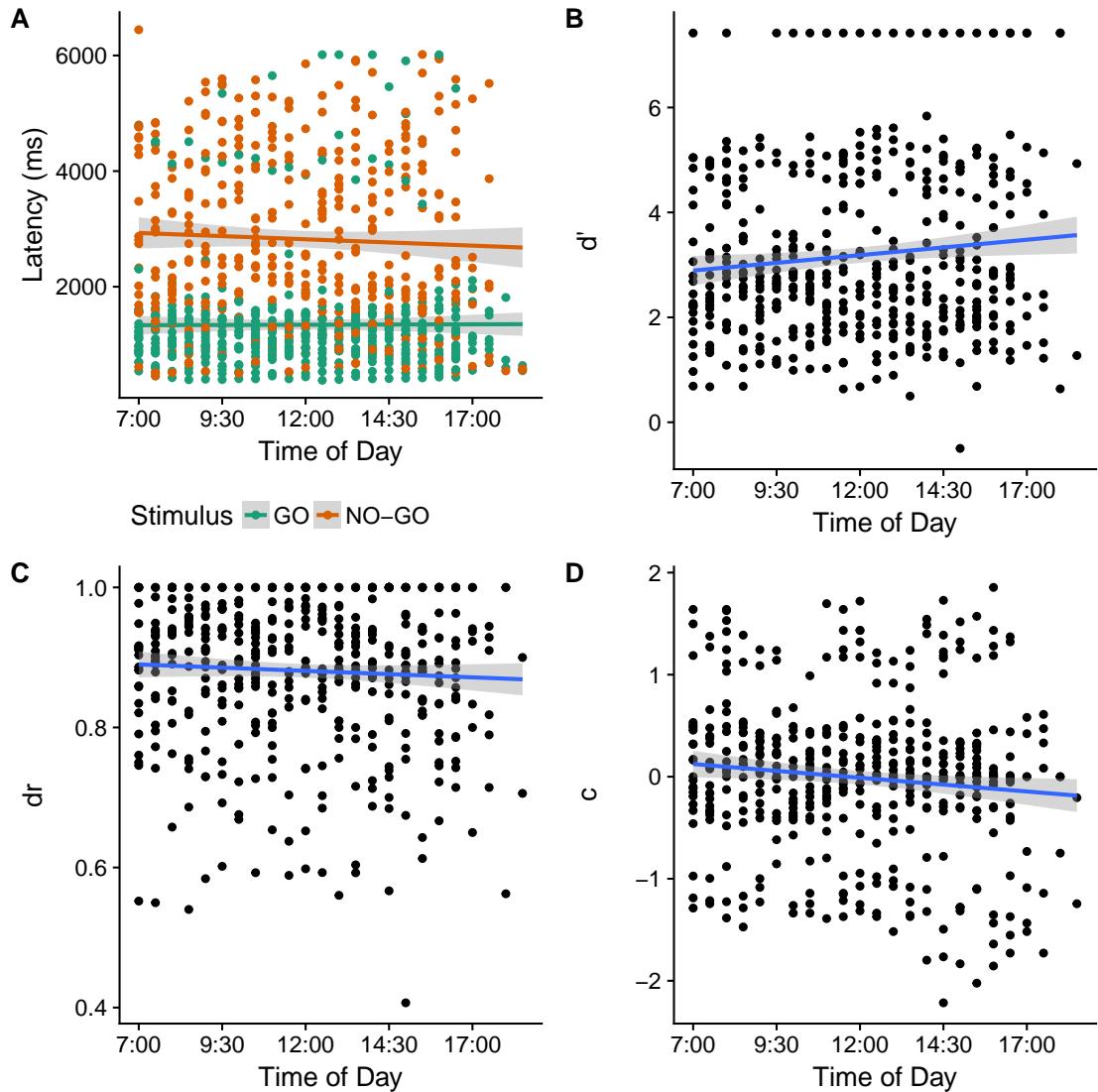


Figure 3.7: Four metrics of behaviour through the day. A) Response latencies to Go and No-Go stimuli. B) Accuracy (d'). C) Accuracy (discrimination ratio). D) Bias (c). All lines of best fit are linear regressions with standard error shading.

as the minimum 100-trial bin number when the birds first reached a discrimination ratio of 0.80. Therefore, larger values for learning rate indicate slower learners. Learning rates were correlated with overall bias, change in bias throughout the day, and two measures of activity timing during the maintenance stage. The maintenance stage was chosen as trials during this period would be less affected by the novelty of the sound attenuation chamber, and therefore provide a cleaner indication of the birds' natural activity in the operant experiment. Neither overall bias (Figure 3.8, Panel A; $\rho = 0.19$, $p = 0.39$) nor change in bias throughout the day, measured as the slope of the linear regression of time bin against bias during that time bin (Figure 3.8, Panel B; $\rho = -0.02$, $p = 0.92$).

Time of day activity was operationalised in two ways: peak activity was defined as the half hour time bin during which the bird initiated the highest number of trials, and median activity was defined as the half hour time bin during which the bird reached half of its total daily trials. Peak activity was not correlated with learning rate (Figure 3.8, Panel C; $\rho = -0.34$, $p = 0.12$), but median activity was moderately significantly negatively correlated with learning rate (Figure 3.8, Panel D; $\rho = -0.45$, $p = 0.034$). This indicates that the birds that were slower learners initiated a greater proportion of their trials during the morning than faster learners.

3.4 Discussion

We found that Go and No-Go stimuli are learned at different rates, with 80% accuracy in response to Go stimuli being achieved much earlier in training than 80% accuracy to No-Go stimuli. These varying learning rates are reflected in the birds' response bias during early learning: birds have a Go response bias during early training, which is not reliably found after birds reach criterion. We also found that response latencies to Go stimuli subtly shorten after learning, whereas response latencies to No-Go stimuli are qualitatively different during learning and maintenance. Birds were most active in the morning, with activity levels declining throughout the day, but there were dramatic individual differences in the timing of trial initiations. We found that the time of day negatively correlated with bias, suggesting that the group-level No-Go bias in the morning diminished through the day. We also found a correlation between learning rate and individual differences in the time of day the birds are preferentially active; slower learning birds tended to be more active early in the day than fast learning birds.

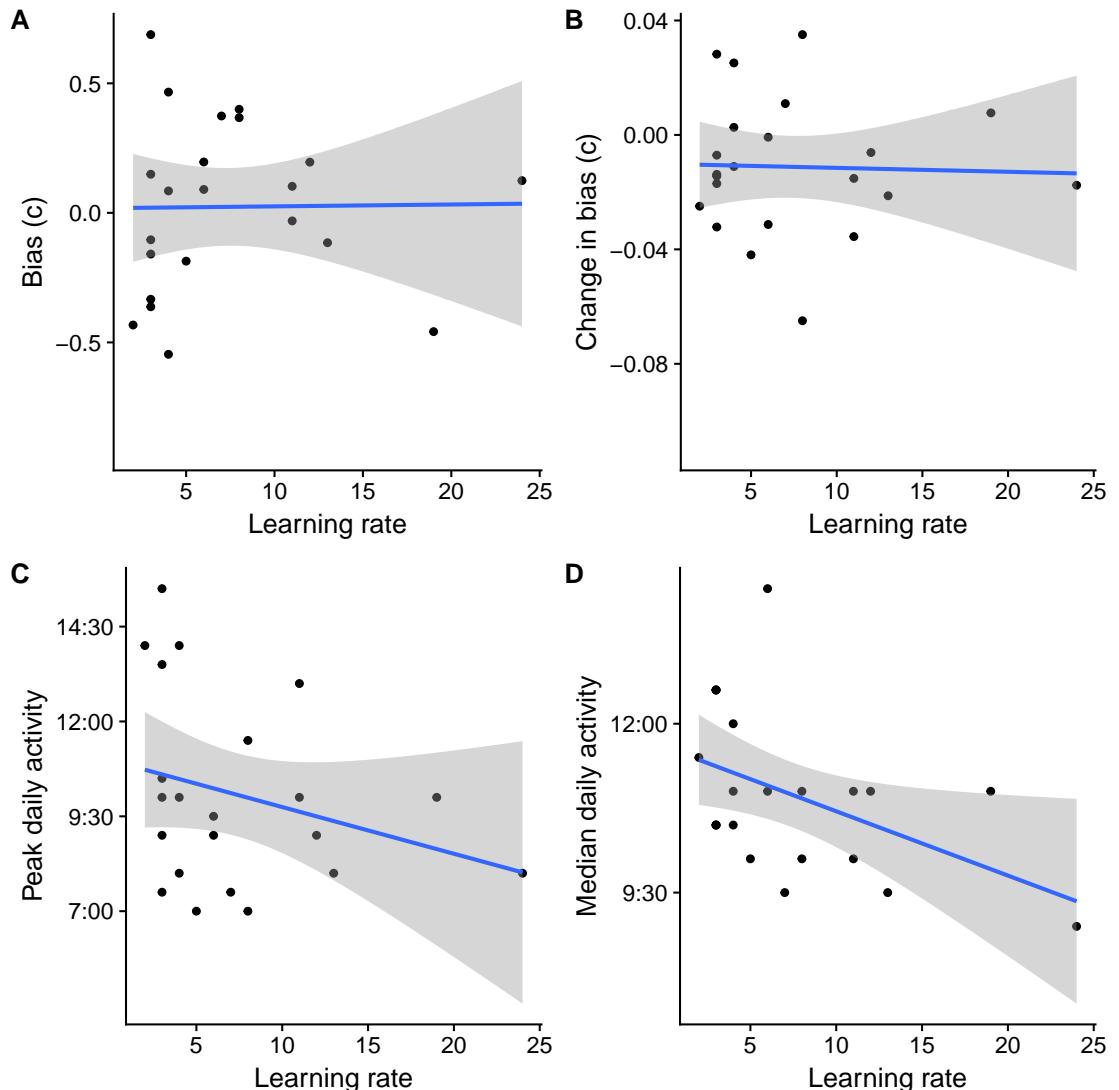


Figure 3.8: Relationship between learning rate, where larger values indicate slower learners, and possible predictors. A) Bias. B) Change in bias through the day. C) Peak activity half-hour time bin. D) Median activity half-hour time bin. Lines of best fit are all linear models with standard error shading.

3.4.1 Go/No-Go response learning rates and bias

The finding of a differential rate of learning of the correct responses to Go and No-Go stimuli was expected for multiple confounded reasons. First, human Go/No-Go literature suggests that withholding the Go response is more effortful than producing the Go response (Gao & Mingming, 2017). Second, one stage in our training procedure requires all birds to learn to Go in response to a conspecific song and to No-Go in response to a tone. Therefore, when the stimuli were swapped to two conspecific songs, birds may have initially responded to a large proportion of both Go and No-Go stimuli because they were generalising from the training conspecific song to all conspecific songs. Third, the birds' initial bias to Go could reflect a change in the decision criterion based on a risk/reward analysis, whereby the birds know that they must Go to receive a food reward, and are willing to risk the darkness punishment to receive that reward.

It is therefore critical to recognise that the response data, even assessed using bias metrics, do not necessarily reflect the active learning of the two stimuli, as is often assumed. For example, a group-level Go bias during learning does not necessarily mean that the birds learned the Go stimulus faster than the No-Go stimulus. Indeed, Bengalese finches preferentially learn a No-Go stimulus (Morisaka & Okanoya, 2009), and this could be the case for our zebra finches as well. If the decision criterion is initially, and on the basis of factors not related to stimulus discrimination, set very far towards the Go stimulus, this bias would only be reduced when the birds learned to both recognise the No-Go stimulus and to associate the No-Go stimulus with the No-Go response. Unfortunately, with no probe stimuli in this experiment, we cannot distinguish between these possibilities. However, our behavioural response data, along with others (Gess, Schneider, Vyas, & Woolley, 2011), do suggest that the learning of Go and No-Go stimuli is not performed at the same rate. We further recommend that future studies that use Go/No-Go operant conditioning as a method to test the generalisation abilities of subjects do so only after confirming that birds have learned both the Go and the No-Go stimuli to an equal criterion, and that they do not have an overall Go or No-Go bias. This might take a few hundred trials longer than previous criterion targets, but would aid in the analysis of probe stimuli.

3.4.2 Response latencies

Further evidence for the dissociation of Go and No-Go learning is found in our response latency results. We show that, for both learning and maintenance stages, (correct) response latencies to Go stimuli follow a logarithmic distribution as is frequently the case with reaction time data (Baayen & Milin, 2010; but see Whelan, 2008). In contrast, (incorrect) response latencies to No-Go stimuli are not easily modelled with any frequently used transformation. This is especially the case for response latencies during the maintenance stage, where longer response latencies become increasingly frequent. It is our view that response latencies after \sim 3000 ms do not reflect a false alarm in the traditional sense of signal detection theory. Instead, these long latencies represent some other psychological process, such as the inability of the zebra finch to withhold a pecking response, as is suggested by the effortfulness literature (e.g. Gao & Mingming, 2017) or the impatience of the zebra finch to initiate another trial, as is suggested by theoretical work on the asymmetry of the Go/No-Go task (Shenoy & Yu, 2002).

Further work could dissociate these possibilities. Our software intentionally did not record any key pecks to the left (initiator) sensor after the stimulus was triggered, but an alteration to record all key pecks would permit the analysis of the timing of all key pecks. For example, if long-latency incorrect pecks to the right (response) sensor could be predicted by un-reinforced pecks to the left (initiator) sensor through cross-correlation, that would suggest that the birds produce a range of pecking behaviours to attempt to more quickly initiate another trial. Further work on characterising the No-Go response latencies could aid in our understanding of the cognitive process underlying these responses; longer windows for responding would specifically help with the modelling of the long latencies. Regardless of the cause of the No-Go response latency bimodal distribution, we recommend that future studies involving zebra finch Go/No-Go operant conditioning use a cutoff time of 3000 ms in order to reduce the number of “false alarm” false alarms.

3.4.3 Time of day

We analysed the patterns of trial initiation throughout the day to inform the improvement of our protocol for animal welfare purposes. Human children learn best when they study during their preferred time of day, suggesting that individual differences in attention through the day may affect learning rate (Ammons, Booker, & Killmon, 1995). For university students, memories stored in the evening appear

to be more easily recalled the next day than memories stored in the morning (Payne et al., 2012). Additionally, female zebra finches are likely to be accustomed to exposure to male song primarily in the morning (Jha & Kumar, 2017) and will of course have their own patterns of daily activity (Dall & Witter, 1998). Therefore, we were interested in determining if there is an ideal time of day to administer the operant training in order to reduce the total duration spent in the isolation chamber, and also interested in whether individual differences in the timing of trial initiation correlate with learning rate.

During maintenance, we found great individual differences in trial initiation activity, with some birds initiating large numbers of trials in the afternoon. The vast differences between when individuals triggered their middle daily trial (i.e. from 9am to 2pm) illustrate this. We also found that response latencies, sensitivity (d') and discrimination ratio did not vary according to the time of day, but bias did. The birds, on average, began the day with a No-Go bias. This is difficult to explain, given that hunger motivation would lead to a Go bias. We believe that our specific protocol, which allowed for birds to feed freely during the first 10 minutes of the daily photoperiod, may have alleviated hunger motivation in the morning. If satiated, the female zebra finches may have engaged with the operant conditioning apparatus to receive the male song stimulus (e.g. Holveck & Riebel, 2007), although this is unlikely as we do not see this same bias during the afternoon. Further work on the fine temporal structure of peck initiation and clustering of trials may help with understanding this daily shift in bias.

We also found evidence that learning rate is related to the pattern of trial initiation, even when the bird has finished learning. Specifically, slower learning birds initiate trials earlier in the day during maintenance. B. A. Bell et al. (2015) found that fast learners exhibited larger neural responses to stimuli after learning, and we wanted to characterise our own birds' learning rates for gene expression analyses. We hypothesised that the learning rate effect on neural activity in response to song playback might be mediated by a time of day effect. That is, birds who prefer to be active in the morning (when our apparatus was always available to the birds) might learn faster (as in Ammons et al., 1995), and would also exhibit greater gene expression in response to morning playbacks. However, our data does not support this hypothesis, as we found a negative correlation between learning rate and time of day activity. We theorise that birds that are preferentially active in the morning are slower learners because they have a longer gap between the bulk of their trials and the next morning, although we did not find a relationship between trial initiation time and a change in bias through the day. Future experiments using this protocol should be sensitive to these diurnal patterns and experimenters

may wish to extend the testing period for particularly morning-active individuals in order to decrease the total number of days spent in the chamber.

3.4.4 Conclusion

Here we found differential learning of the Go and No-Go stimuli, which we suggest supports the notion that Go and No-Go stimuli are learned separately. This differential learning could be caused by a range of factors, and advocate conservative metrics for establishing a learning criterion. Additionally, we posit that the No-Go responses likely reflect two separate cognitive processes and recommend that in future, researchers limit the response window to 3000 ms after stimulus presentation. We also found great individual differences in trial initiation timing patterns and that slower learning birds preferentially initiate trials in the morning compared to faster learning birds. The causal relationship between learning rate and photoperiodic activity remains unclear.

