

Operant conditioning of song associations  
in the zebra finch: molecular, anatomical  
and behavioural characterisations

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Doctor of Philosophy*

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Chapter 2: Dr Julia George helped with the sectioning.

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Chapter 5: Dr Julia George helped with bird training.

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# Abstract

Zebra finches are a well-established animal model for probing the molecular and neuroanatomical mechanisms underlying several forms of learning. Much of the past research has focused on highly specialised forms of learning during juvenile critical periods, especially male song learning. However, birds must continue learn from daily experience even in adulthood. Here I develop and apply operant conditioning techniques to gain insight into the behavioural, molecular and neural mechanisms that allow zebra finches to form and maintain different associations with specific songs they hear in adulthood. Song associations were shaped using a Go/No-Go operant conditioning paradigm. I then compared the anatomical pattern of *ZENK* expression after conditioning was complete. I detected no significant difference in the overall magnitude of *ZENK* when birds heard either category of stimulus, but a statistical analysis of local variations in gene expression within the auditory forebrain suggests that stimulus categories may be represented by the engagement of different network structures. To characterise the formation of Go/No-Go associations at a deeper behavioural level, I developed a new hardware/software system for performing operant conditioning efficiently (Operanter). Using this system I found differences in the dynamics of how birds learn Go vs No-Go associations. I also observed large individual differences in daily patterns of activity, and found a relationship between learning rate and when birds prefer to be active. Finally, I tested whether passive unreinforced exposure to previously conditioned No/No-Go stimuli triggered differences in gross motor behaviour that might influence patterns of gene expression in the auditory forebrain but found no evidence to support this. In sum, these results suggest that Go/No-Go operant conditioning may drive two distinct types of learning, which may be reflected in subtle variations in gene expression in the auditory forebrain.

# Contents

<b>List of Abbreviations</b>	<b>12</b>
<b>Acknowledgements</b>	<b>14</b>
<b>1 Introduction</b>	<b>16</b>
1.1 Classical models of learning mechanisms in zebra finches and other songbirds . . . . .	17
1.1.1 The zebra finch as a model species for vocal learning and auditory communication . . . . .	17
1.1.2 Song copying in the juvenile male zebra finch . . . . .	17
1.1.3 Habituation to song presentation in adult zebra finches . .	18
1.1.4 Song perception in both sexes . . . . .	19
1.2 Use of operant conditioning to study adult learning and perception	21
1.2.1 Operant conditioning in songbird research . . . . .	22
1.3 Use of ZENK to probe neural activity patterns during and after learning . . . . .	23
1.3.1 Role of the auditory forebrain in acoustic processing . . . .	25
1.3.2 Associative learning in the auditory forebrain . . . . .	27
1.4 Aims and objectives . . . . .	28
<b>2 ZENK gene expression in auditory forebrain after exposure to stimuli with different learned associations</b>	<b>30</b>
2.1 Introduction . . . . .	32
2.1.1 Immediate early genes are a valuable tool for investigating gene expression in response to the environment . . . . .	32
2.1.2 Auditory forebrain as a collection of high level auditory processing areas . . . . .	34
2.1.3 Aims and hypotheses . . . . .	38
2.2 Methods . . . . .	39
2.2.1 Animals . . . . .	39
2.2.2 Operant conditioning . . . . .	40

<i>CONTENTS</i>	4
2.2.3 Operant conditioning apparatus . . . . .	40
2.2.4 Experimental design . . . . .	41
2.2.5 Stimuli . . . . .	42
2.2.6 Tissue collection . . . . .	43
2.2.7 Tissue sectioning . . . . .	43
2.2.8 In situ hybridisation . . . . .	44
2.2.9 Image analysis . . . . .	45
2.2.10 Graph theory . . . . .	47
2.3 Results . . . . .	47
2.3.1 Zebra finches learn to discriminate Go from No-Go stimuli	47
2.3.2 Visual inspection of matched sections . . . . .	48
2.3.3 Quantitative analysis of <i>ZENK</i> signal intensities in the auditory forebrain . . . . .	52
2.3.4 Graph theory analysis of regional connectivity . . . . .	55
2.4 Discussion . . . . .	57
2.4.1 Individual differences bear no relationship to condition . .	57
2.4.2 All conditions elicit similar levels of <i>ZENK</i> expression in the auditory forebrain . . . . .	58
2.4.3 Connectedness of the auditory forebrain varies by condition	61
2.4.4 Conclusion . . . . .	62
<b>3 Operanter: open source hardware and software for avian operant conditioning</b>	<b>63</b>
3.1 The need for improved operant conditioning apparatus . . . . .	64
3.2 What is Operanter . . . . .	65
3.2.1 Hardware: Raspberry Pi . . . . .	66
3.2.2 Hardware: Peripheral components . . . . .	66
3.2.3 Software . . . . .	70
3.2.4 Ease of use . . . . .	72
3.3 Conclusion . . . . .	73
<b>4 Initial validation of the Operanter system</b>	<b>74</b>
4.1 Introduction . . . . .	74
4.2 Methods . . . . .	74
4.2.1 Animals . . . . .	75
4.2.2 Apparatus . . . . .	75
4.2.3 Stimuli . . . . .	75
4.2.4 Operant conditioning . . . . .	76
4.2.5 Final playback . . . . .	77
4.2.6 Statistics . . . . .	77

4.3	Results . . . . .	77
4.4	Discussion . . . . .	78
<b>5</b>	<b>Characterising Go/No-Go learning and maintenance behaviour in the zebra finch</b>	<b>80</b>
5.1	Introduction . . . . .	81
5.1.1	Motivational factors in operant conditioning . . . . .	82
5.1.2	Response behaviours to Go/No-Go tasks . . . . .	84
5.1.3	Aims and hypotheses . . . . .	85
5.2	Methods . . . . .	86
5.2.1	Animals . . . . .	86
5.2.2	Apparatus . . . . .	86
5.2.3	Stimuli . . . . .	86
5.2.4	Operant conditioning . . . . .	87
5.2.5	Final playback . . . . .	88
5.2.6	Statistics . . . . .	88
5.3	Results . . . . .	89
5.3.1	Go and No-Go stimuli are learned at different rates . . . . .	89
5.3.2	Birds have a Go response bias during early training . . . . .	89
5.3.3	Response latencies during learning and maintenance . . . . .	90
5.3.4	Activity levels, but not accuracy or bias, vary according to the time of day . . . . .	93
5.3.5	Early birds are slow learners . . . . .	94
5.3.6	Response accuracy during maintenance is affected by time of day and recent preceding behaviour . . . . .	96
5.4	Discussion . . . . .	101
5.4.1	Go/No-Go response learning rates and bias . . . . .	103
5.4.2	Response latencies . . . . .	104
5.4.3	Time of day . . . . .	105
5.4.4	Complex interactions predict response accuracy during main- tenance . . . . .	106
5.4.5	Conclusion . . . . .	109
<b>6</b>	<b>Birds respond similarly to passive acute playback of songs asso- ciated with reward and punishment</b>	<b>110</b>
6.1	Introduction . . . . .	111
6.1.1	Aims and predictions . . . . .	112
6.2	Methods . . . . .	113
6.2.1	Animals . . . . .	113
6.2.2	Apparatus . . . . .	113

6.2.3	Stimuli . . . . .	114
6.2.4	Operant conditioning . . . . .	115
6.2.5	Final playback . . . . .	115
6.2.6	Video analysis . . . . .	115
6.2.7	Statistics . . . . .	116
6.3	Results . . . . .	116
6.3.1	Overall activity is similar for both Go and No-Go playbacks	116
6.3.2	A linear discriminant analysis does not successfully classify playback conditions . . . . .	118
6.3.3	Principal components do not discriminate between activity- related states . . . . .	118
6.3.4	No individual behaviours vary by condition . . . . .	120
6.3.5	Individual differences in behavioural responses . . . . .	120
6.3.6	Power analysis . . . . .	120
6.4	Discussion . . . . .	121
6.4.1	No evidence for an acute response to song presentation . .	121
6.4.2	No evidence for clusters of behaviours . . . . .	122
6.4.3	Implications for interpretation of gene expression studies .	123
6.4.4	Conclusion . . . . .	123
<b>7</b>	<b>Discussion</b>	<b>124</b>
7.1	How are learned auditory associations encoded in the brain? . . .	125
7.2	Can behaviour during operant conditioning enhance our under- standing of the learning process? . . . . .	127
7.3	Do behaviours in non-reinforced contexts correlate with neural processes in the same context? . . . . .	130
7.4	Integrating findings across three aims . . . . .	130
7.5	Conclusion . . . . .	132
	<b>References</b>	<b>134</b>
	<b>Appendix A</b>	<b>154</b>

# List of Tables

1.1	Outcomes of previous studies into female preference for song types using IEG expression in the auditory forebrain. . . . .	26
1.2	Outcomes of previous studies into associative learning in songbirds.	28
2.1	Go and No-Go training and playback stimuli for all conditions. . .	42
2.2	Song pairs for training, where subscripts denote different male directed songs. . . . .	43
2.3	LMMs for median pixel intensity of all target brain regions. . . .	53
5.1	GLMMs for modelling accuracy of response during maintenance trials. . . . .	98
6.1	Training and playbacks for all ten individuals. Each song was recorded from a different male. . . . .	114
6.2	GLMMs for total incidences of all behaviours. . . . .	117
6.3	GLMMs for individual behaviour types. . . . .	120



# List of Figures

1.1	Parasagittal cartoon of song perception regions in the zebra finch. MLd - dorsal lateral nucleus of the mesencephalon. Ov - nucleus ovoidalis. L2 - Field L2. NCM - caudomedial nidopallium. CMM - caudomedial mesopallium. CLM - caudolateral mesopallium. . . .	20
2.1	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. . . . .	41
2.2	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	46
2.3	Learning and maintenance of Go/No-Go discrimination for all four conditions. X-axis is 100-trial bin number normalised across birds by dividing 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.	48
2.4	Right hemisphere parasagittal sections from each individual, 1.2 mm from midline. . . . .	49
2.5	Dense staining in the granule cell layer of folia VIII/IX of medial cerebellum, 0.5 mm from midline, right hemisphere. . . . .	50
2.6	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. . . . .	51
2.7	Proportion of individuals in each condition exhibiting clear <i>ZENK</i> expression in each brain region. . . . .	52

2.8	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.	53
2.9	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. . . . .	54
2.10	Graph of all ROI correlations where $p < 0.10$ , across all conditions.	55
2.11	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. . . . .	56
2.12	Normalised counts of <i>ZENK</i> gene expression in the auditory fore-brain from two experiments. Aviary (Avi) and Isolated (Iso) are from George & Clayton, 2018. Go and No-Go are from the birds characterised in Chapter 4. Figure produced by J. George. . . . .	59
3.1	Operanter hardware, Raspberry Pi and electronics. A) The chamber with individual Raspberry Pi on top. B) Back of Raspberry Pi with GPIO connections to peripheral components. C) Infrared sensors and food hatch inside the cage. . . . .	67
3.2	The sensor and LED component. The bird's beak breaks the infrared beam from the emitter to the detector ends of the infrared sensor, and an LED component indicates when the sensor is active and provides an illuminated target for the bird's beak. . . . .	69
3.3	Operanter software. A) Schedule tab. B) Operant Experiment tab. C) Log tab. D) Stats tab. . . . .	71
4.1	Learning curves for Leiden birds (proprietary system) and London birds (Operanter system). . . . .	78
5.1	Diagram of the operant conditioning apparatus in the sound attenuation chamber. The setup includes two infrared detectors with green LEDs and a horizontally mounted motorised food hopper opening. . . . .	87
5.2	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. . . . .	90

5.3	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). . . . .	91
5.4	Response latencies (in milliseconds) to stimuli throughout learning and maintenance. Panel A is correct responses to Go stimuli; Panel B is incorrect responses to No-Go stimuli. . . . .	91
5.5	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). . . . .	92
5.6	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. . . . .	93
5.7	Activity levels for individual birds throughout the day, in half hour bins, during the maintenance stage. Lines of best fit are loess regression lines fit to the mean proportion of trials during half hour bins for each individual bird, across all days of maintenance. . . . .	94
5.8	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. . . . .	95
5.9	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. . . . .	97
5.10	Line/elbow graph of AICs by number of splines describing time of day in the nested generalised linear mixed models. . . . .	99
5.11	Visualisation of the three-way interaction between stimulus type, preceding trial accuracy, and lag. For this figure, lags have been grouped into long or short based on the median lag duration; lags were modelled as continuous data in the GLMMs. . . . .	100
5.12	Bar chart of correct responses during early and late parts of the day to A) Go and No-Go stimuli and B) stimuli to which the preceding responses were either accurate or inaccurate. The times of day have been divided by a median split for this visualisation, but the GLMMs model time of day using two automated splines, which are unlikely to be knotted at the median time of day. . . . .	102

6.1	Number of times each behaviour was performed during and after playback, by condition. . . . .	116
6.2	Total activity level by condition and period. . . . .	117
6.3	Loadings for the PCA. . . . .	119
6.4	Principal components plotted against each other. A) PC1 plotted against PC2. B) PC1 plotted against PC3. . . . .	119
6.5	Individual differences in behavioural response to song playback. . .	121