Chapter 4

Birds respond similarly to passive acute playback of songs associated with reward and punishment

Responses to Go/No-Go stimuli in the context of operant conditioning can be simply assessed with whether the subject produced the Go or the No-Go response. However, this does little to inform us of the effect of the stimulus on the behavioural state of the subject. Here we train 10 female zebra finches on a Go/No-Go task; after training and four days of maintenance of the Go/No-Go discrimination, we expose the birds to 10 minutes of acute song playback of either the reinforced or the punished stimulus. During this song playback, we video record the birds' behaviours, and analyse these using an array of statistical techniques. We find no evidence for differential behavioural response to the Go and No-Go songs through linear discriminant analysis, principal components analysis, or by comparing nested generalised linear mixed models. We conclude that motor/behavioural responses to acute song playback are therefore unlikely to be a major factor in differential gene expression studies using the same playback assay.

4.1 Introduction

In the previous chapter, I characterised the learning and maintenance of Go/No-Go discrimination in female zebra finches. In this chapter, I explore whether there are lasting differences in the spontaneous behavioural responses to the learned stimuli when they are encountered passively, in an unreinforced context. Behavioural responses to acute playback, without the need for birds to engage in operant conditioning for a food reward, could provide an understanding of the birds' affective or cognitive state. For example, an increase in behaviours associated with stress, such as puffing and flying towards the wall, could indicate a learned subjective valence if associated with just the No-Go song, or an effect of playback novelty if associated with both the Go and No-Go songs. Therefore, discriminable patterns of responses during exposure to an unreinforced, but previously learned, stimulus could aid in understanding the state of the subject, albeit with consideration that this approach has its limitations (Paul, Harding, & Mendl, 2005)

Behavioural responses to acute playback, without the presence of operant conditioning apparatus, can also aid in understanding the associations formed between the stimulus, response, and outcome. As described in Chapter 3, Go/No-Go conditioning goes beyond the simplest form of operant conditioning (i.e. a response-outcome association) and also includes the learning of a stimulus-response association, which is generally associated with classical conditioning (Kirsch et al., 2004). Therefore, we are interested in whether the stimulus-response association continues when there is no immediate response-outcome pairing nor the hunger motivation to engage with the operant apparatus; evidence, for example, that birds peck at the sensor in response to the Go but not the No-Go stimulus would provide support for this hypothesis.

Many studies have established that female zebra finches learn song preferences based on early life experiences (N. S. Clayton, 1988; Holveck & Riebel, 2014; Lauay et al., 2004), but it is unclear whether adult life experiences can also shape song preference. Avoidance learning through operant tasks has been shown to strongly alter response to stimuli (Dalla & Shors, 2009) and for humans, reinforcement of stimuli with low subjective value can lead to subjects preferring the low-value stimulus in later choice trials (Schonberg, Bakkour, Hover, Mumford, & Poldrack, 2014). However, changes in preference for sexual stimuli such as songs, have not, to our knowledge, been demonstrated in adult female zebra finches. Behavioural evidence that females respond differentially to the Go and No-Go stimuli might

reflect a change in subjective valence, learned through the Go/No-Go task.

A secondary motivation arose from an allied analysis of brain gene expression patterns in those birds characterised in Chapter 3. Using RNA-Seq, George & Clayton (n.d.) found an upregulated oxidative phosphorylation gene expression signature in the auditory forebrain when birds were exposed to the No-Go stimulus compared to the Go stimulus, in an unreinforced context just prior to euthanasia. Oxidative phosphorylation drives cellular energy provision (C. N. Hall, Klein-Flugge, Howarth, & Attwell, 2012), and we reasoned that there might be differences in metabolic demand across the brain as a whole if there were gross differences in overt behavioural activity when birds encounter the two different stimuli. Alternatively, if the spontaneous behaviour patterns are similar in the two contexts, then the differences in gene expression may more specifically reflect the learned perceptual associations.

4.1.1 Aims and objectives

The differential behavioural response to Go and No-Go stimuli (i.e. pecking a sensor, or withholding that response) during training and active maintenance is evident. Here we aim to characterise the response to acute unsolicited playback of these stimuli after training occurs. First, we hypothesised that acute playback of the trained stimuli would result in different activity levels to silence. Second, we hypothesised that there would be more than one pattern of behaviours, with, for example, a positive correlation between alarm calls and puffing. Finally, we hypothesised that these patterns of behaviours, or behavioural states, would be related to whether the bird heard the Go or the No-Go stimulus.

4.2 Methods

4.2.1 Animals

10 female zebra finches (*Taeniopygia guttata*) bred at Queen Mary University of London were housed in a single sex aviary for at least a week before being placed singly into a sound attenuation chamber with an operant conditioning setup. The birds ranged in age from 1-3 years, but exact hatch dates were not available for most individuals. The birds were kept on a 16:8 light cycle. Animal housing and welfare were in compliance with the European directives for the protection

of animals used for scientific purposes (2010/63/EU) under Procedures Project License PPL70-8183.

4.2.2 Apparatus

The birds were housed in a sound attenuation chamber fitted with an operant conditioning cage (43 cm w x 46 cm d x 42 cm h). The cage had a solid floor and back, with mesh on the remaining four faces. The back of the cage contained the operant conditioning peripheral equipment: a motorised food hopper and two LED/peck detectors. A Jawbone Mini Jambox speaker was placed on top of the chamber. Two Genius WideCam F100TL USB cameras were also placed on top of the chamber to maximise the visible range of the video recordings. A Raspberry Pi automatically controlled the operant conditioning, including the food hopper, LED/peck detectors, speaker, and the chamber light. This apparatus setup was the same as used in the RNA-Seq experiment, with the addition of the cameras.

4.2.3 Stimuli

For all birds, the early training stages used the same male zebra finch song and sine wave tone. The final training stage involved four different songs, and each bird received two of these in a counterbalanced design: one as the Go stimulus and another as the No-Go stimulus (Table 4.1). These songs were matched for duration. All songs were from the population of zebra finches at the University of Leiden, and were therefore novel to the birds in this study. The song recordings were edited in Praat to include a 10ms on and off ramp (Boersma & Weenink, 2018).

Final song playbacks were created using Audacity, and consisted of one of the stimuli (either Go or No-Go) repeated once every 10 seconds for 10 minutes, for a total number of 60 song playbacks. This duration was chosen to balance the need for large changes in gene expression and the possibility that the birds' behavioural, and therefore neurogenomic, response to the song might be extinguished over many non-reinforced presentations. All stimuli were played at an SPL of 70 dB, measured using a Realistic sound level meter (Cat. No 33-2050, RadioShack) on the fast setting at the location where the bird's head would be after pecking the response sensor. Each bird received playback of both their Go and No-Go songs, counterbalanced so half of the birds heard a Go song on Day 1 (most matched to

Table 4.1: Training and playbacks for all ten individuals. Each song was recorded from a different male.

Training		Playback	
Go	No-Go	Day 1	Day 2
A	B	A	B
B	A	A	B
A	B	B	A
B	A	B	A
C	D	C	D
D	C	C	D
C	D	D	C
D	C	D	C
A	B	B	A
B	A	B	A

the original RNA-Seq study), and half of the birds heard a Go song on Day 2, after already having been exposed to a No-Go song on Day 1.

4.2.4 Operant conditioning

The operant conditioning protocol was the same as that used in Chapter 3.

4.2.5 Final playback

The afternoon before final playback, the birds were taken off of the operant conditioning and again allowed *ad libitum* access to food. The following morning, between four and six hours after the lights came on, the camera began recording video of the bird’s activity for at least 10 minutes. Then the 10 minute playback was initiated. The camera stopped recording 20 minutes after the end of song playback, which was the point of death in the RNA-Seq experiment. If the bird had another playback planned for the following day, the bird then resumed operant conditioning until later in the day, when the same pre-playback procedure was followed. If the bird had completed its playbacks, it was returned to the aviary.

4.2.6 Video analysis

The videos were coded using the BORIS software for behavioural observation (Friard & Gamba, 2016). An “ethogram” was designed with 12 behaviours:

pecking at the sensors, feeding, drinking, scooting (a movement along the same horizontal surface), hopping (a vertical movement), freezing, hugging the wall, preening, calling, alarm calling, puffing, and poking (pecking anywhere except the sensors). These behaviours were selected to cover as much of the spectrum of avian behaviour in the sound chambers as possible, with a focus on behaviours that might vary depending on the playback condition. A coder naive to treatment conditions, Joelle Clayton, coded the videos on her MacBook Pro. The BORIS software saved a time stamp for the initiation of each manually coded behaviour along with the behaviour ID. These data were then reformatted for statistical analysis.

The resulting data included the number of incidences of each behaviour, the individual ID, whether the recording was from day 1 or 2 for each bird (day), whether the behaviour was performed before, during or after the playback (period), the song ID, and whether the playback song was a Go or a No-Go song for that individual (condition).

4.2.7 Statistics

All statistics were carried out using the base stats package in R v3.3.3 unless otherwise stated.

4.3 Results

4.3.1 Overall activity is similar for both Go and No-Go playbacks

Across all of the recordings, 33583 unique behaviours were logged (mean per individual = 4061, sd = 1904). Of these, only those falling in the 10 minutes before playback, the 10 minutes during playback, and the 10 minutes immediately after playback were included (mean = 3010, sd = 1494; Figure 4.1).

To determine if there was an overall difference in activity level, all behaviours for each individual/period/condition combination were summed and subjected to a generalised linear mixed model (GLMM) with a Poisson error distribution and log link (lme4 package, R). This provided the best model fit as assessed by visual examination of modified qq-plots of residuals (DHARMA package, R), and also

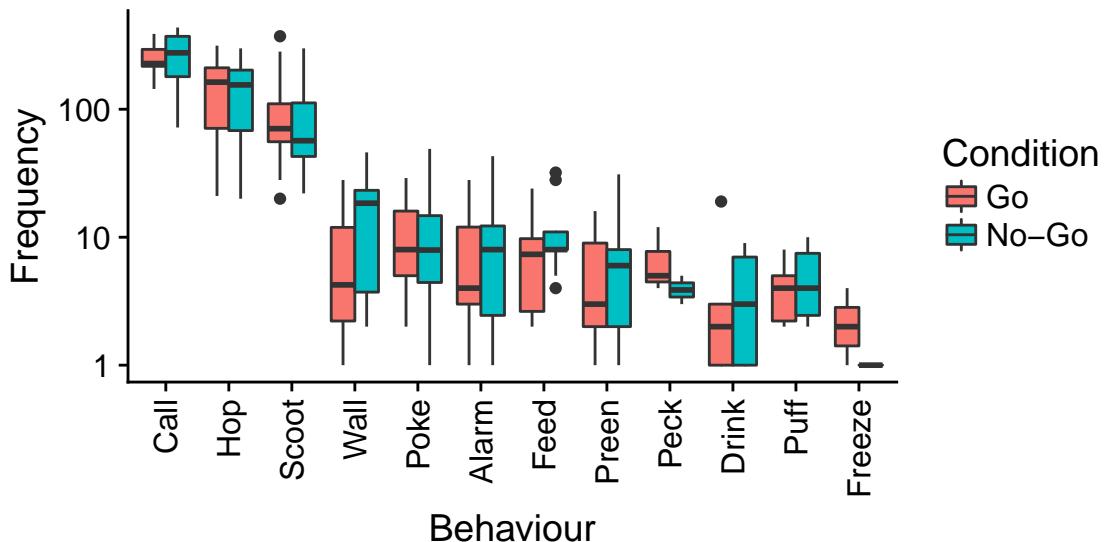


Figure 4.1: Number of times each behaviour was performed during and after playback, by condition.

Table 4.2: GLMMs for total incidences of all behaviours.

Model	Factors	df	AIC	Log-likelihood	Comparator	χ^2 test	P ($> \chi^2$)
NULL	Day + (1 Individual) + (1 Obs)	4	655.8	-323.9			
1	NULL + Condition	5	656.8	-323.4	NULL	>0.99	0.32
2	NULL + Period	6	658.6	-323.3	NULL	1.25	0.53
3	Model 1 + Period	7	659.4	-322.8	Model 1	1.28	0.53
4	Model 3 + Condition:Period	9	662.4	-322.1	Model 3	1.12	0.57

makes statistical sense as Poisson distributions are often used when modelling count data. Overdispersion due to zero-inflation was accounted for by including an observation-level random factor. As modelling zero-inflated data using observation level factors can sometimes lead to an increase in model bias (Harrison, 2014), dual binomial/Poisson models were fitted using an expectation-maximisation algorithm to separately model the zero-likelihood and the Poisson distribution (e.g. Bolker, Brooks, Gardner, Lennert, & Minami, 2012). On the basis of both DHARMA-modified qq-plots of residuals and Aikake information criteria (AIC), these models did not fit the data as well as the simpler models presented below. As well as an observation-level random factor, the null model also contained a fixed effect of day (to control for any effect of the within-subjects counterbalanced design) and a random effect of individual. ANOVA comparisons of GLMMs demonstrate that the inclusion of condition, period, and an interaction between condition and period do not significantly improve the model fit (Table 4.2). Therefore, there are no significant main effects of condition or period, nor is there an interaction between condition and period on behaviour counts.

A Levene's test on the log-transformed data did not support the visual suggestion (see Figure 4.2) of less variance during the playback period than before or after

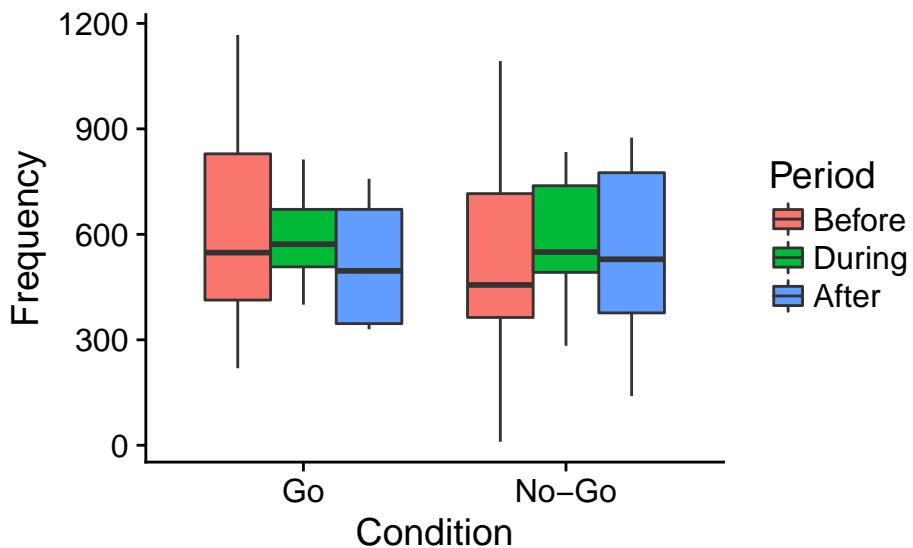


Figure 4.2: Total activity level by condition and period.

after playback ($F(2, 42) = 1.81, p = 0.18$; car package, R).

4.3.2 A linear discriminant analysis does not successfully classify playback conditions

In order to determine if the differences in overall levels of activity were being driven by a subset of behaviour types, a linear discriminant analysis (LDA) was applied to the behaviours performed during and after playback (MASS package, R). On a correct cross validation challenge, the LDA performed slightly worse than chance, correctly categorising the individuals by condition 43.3% of the time (chance = 50%). Given that the activity levels during the period before playback varied by condition, another LDA was run on the interaction between period (including before, during and after playback) and condition. This LDA also performed worse than chance at cross validation, categorising to the correct combination of period and condition 13.3% of the time (chance = 16.7%). These correct cross validation scores indicate that there is no difference in condition between behaviours.

4.3.3 Principal components do not discriminate between activity-related states

In order to determine if the 12 individually coded behaviours could be reduced to fewer dimensions, a principal component analysis (PCA) was carried out on the

Table 4.3: GLMMs for individual behaviour types.

Model	Factors	df	AIC	Log-likelihood	Comparator	χ^2 test	P ($> \chi^2$)
NULL	Day + (1 Individual) + (1 Obs)	4	3356	-1774			
1	NULL + Behaviour	15	3001	-1485	NULL	577	<2e-16
2	NULL + Condition	5	3558	-1774	NULL	0.0042	0.95
3	NULL + Period	5	3560	-1774	NULL	0.11	0.94
4	Model 1 + Condition	16	3002	-1485	1	0.0001	0.99
5	Model 4 + Condition:Behaviour	27	3014	-1480	4	11.2	0.43
6	Model 1 + Period	17	2999	-1483	1	5.4	0.068

log-transformed and scaled behaviour counts for each subject/condition/period combination. The first principal component (PC) (negatively loading calling, feeding, hopping, pecking, poking and scooting) explains only 28.7% of the variance (Figure 4.3). The second PC (negatively loading preening, puffing, scooting and wall, and positively loading feeding) explained 18.1% of the variance. 7 PCs were needed to account for 90% of the variance, suggesting that PCA does not provide simple effective dimensionality reduction for this data set. Further, the loadings for the PCs did not lend themselves to intuitive interpretation (e.g. PC1 strongly positively loading behaviours associated with stress, such as wall, alarm or puff).