# List of Abbreviations

2-AFC — 2-alternative forced choice  
ABX / AXB — operant conditioning  
AIC — Aikake information criterion  
ANOVA — analysis of variance  
BDNF — brain-derived neurotrophic factor  
BOLD — blood-oxygen-level dependent  
BOS — bird's own song  
BSA — bovine serum albumin  
c — response bias  
cDNA — complementary DNA  
CLM — caudolateral mesopallium  
CMM — caudomedial mesopallium  
cNCM — caudal NCM  
CPU — central processing unit  
CSV — comma separated value  
d' — d prime, measure of sensitivity  
dB — decibels  
DLM — dorsolateral nucleus of the anterior thalamus  
dNCM — dorsal NCM  
dr — discrimination ratio  
DTT — dithiothreitol  
fMRI — functional magnetic resonance imaging  
GLMM — generalised linear mixed model  
GPIO — general purpose input output  
GUI — graphical user interface  
HVC — used as proper name  
Hz — hertz  
IEG — immediate early gene  
LB — lysogeny broth  
LDA — linear discriminant analysis

LED — light emitting diode  
LMM — linear mixed model  
MANOVA — multivariate analysis of variance  
MB — megabyte  
miRNA — micro RNA  
MLd — dorsal lateral nucleus of the mesencephalon  
MRI — magnetic resonance imaging  
mRNA — messenger RNA  
ms — milliseconds  
NCL — caudolateral nidopallium  
NCM — caudomedial nidopallium  
OCT — optimal cutting temperature compound  
Ov — nucleus ovoidalis  
PBS — phosphate-buffered saline  
PC — principal component  
PCA — principal component analysis  
PCR — polymerase chain reaction  
RA — robust nucleus of the arcopallium  
RNA-Seq — RNA sequencing  
ROI — region of interest  
SPL — sound pressure level  
TIFF — tagged image file format  
USB — universal serial bus  
USD — United States dollars  
vNCM — ventral NCM  
XML — eXtensible markup language  
ZENK — zif268, egr-1, NGFI-A, Krox24

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# Chapter 1

## Introduction

Zebra finches are a well-established animal model for probing the molecular and neuroanatomical mechanisms underlying several forms of learning (Zann, 1996). Much of the past research has focused on highly specialised forms of learning during juvenile critical periods, especially male song learning (Pfenning et al., 2014). However, birds must continue to learn from daily experience even in adulthood.

For this thesis, I develop and apply operant conditioning techniques to gain insight into the molecular and neural mechanisms that allow zebra finches to form and maintain different associations with specific songs they hear in adulthood. In this introduction, I begin by reviewing classical models of learning mechanisms in the zebra finch, including male song copying/learning, habituation, and female preference. I then describe the use of operant conditioning to study adult learning across multiple species. I continue by reviewing the use of immediate early genes (IEGs) to probe neural activity patterns during and after learning, and discuss literature suggesting that IEGs may be involved in the acute storage of salient information and in the long-term maintenance of memories. I then lay out the questions that I will address in this thesis.

## 1.1 Classical models of learning mechanisms in zebra finches and other songbirds

### 1.1.1 The zebra finch as a model species for vocal learning and auditory communication

Songbirds (oscines of Order Passeriformes) provide a unique opportunity to study the mechanisms that underlie communication through learned vocalisations — an ability lacking in more familiar laboratory animals like the mouse (*Mus musculus*). Among the thousands of songbird species, the zebra finch (*Taeniopygia guttata*) has emerged as the primary focus for laboratory based studies (Zann, 1996), in part because it is hardy in captivity, breeds rapidly, and has been domesticated over the past 50-100 years (Griffith et al., 2017; Olson, Wirthlin, Lovell, & Mello, 2014). Reflecting its primary status as an emerging model organism, the zebra finch was the second avian species (after the chicken) chosen for whole genome sequencing (Warren et al., 2010).

### 1.1.2 Song copying in the juvenile male zebra finch

The behaviour and neurobiology of zebra finch song learning has been investigated by a long line of researchers starting with Immelmann (1969). Much of this research has focused on the process by which a juvenile male learns to produce a unique song, by approximately copying the song of one or more adult tutors (for some representative reviews of this large literature, see Bolhuis, Okanoya, & Scharff, 2010; Doupe & Kuhl, 1999; Gobes, Jennings, & Maeda, 2017; Marler & Doupe, 2000). In brief, this learning process proceeds in two phases during a limited juvenile “critical period”. During an initial sensitive/sensory phase, male zebra finches learn the sound of a “tutor song”, which they will learn to reproduce through practice during a subsequent sensory-motor phase. These two phases overlap, but once the sensory phase ends, around 65 days post hatch, normally reared juvenile male zebra finches will not learn new songs. The neural circuitry responsible for male song production has been worked out in considerable detail, and involves a network of discrete, interconnected, sexually dimorphic brain nuclei that are unique to songbirds (reviewed in Bolhuis & Gahr, 2006; Doupe, Perkel, Reiner, & Stern, 2005; Mooney, 2009a). A caudal descending sensorimotor pathway from HVC (used as a proper name) to the robust nucleus of the arcopallium (RA) controls the motor production of song by driving activity

in the tracheosyringeal portion of the hypoglossal nucleus (nXIIIts), which controls the syrinx. An anterior pathway from HVC through the striatum, to the thalamus, and back to HVC via the lateral magnocellular nucleus of the anterior nidopallium (IMAN) is necessary for producing variability during juvenile song learning (Marler & Doupe, 2000), which enables the bird to produce increasingly accurate copies of the tutor's song during the sensory-motor phase (Goldberg & Fee, 2012); lesioning this pathway in adulthood, once the song has crystallised, has little effect on the song structure (Brainard, 2004).

In terms of behaviour, research has been conducted into factors including the accuracy and precision of learning (Brumm, Zollinger, & Slater, 2009) and the role of the song tutor (N. S. Clayton, 1987; H. Williams, 1990). These factors have also been assessed in a neurobiological framework; for example, though brood size has an effect on the accuracy of song learning, it does not correlate with the volume of brain regions involved in song production (Gil, Naguib, Riebel, Rutstein, & Gahr, 2006). In contrast, the accuracy of song copying may be reflected in brain activity in song production brain regions (Bolhuis, Gobes, Terpstra, Boer-Visser, & Zandbergen, 2012).

### **1.1.3 Habituation to song presentation in adult zebra finches**

Both male song production and female song preference are rooted during juvenile development, but the zebra finch has also served as a model species for a form of learning that continues through the lifespan: habituation (Dong & Clayton, 2009). Habituation is a form of non-associative learning whereby repeated presentation of a stimulus leads to a reduced response to that stimulus (S. Schmid, Wilson, & Rankin, 2015). Habituation to song playbacks has been studied in wild songbirds such as white-crowned sparrows (Verner & Milligan, 1971) and great tits (Krebs, 1976). In those studies, behaviours that indicate a response to the song, such as aggressive attacks and vocalisations, were measured and shown to reduce upon repeated song presentation. In the lab, neuromolecular correlates of behavioural habituation have been measured in the zebra finch, improving our understanding of the genomic and neuroanatomical underpinnings of habituation to auditory stimuli (Mello & Clayton, 1994; Mello, Vicario, & Clayton, 1992). This will be further discussed in the framework of IEG research in Section 1.3.

### 1.1.4 Song perception in both sexes

Only male zebra finches learn to sing. However, both sexes produce other unlearned vocalisations, and both sexes attend to and discriminate individuals based on their vocalisations (Riebel, 2003; Riebel, Smallegange, Terpstra, & Bolhuis, 2002). Female song perception and male song production share similarities, including that both are driven by early life experiences (N. S. Clayton, 1988; Eales, 1985; Holveck & Riebel, 2014; Lauay, Gerlach, Adkins-Regan, & Devoogd, 2004; M. I. M. Louder, Hauber, & Balakrishnan, 2018). The development of song preferences in females is a process sometimes placed in an imprinting framework (Bischof, 1994; N. S. Clayton, 1987; Riebel, 2003). Though male song production has been studied more extensively than female song perception, female preference for male songs may be a key driver in male song production (Carouso-Peck & Goldstein, 2019). Adult females prefer louder songs (Ritschard, Riebel, & Brumm, 2010) and directed rather than undirected songs (S. C. Woolley & Doupe, 2008). Female song preference has been hypothesised to be driven by the quality of male song, which reflects the early developmental conditions of the male, and therefore male quality (Holveck, Vieira, Lachlan, Cate, & Riebel, 2008). Though male song quality is driven by the developmental conditions of the male, early developmental stress does not appear to affect female song preference (Woodgate et al., 2011). Despite the recent research into female song preference, little remains known about whether song preference can be modified during adulthood.

Beginning with the discovery of the *ZENK* gene response to song playbacks (Mello et al., 1992), the neural circuitry involved in song perception has been gradually worked out (Mello, Velho, & Pinaud, 2004). 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, & Linden, 2009) (Figure 1.1). The MLd projects to the nucleus ovoidalis (Ov), 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 (F. E. 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 zebra finches.

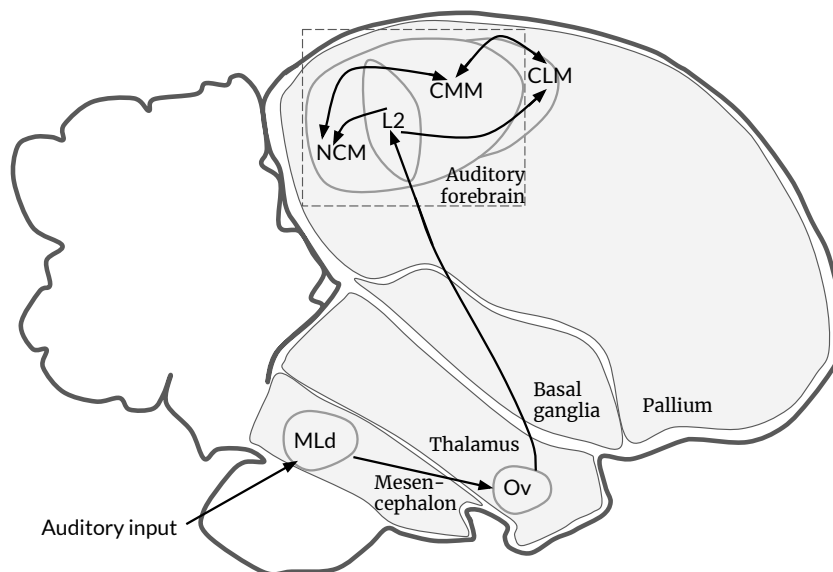


Figure 1.1: Parasagittal cartoon of song perception regions in the zebra finch. MLd - dorsal lateral nucleus of the mesencephalon. Ov - nucleus ovoidalis. L2 - Field L2. NCM - caudomedial nidopallium. CMM - caudomedial mesopallium. CLM - caudolateral mesopallium.

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).

In sum, evidence suggests that circuits involving NCM and CMM in particular

are involved in generating higher-order representations of complex, salient songs. However, the details of those representations and the mechanisms by which they are generated, stored and recalled remain unknown. One of the aims of this thesis will be to seek evidence that representational activity patterns may shift according to the context in which those songs are experienced.

## 1.2 Use of operant conditioning to study adult learning and perception

Operant conditioning is a form of associative 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, another form of associative learning, 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, the role of complex neural processes, such as choice (Washburn, Hopkins, & Rumbaugh, 1991) and motivation (Lawrence & Illius, 1989), in operant responding has also been investigated. As much of the early work into operant conditioning was conducted on pigeons (Brown & Jenkins, 1968), 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 (Evans, 1970). 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 (Rose & Schmidt, 2012).

### 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, & 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-alternative forced choice, 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, I will use Go/No-Go operant conditioning solely as a tool to train the birds to associate one song with reward (dependent on a pecking behaviour) and the other with punishment (if pecking behaviour is not suppressed). 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), I 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.

It is also worth noting that many previous studies into associative learning of previously novel songs involved training male zebra finches (Gentner & Margoliash, 2003; Gentner, Hulse, & Ball, 2004; Jarvis, Mello, & Nottebohm, 1995). However, testing song perception using male zebra finches risks confounding perception of the target stimulus with perception of bird's own song (BOS) (Pytte & Suthers, 1999; S. M. Woolley, Hauber, & Theunissen, 2010). Though female zebra finches' preference for father's song influences conspecific song preference (Terpstra, Bolhuis, Riebel, Burg, & Boer-Visser, 2006), it does not involve reciprocal connectivity between song production and song perception pathways, thereby simplifying interpretation of behavioural and neuroanatomical findings. For these reasons, in the operant training experiments described in this thesis, I have chosen to use female zebra finches.