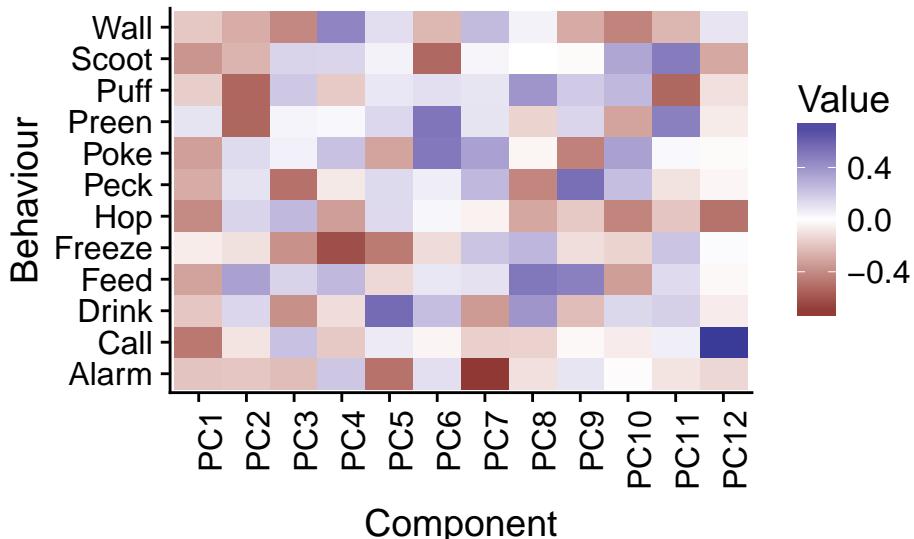


Figure 4.3: Loadings for the PCA.

A MANOVA on the first five PCs (selected on the basis of the standard deviations of the PCs) indicates a main effect of period ($F(10, 72) = 2.22, p = 0.026$), no main effect of condition ($F(5, 35) = 0.262, p = 0.93$) and no interaction between condition and period ($F(10, 72) = 0.179, p > 0.99$). A plot of PC1 versus PC2 (Figure 4.4; Panel A) and of PC1 versus PC3 (Panel B) demonstrates that there is no clear separation between conditions.

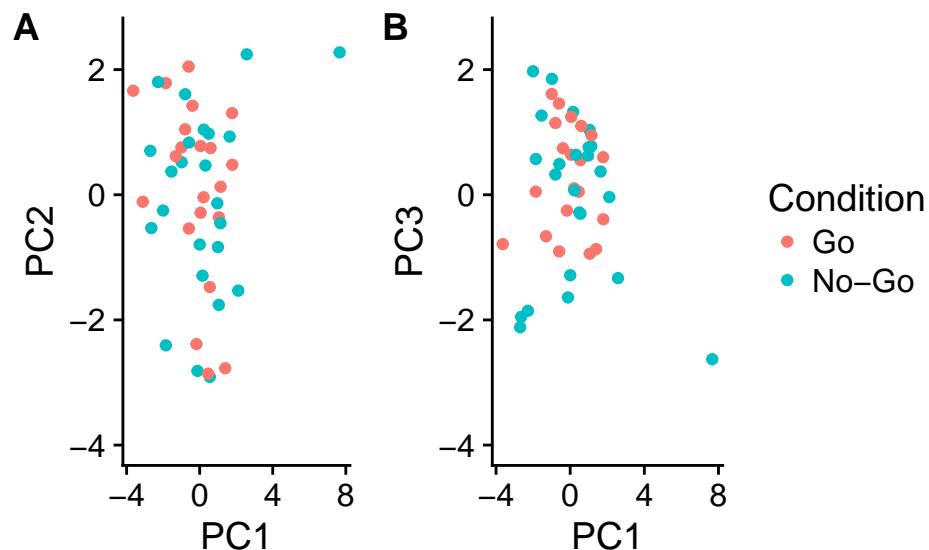


Figure 4.4: Principal components plotted against each other. A) PC1 plotted against PC2. B) PC1 plotted against PC3.

4.3.4 No individual behaviours vary by condition

Finally, to determine if any individual behaviours varied by condition, a GLMM with a Poisson error distribution on the number of instances of each behaviour was carried out, with fixed effects of behaviour type (i.e. alarm, call, etc.), condition and period, and random effects of individual and observation-level to reduce the bias caused by overdispersion (lme4 package, R). Nested model comparisons indicated a main effect of behavior, no main effect of condition, no main effect of period, and no interaction between condition and behavior (Table 4.3). We were unable to test for an interaction between period and behaviour or for a three-way interaction between period, behavior and condition due to our sample size causing rank deficiency. The main effect of behaviour was driven by calling, hopping and scooting all occurring more frequently than any of the other behaviours.

4.3.5 Individual differences in behavioural responses

Although we did not find any significant differences in patterns of behaviour between conditions, we did find individual differences in behavioural responses to the song playback (Figure 4.5). For example, the most active individual (Bird 16_1) had over twice as many recorded behaviours during the song playback as the least active individuals (Birds 16_6 and 51_15). Additionally, Bird 15_16 was unique in scooting along the same perch more than hopping from one perch to another.

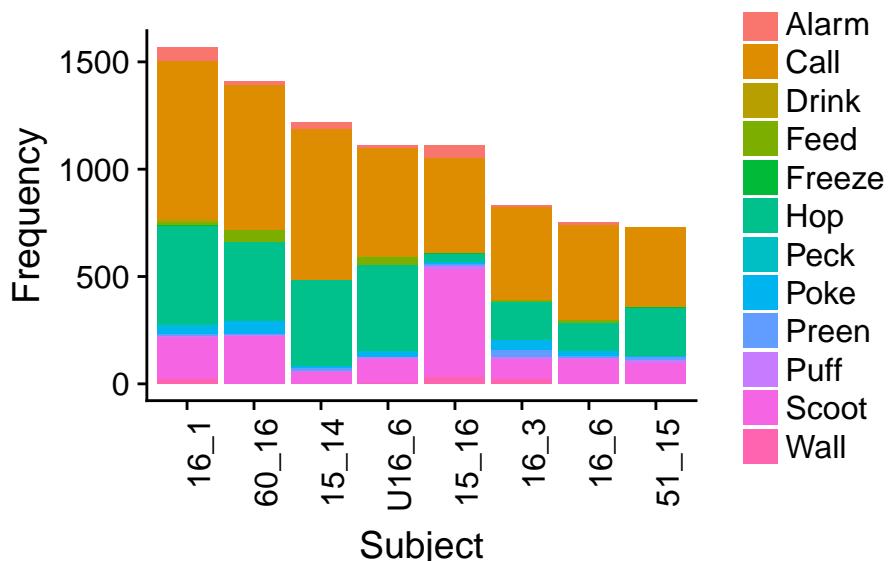


Figure 4.5: Individual differences in behavioural response to song playback.

4.3.6 Power analysis

In order to determine how much power our experiment had to find differences between conditions, we ran a power analysis on Model 1 from Table 4.2 ($\alpha = 0.05$, 1000 iterations; simr package, R). We found that with our sample size we had 78% power to detect a medium effect size (0.5) of condition and 99% power to detect a large effect size (0.8) of condition. Therefore, we did not have enough power to detect small effects of the total number of behaviours.

4.4 Discussion

Here we found no evidence to suggest that overall activity levels in response to song playback vary depending on the previously learned association of that song. Therefore, activity levels are unlikely to have driven the difference in oxidative phosphorylation-related gene expression in a previous study. Given the relatively small sample size tested, we cannot rule out a small but significant effect of Go versus No-Go condition; however, the methods employed here would have been likely to discover a medium or large effect, which was not supported.

4.4.1 No evidence for an acute response to song presentation

Our most fundamental finding is that there is no overall change in activity levels during presentation of a conspecific song from the previous silence. Further, there is no interaction between behaviour type and period, suggesting that no individual behaviours change in response to acute song presentation. This finding is in contrast to a previous study, where birds were found to freeze for 1-2 minutes upon presentation of a conspecific song after previous habituation (Stripling, Milewski, Kruse, & Clayton, 2003). This should perhaps not be surprising, as the habituation procedure used by Stripling et al. (2003) differed from ours. In their case, birds were placed in a sound attenuation chamber overnight before being presented with three hours of song playback. The following day, birds were again presented with the same song. In contrast, the zebra finches in the present study had been placed in the sound attenuation chamber for at least two weeks, and had been exposed to the songs for at least five days. We therefore propose that the birds in the present study were more behaviourally habituated to the song presentation than are birds in traditional habituation experimental designs (e.g. Kruse et al., 2004), despite the change in song initiation (i.e. from bird-solicited during training to passive exposure during the playback).

4.4.2 No evidence for clusters of behaviours

We also found no evidence that, with our set of behaviour definitions, birds perform patterns of behaviour that can be interpreted as discrete behavioural states. Further, a dimensionality reduction approach did not indicate that behaviours traditionally associated with a positive or neutral state (i.e. feeding, calling, drinking) cluster separately from behaviours traditionally associated with a negative or stressed state (i.e. flying to the wall, puffing). This can be contrasted with the playback of conspecific dominance interactions to corvids, where dimensionality reduction produces components associated with activity, vocalisation, and stress that vary by treatment type (Massen, Pašukonis, Schmidt, & Bugnyar, 2014). Instead, we find that after being socially isolated in a sound attenuation chamber for a few weeks, patterns of behaviour do not vary consistently between individuals.

Additionally, we found no evidence that the presentation of a song associated with reinforcement elicits a different pattern of behaviours than a song associated with punishment. Though we did not explicitly test birds' preference for the songs,

the birds' behaviour does not indicate that they learned a preference for the Go song over the No-Go song. Future experiments should explicitly test whether the birds learn a preference (e.g. through a stereotaxic or operant design), and therefore whether the songs can be said to have taken on a valence. However, to date, there is no evidence that operant conditioning alters the perceived valence of a conspecific song for female zebra finches. Further, we did not find any evidence for a classical conditioning-like effect on behaviours as the Go playback did not elicit an increase in pecking and/or feeding behaviours, and the No-Go playback did not elicit an increase in freezing behaviour.

4.4.3 Implications for interpretation of gene expression studies

We suggest that our findings indicate that our operant conditioning training and maintenance experimental design, followed by passive exposure to a trained conspecific song, does not drive behaviour during song presentation. Therefore, gene expression studies that rely on this assay can conclude that it is unlikely that any differences between the Go and the No-Go condition are due to behavioural confounds. Specifically, an upregulation in oxidative phosphorylation in response to No-Go song playback can be interpreted as reflecting neural activity and not whole body activity (George & Clayton, n.d.). Additionally, ZENK *in situ* hybridisation on animals tested using the same methodology is unlikely to be affected by basal stress levels (Park & Clayton, 2002), as we've found no evidence for increased stress behaviours in response to No-Go song playback.

4.4.4 Conclusion

Here we found no evidence for discrete behavioural states among female zebra finches exposed to previously learned conspecific songs. We also found no evidence for an acute response to song playback. We suggest that the birds experienced the passive song playback passively, with no large shifts in behaviour during or after the song presentation. Further, we found that behaviour did not depend on the the previously learned association (i.e. the reinforcement or punishment) of the song. We conclude that neurogenomic shifts in response to this form of song presentation are unlikely to be driven by behaviour and instead represent a neural response to hearing previously learned song.

Chapter 5

***ZENK* gene expression in auditory forebrain after exposure to stimuli with different learned associations**

Increased expression of the immediate early gene *ZENK* has been used as a marker of both new memory formation, and recall or reconsolidation of old memories. The neuroanatomical pattern of *ZENK* expression following exposure to a particular stimulus may thus give insight into how that stimulus is represented in the brain. Here we ask whether the same acoustic stimulus might be linked to different patterns of *ZENK* activity in the auditory forebrain, depending on the associations the animal has already formed through previous exposure to that stimulus. 24 female zebra finches were trained using Go/No-Go operant conditioning to associate a song with either a food reward or a darkness punishment. After the animals learned to discriminate these songs, we analysed the neuroanatomical pattern of *ZENK* expression following passive exposure to either the Go (reinforced) song, the No-Go (punished) song, a novel song, or a song made familiar through repeated unreinforced exposure. Visual analysis of *in situ* hybridisation images revealed no consistent differences in the gross pattern of gene expression, nor did we detect any main effect of condition by quantitative analysis of pixel intensities in eight target regions within the auditory forebrain. However, applying a network analysis of covariance of *ZENK* expression across those eight regions of the auditory forebrain, we observed a more correlated pattern of expression in response to exposure to the Go stimulus compared to the three other stimuli. These results lead to two main conclusions. First, simple passive exposure to a

novel acoustic stimulus does not necessarily induce significantly greater *ZENK* gene expression than habituated or previously trained stimuli, if the stimulus presentation occurs in a neutral and familiar context. Second, the same stimulus may elicit subtle variations in the neural networks within the responsive brain regions, depending on the valence of previously learned associations.

5.1 Introduction

Many levels of neurobiological activity contribute to the encoding of experiences. Historically, studies have focused on synaptic plasticity (Dubnau et al., 2003), but recent research has highlighted the role of gene expression in memory formation (D. F. Clayton, 2000). Gene expression can be studied as either changing patterns of large ensembles of genes across a region (e.g. Dong et al., 2009), or as the fine anatomical distribution of single genes (e.g. Mello et al., 1992). Evidence shows that maps of single genes can tell us, for example, whether a canary heard a whistle or a guitar note (Ribeiro et al., 1998). Indeed, the same stimulus can induce differential patterns of gene expression in different contexts (Jarvis et al., 1995; e.g. Mello et al., 1995), and the distribution of the expression of a single gene can tell us about a recent exposure to a learned association (Wheeler et al., 2013). Could the neuroanatomical pattern of gene expression encode or reflect a previously established memory based on the valence of its association?

5.1.1 Immediate early genes are a valuable tool for investigating gene expression in response to the environment

The genomic action potential theory posits that immediate early gene (IEG) expression levels determine the likelihood of memory formation by mediating the translation of proteins involved in synaptic plasticity necessary for long-term memory storage (D. F. Clayton, 2000). These ideas are now well established among memory researchers, and the role of gene expression in the production of the memory engram is noncontroversial (Poo et al., 2016). The engram, or the physical changes in the brain that encode memories in response to external stimuli, has long been sought in individual brain regions (Josselyn, Kohler, & Frankland, 2015). However, there is little evidence that most memories are localised to one brain region and studies have shown that multiple brain regions are involved in the recall of fear memories (Tanaka et al., 2014). The development of new methods in recent years have allowed researchers to map cells that are known, on the basis of their IEG activity, to be active during fear memory formation (X. Liu et al., 2012). These same cells, if then simultaneously stimulated using optogenetics, can induce a freezing response in the subject without presentation of the initial fear-inducing stimulus (X. Liu et al., 2012). This study, and others like it (e.g. Tanaka et al., 2014), highlight the role of IEG-expressing cells in both memory

formation and recall.

The relationship between IEG expression and electrophysiology is often considered to be close enough to allow for the use of IEG expression as a proxy for neural activity (Kubik, Miyashita, & Guzowski, 2007). In songbird NCM, both electrophysiological activity and IEG expression habituate in response to repeated playbacks of the same stimulus, although the electrophysiological activity does not habituate to near-zero levels as does the IEG expression (Chew et al., 1996; Mello et al., 1995; Stripling et al., 1997). IEG expression also correlates with fMRI-measured BOLD responses to song stimuli, with similar patterns found in female zebra finch brains in response to male conspecific songs (Ruijssevelt et al., 2018). Given the relationship between electrophysiological activity and IEG expression, the neuroanatomical distribution of IEGs or their protein products has been used in many studies as a “read-out” of neural activity, which is sometimes referred to as IEG imaging (e.g. Ribeiro et al., 1998; Terpstra et al., 2006).

Given the role of IEG expression in learning, IEG imaging should perhaps be thought of as a proxy for plasticity-related activity (Minatohara et al., 2016). IEG expression, in high-level neuroanatomical regions, appears to represent the salience, or ethological relevance of a stimulus (Smulders & Jarvis, 2013). For example, in the auditory forebrain, expression of the IEG *ZENK* is higher when a stimulus is paired with a shock rather than when the stimulus/shock are presented independently (Jarvis et al., 1995). Additionally, conspecific songs induce higher levels of *ZENK* expression in the zebra finch auditory forebrain than heterospecific songs, which in turn induce higher levels of *ZENK* expression than sine wave tones (Mello et al., 1992). In contrast to these studies, where IEG expression is associated with the proposed salience of the stimulus in the context of active learning, IEG expression can also be induced by previously experienced stimuli: in one auditory forebrain region, the IEG protein product response to the presentation of the bird’s tutor’s song correlates with how accurately the bird learned the tutor song (Bolhuis, Hetebrij, Boer-Visser, De Groot, & Zijlstra, 2001), and in another auditory forebrain region IEG expression is higher when females hear their father’s song than when they hear novel songs (Terpstra et al., 2006). These studies indicate that as well as being elicited by novel stimuli, IEG expression may be elicited by exposure to previously learned stimuli that are no longer novel, but remain salient. This is in keeping with the evidence that IEG-expressing cells are involved in both memory formation and recall.

The precise role of IEG expression may vary across the brain, but the study of whole-brain patterns of IEG expression can highlight networks of brain regions

involved in responses to the stimulus of interest (Z. J. Hall et al., 2014; Teles, Almeida, Lopes, & Oliveira, 2015). The development of graph theory approaches to study the relationships between brain regions has allowed researchers to uncover statistical networks that may represent actual neural connectivity (Wheeler et al., 2013). For example, recognition of a well-known conspecific elicits denser connectivity among brain regions than recognition of a less well-known conspecific (Tanimizu et al., 2017). Additionally, graph theory approaches can highlight differences in functional networks even where linear modelling finds no main effect of condition on the gene expression for all regions of interest (Tanimizu et al., 2017). Within the zebra finch auditory forebrain, where there are a large number of reciprocal projections between regions (Vates et al., 1996), the use of graph theory can elucidate which of the regions respond in tandem.

5.1.2 Auditory forebrain as a collection of high level auditory processing areas

The auditory forebrain is a medial neuroanatomical region in the songbird brain shaped like a teardrop (Kruse et al., 2004). From rostral to caudal, it contains the caudomedial mesopallium (CMM), Field L2, and the caudomedial nidopallium (NCM). CMM and NCM function as auditory associative areas and are generally considered to store, at least partially, memory for conspecific song (Gobes & Bolhuis, 2007; Terpstra et al., 2006; S. C. Woolley & Doupe, 2008). Additionally, there are no clear boundaries between the medial CMM and the more lateral caudolateral mesopallium (CLM) nor between the NCM and caudolateral nidopallium (NCL) (Ikeda, Krentzel, Oliver, Scarpa, & Remage-Healey, 2017). Like CMM, CLM shows selective auditory responses (Gill, Woolley, Fremouw, & Theunissen, 2008), but NCL is sometimes considered to be less specifically involved in auditory perception and more generally involved in cognitive function (Güntürkün, 2005). Analysis of the entire auditory forebrain has highlighted large-scale shifts in gene expression in response to conspecific song (Dong et al., 2009; Gunaratne et al., 2011), but the formation and recall of operantly trained associative auditory memories may be mediated by any or all of the regions within the auditory forebrain.

5.1.2.1 Caudal mesopallium

The IEG response in CMM is known to respond to conspecific songs over heterospecific songs, and to show very little response to tones (Mello et al., 1992). Additionally, there is a greater *ZENK* response in CMM when female canaries are exposed to sexy syllables than non-sexy syllables (Leitner et al., 2005), and there is also a greater *ZENK* response in CMM when female zebra finches are exposed to female-directed song than undirected song (S. C. Woolley & Doupe, 2008). These studies indicate that CMM preferentially responds to preferred stimuli. However, this preference for high-quality song in CMM might require previous exposure to high-quality songs, and may not be an inherent part of the female song perception system (Lynch et al., 2017; Tomaszycki, Sluzas, Sundberg, Newman, & DeVoogd, 2006). Father's song induces greater *ZENK* expression in female zebra finch CMM than novel song, which may reflect either preference or novelty (Terpstra et al., 2006). Indeed, previous experience can dramatically modulate IEG expression in CMM. For zebra finches, the *ZENK* response in CMM habituates upon repeated presentation of the same conspecific song (Mello et al., 1995), but exposure to a novel conspecific song, or even to a change in the perceived spatial location of the previously habituated song, is sufficient to re-induce the *ZENK* response (Kruse et al., 2004). Additionally, pairing a song with lights that turn on and off in time with the song can re-induce the *ZENK* response, demonstrating that CMM is involved in more than purely auditory responses (Kruse et al., 2004). Visual presentation of a courtship stimulus, with no auditory component, can induce an intermediate *ZENK* protein response in CMM, which may be due to previously learned associations between the visual and auditory components of a courtship display (Avey, Phillmore, & MacDougall-Shackleton, 2005).

A series of studies have explicitly tested the role of CMM in processing previously learned stimuli. Gentner et al. (2004) found that, after learning to discriminate between rewarded and punished songs, starling CMM expressed the greatest *ZENK* induction in response to novel songs, followed by rewarded/punished songs. The authors argue that this indicates that CMM is involved in associative learning, but the results could also be explained by the familiarity of the stimulus. In contrast, Gentner & Margoliash (2003) found that the electrophysiological response in starling CMM was greater to familiar songs than novel songs, but that the response to songs associated with reward was also greater than to songs associated with punishment. More than simply the absolute response to rewarded/punished songs, starling CMM neurons encode more information about song motifs from rewarded songs than from punished or novel songs (Jeanne et al., 2011). In contrast, the

male zebra finch CMM electrophysiological response is greater to rewarded and punished songs than to novel songs, with no difference in the magnitude of the response between rewarded and punished songs (B. A. Bell et al., 2015). It is unknown whether these differences in CMM response to trained and novel songs are due to small differences in experimental design/statistical analysis or the species of the subject.

The boundary between CMM and CLM is as yet undefined, with the region between 1.0 mm and 2.7 mm from the midline especially unclear (Ikeda et al., 2017). In contrast to CMM, CLM has been studied in far less detail, but it does have a similar pattern of IEG responses as CMM to presentation of conspecific song (Mello & Clayton, 1994). CLM neurons receive projections from Field L1 and L3 (Vates et al., 1996) and other parts of the auditory forebrain (Shaevitz & Theunissen, 2007) and are therefore likely to preferentially process conspecific information or at least reflect the processing that occurs in other regions of the auditory forebrain. Where it has been explicitly studied, CLM has been shown to encode stimulus surprise, and it therefore might function “as a mediator of bottom-up attention” (Gill et al., 2008, p 2818). In contrast to CMM neurons, CLM neurons encode less information about whether songs were previously rewarded or punished (Jeanne et al., 2011). The specific role of CLM among the numerous reciprocal projections of the auditory forebrain has yet to be determined, but evidence does suggest a role for it in the mediation of attention to salient stimuli.

5.1.2.2 Caudal nidopallium

On the caudal side of Field L in the auditory forebrain lies the NCM. NCM, like CMM, exhibits greater *ZENK* induction in response to conspecific song than to heterospecific song or silence (Mello et al., 1992), and habituates in response to repeated presentation of the same conspecific song (Chew et al., 1995; Mello et al., 1995). But unlike CMM, NCM is posited to be the home of the tutor’s song engram for male songbirds (Pinaud & Terleph, 2008), and normal NCM function is necessary for female zebra finches to prefer high quality males (Tomaszycki & Blaine, 2014). For female zebra finches, familiarity, but not song quality, drives the *ZENK* expression in NCM, with unfamiliar songs eliciting greater *ZENK* expression (S. C. Woolley & Doupe, 2008). Similarly, for female canaries, *ZENK* expression in NCM is not driven by the sexiness of syllables (Leitner et al., 2005). Electrophysiological activity in NCM is greater in response to unfamiliar songs than it is to songs that have been previously trained to be associated with a reward or a punishment (Thompson & Gentner, 2010). It therefore appears as

though NCM preferentially responds to unfamiliar or novel stimuli.

However, NCM is a large region and many studies have highlighted differential patterns of response throughout. Most fundamentally, different syllables elicit different patterns of *ZENK* expression in subregions of canary NCM, with natural stimuli eliciting more easily discriminable patterns than artificial stimuli (Ribeiro et al., 1998). Dorso-caudal NCM neurons habituate more to repeated presentations of the same song than ventro-rostral NCM neurons (Chew et al., 1995). Additionally, for female white-throated sparrows exposed to conspecific song, *ZENK* expression is higher in dorsal NCM (dNCM) than ventral NCM (vNCM) and higher in medial NCM than lateral NCM (Sanford, Lange, & Maney, 2010). In one study of associative learning, vNCM neurons showed a strong increase in activity in response to unfamiliar songs over learned songs, whereas some dNCM neurons preferred familiar songs and others preferred learned (Thompson & Gentner, 2010). However, discriminable activity within NCM is not found in all studies; Gentner et al. (2004) found no significant change in *ZENK* expression in starlings along either the medio-lateral or the ventro-dorsal axis.

Studies of associative memory in songbirds have sought to address the role of NCM in the formation and recall of these memories. Thompson & Gentner (2010) found that electrophysiological activity in starling NCM correlates with the amount of exposure birds had to the associative conditioning, with neurons responding less to trained songs; novel and habituated songs elicited the same amount of firing, suggesting that NCM neurons “groove” to songs with associations, and that simple familiarity does not drive their activity. B. A. Bell et al. (2015) found that male zebra finch NCM responds differently: songs associated with a reward elicited a greater magnitude electrophysiological response than songs associated with a punishment, and novel songs elicited a somewhat intermediate response. And in another study of starling NCM, *ZENK* expression was greatest in response to novel song, with *ZENK* expression similarly lower for previously trained songs and silence (Gentner et al., 2004). Therefore, a range of evidence suggests that NCM may be involved in encoding or recalling associative memories, but due to variations in experimental design, it is unclear whether familiarity interacts with the valence of the associated memory (i.e. whether the stimulus was associated with a reward or punishment), and whether different subregions of NCM may have independent patterns of response.

Along the medio-lateral axis, there is no clear boundary between NCM and NCL (Ikeda et al., 2017). However, lateral to NCM (presumably ~ 1.0-1.5 mm from the midline) is a region (caudocentral nidopallium, NCC) where female-directed

song induces greater *ZENK* expression than undirected song; more lateral and more medial parts of the nidopallium do not show this distinction (Ruijssevelt et al., 2018). Lateral to the NCC is NCL, which is frequently likened to the mammalian pre-frontal cortex (Güntürkün, 2005) and is necessary for working memory in pigeons (Diekamp, Gagliardo, & Güntürkün, 2002). Therefore, careful consideration of the laterality of the *ZENK* expression signal is necessary in order to determine whether the region under investigation is involved in auditory or more general functioning.

To summarise, there is greatly conflicting evidence about the function of subregions in the auditory forebrain. In response to extreme treatment (e.g. silence versus repeated song), shifts in activity can be seen across the whole of the auditory forebrain (Dong et al., 2009; Mello et al., 1992). However, more subtle manipulations drive the regions differentially. Across a range of studies, CMM has been shown to respond more to high-quality songs than to low-quality songs (Leitner et al., 2005; S. C. Woolley & Doupe, 2008). However, this does not capture the range of CMM's processing capability, as it responds in complex ways to familiar songs (Terpstra et al., 2006) and songs that have been trained to have a positive or negative association (B. A. Bell et al., 2015; Gentner et al., 2004). NCM, in contrast, is generally not driven by the quality of songs (Leitner et al., 2005; S. C. Woolley & Doupe, 2008), but responds differentially based on familiarity (Thompson & Gentner, 2010). Additionally, there is evidence for a role of NCM in associative learning (Gentner et al., 2004). In order to determine if stimuli that have previously been trained to be associated with a reward or a punishment elicit different patterns of IEG expression in subregions of the auditory forebrain, it is necessary to carefully control both the training and the eventual presentation of the stimuli.

5.1.3 Aims and objectives

Here we will use Go/No-Go operant conditioning to train female zebra finches to associate one conspecific song with a reward and another conspecific song with a punishment. The presentation of conspecific songs is, itself, rewarding to female zebra finches (Holveck & Riebel, 2007), but the acute presentation of a food reward or darkness punishment will also become associated with the song stimuli. In contrast to previous studies (e.g. Gentner et al., 2004), we will not test birds during the ongoing operant conditioning procedure, but will instead present a passive playback following discrimination training. In this way, we aim to test the IEG response to the song presentation and not its involvement in discrimination

learning. Additionally, familiarity and song preference will not be confounded (as in e.g. Terpstra et al., 2006), and the rewarded and punished songs will be fully counterbalanced so that any effects are due to the learned association, and not due to acoustic parameters.

We aim to investigate whether the association of a song with reward or punishment determines the neuroanatomical distribution of *ZENK* gene expression in the zebra finch brain. We hypothesised that memory for differential Go/No-Go reinforcers would be stored as different patterns of neuroanatomical gene expression, which could be detected using *in situ* hybridisation on *ZENK*. We predicted that brain regions associated with reward and stress networks would differentially express *ZENK*. We further predicted that *ZENK* gene expression would vary by condition in regions in the auditory lobule. Specifically, we hypothesised that *ZENK* gene expression in the auditory lobule would be very low for birds in the habituated condition, and high for birds in the novel condition. We predicted that overall levels of *ZENK* gene expression in the auditory lobule for the Go and the No-Go conditions would fall in between that of the habituated and novel conditions, but that these would be separable by consistent patterning in CMM and NCM. Finally, we hypothesised that in the absence of consistent patterning of *ZENK* in response to Go and No-Go songs, we would find differential patterns of recruitment of regions within the auditory lobule, which could be detected using graph theoretical approaches.

5.2 Methods

5.2.1 Animals

24 female zebra finches were operantly trained, tested, and decapitated for *in situ* hybridisation at the University of Leiden. All birds were aged between 246 and 424 days post hatch and had not participated in previous experiments. The birds were housed in a single sex aviary on a 13.5:10.5 light:dark schedule. Throughout the experiment, water and cuttlebone were available *ad libitum*. Access to food was restricted to reinforcement of correct Go responses; the birds' health was monitored to ensure sufficient eating. The study was approved by the University of Leiden and complied with Dutch animal welfare regulations.

5.2.2 Operant conditioning

Zebra finches were trained using the same procedure as described in Chapter 3. Briefly, this entailed handshaping and/or autoshaping the birds to peck at both sensors, training the birds to peck first at the left sensor then the right sensor, and finally training the birds to learn the Go/No-Go discrimination using a song recorded at the University of Leiden as the Go stimulus and a 440 Hz sine wave tone as the No-Go stimulus. After learning the song/tone Go/No-Go discrimination, for three conditions, the Go and No-Go stimuli were swapped to the two final experimental songs. The birds were left to re-learn the discrimination until they reached a discrimination ratio (proportion correct responses to Go stimuli divided by the sum of the proportion correct responses to Go stimuli and the proportion incorrect responses to No-Go stimuli) of 0.80. For the fourth condition (habituated) the birds continued with visual operant conditioning with no stimulus swap (see Experimental design). They were then required to maintain this discrimination for 4 days to ensure that learning was well established and could not easily be extinguished.

5.2.3 Operant conditioning apparatus

Birds were housed for 3-4 weeks in mesh and plywood cages that contained operant conditioning apparatus (70 cm w x 30 cm d x 45 cm h). Each cage included a light controlled by the operant conditioning software, two LED/buttons, a food hopper to which access was limited by a motorised cover, and a water container (Figure 5.1). The cage was placed in a small sound attenuated room.

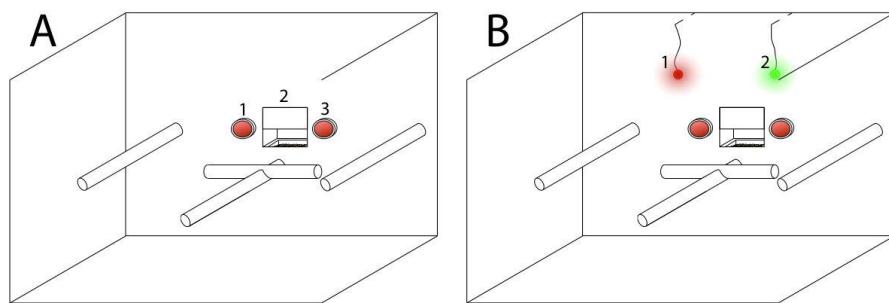


Figure 5.1: Diagram of the operant conditioning apparatus in the sound attenuation chamber. A) Setup for Go, No-Go, and novel conditions. 1 & 3 are sensors. 2 is the food hatch. B) Setup for habituated condition. Sensors and food hatch same as three other conditions. 1 & 2 are the stimulus lights.

Table 5.1: Go and No-Go training and playback stimuli for all conditions.