### 1.3 Use of ZENK to probe neural activity patterns during and after learning

IEGs are genes that respond rapidly to stimuli and have a broad range of downstream effects, and have been widely used in neuroscience to measure activity and learning in the brain (Clayton, 2000; Minatohara, Akiyoshi, & Okuno, 2016). IEG expression can be measured using *in situ* hybridisation, a 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). The first application of IEGs to songbird research yielded the initial identification of brain areas specifically and selectively activated by the sound of birdsong, i.e. NCM and CMM (Mello et al., 1992). This study also made use of a gene known in songbirds as *ZENK*, the avian homologue of and an acronym for *zif268*, *egr-1*, *NGFI-A*, and *Krox24* (Mello et al., 1992). (Note that 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.)

The initial discovery was that 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). Within the auditory forebrain, expression



of *ZENK* was shown to correlate strongly with electrophysiologically measured activity in response to songs (Chew, Mello, Nottebohm, Jarvis, & Vicario, 1995; Chew, Vicario, & Nottebohm, 1996; Stripling, Volman, & Clayton, 1997). 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, Schwabl, Ribeiro, & Mello, 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).

*ZENK* expression in NCM and CMM varies depending on the salience of features, making it especially useful for probing how the salience or significance of a particular stimulus may be represented in neural activity patterns. 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). Additionally, 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 (Beckers et al., 2003), novelty (Horstmann, Becker, & Ernst, 2016), and perceived spatial location (D. A. Hall & Moore, 2003) are all examples of varying levels of perceptual salience, and there is ample evidence that the *ZENK* response in the auditory forebrain encodes this.

Other IEGs, such as *c-fos*, are regularly used as indicators of activity in songbird spatial memory studies (Z. J. Hall, Bertin, Bailey, Meddle, & Healy, 2014; Mayer, Watanabe, & Bischof, 2010). *c-fos* has also been used in some auditory perception studies, and *c-fos* and *ZENK* sometimes display similar patterns of activation (Monbureau, Barker, Leboucher, & Balthazart, 2015), but *ZENK* expression contrasts with other IEGs in multiple ways; for example, *c-fos*, but not *ZENK*, is induced in male zebra finch HVC in response to food aversion training (Tokarev, Tiunova, Scharff, & Anokhin, 2011). Additionally, developmental trajectories for IEG expression in response to song playback may vary by sex (D. J. Bailey & Wade, 2005). Therefore, it is fruitful to select the IEG used in the greatest number of similar previous studies in order to minimise the number of extraneous variables and allow direct comparison to previous literature. To that end, the levels of the IEG *ZENK* will be measured throughout this thesis.

### 1.3.1 Role of the auditory forebrain in acoustic processing

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 (P. H. 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.

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, 2009b; F. E. 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 an overview of relevant studies into female preference for song stimuli, using IEG expression in the auditory forebrain, see (Table 1.1). 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

Table 1.1: Outcomes of previous studies into female preference for song types using IEG expression in the auditory forebrain.

Reference	Species	Brain region	Assay	Outcome
Leitner et al. (2005)	Canary	CMM	ZENK gene	Sexy > nonsexy syllables
Gentner et al. (2005)	Starling	CMM	ZENK gene	Longer = shorter songs
Gentner et al. (2000)	Starling	Ventral NCM	ZENK gene	Longer > shorter songs
Terpstra et al. (2006)	Zebra finch	CMM	ZENK protein	Father's song > novel songs
Woolley & Doupe (2008)	Zebra finch	CMM	ZENK gene	Directed > undirected songs
Lampen et al. (2004)	Zebra finch	NCM, CMM	ZENK gene	Time distorted > natural songs
Woolley & Doupe (2008)	Zebra finch	NCM	ZENK gene	Familiar > novel songs

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  $\mu\text{m}$  from the midline (S. C. Woolley & Doupe, 2008), and a frequently used zebra finch atlas shows CMM from 200-1700  $\mu\text{m}$  from the midline (Oregon Health & Science University, 2013). Multiple studies provide no clear indication of laterality (e.g. Jarvis et al., 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.

### 1.3.2 Associative learning in the auditory forebrain

Features that alter motivational salience, or salience that has been learned through repeated association (Puglisi-Allegra & Ventura, 2012), are also reflected in the *ZENK* response (Table 1.2). This is in line with previous evidence suggesting that both perceptual and motivational salience predict the rate of associative learning (Treviño, 2016). Stimuli with no differences in perceptual salience, but that have been associated with a stimulus with perceptual 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 darkness punishment elicit similarly low levels as silence 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

Table 1.2: Outcomes of previous studies into associative learning in songbirds.

Reference	Species	Sex	Paradigm	Brain region	What was measured	Outcome
Jarvis et al. (1995a)	Canary	Male	Classical conditioning	NCM, CMM	ZENK gene	Paired shock/song > unpaired
Gentner et al. (2004)	Starling	Male	Operant conditioning	NCM	ZENK protein	Novel > rewarded Novel > punished Novel > silence
Gentner et al. (2004)	Starling	Male	Operant conditioning	CMM	ZENK protein	Novel > rewarded Novel > punished Novel/rewarded/punished > silence
Gentner & Margoliash (2003)	Starling	Both	Operant conditioning	CMM	Electrophysiology	Rewarded > punished Punished > novel
Bell et al. (2015)	Zebra finch	Male	Operant conditioning	NCM	Electrophysiology	Rewarded > novel Novel > punished
Bell et al. (2015)	Zebra finch	Male	Operant conditioning	CMM	Electrophysiology	Rewarded > punished Punished > novel

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.

## 1.4 Aims and objectives

Throughout this thesis, I aim to answer the following questions:

1. Are there differences in the neuroanatomical patterns of activity, as revealed by *ZENK*, when birds hear Go versus No-Go conditioned stimuli? That is, can we gain insight into the underlying neural and/or genomic architecture responsible for encoding memories?
2. Are there differences in how birds learn Go and No-Go stimuli, or is Go/No-Go operant conditioning a unitary task?
3. Are there differences in the gross motor behaviours displayed by birds when they passively hear previously conditioned Go and No-Go stimuli?

First, I will do this by combining 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 the assessment of neural gene expression with high spatial resolution. I will train birds to discriminate between one song (Go stimulus) and a second song (No-Go

stimulus). Then I will play one of those two songs immediately before collecting tissue for *ZENK in situ* hybridisation. I will use the pattern of *ZENK* induction to assess which brain regions are involved in the perception of previously learned stimuli.

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

Third, I will train a second set of birds using operant conditioning, and I will use a fine-grained analysis of operant conditioning learning and maintenance behaviours to characterise individual differences in Go/No-Go learning, and to better understand the processes underlying the Go and No-Go responses.

Finally, I will train a third set of birds using operant conditioning, after which I will video record their behaviours in response to passive playback of either the Go or the No-Go song. I will use an array of statistical techniques to test if behaviours to passive playbacks might reflect, or even cause, changes in brain gene expression found during an allied study.

## Chapter 2

# ***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 I 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, I 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 I 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, I 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.



## 2.1 Introduction

Many levels of neurobiological activity contribute to the encoding of experiences. Historically, studies have focused on synaptic plasticity (Dubnau, Chiang, & Tully, 2003), but recent research has highlighted the role of gene expression in memory formation (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? In this chapter I will use operant conditioning to test this hypothesis.

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

The genomic action potential analogy 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 (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 (Clayton et al., n.d.; 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.

### **2.1.2 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, & Ramage-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; P. H. 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.

### 2.1.2.1 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 innate part of the female song perception system (Lynch et al., 2017; Tomaszycski, Sluzas, Sundberg, Newman, & DeVogd, 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.

### 2.1.2.2 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 (S. E. 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  $\sim 1.0$ - $1.5$  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.

### 2.1.3 Aims and hypotheses

Drawing together the precedents above, I hypothesise that the learned valence of an acoustic stimulus is encoded by, or represented in, different patterns of *ZENK* expression within NCM and CMM. If true, this would provide new insight into the mechanisms by which integrated representations of salient experience are formed in the brain. To test this hypothesis I 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), I will not test birds during the ongoing operant conditioning procedure, but will instead present a passive playback following discrimination training. In this way, I 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.

From my hypothesis, I make the following predictions: 1) after Go/No-Go conditioning, subsequent exposure to either class of conditioning stimuli will result in different neuroanatomical patterns of *ZENK* expression, as revealed by *in situ* hybridisation. 2) *ZENK* gene expression in the auditory forebrain will be very low for birds in the habituated condition, and high for birds in the novel condition. 3) Overall levels of *ZENK* gene expression in the auditory forebrain for the Go and the No-Go conditions will fall in between that of the habituated and novel conditions. 4) Brain regions associated with reward and stress networks will differentially express *ZENK* when the animal is re-exposed to a conditioned stimulus. 5) Finally, I predict that in the absence of consistent patterning of *ZENK* in response to Go and No-Go songs, I will find differential patterns of recruitment of regions within the auditory lobule that can be detected using graph theoretical approaches.

## 2.2 Methods

### 2.2.1 Animals

24 female zebra finches were operantly trained, tested, and decapitated for *in situ* hybridisation at the University of Leiden. All birds were bred and reared at the Leiden University animal breeding facility and at the start of the experiment 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 kept at 20-22°C; they were removed from this single sex aviary in groups of four and placed into the operant conditioning apparatus (described below). 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.



### 2.2.2 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 days before initiation of the final playback.

### 2.2.3 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). The floor was covered with sand. Each cage included two red LED/buttons, a food hopper to which access

was limited by a vertical motorised cover, and a water container (Figure 2.1). The cage was singly placed in a small sound attenuated room kept at the same 20-22°C as the single sex aviary. The room was illuminated by a fluorescent tube that emitted daylight spectrum light on the same 13.5:10.5 schedule as the single sex aviary placed on top of the cage and controlled by the operant conditioning software. A Vifa 10BGS119/8 speaker was located 0.6 m above the top centre of the cage.

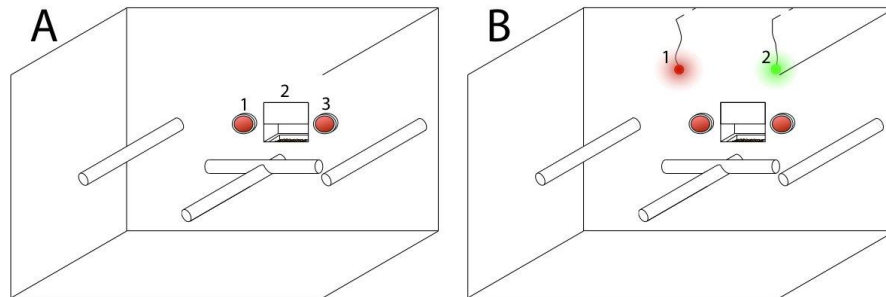


Figure 2.1: Diagram of the operant conditioning apparatus in the sound attenuation chamber. A) Setup for Go, No-Go, and novel conditions. 1 & 3 are sensors. 2 is the food hatch. B) Setup for habituated condition. Sensors and food hatch same as three other conditions. 1 & 2 are the stimulus lights.

#### 2.2.4 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 2.1). 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

Table 2.1: Go and No-Go training and playback stimuli for all conditions.

		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

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.

### 2.2.5 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 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  $\pm 10\%$ ) and to maximise human-perceived differences in syllable content. 12 songs were selected (4 for each condition) (Table 2.2). 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.

Table 2.2: Song pairs for training, where subscripts denote different male directed songs.

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

### 2.2.6 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  $-80^{\circ}\text{C}$  for long-term storage. The process of catching, dissecting and freezing took no more than 6 minutes.

### 2.2.7 Tissue sectioning

The right hemispheres of OCT-mounted brain tissue were removed from  $-80^{\circ}\text{C}$  storage, placed in a Leica cryostat and allowed to equilibrate to  $-20^{\circ}\text{C}$ . Parasagittal sections were cut on the cryostat (with the assistance of Dr George). Three sections from each 100  $\mu\text{m}$  were collected from the midline to the distal edge. A total of  $\sim 144$  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  $-80^{\circ}\text{C}$ .

### 2.2.8 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 $\alpha$  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  $\mu$ m of plasmid DNA was linearised using a BssHII digestion. A PCR purification 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  $\mu$ g T3 RNA polymerase, 1X digoxigenin(DIG)-11-UTP RNA labelling kit (Roche), 2 U/ $\mu$ L recombinant RNAsin, 1  $\mu$ g/ $\mu$ L BSA, 10mM DTT, and 1  $\mu$ 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  $\mu$ L of hybridisation solution (6.25% v/v purified riboprobe at 1 ng/ $\mu$ L, 1  $\mu$ g/ $\mu$ L PolyA, 1  $\mu$ g/ $\mu$ L BSA, 2  $\mu$ g/ $\mu$ L 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  $\mu\text{m}$  syringe. 200  $\mu\text{L}$  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  $\mu\text{L}$  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  $\text{MgCl}_2$ ) 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 ddH<sub>2</sub>O for 1 hour with agitation. Slides were then allowed to air dry before being coverslipped with VectaMount AQ mounting media.