	Condition	Stimulus		
		Training	Testing	
		Go	No-Go	Playback
Condition	Go	A	B	A
	No-Go	B	A	A
	Novel	C	D	A
	Habituated	Red	Green	A

5.2.4 Experimental design

24 birds were allocated into 4 conditions, ensuring an even spread of ages in all conditions, for a total of 6 birds per condition. 4 birds, 1 from each condition, formed a set, and all birds within a set heard the same final playback. The conditions were defined by the final playback: Go, No-Go, Novel, and Habituated (Table 5.1). For example, for set 1, the Go condition bird was trained on Song A as the Go stimulus and Song B as the No-Go stimulus. Inversely, the No-Go condition bird was trained on Song B as the Go stimulus and Song A as the No-Go stimulus. Birds in the Novel condition learned songs C and D as the Go and No-Go stimuli. The Habituated condition varied from the previous 3 conditions in that the Go/No-Go stimuli were red and green LEDs, and not songs. The sound from either the paired Go or No-Go bird's chamber was live piped into the "yoked" Habituated bird's chamber. This ensured that the Habituated bird was exposed to the same acoustic environment as the paired Go or No-Go bird, but that those songs were not associated with reward or punishment. The LED-based operant conditioning ensured that the Habituated birds were in a similarly cognitively enriched environment as the birds in the other conditions. The final song playback for all birds in set 1 was Song A. Therefore, 4 birds (i.e. one bird per condition) all heard the same Song A playback, ensuring that any differences in behavioural or neural activity were due to the experience the bird had with that song and not with the acoustic structure of the song. 6 different playback songs were used to reduce pseudoreplication.

5.2.5 Stimuli

All songs were recorded in the Clayton aviary by McMahon and Dr Lachlan in 2014. In a two-sided cage with an opaque barrier down the middle, one male was placed in the left half and one female was placed in the right half. This cage was then moved into a large sound attenuated chamber fitted with sound recording

Table 5.2: Song pairs for training, where subscripts denote different male directed songs.

		Set					
		1	2	3	4	5	6
Condition	Go	A ₁ B ₁	C ₁ D ₁	A ₂ B ₂	C ₂ D ₂	A ₃ B ₃	C ₃ D ₃
	No-Go	B ₁ A ₁	D ₁ C ₁	B ₂ A ₂	D ₂ C ₂	B ₃ A ₃	D ₃ C ₃
	Novel	C ₁ D ₁	A ₁ B ₁	C ₂ D ₂	A ₂ B ₂	C ₃ D ₃	A ₃ B ₃
	Habituated	A ₁ B ₁	C ₁ D ₁	A ₂ B ₂	C ₂ D ₂	A ₃ B ₃	C ₃ D ₃

equipment. When the opaque barrier was removed, in order to allow the two birds to physically interact, the sound chamber was closed and the recording began. This elicited directed song from the male towards the female.

All of the songs were novel to the Leiden birds. Matched songs were selected to have equal durations (no more than +/- 10%) and to maximise human-perceived differences in syllable content. 12 songs were selected (4 for each condition) (Table 5.2). Praat software was used to introduce a 10 ms ramp up and down at the beginning and end of each song and to normalise the average intensity of the sound recording to 70 dB SPL (Boersma & Weenink, 2018). All songs were played at 70 dB SPL, measured using a Realistic sound level meter (Cat. No. 33-2050, RadioShack) on the fast setting at the location of the bird’s head after pecking a sensor. Final playback recordings were produced using Audacity 2.0.5. Each song was repeated once every 10 seconds for 10 minutes, for a total of 60 repetitions.

5.2.6 Tissue collection

To minimise between-condition differences of behavioural startling in response to song playback, the operant apparatus was turned off the afternoon before tissue collection and birds were given *ad libitum* access to food. On the morning of tissue collection, the final playback recording was initiated between 3 and 4 hours after the lights came on. The playback lasted for 10 minutes, followed by 20 minutes of silence. The 10 minute playback minimised the risk of extinction of the operantly-learned association due to repeated unsolicited song playback, and the total 30 minute duration from start of playback to decapitation maximised ZENK mRNA in response to song (Mello et al., 1995). After the period of silence, the birds were captured, decapitated, and the brain tissue was bisected laterally and placed with the medial side down into a mould containing OCT. The brain was covered with more OCT and the mould was immediately frozen in a dry ice and isopropanol slurry before being placed in -80°C for long-term storage. The

process of catching, dissecting and freezing took no more than 6 minutes.

5.2.7 Tissue sectioning

The right hemispheres of OCT-mounted brain tissue were removed from -80°C storage, placed in a Leica cryostat and allowed to equilibrate to -20C. Parasagittal sections were cut on the cryostat (with the assistance of Dr George). Three sections from each 100 μ m were collected from the midline to the distal edge. A total of ~144 sections were collected per hemisphere (i.e. 12 slides with 4 sections per slide, and 3 series of 12 slides) onto Superfrost Plus slides. Slides were fixed in a 3% w/v paraformaldehyde in PBS (pH 7.4) solution for 5 minutes before being briefly rinsed in PBS (pH 7.4), dehydrated in an ascending ethanol series (70%, 95%, 100%) for 2 minutes each, air-dried, and stored at -80°C.

5.2.8 In situ hybridisation

A well-established *in situ* hybridisation protocol was followed for the *ZENK* hybridisation (Carleton et al., 2014).

Riboprobes were prepared by obtaining plasmid (containing zebra finch *ZENK* cDNA from laboratory stocks). Plasmids were amplified in DH5 α cells using heat shock. Cells were then streaked onto LB agar plates with ampicillin, which were incubated at 37°C for 16 hours. Single colonies were selected using a pipette tip and used to inoculate a 5 mL LB/ampicillin media. The culture tubes were placed on a shaker for 12-16 hours at 37°C. Fresh *ZENK* stock was obtained from cell cultures using a plasmid purification kit (QIAPrep Spin Miniprep Kit). Plasmid samples were then tested on a Nanodrop to determine concentration and for quality control checking. Plasmid DNA was then sequenced using the Eurofins sequencing service and confirmed by BLAT-alignment against a recent zebra finch genome assembly using the UCSC genome browser.

20 μ m of plasmid DNA was linearised using a BssHII digestion. A PCR puification kit (GENEJet) was used to purify the cDNA from enzymes and salts. Antisense riboprobes were generated from the cDNA template in a solution containing 1 μ g T3 RNA polymerase, 1X digoxigenin(DIG)-11-UTP RNA labelling kit (Roche), 2 U/ μ L recombinant RNAsin, 1 μ g/ μ L BSA, 10mM DTT, and 1 μ g digested clone at 37°C for 2-3 hours. The riboprobe synthesis reaction was then equilibrated on a Sephadex G-50 column and stored at -80°C.

Slides were removed from -80°C and allowed to briefly thaw at room temperature. Each 24-slide hybridisation batch contained one slide from each bird. 14 total batches were conducted; three of these batches contained a *ZENK* sense riboprobe control. The slides were acetylated (TEA 1.35% v/v, acetic anhydride 0.25% v/v) for 10 minutes, rinsed three times in a 2X SSPE buffer, and dehydrated in an ascending ethanol series (70%, 95%, 100%; 2 minutes each) before being allowed to air dry. 16 µL of hybridisation solution (6.25% v/v purified riboprobe at 1 ng/µL, 1 µg/µL PolyA, 1 µg/µL BSA, 2 µg/µL tRNA, 2X SSPE, 50% v/v deionised formamide) was pipetted onto each section and sections were then coverslipped. Slides were loaded into a vertical slide rack and immersed into 65°C-equilibrated heavy paraffin oil. Hybridisation proceeded for 12-18 hours.

Following hybridisation, the slide rack was removed from the paraffin oil and transferred to three chloroform baths (2 minutes each) to remove remaining paraffin oil. Slides were left to slightly air dry before being placed into 2X SSPE for a few minutes to aid in coverslips falling off without damaging the tissue. The slides were then transferred into a solution containing 50% v/v 2X SSPE and 50% v/v formamide for 90 minutes with regular agitation. Slides were transferred into 65°C 0.1X SSPE for 30 minutes with regular agitation. This last step was repeated with fresh SSPE. Slides were then transferred to TNT buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.3% v/v Triton-X).

Slides were removed from TNT buffer, dried where necessary using cotton buds, and the area with sections was encircled with a PAP pen. TNB blocking buffer (100 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.36% w/v bovine serum albumin) with 0.1% v/v skim milk was filtered using a 0.22 µm syringe. 200 µL TNB was pipetted onto each slide. Slides were incubated in a humidified chamber at room temperature for 30 minutes. The blocking buffer was tipped off and 200 µL TNB blocking buffer with 0.1% v/v skim milk and anti-digoxigenin antibody (1:600) was pipetted onto each slide. Slides were incubated in a humidified chamber at room temperature for 2 hours. The antibody solution was tipped off and slides were washed twice in TMN (100 mM Tris-HCl (pH 9.5), 150 mM NaCl, 5mM MgCl₂) for 15 minutes each. Slides were then placed in Coplin jars containing 30 mL of filtered NBT/BCIP. The jars were protected from light and agitated for 12-20 hours. Colour development was checked, and when sufficient, slides were transferred to ddH₂O for 1 hour with agitation. Slides were then allowed to air dry before being coverslipped with VectaMount AQ mounting media.

5.2.9 Image analysis

Slides were digitally photographed using a Hammamatsu NanoZoomerslide scanner (objective x40). All remaining image processing was conducted using the Fiji distribution of ImageJ (Schindelin et al., 2012; C. A. Schneider, Rasband, & Eliceiri, 2012). Whole slide images were automatically segmented into 4 TIFF images, each with one brain section at object x10, using the ndpsisplit command (NDPITools plugin, Deroulers et al., 2013). Sections were manually selected to best represent regions of interest (ROI) within the auditory forebrain (a medial song-responsive region containing CMM, NCM and Field L), at 0.5 mm and 1.2 mm from the midline using the ZEBRA histological atlas as a reference (Oregon Health & Science University, 2013). Individual ROIs were specified using the base ImageJ ROI Manager (Figure 5.2).

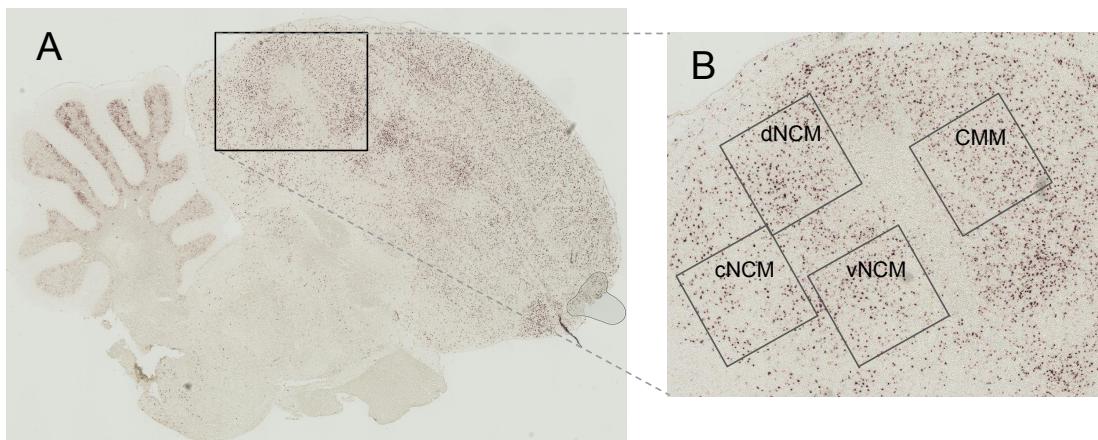


Figure 5.2: Neuroanatomy for region of interest selection. A) Parasagittal whole brain section, 1.2 mm from midline, where right is towards the beak. B) A zoomed-in image of the auditory forebrain region, with rectangular regions of interest placed as for the image analysis

CMM was represented by a ROI defined as a square (0.5 mm from midline: 400 μm x 400 μm ; 1.2 mm from midline: 600 μm x 600 μm) placed halfway along the rostral length of Field L, with one edge perpendicular to the long axis of Field L. Three ROIs, captured with squares (0.5 mm from midline: 400 μm x 400 μm ; 1.2 mm from midline: 600 μm x 600 μm), were placed within NCM to capture dorsal, ventral, and caudal regions. The dorsal NCM (dNCM) ROI was placed as dorsally as possible within NCM, with one edge perpendicular to the caudal long axis of Field L. The ventral NCM (vNCM) ROI was placed ventrally within NCM, with one edge perpendicular to the caudal long axis of Field L and with the ventral corner of the ROI placed at the ventral edge of Field L. For sections 1.2 mm from the midline, the caudal NCM (cNCM) ROI was placed

halfway between the dNCM and vNCM ROIs, with its most rostral edge aligned with the caudal edges of the dNCM and vNCM ROIs. For sections 0.5 mm from the midline, the cNCM ROI was placed halfway between the dNCM and vNCM with its most caudal edge placed along the caudal edge of the teardrop shaped auditory forebrain. The whole telencephalon was selected using the polygon tool. 25-35 points were manually selected around the visually identified edges of the whole telencephalon; these points erred on the internal side of the edge so as not to select slide background, and a straight line was drawn from the indentation under the occipital membrane to the indentation under the medial striatum in order to minimise the error associated with manually determining where the telencephelon/diencephalon boundary occurs.

Using the ImageJ Measure tool, the area of the ROI, mean/standard deviation/min/max/median pixel intensity (from 0 to 255, where 0 is black and 255 is white), and the skewness and kurtosis of pixel intensity were calculated. Pixel intensity measurements were then subtracted from 255 (the maximum possible pixel intensity) for ease of interpretation; in subsequent analyses, higher numbers for pixel intensity reflect more intense staining. These measurements were imported into R (v3.3.3; RStudio v1.0.136) for further data processing.

5.2.10 Graph theory

For each condition (i.e. Go, No-Go, Novel, Habituated), an undirected graph was produced (igraph package; R). The residuals from the null linear mixed model (the remaining variance once the data was normalised) from each ROI were correlated with model residuals from all other ROIs. Each ROI was modelled as a node, and for all correlations where $p < 0.10$, weighted edges were created between ROIs with the correlation coefficient (r) as the edge weight.

5.3 Results

5.3.1 Zebra finches learn to discriminate Go from No-Go stimuli

Zebra finches learn to discriminate between two conspecific songs when one is presented as a Go stimulus and the other as a No-Go stimulus (Figure 5.3). d' (a measure of sensitivity/accuracy from signal detection theory that is robust

to bias; calculated by subtracting the z-score of the false alarm rate from the z-score of the hit rate) reliably increases through learning (Figure 5.3; Panel A). The discrimination ratio (dr , a measure of accuracy used by the ten Cate lab at the University of Leiden; the hit rate divided by the sum of the hit rate and the false alarm rate) also increases through learning (Figure 5.3; Panel B). Habituated birds, who were trained using lights, appear to have a flat learning curve because they had already reached criterion at the time point when their paired bird was first presented with two conspecific songs. There is no significant difference between conditions in final discrimination performance (ANOVA on d' scores for the final 5 100-trial bins for each bird, by condition; $F(3, 19) = 0.27$, $p = 0.85$; ANOVA on dr scores for the final 5 100-trial bins for each bird, by condition; $F(3, 19) = 0.85$, $p = 0.48$).

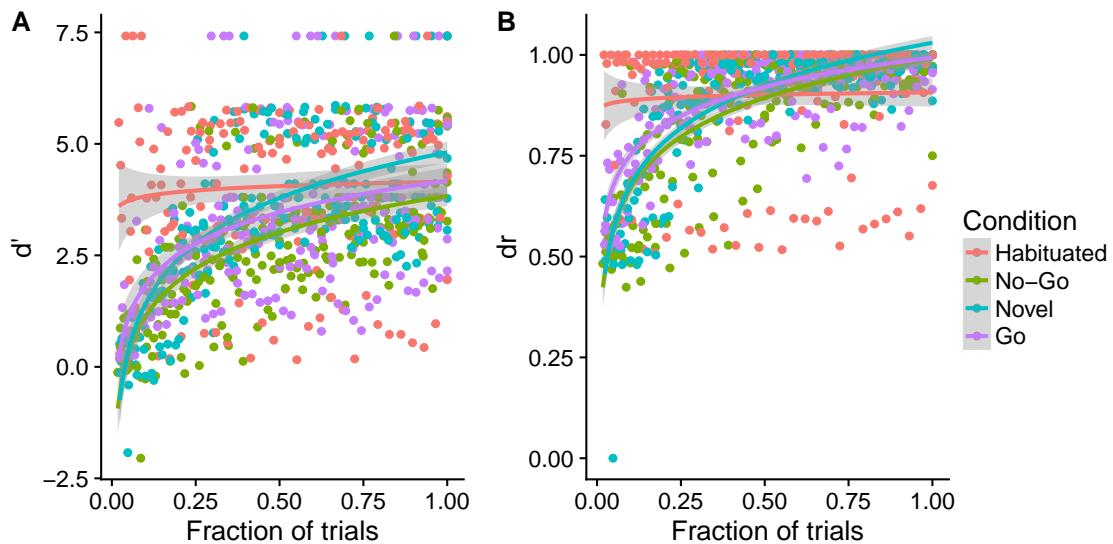


Figure 5.3: Learning and maintenance of Go/No-Go discrimination for all four conditions. X-axis is 100-trial bin number normalised across birds by divididng the bin number by the maximum number of bins for each individual bird. Y-axis is A) d' and B) discrimination ratio. Lines of best fit are logarithmic functions with standard error shading.

5.3.2 Visual inspection of matched sections

The hybridised section closest to 1.2 mm from the midline (matched using the ZEBRA Atlas (Oregon Health & Science University, 2013)) was manually selected for each bird and placed in a montage (Figure 5.4). Careful visual inspection of this selection of images did not reveal any obvious between condition differences. Subtle variations in the anatomical pattern of labeling throughout the brain are apparent when comparing sections. However, these variations do not visibly correlate with treatment conditions.

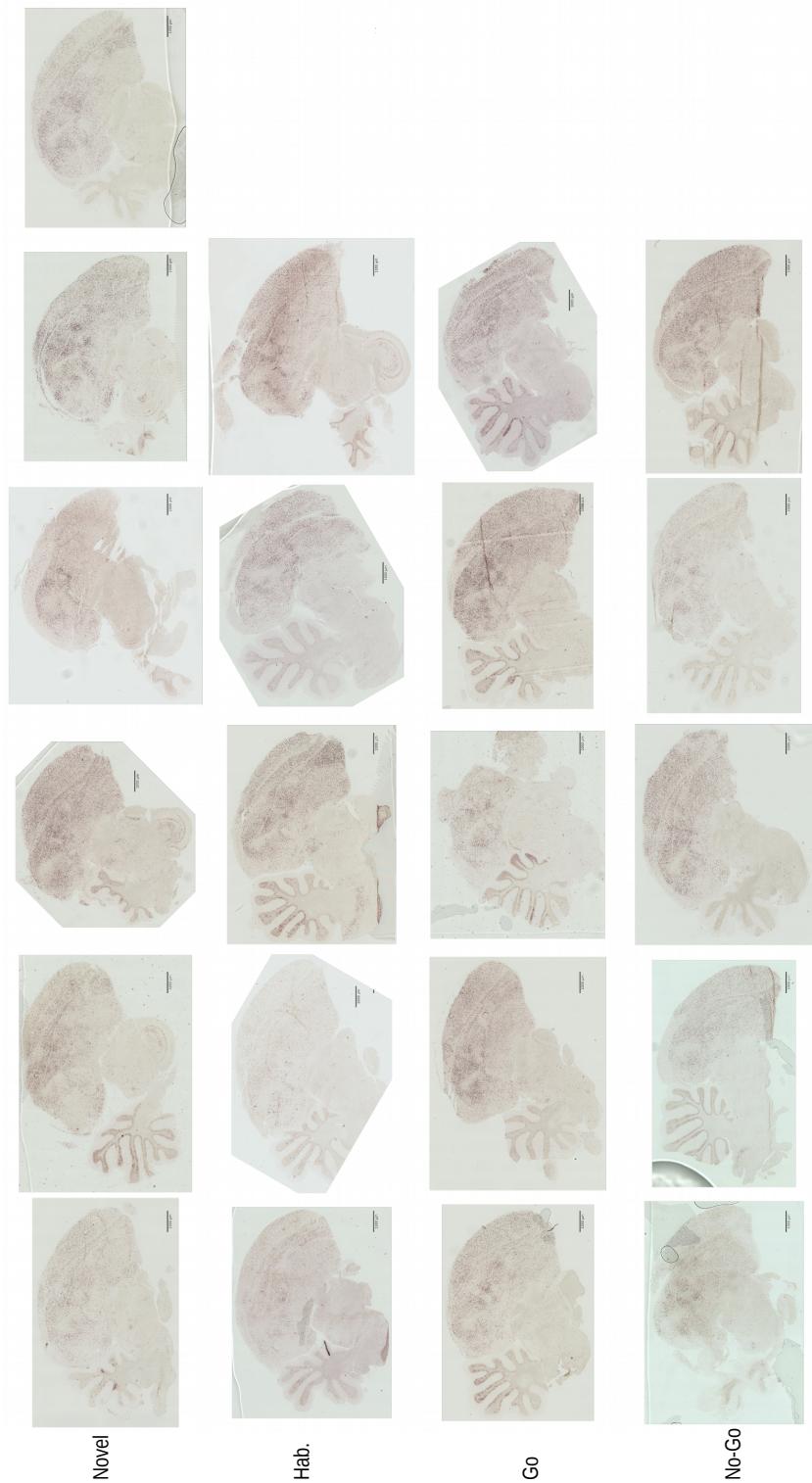


Figure 5.4: Right hemisphere parasagittal sections from each individual, 1.2 mm from midline.

For example, the pattern of expression in NCM is in some birds patchy (e.g. Novel column 5 and No-Go column 5) and in others more consistent throughout (e.g. Novel column 3 and Go column 1); these patterns of expression do not bear any obvious relationship to condition. Other regions at this level that varied between individuals, but not between conditions, were the dorsal medial arcopallium (ventral to NCM), medial striatum, and lateral striatum. Additionally, some individuals exhibited a distinctive pattern of staining in the granule cell layer in folia VIII/IX of the cerebellum, which was not explained by condition or song ID.

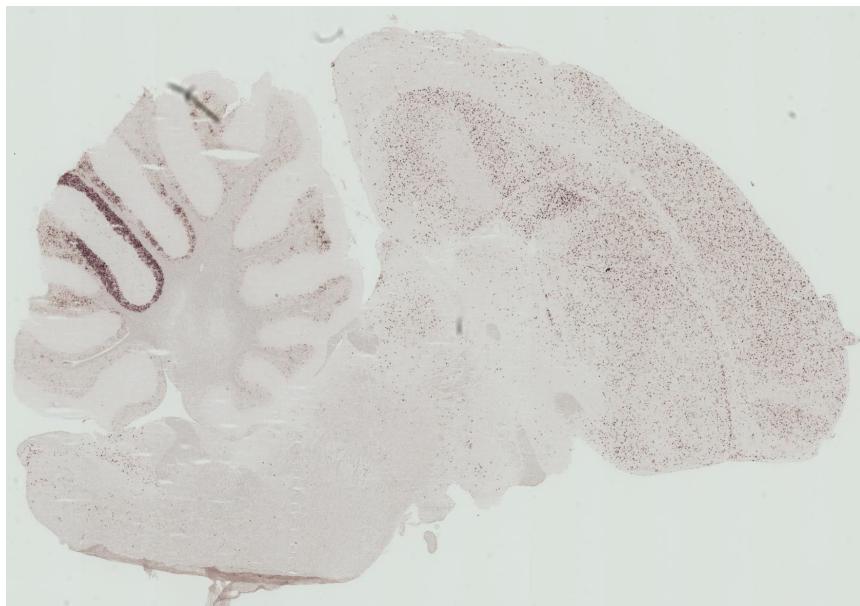


Figure 5.5: Dense staining in the granule cell layer of folia VIII/IX of medial cerebellum, 0.5 mm from midline, right hemisphere.

Unexpectedly, we found no visually discernable difference in the strength of the staining in the auditory forebrain between the novel and habituated conditions. Instead, all sections were similarly densely stained in CMM, and somewhat less densely in NCM, albeit with some individual differences that did not appear to relate to condition. Birds in the two experimental conditions (Go and No-Go) appeared to have the same overall level of staining in the auditory forebrain as the birds in the control conditions (Figure 5.6).

To evaluate the range of brain regions that expressed *ZENK*, a semi-quantitative assessment of regional staining was conducted for a subset of individuals ($n = 14$; 3 Novel, 4 Habituated, 3 Go, 4 No-Go); all hybridised sections for that individual were viewed and if any of those regions showed staining such that it caused that region to be identifiable (using the ZEBRA Atlas as a reference (Oregon Health & Science University, 2013)), that region was coded as expressing *ZENK*. If a region was not easily identifiable through its *ZENK* expression, then it was

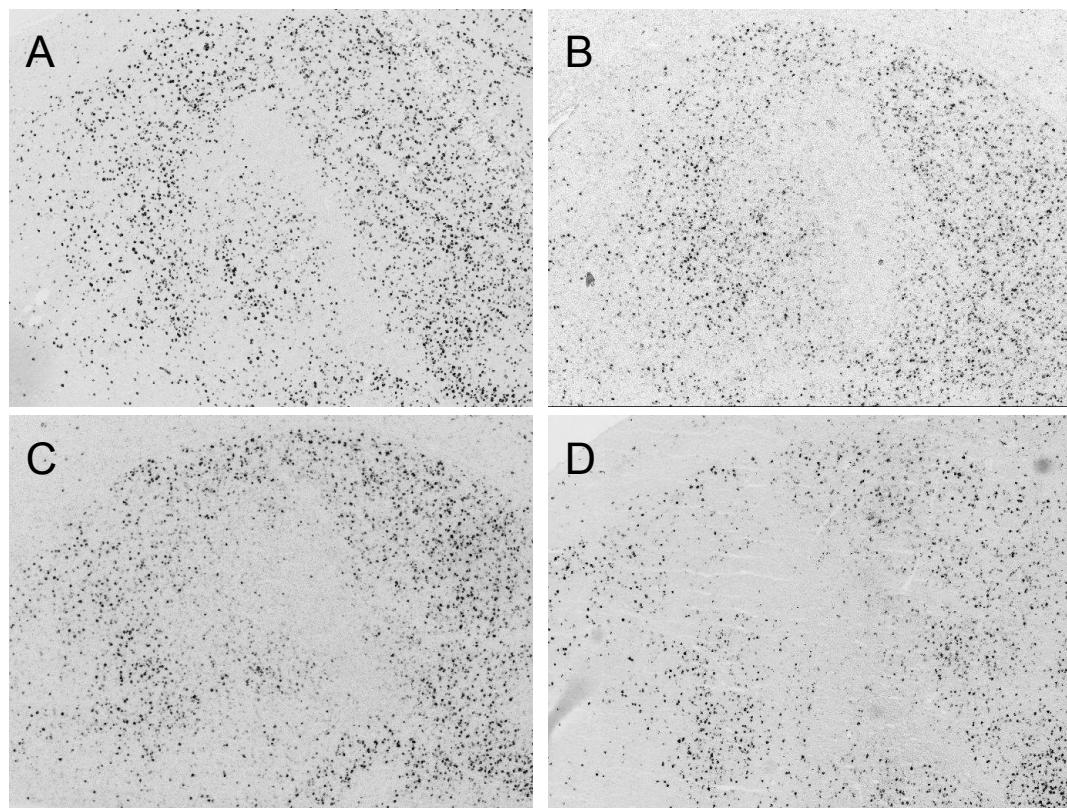


Figure 5.6: Right hemisphere auditory forebrain, 1.2 mm from midline. A) Go. B) No-Go. C) Novel. D) Habituated. All images are from representative birds, where overall staining levels are average for that condition.

coded as not expressing *ZENK*. The 16 regions of interest were: CMM, NCM, hippocampus, parahippocampus, HVC, nidopallium, lateral striatum, medial striatum, globus pallidus, dorsolateral corticoid area, entopallium, robust nucleus of the arcopallium, nucleus taeniae, dorsolateral nucleus of the anterior thalamus (DLM), intercollicular nucleus, and folia VIII/IX of medial cerebellum (Figure 5.7). With such a small sample size it is impossible to draw robust conclusions, but only the parahippocampus revealed “all-or-nothing” staining for one condition and not another (all birds in the novel condition exhibited parahippocampal staining, and no birds in the habituated condition exhibited parahippocampal staining).

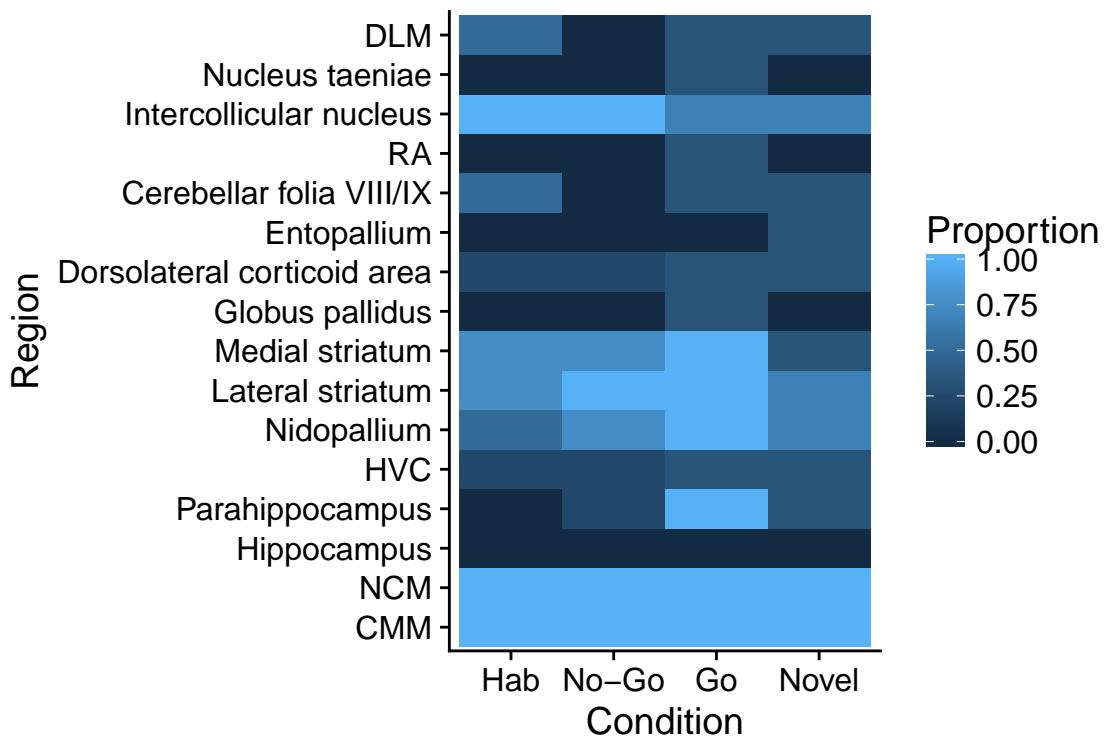


Figure 5.7: Proportion of individuals in each condition exhibiting clear *ZENK* expression in each brain region.

5.3.3 Quantitative analysis of *ZENK* signal intensities in the auditory forebrain

The distribution of pixel intensities for each ROI was determined to be non-parametric. For example, skewness values (third order moment about the mean) for each ROI were z-transformed and plotted against a red box indicating an acceptable range of skewness (H.-Y. Kim, 2013). As the vast majority of skewness scores fall outside the acceptable range, median pixel intensity values for each ROI were used as the response variable (Figure 5.8).

Nested linear mixed effects models (LMMs) on median pixel intensity for each ROI were carried out using lme4 (R package). The null model included median pixel intensity of the whole telencephalon (WholeIntensity) and a random effect of SongID (6 levels, each representing a different male's song). The inclusion of WholeIntensity as a fixed effect served to normalise the ROI pixel intensity to the overall telencephalon signal level. As the median pixel intensity of ROIs has a strong linear relationship to the WholeIntensity of the relevant image ($r^2 = 0.75$, $p < 0.0001$; Figure 5.8, Panel B), whole telencephalon median pixel density can be included as a linear predictor variable. Post-hoc tests indicated a significant main effect of median telencephalon pixel intensity ($t = -21.0$, $p < 0.0001$; lsmeans function from lmerTest package) but not of song ID ($\chi^2 = 2.21$, $p = 0.10$; rand function from lmerTest package).