### 2.2.9 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 2.2).

CMM was represented by a ROI defined as a square (0.5 mm from midline: 400  $\mu\text{m}$  x 400  $\mu\text{m}$ ; 1.2 mm from midline: 600  $\mu\text{m}$  x 600  $\mu\text{m}$ ) placed halfway along

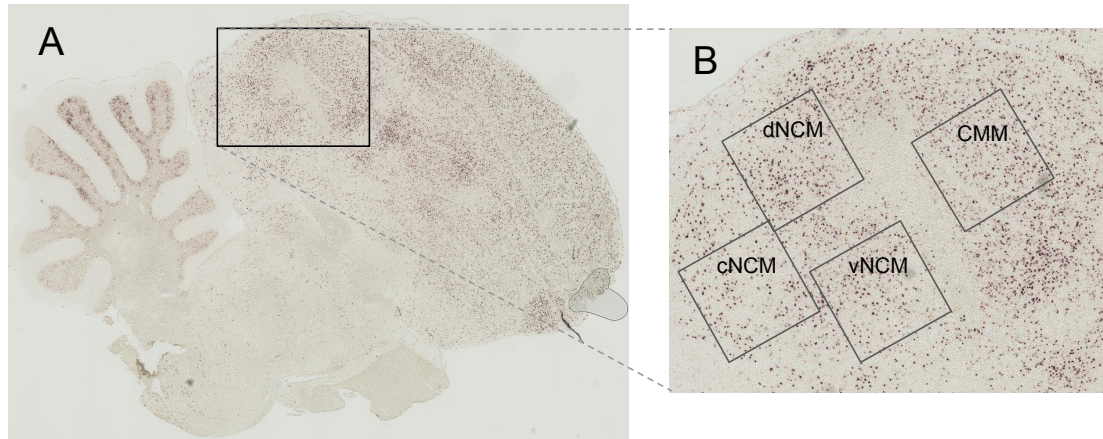


Figure 2.2: Neuroanatomy for region of interest selection. A) Parasagittal whole brain section, 1.2 mm from midline, where right is towards the beak. B) A zoomed-in image of the auditory forebrain region, with rectangular regions of interest placed as for the image analysis

the rostral length of Field L, with one edge perpendicular to the long axis of Field L. Three ROIs, captured with squares ( $0.5\text{ mm}$  from midline:  $400\ \mu\text{m} \times 400\ \mu\text{m}$ ;  $1.2\text{ mm}$  from midline:  $600\ \mu\text{m} \times 600\ \mu\text{m}$ ), 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  $1.2\text{ mm}$  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  $0.5\text{ mm}$  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 telencephalon/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 in-

tensity 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.

### 2.2.10 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.

## 2.3 Results

### 2.3.1 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 2.3). The total number of 100-trial bins of final song presentations ranged from 18-63 (mean = 36.8, sd = 9.2) depending on the bird's learning rate.  $d'$  (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 2.3; 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 2.3; 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;  $F(3, 19) = 0.85$ ,  $p = 0.48$ ). In (Figure 2.3), the beginning of the final 5 100-trial bins ranged from 0.72



to 0.92 (mean = 0.86) on the x-axis.

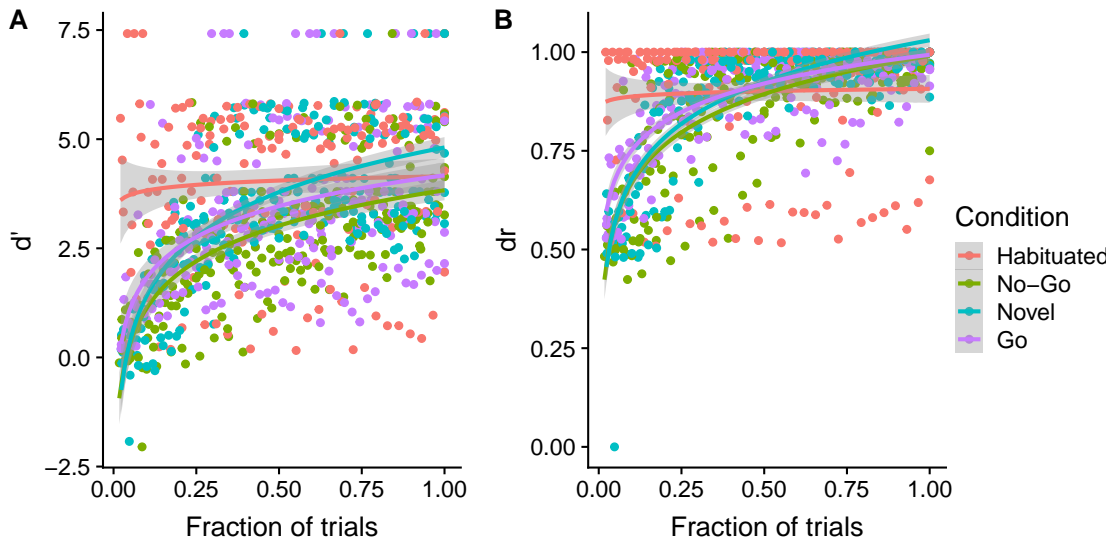


Figure 2.3: Learning and maintenance of Go/No-Go discrimination for all four conditions. X-axis is 100-trial bin number normalised across birds by dividing the bin number by the maximum number of bins for each individual bird. Y-axis is A)  $d'$  and B) discrimination ratio. Lines of best fit are logarithmic functions with standard error shading.

### 2.3.2 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 2.4). 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.

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.

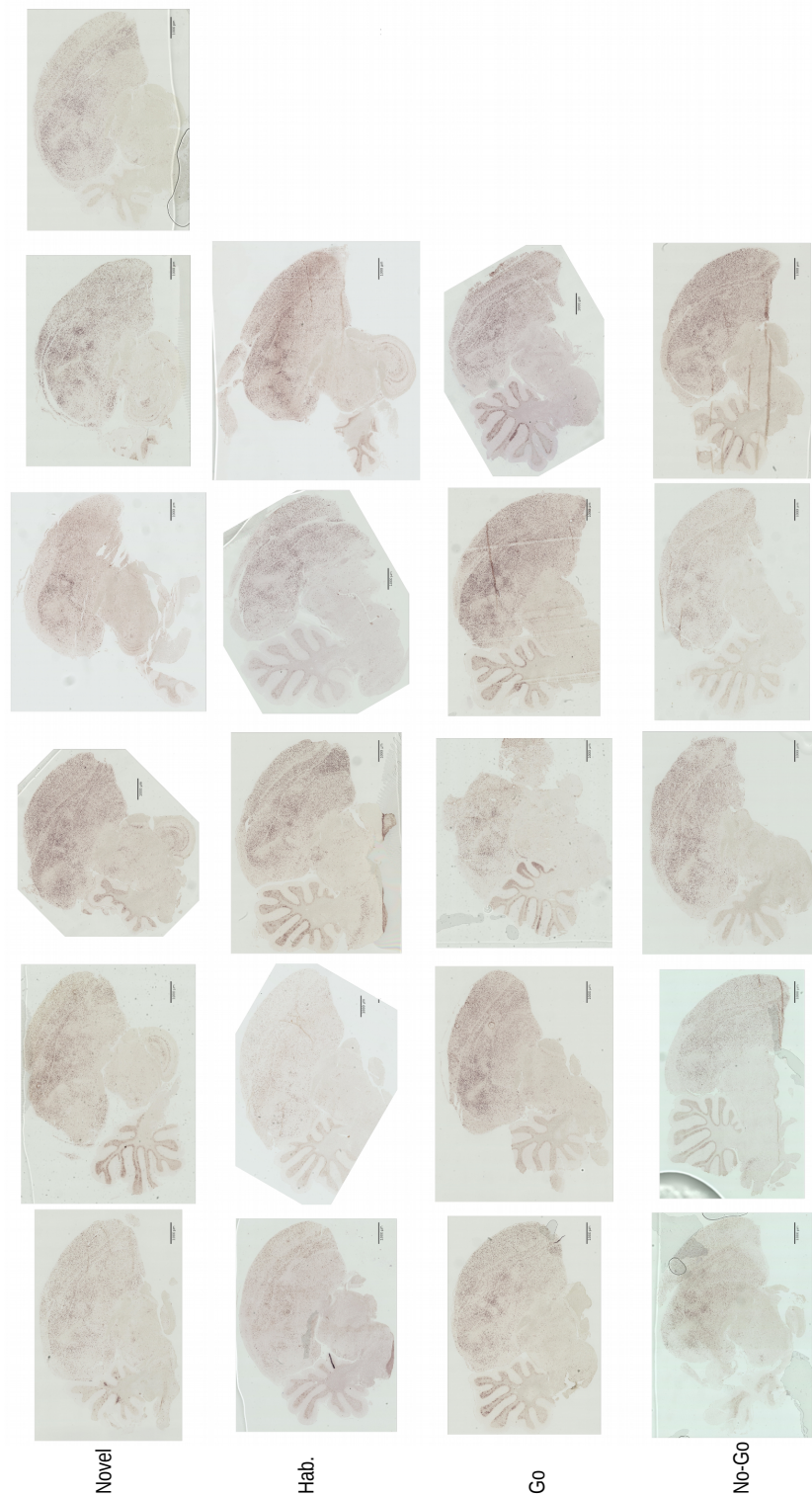


Figure 2.4: Right hemisphere parasagittal sections from each individual, 1.2 mm from midline.

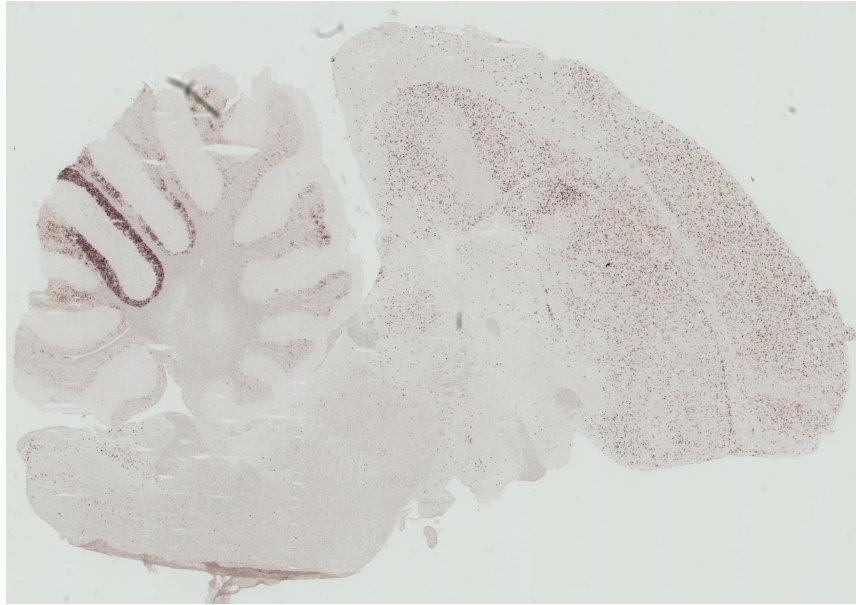


Figure 2.5: Dense staining in the granule cell layer of folia VIII/IX of medial cerebellum, 0.5 mm from midline, right hemisphere.

Unexpectedly, I 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 2.6).

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 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 2.7). 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

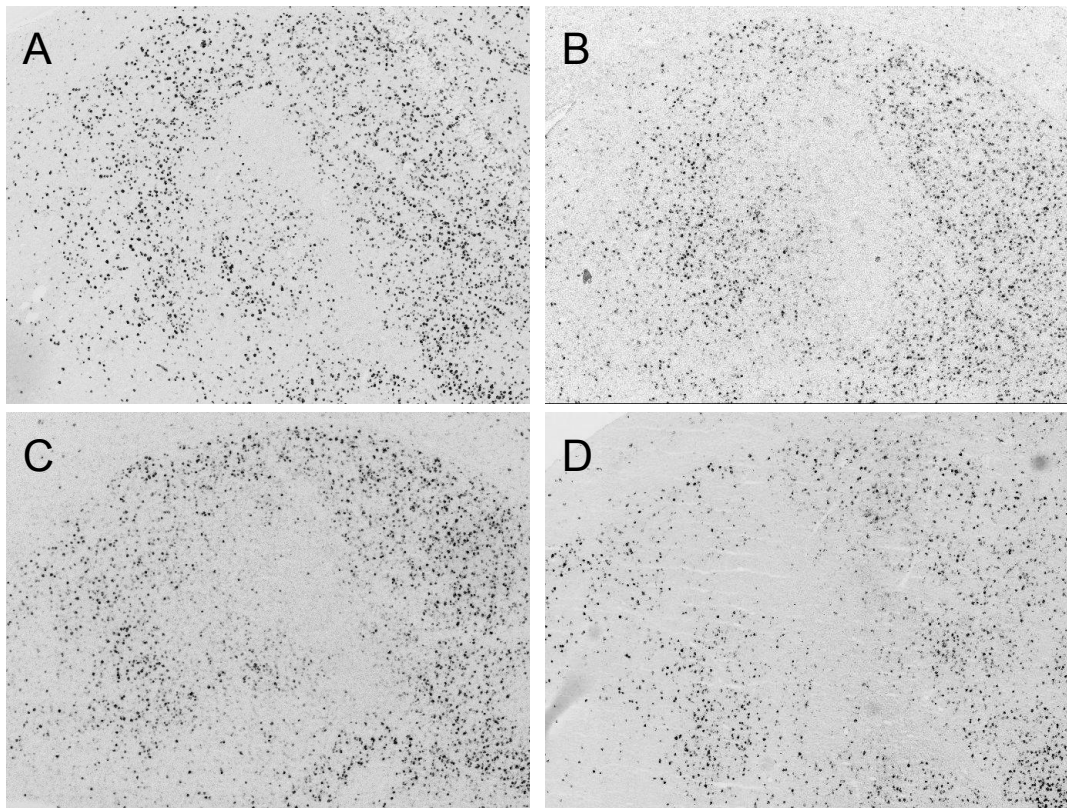


Figure 2.6: Right hemisphere auditory forebrain, 1.2 mm from midline. A) Go. B) No-Go. C) Novel. D) Habituated. All images are from representative birds, where overall staining levels are average for that condition.

no birds in the habituated condition exhibited parahippocampal staining).

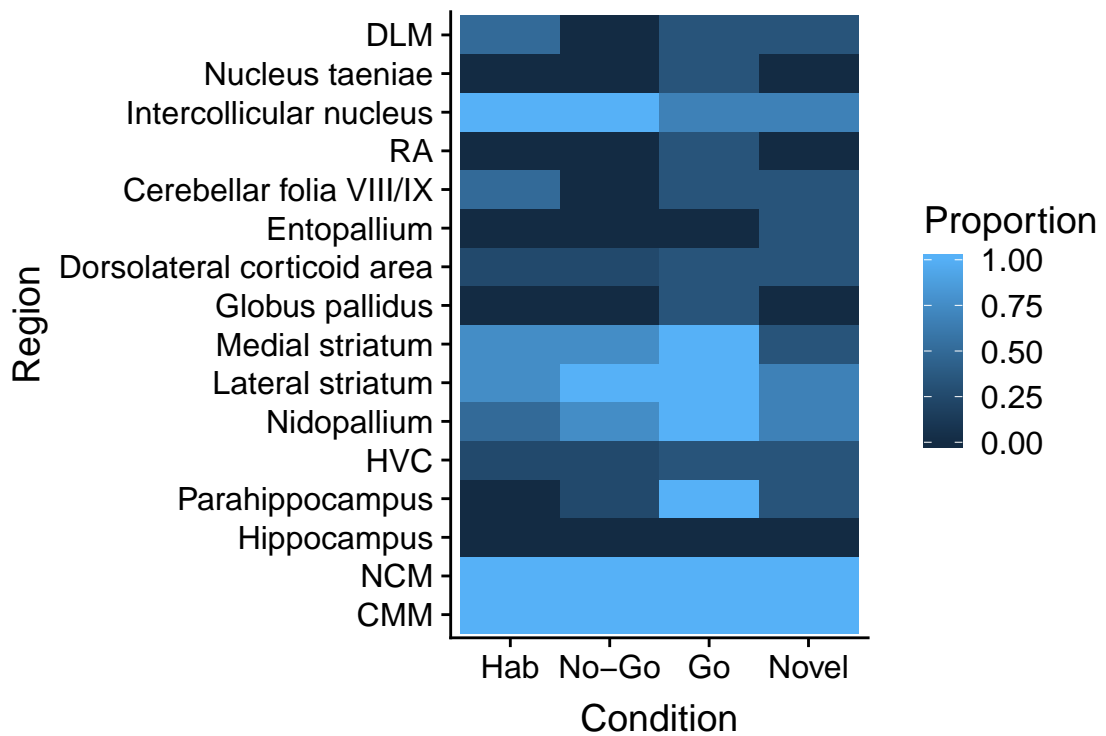


Figure 2.7: Proportion of individuals in each condition exhibiting clear *ZENK* expression in each brain region.

### 2.3.3 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 2.8).

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 2.8, Panel B), whole telencephalon median pixel density can

Table 2.3: LMMs for median pixel intensity of all target brain regions.

Model	Factors	df	AIC	Log-lik.	Comparator	$\chi^2$ 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

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).

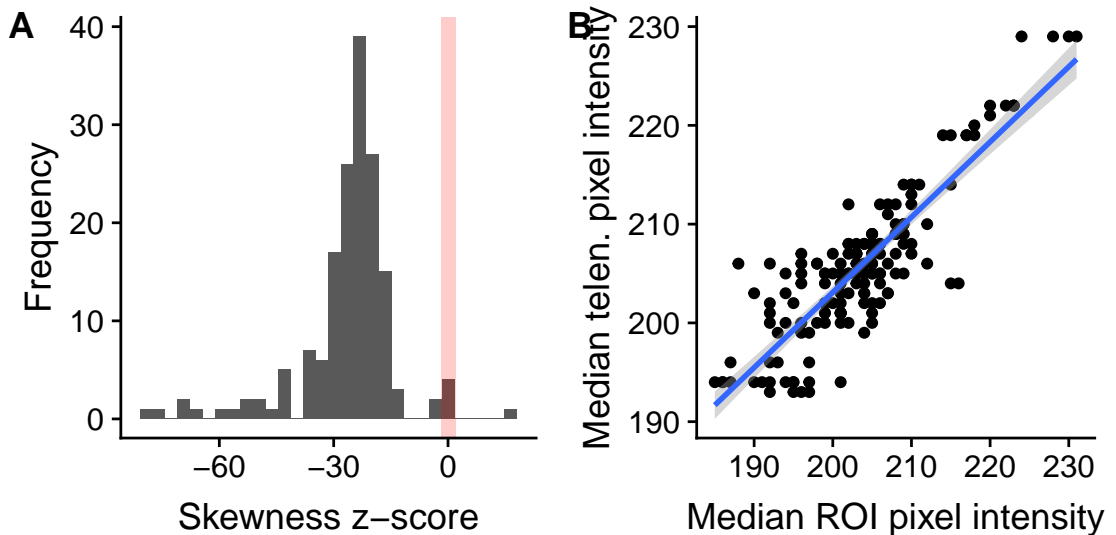


Figure 2.8: Model validation of GLMM. A) Distribution of skewness z-scores for ROI pixel intensity. The red rectangle indicates the acceptable range of skewness for small sample sizes. B) Linear relationship between median pixel intensity of ROI and of whole telencephalon.

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 2.3, Model 4; see also Figure 2.9). 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 2.3; Model 2) show significant ROI differences between 14 of the 28 possible contrasts (all  $p < 0.05$ ; Figure 2.9).

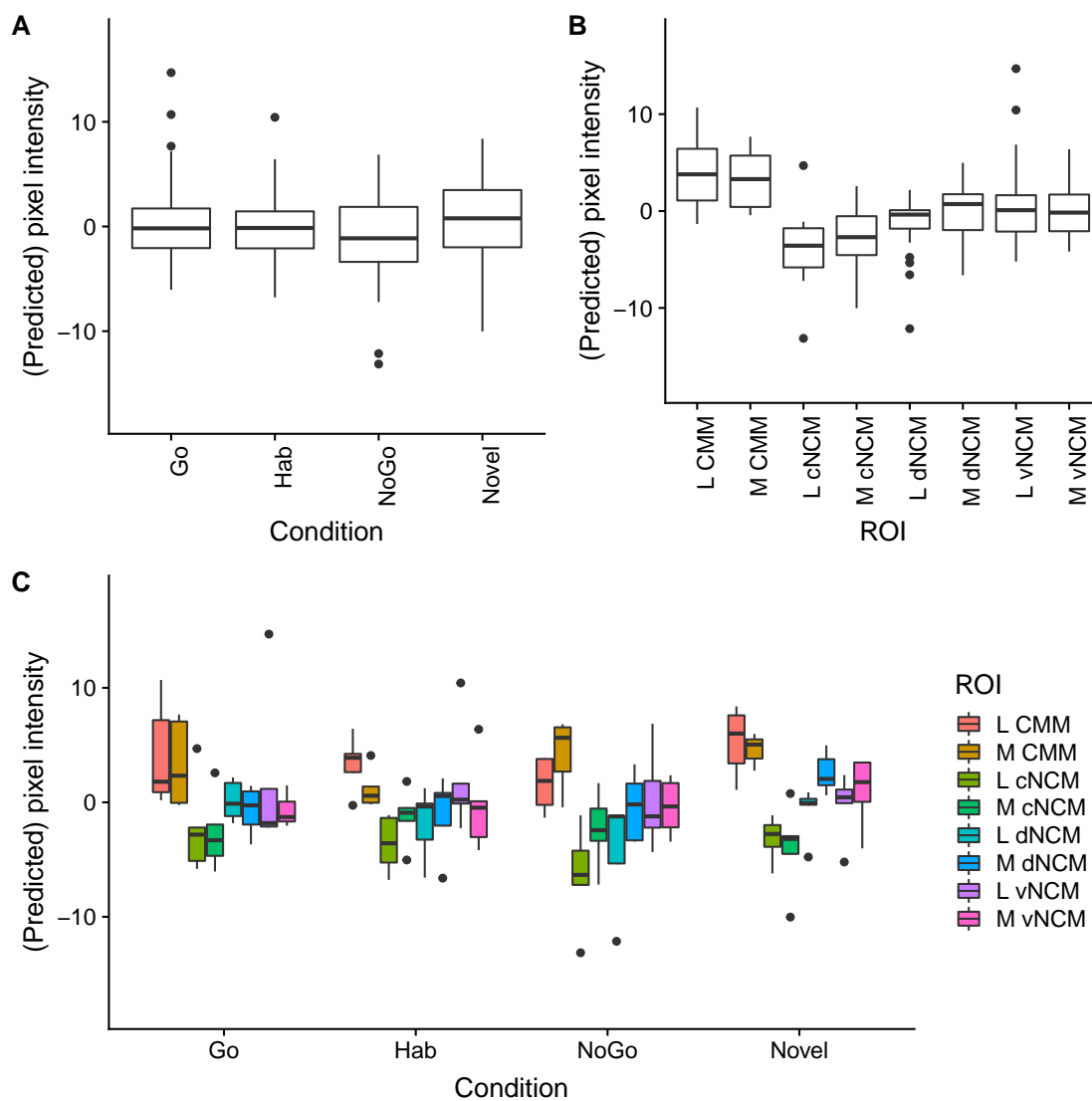


Figure 2.9: Median predicted pixel intensity (i.e. model residuals). A) Pixel intensity across all ROIs by condition. B) Pixel intensity across all conditions by ROI. C) Pixel intensity by ROI and condition.

### 2.3.4 Graph theory analysis of regional connectivity

Using a linear mixed model on pixel intensity, I found no significant main effect of condition, nor an interaction between condition and ROI. However, by visual inspection, I 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, I therefore turned to graph theory to determine if the different conditions elicited different patterns of ZENK. I 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 2.10). The edges were weighted such that the edge weights were set equal to the correlation coefficients. I 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.

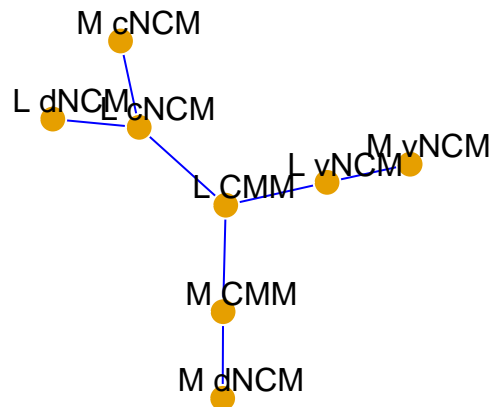


Figure 2.10: Graph of all ROI correlations where  $p < 0.10$ , across all conditions.

I then produced, for each condition, a graph using the same parameters (Figure 2.11). I 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).