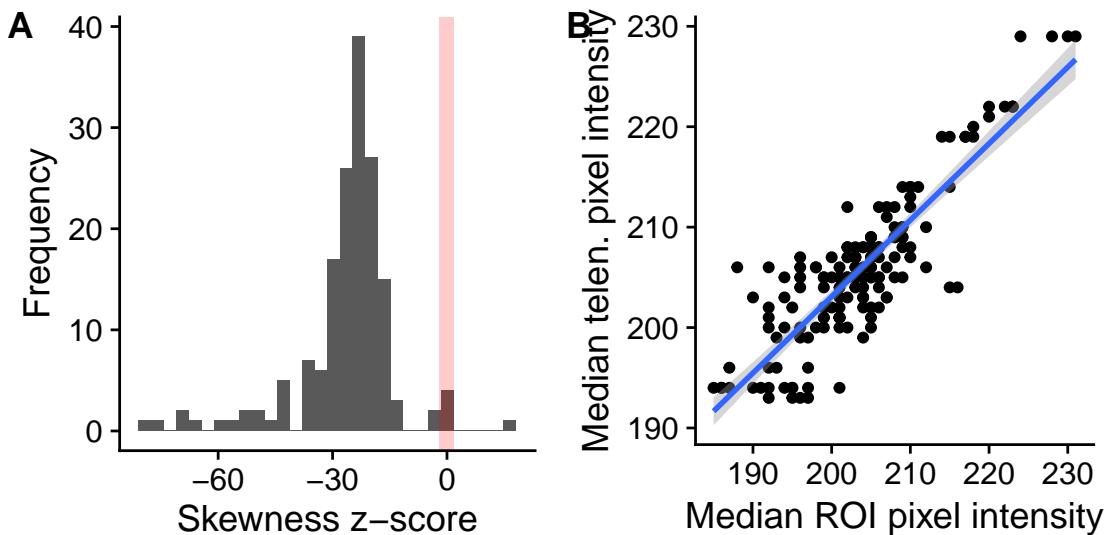


Figure 5.8: Model validation of GLMM. A) Distribution of skewness z-scores for ROI pixel intensity. The red rectangle indicates the acceptable range of skewness for small sample sizes. B) Linear relationship between median pixel intensity of ROI and of whole telencephalon.

LMMs including main fixed effects of condition (4 levels: Go, No-Go, Novel, Habituated), ROI (8 levels: medial CMM, medial dNCM, medial vNCM, medial cNCM; and lateral CMM, lateral dNCM, lateral vNCM, lateral cNCM), and an interaction between condition and ROI were also conducted. The best fitting model included a main effect of ROI, but not a main effect of Condition nor an interaction between the two (Table 5.3, Model 4; see also Figure 5.9). Nested model comparisons indicated only ROI increased the goodness-of-fit of the model; therefore, ROI is the only significant predictor of median pixel density. Post-hoc tests (lsmeans package, Tukey correction) on the best fitting model (Table 5.3; Model 2) show significant ROI differences between 14 of the 28 possible contrasts (all $p < 0.05$; Figure 5.9).

Table 5.3: LMMs for median pixel intensity of all target brain regions.

Model	Factors	df	AIC	Log-lik.	Comparator	χ^2 test	P ($> \chi^2$)
NULL	WholeMed + (1 SongID)	4	945.9	-469.0			
1	NULL + Condition	7	948.0	-467.0	NULL	3.92	0.27
2	NULL + ROI	11	886.0	-432.0	NULL	73.9	2.4e-14
3	Model 2 + Condition	14	885.2	-428.6	Model 2	6.80	0.079
4	Model 3 + Condition:ROI	35	906.0	-418.0	Model 3	21.2	0.45

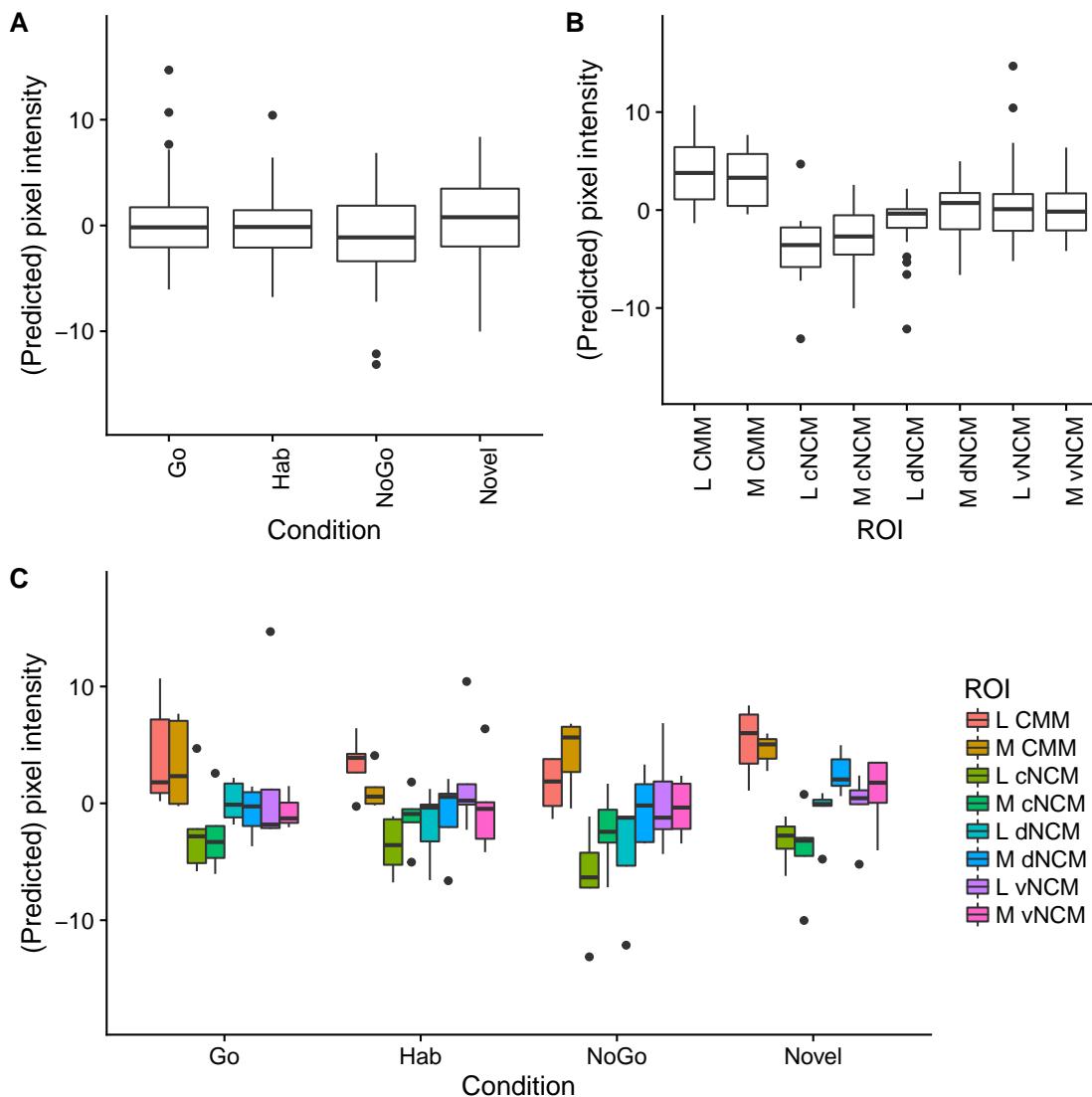


Figure 5.9: Median predicted pixel intensity (i.e. model residuals). A) Pixel intensity across all ROIs by condition. B) Pixel intensity across all conditions by ROI. C) Pixel intensity by ROI and condition.

5.3.4 Graph theory analysis of regional connectivity

Using a linear mixed model on pixel intensity, we found no significant main effect of condition, nor an interaction between condition and ROI. However, by visual inspection, we noted subtle but apparent variations in the fine anatomical pattern of ZENK labelling, despite the absence of evident effects on overall median intensities. To formally evaluate this, we therefore turned to graph theory to determine if the different conditions elicited different patterns of *ZENK*. We first created a graph from all conditions averaged together; vertices (nodes) were defined as the eight ROIs and edges (connections) were only those correlations between ROIs that were significant at $\alpha = 0.10$ (Figure 5.10). The edges were weighted such that the edge weights were set equal to the correlation coefficients. We found a sparsely connected network (with edge connectivity of 1) with seven edges, with lateral CMM and lateral cNCM as the most central vertices (edge_connectivity and degree functions, igraph package, R). All of the correlations between significantly correlated ROIs were positive.

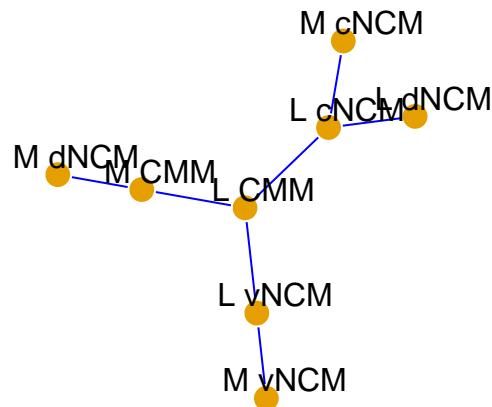


Figure 5.10: Graph of all ROI correlations where $p < 0.10$, across all conditions.

We then produced, for each condition, a graph using the same parameters (Figure 5.11). We found that the graph for birds in the Go condition was the most connected (edge connectivity = 2) and the novel and habituated conditions were the least connected (edge connectivity = 0). Lateral CMM was again the most central vertex for the Go condition. For the No-Go condition, lateral cNCM, medial CMM and lateral dNCM were the most central vertices. For the habituated condition, medial dNCM and lateral vNCM were the most central vertices. And for the novel condition, lateral cNCM, medial dNCM and medial vNCM were the most central vertices. All of the graphs were somewhat sparsely connected, with the Go condition easily the most connected (number of edges, Go: 15; No-Go: 4; Habituated: 4; Novel: 7).

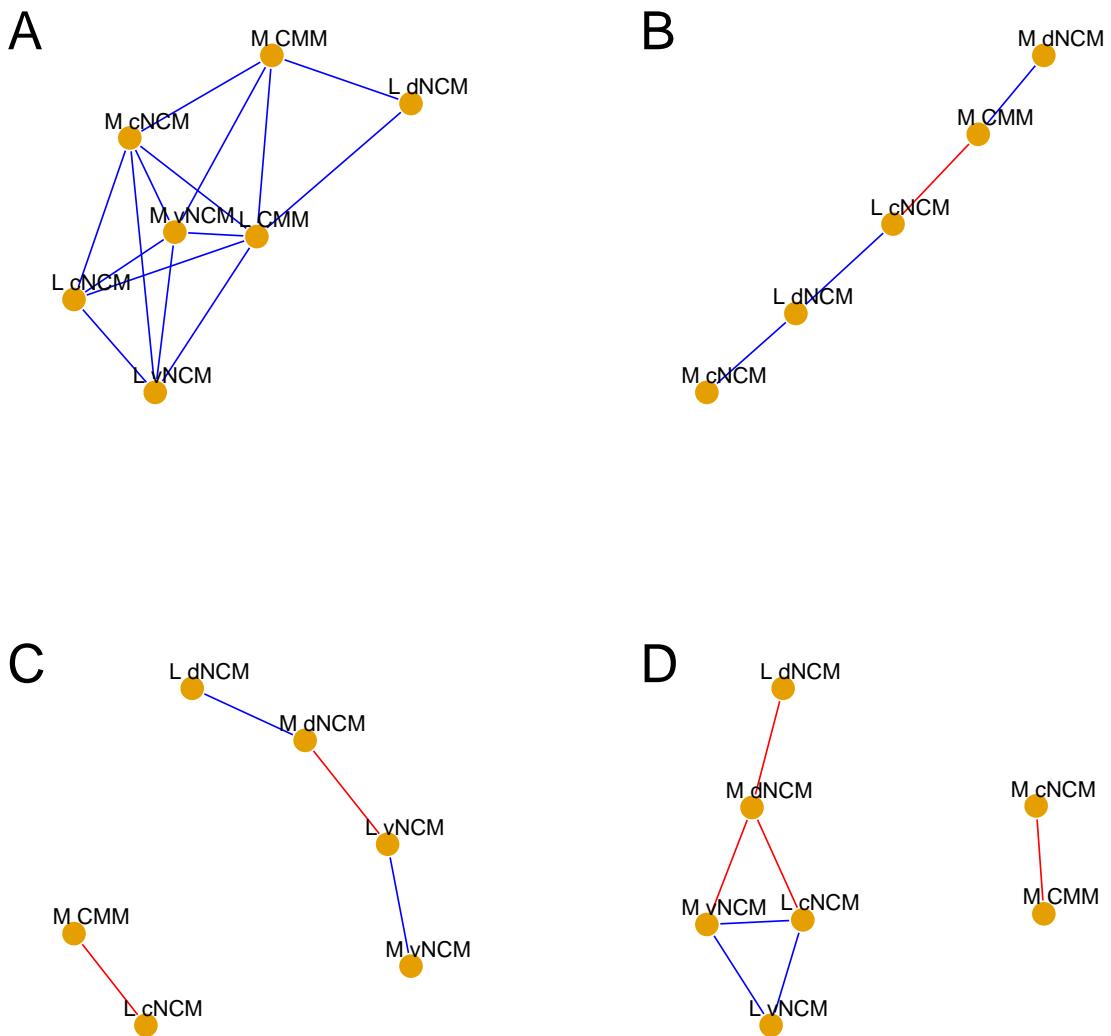


Figure 5.11: Graphs for each condition of all ROI correlations where $p < 0.10$. A) Go. B) No-Go. C) Habituated. D) Novel. Positive correlations have blue edges and negative correlations have red edges.

5.4 Discussion

Here we trained female zebra finches to associate one conspecific song with a food reward and another conspecific song with a darkness punishment. We then analysed the neuroanatomical pattern of *ZENK* expression following passive exposure to one of the two songs. We first demonstrated that birds did learn to discriminate between the Go and No-Go songs. We then highlighted patterns of individual differences in *ZENK* expression throughout the brain. However, using quantitative analysis, we found that these individual differences did not relate to the condition; that is, there is no clear difference in the overall level of *ZENK* expression in the auditory forebrain between the Go, No-Go, novel and habituated conditions. Finally, using simple graph theory, we did find evidence that the Go condition elicited a more coordinated response across the auditory forebrain than the three other conditions.

5.4.1 Individual differences bear no relationship to condition

Visual inspection of *in situ* hybridisation images revealed multiple regions where apparent individual differences were not explained by the condition. These included staining in the medial and lateral striatum, and the granule cell layer in folia VIII/IX of the cerebellum. *c-fos* expression in the striatum has been shown to be associated with nest building behaviours in male zebra finches (Z. J. Hall et al., 2014), and *ZENK* expression there may reflect planned motor behaviours, but if so, those behaviours are not produced in response to the song playback condition. The remarkably dense staining in folia VIII/IX of the medial cerebellum for some individuals, has, to our knowledge, not been previously characterised (but see Feenders et al., 2008 for evidence that widespread cerebellar staining is involved in hopping movements). Folia VIII/IX receive trigeminal (i.e. facial) input (Arends & Zeigler, 1989) and zebra finches have averaged sized folia, compared to other bird species (Iwaniuk, Hurd, & Wylie, 2007). The presence of *ZENK* expression in this part of the cerebellum could not be explained by condition or song ID, but could perhaps be related to pecking or feeding behaviour during song playback, which was not assessed here.

Additionally, the visual patchiness in NCM was unexpected, as many studies of conspecific playback find a more uniform distribution of cells expressing *ZENK* (Kruse et al., 2004; Lampen et al., 2014; Stripling, Kruse, & Clayton, 2001).

However, this finding is in keeping with the wealth of evidence for the non-uniformity of activity in NCM (Chew et al., 1995; Ribeiro et al., 1998; Sanford et al., 2010). We suggest that the non-uniformity of activity in NCM reflects the complex environment in which the birds were exposed to the song presentation. Indeed, the patchiness is more similar to that seen in response to heterospecific song (Stripling et al., 2001), noise (Park & Clayton, 2002), or unpaired shocks and conspecific songs (Jarvis et al., 1995). Additionally, the difficulty in selecting matched sections may have added to the perceived non-uniformity of *ZENK* expression in NCM across birds.

5.4.2 All conditions elicit similar levels of *ZENK* expression in the auditory forebrain

Quantitative analysis revealed that the intensity of *ZENK* staining in the auditory forebrain was consistent across all conditions, though there was a non-significant trend for low levels of *ZENK* expression in the No-Go condition compared to the three other conditions. The lack of significant difference in overall *ZENK* staining in the auditory forebrain between the novel and habituated condition was especially surprising, as we initially conceived the novel and habituated conditions to act as positive and negative controls, respectively. Previous literature has almost uniformly found a difference in *ZENK* expression in the auditory forebrain between novel and habituated song, where very little *ZENK* staining can be found in response to habituated song (Jarvis et al., 1995; Kruse et al., 2004; Mello et al., 1995; S. C. Woolley & Doupe, 2008). Unlike previous studies assessing habituation by direct repetition of the same stimulus in the same context, here our “habituated” stimulus was presented in a subtly novel context as it had a novel temporal organisation (i.e. one song steadily repeated every 10 seconds). In a post-hoc comparison using RNA-Seq methods, George (2018, pers. comm.) found that a separate cohort of female zebra finches exposed to Go and No-Go songs (the birds characterised in Chapter 3) had intermediate levels of *ZENK* gene expression compared to female zebra finches in overnight social/auditory isolation and females in an aviary (Figure 5.12). Though the data is from different birds, this provides evidence that all of the birds in the present study, including the habituated condition, exhibit an actual *ZENK* response to the song playback. We posit that the habituated condition may have been sufficiently novel to the birds, given the overnight silence and acute nature (i.e. one song every 10 seconds) of the playback. Though the birds in the habituated condition were accustomed to unsolicited playback of the song, the timing of those playbacks would have

been less frequent and more irregular. This change in context may have driven the *ZENK* response to habituated playback here (as in Kruse et al., 2004).