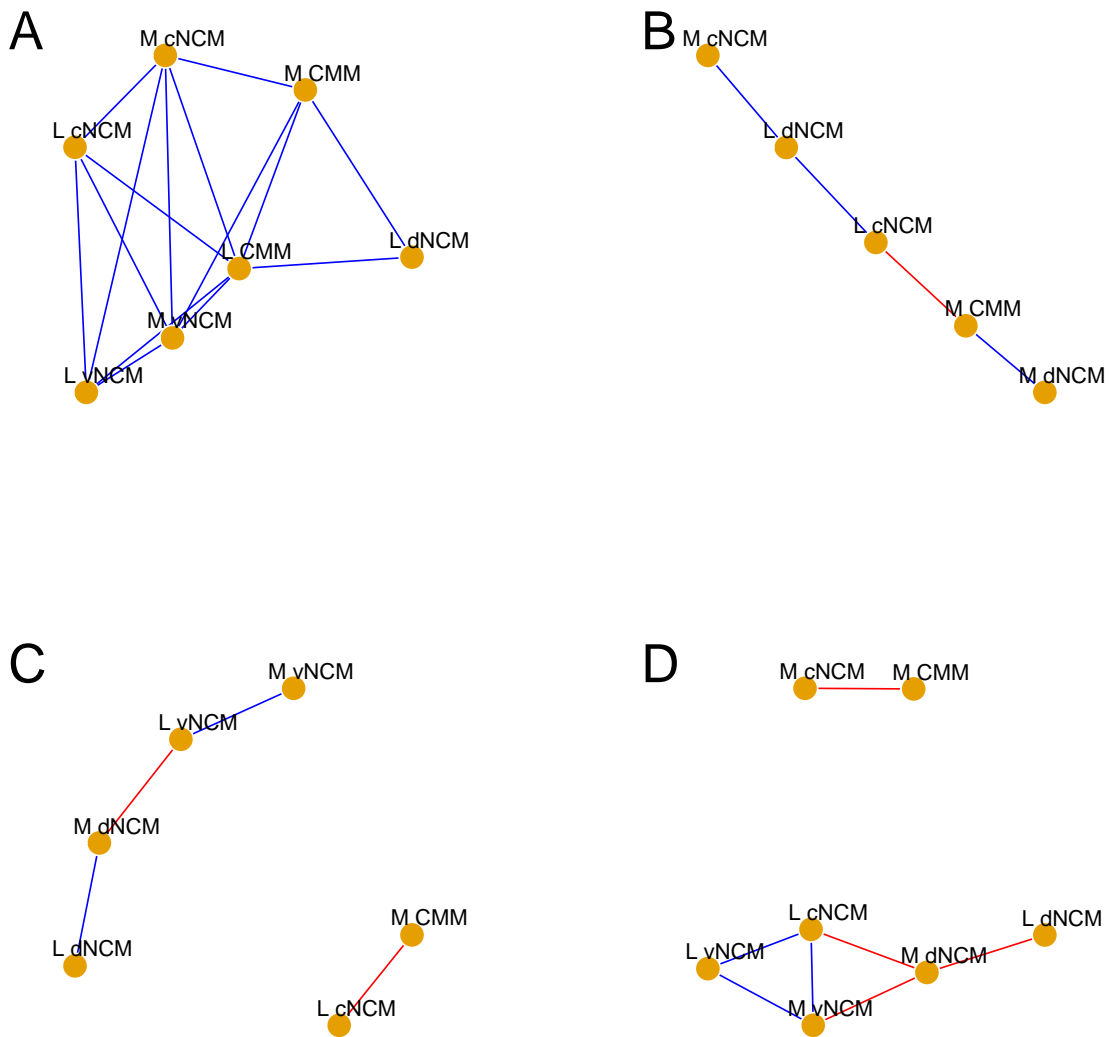


Figure 2.11: Graphs for each condition of all ROI correlations where  $p < 0.10$ . A) Go. B) No-Go. C) Habituated. D) Novel. Positive correlations have blue edges and negative correlations have red edges.

## 2.4 Discussion

Here I tested whether the learned valence of an acoustic stimulus is encoded by, or represented in, different patterns of *ZENK* expression within NCM and CMM. Using *in situ* hybridisation, I found patterns of individual differences in *ZENK* expression throughout the brain. However, using quantitative analysis, I 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, I did find evidence that the Go condition elicited a more coordinated response across the auditory forebrain than the three other conditions.

### 2.4.1 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; S. E. Sanford et al., 2010). I 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.

### 2.4.2 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 reduced levels of *ZENK* expression in the No-Go condition compared to the three other conditions. Previous literature has demonstrated aspects of song processing that are lateralised to either the left or right hemisphere (Lampen, McAuley, Chang, & Wade, 2017; Ruijssevelt et al., 2018; Voss et al., 2007). Here I only assessed the right hemisphere, so it is therefore possible that a Go/No-Go discrimination might be mediated by the left hemisphere. However, a separate RNA-Seq analysis following Go or No-Go acute song playback, which incorporated data from the auditory forebrain region in both hemispheres, found no significant difference in *ZENK* expression between the Go and No-Go conditions (Figure 2.12; Go and No-Go bars).

The lack of significant difference in overall *ZENK* staining in the auditory forebrain between the novel and habituated condition was especially surprising, as I 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 4) had intermediate levels of *ZENK* gene expression compared to female zebra finches in overnight social/auditory isolation and females in an aviary (Figure 2.12).

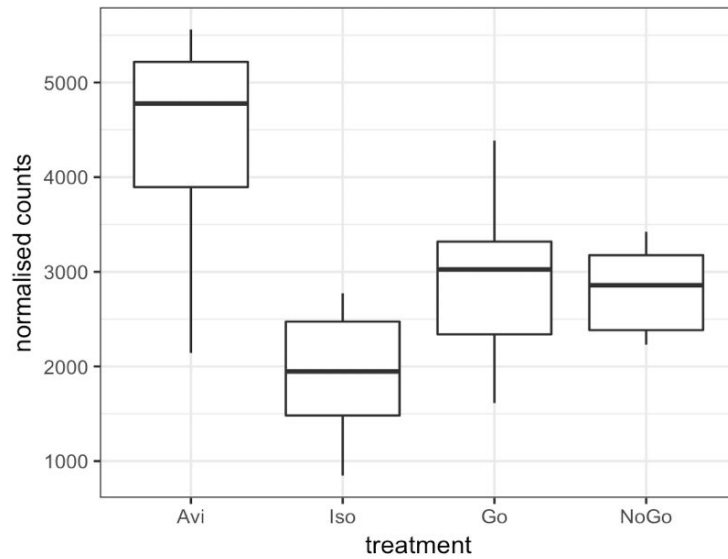


Figure 2.12: Normalised counts of *ZENK* gene expression in the auditory forebrain from two experiments. Aviary (Avi) and Isolated (Iso) are from George & Clayton, 2018. Go and No-Go are from the birds characterised in Chapter 4. Figure produced by J. George.

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. I 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).

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, I 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. I 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. I 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; S. E. 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 I still have no clear indication as to the cause or function of these.

I 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, I 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. I 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). I 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 I presented playbacks in a passive context where, as much as possible, I did not encourage any active learning about the stimulus, although I 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.

### 2.4.3 Connectedness of the auditory forebrain varies by condition

Though I found no main effect of condition, nor an interaction between condition and region of interest, I had predicted 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 (Clayton, 2000).

Network analyses often attempt to find central vertices, or regions that correlate with many other regions. For the Go response, I 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.

#### 2.4.4 Conclusion

Here I designed an experiment where I minimised, as much as possible, the confound of active learning in order to investigate passive perception of previously learned conspecific songs in adult female zebra finches. I 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, I 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. I saw evidence that the Go song playback drives a more coordinated response across the auditory forebrain than do the three other conditions. I 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. The subtlety of the differences between conditions suggests that there may be a role for subtle differences in learning behaviours, which I will examine in the following chapters.

## Chapter 3

# Operanter: open source hardware and software for avian operant conditioning

The experiments in Chapter 2 suggest there may be subtle differences in the pattern of correlated neural activities in the auditory forebrain elicited by a song stimulus, depending on whether the stimulus has been Go or No-Go conditioned. These observations motivated further study of how the Go and No-Go stimuli are learned (as described in this thesis), as well as a related project to look more deeply for transcriptional signatures that may distinguish Go and NoGo activity patterns in the auditory forebrain (George & Clayton, n.d.). Both of these aims require ready access to operant conditioning apparatus and populations of healthy adult zebra finches. As the operant conditioning experiments in Chapter 2 were conducted in a laboratory in the Netherlands, my next aim therefore was to develop an operant conditioning apparatus for the Clayton lab in London where I was based. In consultation with Prof. Clayton and colleague Dr. Robert Lachlan, we decided to build, from scratch, an operant conditioning hardware/software system that could be readily modified for different experimental designs and purposes, and might also be of wider utility to other laboratories. In this Chapter I describe the result: development of Operanter, a new open source hardware and software system. Then in the following chapter (Chapter 4), I describe my validation of the system, and in Chapters 5 and 6 I describe experiments using the system for close characterisation of Go/No-Go learning in the zebra finch.



### 3.1 The need for improved operant conditioning apparatus

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 interacting 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.). The most flexible operant conditioning system for birds is ARTSy, but this requires an expensive National Instruments I/O card, Windows OS, and Matlab, and the necessary Matlab code does not appear to be currently openly available (Gess, Schneider, Vyas, & Woolley, 2011).

Given these considerations, Prof. Clayton and I decided to work with Dr. Lachlan to implement a new open source hardware/software system that would immediately support the aims of my research but would also be readily adaptable for other purposes. Dr. Lachlan had already begun to develop Java-based software for operant conditioning based on an Arduino computer. My role was to work with him and Dr. Julia George to design and build the necessary Raspberry Pi-based hardware including the electronics and enclosures for the electronics, and to refine and extend the software. In the rest of this chapter, I provide a detailed description of the resulting system, “Operanter”.

## 3.2 What is Operanter

Operanter is a flexible and intuitive operant conditioning system. 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, the Operanter software 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>). The Operanter software controls multiple peripheral components using a Raspberry Pi running Raspbian.

### 3.2.1 Hardware: Raspberry Pi

Operanter was developed on a Raspberry Pi B+ running Raspbian Jessie (Figure 3.1). 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. The Operanter software 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.

### 3.2.2 Hardware: Peripheral components

The Operanter software is designed to work with the do-it-yourself (DIY) hardware on our GitHub Wiki. I describe how to use inexpensive and manufacturer-independent parts to build three types of components:

The Operanter software 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.

Though with adequate programming, any peripheral components can be built and controlled with Operanter, I developed the following 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.

#### 3.2.2.1 Light component

Automated control of light in the chamber is required for both the daily light/dark schedule, and control of punishment in our operant conditioning procedure. I considered two approaches to control the light: a remote/radio solution from Energenie, and a solid-state relay. I initially developed a system based on the

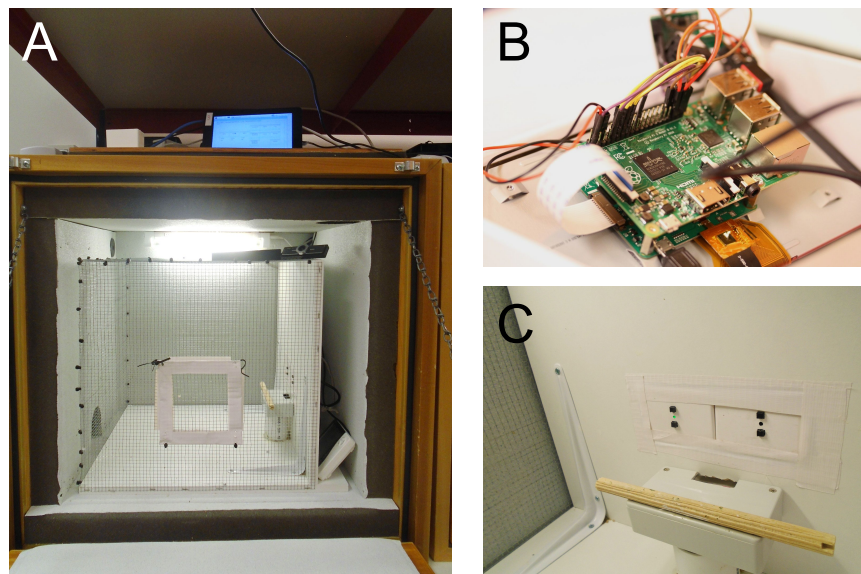


Figure 3.1: Operanter hardware, Raspberry Pi and electronics. A) The chamber with individual Raspberry Pi on top. B) Back of Raspberry Pi with GPIO connections to peripheral components. C) Infrared sensors and food hatch inside the cage.

Energenie Pi-mote (<https://energenie4u.co.uk/>), a radio-controlled on/off outlet that integrates with a Raspberry Pi. This control system simply involved wiring a light to a standard 13A UK mains plug, which was then plugged into the Energenie socket. Energenie supplies an add-on for the Raspberry Pi, which sends radio signals via the Raspberry Pi's GPIO pins, effectively turning the Raspberry Pi into a remote. Using sample code supplied by Energenie, Operanter controlled the light with sufficiently short lag times to enable a bird to learn the Go/No-Go procedure (data not shown). However, when eight chambers were placed in the same room, the radio signals were not sufficiently differentiated, and the radio signals from one Raspberry Pi/Energenie unit interfered with the signals from another unit. I estimated that a maximum of four chambers can be used in the same location without this issue arising. Therefore, for small laboratories where there will not be many chambers in use at the same time, the Energenie-based control method may be useful for controlling peripheral components without a direct/wired link to the Raspberry Pi.

However, the project for which Operanter was developed required eight chambers to run in tandem. I therefore developed a simpler wired control system based on a solid-state relay device. The advantage of the solid-state relay is its precise control of the electrical component to which it is wired, with no risk of radio interference between chambers. In contrast to the Energenie remote approach, developing the solid-state relay requires more confidence with electrical wiring as it involves

directly wiring a 13A UK mains plug to the solid-state relay device: the power source is much greater than the few hundred milliamps used for the remainder of the electrical components used by Operanter. Additionally, the correct solid-state relay must be purchased: the default state of the relay can be on or off, and if the Raspberry Pi fails, the solid-state relay will default to either open or closed. I therefore selected a default-on solid-state relay so that birds would not be in darkness in the case of software/hardware failure. I selected the solid-state relay as the final control unit for the light in the operant conditioning setup as it had none of the radio interference issues, and was also faster at switching the chamber light on and off (McMahon, pers. obs.).