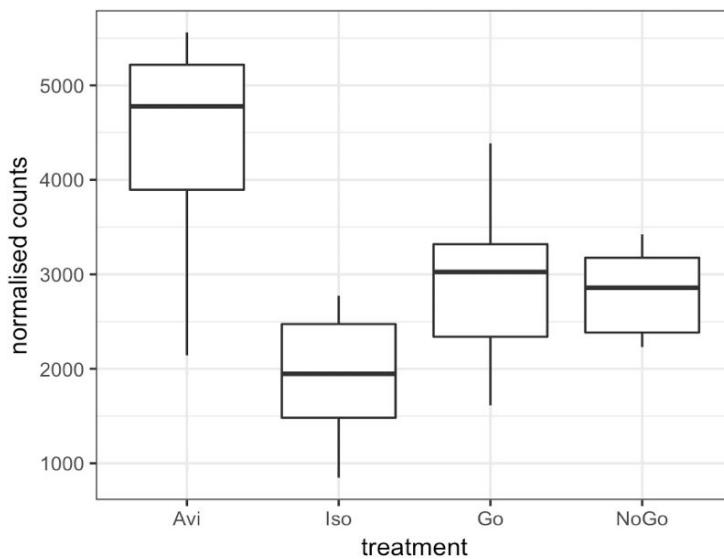


Figure 5.12: Normalised counts of *ZENK* gene expression in the auditory forebrain from two experiments. Aviary (Avi) and Isolated (Iso) are from George & Clayton, 2018. Go and No-Go are from the birds characterised in Chapter 3. Figure produced by J. George.

There was, however, across all conditions, a main effect of region of interest, where *ZENK* expression was highest in the lateral and medial CMM and lowest in lateral and medial cNCM. Along the medio-lateral axis, we found little evidence that medial (0.5 mm from the midline) and lateral (1.2 mm from the midline) parts of the same region varied. We therefore suggest that the region 1.2 mm from the midline is still part of the auditory forebrain, and that NCL/CLM begin more laterally. We did, however, find evidence that there is less of a *ZENK* response to all conditions in cNCM than rostral NCM (i.e. dNCM and vNCM). This is a similar pattern of response as found by Terpstra et al. (2006) when female zebra finches were passively exposed to their father's song, but it differs from the pattern of response they found when female zebra finches were passively exposed to novel song. The pattern of *ZENK* expression found here also does not match with that found in female white-throated sparrows in response to acute conspecific male song; Sanford et al. (2010) found greater expression in cNCM than in dNCM and vNCM, which is opposite to the pattern seen in the present study. It also contrasts with a study of conspecific calls in cowbirds, where *ZENK* expression was greater in NCM than CMM (Lynch et al., 2017). Our findings do, however, agree with two other studies of conspecific song playback to female zebra finches, where *ZENK* expression was denser in CMM than NCM (Lampen et al., 2014;

S. C. Woolley & Doupe, 2008). These diverse patterns of responses imply that the avian forebrain can recruit different gross patterns of activity in response to conspecific playback, but we still have no clear indication as to the cause or function of these.

We did not replicate the results of Gentner et al. (2004), where, for starlings, *ZENK* expression was greater in both NCM and CMM in response to novel song playback than in response to trained songs. In contrast to that study, we presented the songs passively, in a context where the birds were not being reinforced or punished for their behaviours. The starlings in Gentner et al. (2004) were engaged with the operant apparatus, and all of the stimuli, including the novel songs, were reinforced or punished using a Go/No-Go methodology. We believe that the increased *ZENK* expression in response to novel songs found by Gentner et al. (2004) may have been due to a combination of both active discrimination and exposure to novel conspecific songs, whereas our birds solely had exposure to novel conspecific songs. Multiple studies have conducted electrophysiological investigations of avian forebrain response to song playback after learning. All found that CMM neurons respond with increased firing rates or encode more data for rewarded songs than novel songs (B. A. Bell et al., 2015; Gentner & Margoliash, 2003; Jeanne et al., 2011). We found no evidence that *ZENK* expression is also increased in response to rewarded songs, which may be due to *ZENK*'s role in memory formation. Here we presented playbacks in a passive context where, as much as possible, we did not encourage any active learning about the stimulus, although we recognise that extinction learning may be occurring (e.g. Jarvis et al., 1995). *ZENK* expression may therefore not be increased in response to rewarded songs because the birds were not engaged in the formation or maintenance of memories.

5.4.3 Connectedness of the auditory forebrain varies by condition

Though we found no main effect of condition, nor an interaction between condition and region of interest, we had hypothesised that the regions within the auditory forebrain may respond as different networks, depending on the condition. An analysis of the statistical correlations of *ZENK* expression revealed that regions within the auditory forebrain responded in the most coordinated way to the Go songs. Compared to the three other conditions, the Go condition produced a more connected network; that is, in response to Go stimuli, the auditory forebrain

responded in a more uniform way. If *ZENK* expression was high in one brain region for one bird, it tended to be high in the other regions. Similarly, if *ZENK* expression was low in one brain region for one bird, it tended to be low in the other regions. Therefore, despite there being no overall increase in *ZENK* expression in response to the Go song, there was an increased tendency for the regions within the auditory forebrain to respond in sync with one another. In contrast, the three other conditions had fewer regions whose activity correlated with one another, and many of the correlations were negative. For example, for the No-Go condition, medial CMM activity was negatively correlated with medial dNCM activity. Fewer edges, and combinations of positive and negative correlations, both suggest that the regions in the auditory forebrain act more independently, and do not form a coordinated response to the No-Go, habituated, and novel songs.

One potential mechanism for producing a coordinated response to Go songs across the auditory forebrain is through catecholaminergic innervation. Catecholamines, especially noradrenalin, are hypothesised to modulate the differential IEG response to familiar and novel songs in the auditory forebrain (Matragrano et al., 2012; Velho et al., 2012). Additionally, evidence from a recent master's thesis indicates that experimental manipulation of dopaminergic activity in NCM can alter female zebra finch preference for song (Barr, 2017). Theoretically, widespread catecholamine release across the auditory forebrain in response to a rewarding stimulus could entrain multiple regions to respond with similar levels of IEG expression (D. F. Clayton, 2000).

Network analyses often attempt to find central vertices, or regions that correlate with many other regions. For the Go response, we found that lateral CMM was the most central vertex. Biologically, this indicates that lateral CMM drives or simply reflects the activity in many other regions in response to Go songs. In contrast, the No-Go, habituated, and novel conditions all produce networks that were too sparse to produce particularly central vertices, but lateral CMM did not correlate with any other regions in any of those three conditions.

5.4.4 Conclusion

Here we designed an experiment where we minimised, as much as possible, the confound of active learning in order to investigate passive perception of previously learned conspecific songs. We analysed eight regions in the auditory forebrain, which is the part of the brain most clearly involved in higher-order auditory processing. *ZENK* expression in these eight regions did not vary by condition,

with no difference in overall *ZENK* expression levels between Go, No-Go, novel, or habituated song playback. However, we found evidence for individual differences in *ZENK* expression, and therefore applied a network analysis to look for evidence of correlated shifts in expression associated with the four conditions. We saw evidence that the Go song playback drives a more coordinated response across the auditory forebrain than do the three other conditions. We conclude that although overall *ZENK* expression may not vary across the auditory forebrain, differential networks of activity are induced depending on the valence of the previously learned stimulus' association.

Chapter 6

Conclusion

Starting with the finding that conspecific song playback induces IEG expression in a part of the zebra finch brain now referred to as the auditory forebrain (Mello et al., 1992), researchers have found increasingly complex roles for the auditory forebrain in song representation (Avey et al., 2005; Jarvis et al., 1995; Kruse et al., 2004; Mello et al., 1995). Multiple studies have tested the IEG response to high quality versus low quality song (Leitner et al., 2005; S. C. Woolley & Doupe, 2008). Still more have tested the IEG response when birds are actively engaged in learning (Gentner et al., 2004). What remained untested was the idea that the same auditory stimulus, varying only in its previously learned association, can induce differential patterns of IEG expression.

We therefore identified Go/No-Go operant conditioning as a method for generating different meanings associated with a stimulus, and developed the hardware and software necessary to conduct that conditioning. We then analysed how the birds learned the Go versus No-Go discrimination and, using behavioural data, assessed the idea that this operant conditioning leads to two different representations. Next, we characterised the behavioural response to passive acute song playback after discrimination learning in order to understand whether birds' behavioural responses are shaped by this form of playback. Finally, we exposed birds to passive acute song playback and analysed the resulting patterns of neural *ZENK* expression in order to test our theory that gene expression reflects the neural response to previously learned songs.

6.1 Summary of findings

In Chapter 2, I described Operanter, which is open source hardware and software for avian operant conditioning. In order to conduct the training necessary for the eventual *ZENK* expression analyses, I developed a suite of Java-based software and non-proprietary hardware. I showed that Operanter can be used to successfully train zebra finches to discriminate between two conspecific songs. The software and hardware is versatile, easily extendable, and inexpensive, and I argue that will allow researchers with small budgets or specific requirements to build operant conditioning setups.

For Chapter 3, I trained birds using Operanter, and characterised their learning of the Go/No-Go discrimination. Most fundamentally, I confirmed that Go/No-Go training leads to discrimination learning for female zebra finches. I also found evidence from both learning and maintenance trials that the Go and No-Go stimuli require different psychological processes. I highlighted large individual differences in when birds prefer to be active, and showed that those preferences correlate with learning rate. I concluded that researchers using Go/No-Go operant conditioning for zebra finches should be conservative when setting learning criteria, and should alter the maximum response latency in light of my findings.

For Chapter 4, I trained a separate group of birds, and recorded their responses to acute passive song playback after learning the Go/No-Go discrimination. I found no evidence that the birds respond differentially to Go and No-Go song playback in this context. I also found no evidence for a behavioural response to the song playback at all, as overall activity levels were similar before and during playback. I argued that this suggests that neurogenomic responses to the same type of playback would represent a neural response to the stimuli, and not overall shifts in activity level.

For Chapter 5, I trained birds using the same methodology characterised in the previous two chapters, and analysed the expression of the IEG *ZENK* after passive acute exposure to a Go song, a No-Go song, a habituated song, or a novel song. I described the individual differences of *ZENK* response, but showed that these did not relate to the playback condition. I found that the category of playback did not alter the overall levels of *ZENK* expression in the auditory forebrain, but that the Go song did elicit a more coordinated response in the auditory forebrain than any of the three other conditions. I concluded that in response to previously learned conspecific songs, differential *ZENK* expression patterns are subtle, but can be found through graph theory approaches.

6.2 Neural *ZENK* expression likely reflects neural activity

As a whole, development of the Operanter software and hardware provided the opportunity to train multiple female zebra finches in order to conduct behavioural analyses. The findings from Chapter 3, that Go and No-Go stimuli are learned differentially, inform our understanding of *ZENK* expression after discrimination learning. By showing that the response latencies to Go and No-Go stimuli are different, and that correct responses to Go and No-Go stimuli are learned at different rates, we add increasing evidence to the body of literature (e.g. Simmonds et al., 2008) that states that not only do subjects learn to associate one stimulus with a reward and the other stimulus with a punishment, but that these are also learned in different ways. Therefore, *ZENK* expression in response to the playback of a trained stimulus may reflect either the association (i.e. food reward or darkness punishment), or it may reflect the difference between the processes necessary to respond correctly to Go and No-Go stimuli.

Further, the findings from Chapter 4, that the passive acute song playback does not induce a discrete behavioural state and that there is no evidence for an increase in stress-related behaviours, also inform our understanding of *ZENK* expression in response to the same passive acute playback. We therefore interpret the *ZENK* expression results as reflecting neural activity and not physical activity for two reasons. First, there could be brain-wide changes due to metabolic demand (Tong, Shen, Perreau, Balazs, & Cotman, 2001), so the findings in Chapter 4 allow us to rule out a shift in *ZENK* expression in the auditory forebrain due to large-scale changes. Second, neural *ZENK* expression can be induced by physical activity, but this is limited to motor regions (Clark, Bhattacharya, Miller, & Rhodes, 2011; Feenders et al., 2008; Jarvis & Nottebohm, 1997), and the regions assessed here are auditory regions (see Dong & Clayton, 2008 for evidence that *ZENK* expression in the auditory forebrain is not related to behavioural response). Additionally, stress levels can alter the basal level of *ZENK* (Park & Clayton, 2002), which has likely confounded previous studies of associative learning in the auditory forebrain (Gentner et al., 2004; Jarvis et al., 1995). Though we did not present novel or habituated songs to the birds in Chapter 4, the lack of evidence for any kind of a behavioural response to the unsolicited passive playback of Go or No-Go songs suggests that the playback of any conspecific song to a bird who has previously been engaged in operant conditioning of conspecific songs is not particularly surprising or alarming. With no behavioural evidence for increases in stress in

response to acute playback of the punished song, we can interpret differences between *ZENK* expression patterns to Go and No-Go stimuli as representing the categorical differences between the stimuli, and not simply varying stress levels.

Therefore, the *ZENK* expression results from Chapter 5 can be interpreted with increased confidence to reflect a neural response to the stimulus category. On the basis of this evidence, we believe that the lack of a difference in overall *ZENK* expression levels in the auditory forebrain is due to similar levels of novelty, and therefore salience, of all of the conditions. That is, the unsolicited and rapid nature of all of the song playbacks may have rendered the novel condition insufficiently different from even the habituated condition (Kruse et al., 2004). In this context, where female birds have habituated to social isolation over a period of weeks, novel and habituated conspecific songs give rise to the same overall levels of *ZENK* expression in the auditory forebrain, perhaps reflecting the sparseness of conspecific song exposure over a long period. To our knowledge, this is the first evidence that the novel/familiar difference in *ZENK* expression across the auditory forebrain is not absolute.

6.3 Limitations and future research

It is, of course, necessary to address the limitations of the current research. Most simply, the birds in all three chapters were from different cohorts, and it is therefore impossible to conclude with certainty that the behaviour of the birds used for *ZENK* expression analysis was the same as those in the behaviour analyses. Additionally, with only five birds per condition in the analysis of behavioural response to passive song playback, and five or six birds per condition in the analysis of *ZENK* expression, we did not have the power to detect subtle changes. However, as there were numerous precedents for large effects on *ZENK* expression (Mello et al., 1995, 1992), and previous similar studies regularly use four to six individuals per condition (Gentner et al., 2004; Ribeiro et al., 1998), we suggest that this was an appropriate decision.

One major concern in the interpretation of the enhanced coordination of *ZENK* response to the Go stimulus relates to the question of what *ZENK* expression actually reflects in this context. IEG expression in the zebra finch auditory forebrain has been shown to encode both novel (Mello et al., 1992) and previously learned stimuli (Gentner et al., 2004). In this context, where the stimulus discrimination has been previously learned but is no longer being actively maintained, the *ZENK*

response could be functioning either as a read-out of the memory (e.g. X. Liu et al., 2012) or it could be assisting in the formation of a new memory (Minatohara et al., 2016). Though we specifically chose the number of song repetitions to reduce the likelihood of extinction learning (e.g. Jarvis et al., 1995), we cannot rule out the possibility that the *ZENK* expression in response to these passive playbacks reflects that.

Future research would ideally explicitly address this, by testing some individuals with passive exposure and other individuals still engaged with the operant apparatus, although this would raise complications related to motor behaviours and motivation. Additionally, it remains unclear whether the birds learned to prefer the Go song during the operant conditioning, or whether the coordinated *ZENK* response to Go songs is due to the learning of the Go response. To test this idea, and to therefore be able to interpret the findings in the context of the female song preference literature (Leitner et al., 2005; S. C. Woolley & Doupe, 2008), song preference could be tested before and after Go/No-Go training using the same operant setup (Holveck & Riebel, 2007).

6.4 Does operant conditioning alter the neurogenomic response to song presentation?

This thesis provides the first evidence that in response to passive playback following the operant conditioning of two conspecific songs, *ZENK* expression in the auditory forebrain is more coordinated to Go songs than to No-Go songs. Therefore, we have shown that operant conditioning does subtly alter the neurogenomic response to song presentation. We also demonstrated that acute song playback of novel and habituated songs does not necessarily drive differential expression of *ZENK* in the zebra finch auditory forebrain. We conclude that although there is no evidence that *ZENK* expression varies across the auditory forebrain in response to our stimuli, differential networks of activity can be induced depending on the valence of the previously learned stimulus' association.

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Appendix A

This document was composed using R Markdown in RStudio. A repository containing all raw data, R statistical code, and other text necessary to reproduce this thesis is available at <http://github.com/maevemcmahon/thesis>