### 3.2.2.2 Interactive sensors/LED component

In order for an organism to be automatically operantly conditioned, the apparatus requires a sensor component that allows the bird to indicate its choice (Go or No-Go). Additionally, previous researchers who have trained birds using operant conditioning have found success using lights to indicate when sensors are active (e.g. ten Cate laboratory at Leiden University). I therefore aimed to replicate this approach by integrating an LED light behind a sensor. All sensors described were wired to GPIO ports on the Raspberry Pi via an Ethernet connection.

I first replicated the button/switch type of sensor used at the ten Cate laboratory at Leiden University, but this form of sensor was unreliable due to the mechanical movement of the sensor itself. I then developed a highly sensitive vibration-based sensor (SW-18010P), which requires only 1g of force to trigger. This sensor responded when the bird pecked, but was also unreliable as it was occasionally triggered by a bird's excessive hopping around the chamber. To resolve this, I finally developed an infrared detection-based sensor (Figure 3.2). This sensor (GP1A57HRJ00F) is a U-shape with an infrared emitter at one end and an infrared detector opposite at the other end. When the beam from the emitter end is interrupted by a bird's beak, the detector recognises that it is no longer receiving the the infrared beam and sends a signal to the Raspberry Pi. The infrared sensor has no moving parts so is not susceptible to jamming like the button sensor, and cannot be accidentally triggered by hopping movement like the vibration sensor.

In order to indicate to the bird whether or not the sensor is active, and to provide a target for the bird's beak to peck, an LED needs to be placed in between the emitter and detector arms of the infrared sensor (Figure 3.2). To match the

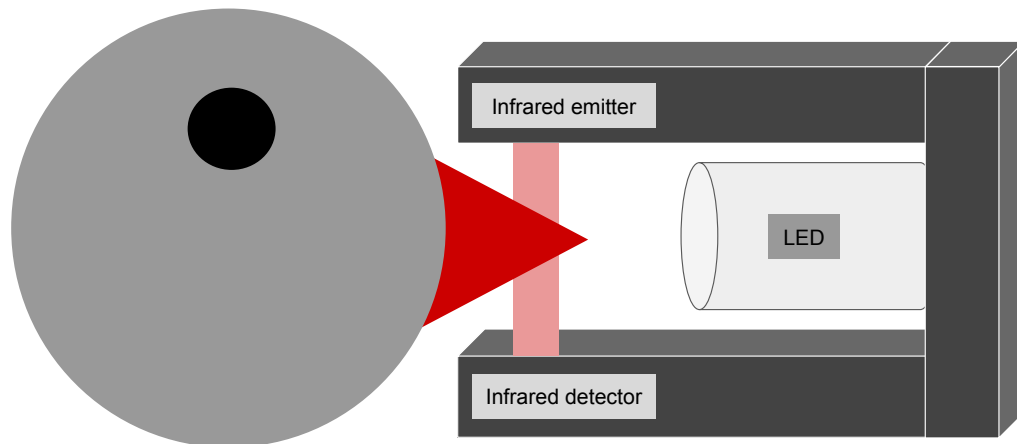


Figure 3.2: The sensor and LED component. The bird’s beak breaks the infrared beam from the emitter to the detector ends of the infrared sensor, and an LED component indicates when the sensor is active and provides an illuminated target for the bird’s beak.

sensor setup at the ten Cate laboratory at Leiden University, I first integrated a red LED into the sensor component. Researchers at Leiden University suggested that the red LED was originally selected because they believed it would attract the attention of zebra finches due to the attractiveness of red beaks (Simons & Verhulst, 2011). Unfortunately, with the red LED in position, the beam from the infrared emitter to the infrared detector could not be broken. This was because the red LED emitted light at wavelength 623 nm, which is between the range detected by the infrared detector (400-1200 nm with maximum sensitivity at 900 nm); that is, the infrared detectors are not just infrared detectors, but they detect wavelengths from violet to infrared. With the red light from the LED exciting the infrared detector, a bird could repeatedly peck at the sensor and not trigger the detector.

A green LED (525 nm) was used to replace the red LED, which attenuated the problem. However, even 525 nm excited the “infrared” detector when the green was bright enough. To resolve this problem, black nail varnish was painted around the sides of the LED cylinder so that the LED could only project light from the end. Black nail varnish was also painted around the inside of the sensor enclosure to reduce the reflectivity and the effect of leaking light from the LED. This reduced the intensity of the green light sufficiently so that a bird’s beak could break the infrared sensor’s beam, and the detector would not be excited by the LED light.

Though no quantitative analysis was conducted on the attractiveness of red versus green LEDs, birds were easily and quickly able to learn to peck at the green LED. Future developers may wish to consider the use of blue LEDs (470 nm) to further ameliorate the problem of residual light from the LED triggering the detector.

### 3.2.2.3 Food hatch motor component

Just as the light component controls the operant conditioning punishment, the food hatch motor component controls the operant conditioning reward: access to seed. Other implementations of avian operant conditioning software use a variety of methods to control access to seed. The ten Cate laboratory at the University of Leiden uses a linear servo motor that raises a vertical opaque window. The bird then pokes its head through the gap to access seed that is stored outside the cage (e.g. Heijningen, Visser, Zuidema, & Cate, 2009). The ARTSy system developed at the Woolley laboratory at Columbia University uses a solenoid motor that drives a food hopper from outside the chamber to inside the chamber where the bird can access the food (Gess et al., 2011). The vertical window system risks the window falling on the bird's neck if the mechanism jams, and the laboratory's pre-built sound isolation chambers did not have enough space to move an entire food hopper in and out of the cage. To optimise the space for birds to move about the cage and to reduce the risk of injury, a linear servo motor was designed that moves a horizontal food hatch cover.

### 3.2.3 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. The Operanter graphical user interface (GUI) comprises a single window with five tabs: Schedule, Operant Experiment, Log, Direct Control, and Stats (Figure 3.3). The entire GUI is optimised for touch-screen interaction; all regularly used buttons are large to enable quick and accurate interaction with the software.

The Schedule tab allows the user to set a daily schedule for when the lights are on

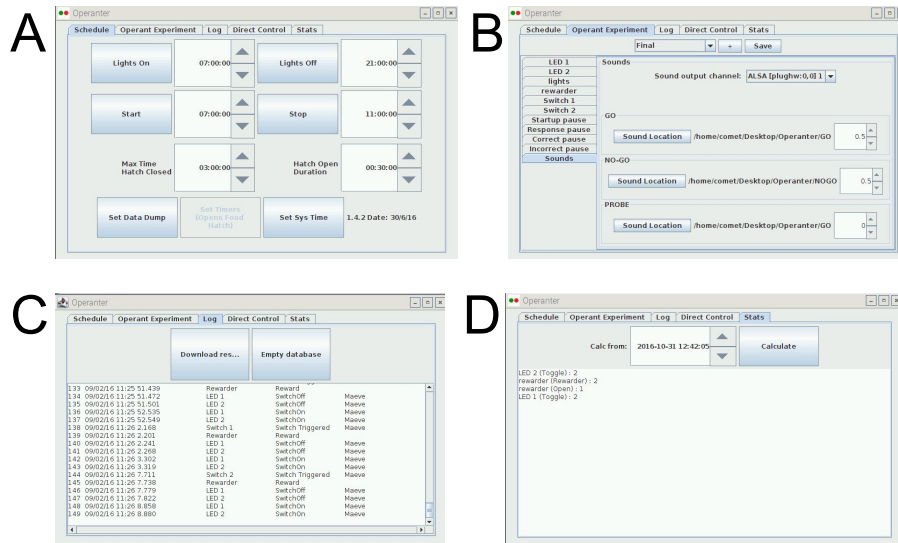


Figure 3.3: Operanter software. A) Schedule tab. B) Operant Experiment tab. C) Log tab. D) Stats tab.

and when the experiment runs. From this tab, the lights can be manually turned on an off, and the experiment can be manually stopped and started. This panel can be used to set a safety mechanism (maximum duration that the food hatch can remain closed); this feature allows our laboratory to comply with Home Office requests to implement technical solutions to reduce the risk of animals not feeding due to inactivity. From the Schedule tab, users can also set the computer system time, and to set the 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. Similarly, if a pre-programmed scheme uses a sound output channel, the Operant Experiment tab will allow the user to switch between available channels.

The Log tab shows a table of all activity. It displays the time of day, the activity (e.g. Switch On, Reward), the component that completed the activity (e.g. LED1, Rewarder) and the scheme name. The Log tab 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. All components referenced in the



current scheme will appear in the Direct Control tab. For example, the LEDs can be flashed and the food hatch can be opened and closed on demand. Large buttons on this panel enable the researcher or technician to view the bird's behaviour on half of the touch-screen and manually control the chamber with the other half.

Finally, the Stats panel shows a summary of how many times each action has been performed by each peripheral component, allowing researchers and animal caretakers to quickly determine the level of an animal's activity and success. It requires a user-input time, which enables the user to view activity from a certain time, such as yesterday morning or from the beginning of the week; the time defaults to the most recent start-up time of the software or scheme.

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. I 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 I 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.

### 3.2.4 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.

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.

### 3.3 Conclusion

Operanter is free open source software and hardware for controlling operant conditioning that runs on a Raspberry Pi computer. Originally designed for avian auditory Go/No-Go training, Operanter 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. 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 systems allows researchers with small budgets or specific needs to carry out operant conditioning experiments. In the next chapter (Chapter 4), I will describe an experiment validating the efficacy of the Operanter system.

# Chapter 4

## Initial validation of the Operanter system

### 4.1 Introduction

After the development of Operanter, chronicled and described in Chapter 3, it was necessary to demonstrate that it could successfully be used to train zebra finches for experiments require Go/No-Go operant conditioning. The Operanter setup will be benchmarked against the Leiden University setup for the following reasons: 1) the Leiden University setup has been used in many published studies over the last decade (e.g. Chen, Rossum, & Cate, 2015; Heijningen et al., 2009; Holveck & Riebel, 2014); 2) the neurogenomic results from birds trained at Leiden University (described in Chapter 2) needed to be compared to neurogenomic results from an allied study that was conducted using Operanter; 3) due to Home Office inspector recommendations, and in contrast to the Leiden University apparatus that ran during all daylight hours, our operant conditioning apparatus will only run while an experimenter is on site. As such, a comparison of Operanter and the proprietary Leiden University apparatus will enable the comparison of neurogenomic data resulting from both laboratories.

### 4.2 Methods

The following methods describe the work undertaken at the Clayton laboratory at Queen Mary University of London. Methods for the data describing the learning of the Leiden cohort of birds can be found in Chapter 2.

### 4.2.1 Animals

23 female zebra finches (*Taeniopygia guttata*) originally from a breeding line at the University of Glasgow were bred at Queen Mary University of London in a large free breeding aviary (20-80 individuals, 3.9 m x 4.3 m). It is unknown which, if any, of the females here had previously been involved in breeding. Prior to the initiation of the experiment, they were then housed in a single sex aviary with 6-24 females at any given time (1.9 m x 2.0 m x 2.0 m high) 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.

### 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. A Raspberry Pi automatically controlled the operant conditioning, including the food hopper, LED/peck detectors, speaker, and the chamber light (as described in Chapter 3). A total of eight chambers, with identical apparatus, were used.

### 4.2.3 Stimuli

For all birds, the early training stages used a novel male zebra finch song and a sine wave tone (440 Hz). 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 and to be maximally acoustically different from each other. 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.

#### 4.2.4 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.

During the first and second stages of training, auto-shaping and hand-shaping was occasionally used to encourage the birds to learn more quickly. During stage one, auto-shaping was completed by navigating to the Direct Control tab of the Operanter GUI, flashing one or both of the LEDs, followed by opening the food hopper. By completing the auto-shaping procedure multiple times, birds learned the association between the LED and the food hopper, which encourages the birds to attempt to peck at the LED more quickly. During stage two, hand-shaping was completed by flashing the right LED after the bird pecked the left sensor, to encourage the bird to attend to the right LED and eventually peck it.

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.

#### 4.2.5 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.

#### 4.2.6 Statistics

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

### 4.3 Results

Both the Leiden ( $n = 18$ ) and the London ( $n = 23$ ) cohorts of birds learned the discrimination between Go and No/Go. Trials were binned into 100-trial bins.  $d'$ , a measure of sensitivity defined as the z-score of the false alarm rate subtracted from the z-score of the hit rate, was calculated for all birds for all bins. These bins are plotted in Figure 4.1. Those  $d'$  scores were linearly modelled on the logarithm of the bin number. That model indicates that the Leiden birds reached a  $d'$  of 2

after 7 100-trial bins, and the London birds reached the same  $d'$  criterion after 8 100-trial bins.

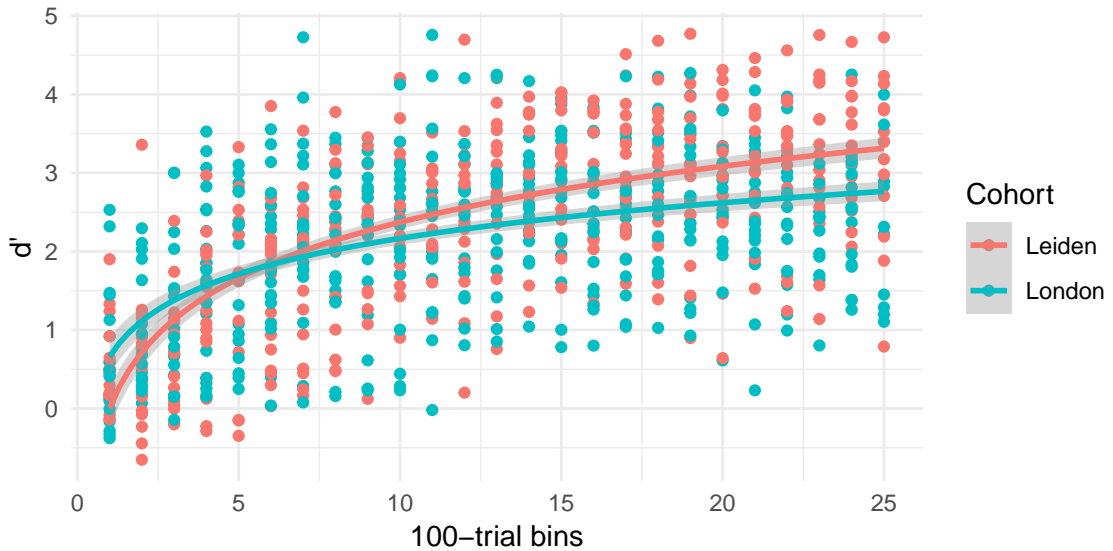


Figure 4.1: Learning curves for Leiden birds (proprietary system) and London birds (Operanter system).

Zebra finches trained using the Operanter software and hardware achieved, on average, a lower asymptotic performance compared to zebra finches trained using proprietary software and hardware at the University of Leiden (Figure 4.1). A two-sample  $t$ -test on bins 20-25 indicates that the final performance of the Leiden cohort is significantly better than the final performance of the London cohort ( $t(34.6) = -3.2, p = 0.003$ ).

## 4.4 Discussion

23 female zebra finches were shown to successfully learn to discriminate two stimuli using Operanter, though the Leiden cohort birds did reach a higher asymptotic performance on average than the London cohort. This is likely due to significant differences in the conditioning protocol. Specifically, London birds were only trained until the experimenter went home, and received food *ad libitum* after this time, to comply with UK Home Office recommendations; in contrast, birds at the University of Leiden never received food *ad libitum* and engaged with the operant apparatus throughout the entire photoperiod. The discrimination performance of the London birds was determined to be sufficient for the purposes of the neurogenomic follow-up studies.

I have developed an integrated hardware/software system for zebra finch operant

conditioning. Operanter is more flexible than most open source or proprietary systems, and is substantially cheaper than all other published systems. To our knowledge, it incorporates more safety and welfare mechanisms than any alternative. It also has the specific advantage of comprising one independent machine per subject, reducing the possibility of accidental interference by other researchers and of multiple systems failing at the same time.

I have validated that Operanter is functional and effective, as demonstrated by its success with controlling operant conditioning training for 23 individuals shown here, as well as 17 additional individuals trained during later studies. As shown here, zebra finches can learn Go/No-Go discrimination in roughly the same number of trials as reported in previous literature (e.g. Gess et al., 2011). Additionally, the rate of learning of discrimination, if not asymptotic performance, is similar to that of birds trained using the proprietary system at our collaborator's facility at the University of Leiden.

In conclusion, Operanter provides a much-needed open source alternative to commercial and proprietary operant conditioning setups. This system will allow us to proceed with the training of zebra finch operant learning of song discriminations, as will be described in the rest of my thesis. Operanter's robust data output can be easily transformed into tidy data (Wickham, 2014), which is a feature I will take advantage of in Chapters 5 and 6.



## Chapter 5

# 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 I avail of a large dataset of simple Go/No-Go discrimination learning of a conspecific song, and long-term maintenance of this discrimination behaviour. I 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. I highlight large individual differences in daily patterns of activity, and demonstrate a relationship between learning rate and when birds prefer to be active. I also find that response accuracy during maintenance can be affected by time of day, inter-trial interval duration, accuracy on the preceding trial and stimulus type. These results have numerous implications for experimenters using Go/No-Go operant conditioning.

## 5.1 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 (Evans, 1970). 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 et al., 2015; M. Long, Jiang, Liu, & Yao, 2015). But despite a long history of investigation of fundamental operant conditioning variables, such as reinforcement frequency (e.g. Herrnstein, 1961; Skinner, 1938), and more recent attempts to understand specific cognitive aspects of Go/No-Go learning, such as working memory and behavioural inhibition (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 (Kirsch et al., 2004). 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 response behaviours can be assessed with signal detection theory in order to quantify those biases (Klink, Bendig, & Klump, 2006). 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). Peak shift is a feature of discrimination learning whereby an organism responds most to probe stimuli that are most displaced from the reinforced stimulus along a dimension opposite to the punished stimulus (Purtle, 1973). In Go/No-Go paradigms, peak shift will cause greater analytical difficulties than in 2-AFC paradigms; only one bias can be calculated for Go/No-Go due to the response behaviour being simply Go or No-Go, whereas three biases can be calculated for 2-AFC (responding left, responding right, and responding left or right when a response is made). Additionally, the Go/No-Go bias can be altered by a wider range of factors, such as motivation, than the 2-AFC biases. A more thorough characterisation of responses during Go/No-Go learning and maintenance without probe stimuli will therefore aid researchers in understanding experiments that use the response to probe stimuli to interrogate perception.

### 5.1.1 Motivational factors in operant conditioning

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). Strong reinforcement schedules using food as a reward may lead to satiation and a decrease in production of the response behaviour (as reviewed in McSweeney & Roll, 1993). 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 associations (Scheiner, Erber, & Page, 1999; Stebbins, Mead, & Martin, 1959), 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 initiate 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. The animal welfare inspector believed that a subject becoming injured during unsupervised conditioning during the evening was a risk we should not take. However, I believe that two factors may outweigh that risk: the lack of enrichment during evening hours due to the inability of a bird to initiate the presentation of intrinsically rewarding stimuli, and the possibility that social isolation might be extended if the birds require more days to reach training criterion. Therefore, a characterisation of trial initiation times could enhance our understanding of response behaviour during training and maintenance, and may also aid in the improvement of our experimental procedure.

Trial timing has been explored in multiple contexts, including the massed versus spaced trial timing framework (as reviewed in Delaney, Verkoeijen, & Spigel, 2010). In one early study of pigeon short-term memory, spacing out the inter-trial interval led to reduced memory retention (Roberts, 1972). However, a wide body of literature has suggested that spaced trials may enhance learning compared to massed trials in classical conditioning (e.g. Spence & Norris, 1950) and in autoshaping (e.g. Gibbon, Baldock, Locurto, Gold, & Terrace, 1977). In the present study design, zebra finches will initiate their own trials, and the inter-trial interval duration may influence the accuracy of the responding.

As well as inter-trial interval timing, the time of day may have an effect on learning and accuracy. 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, I was 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 response accuracy and learning rate.

### 5.1.2 Response behaviours to Go/No-Go tasks

The response behaviours to Go/No-Go tasks 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 with little change in response accuracy for multiple days (i.e. correct responses are subject to a floor effect), 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 fine-grained information on subject performance (Kahana & Loftus, 1999). Though previous studies have not found that response latency is more sensitive than response accuracy (MacLeod & Nelson, 1984), this might not be true for all states of the Go/No-Go operant conditioning procedure,

especially those states at the beginning and end of training when response accuracy sensitivity is extremely low due to floor and ceiling effects.

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). Specifically, it has been theorised that response accuracy measures whether the memory encoding process was efficient for retrieval, whereas response latency measures the number of decoding steps during retrieval (MacLeod & Nelson, 1984). In contrast, Anderson characterised response accuracy as being an indication of the probability of a trace (a connection between stimuli and/or concepts) being formed or the probability of a trace not being able to be activated, and he characterised response latency as the level of activation of a trace (1981). What MacLeod (1984) and Anderson (1981) have in common is the conceptualisation of response accuracy and response latency as measuring two separate aspects of memory. The characterisation of response latencies, particularly by comparing change in response latency with change in response accuracy, during avian Go/No-Go conditioning could be of value to researchers who use this methodology.

### 5.1.3 Aims and hypotheses

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 predicted 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 4, Leiden birds were on average faster to reach our discrimination criterion than London birds, despite using a similar 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. 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. We also aimed to develop a model for predicting response accuracy during maintenance trials to better understand the factors that drive the birds' response decisions after the initial learning of the discrimination.

## 5.2 Methods

### 5.2.1 Animals

24 female zebra finches (*Taeniopygia guttata*) originally from a breeding line at the University of Glasgow were bred at Queen Mary University of London in a large free breeding aviary (20-80 individuals, 3.9 m x 4.3 m). It is unknown which, if any, of the females here had previously been involved in breeding. Prior to the initiation of the experiment, they were then housed in a single sex aviary with 6-24 females at any given time (1.9 m x 2.0 m x 2.0 m high) 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.

### 5.2.2 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 3; Figure 5.1).

### 5.2.3 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

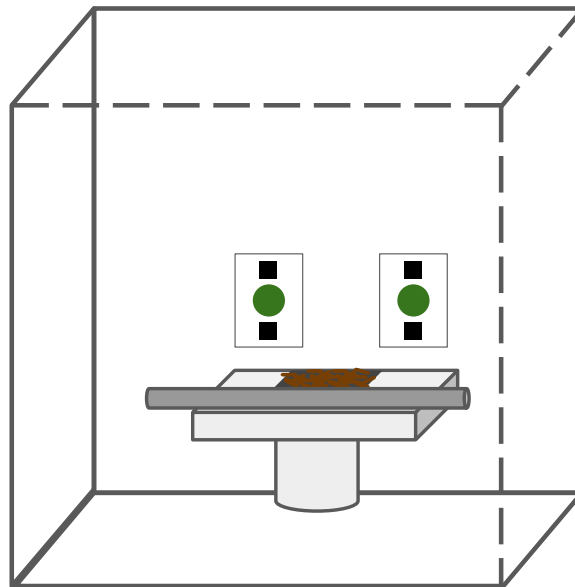


Figure 5.1: Diagram of the operant conditioning apparatus in the sound attenuation chamber. The setup includes two infrared detectors with green LEDs and a horizontally mounted motorised food hopper opening.

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.

#### 5.2.4 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.

### 5.2.5 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.

### 5.2.6 Statistics

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

## 5.3 Results

### 5.3.1 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 5.2. Panel A of Figure 5.2 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 5.2 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.

### 5.3.2 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 5.3 shows that for the first 400 trials, birds had a slight bias towards a Go

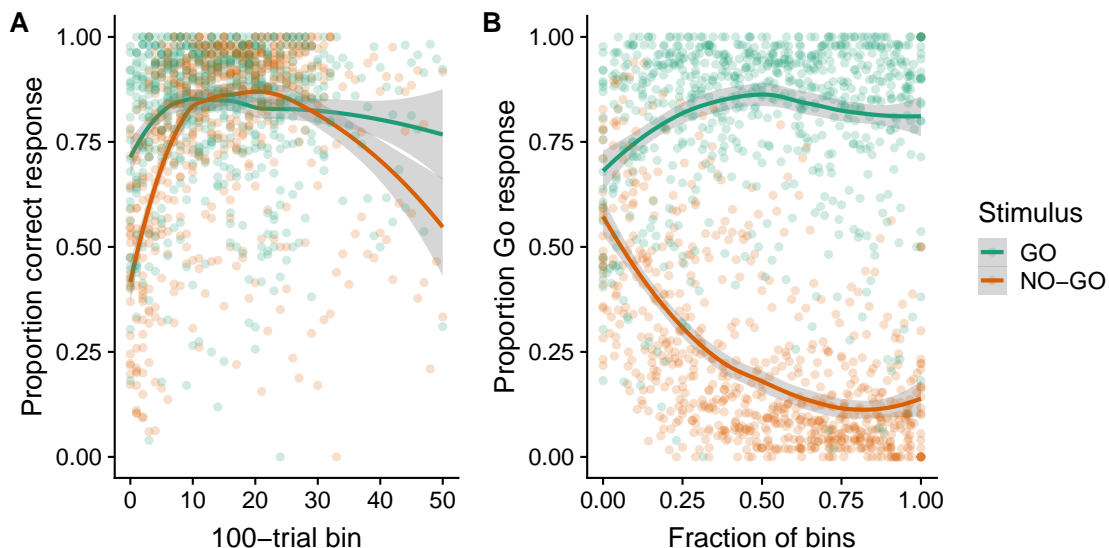


Figure 5.2: Averaged learning curves for all birds. A) Proportion of correct trials for 100-trial bins. B) Proportion of Go responses, normalised for each bird, where bin fraction is the bin number divided by the maximum number of bins for each bird. Lines of best fit are modelled with loess regression, with standard error shading.

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.

### 5.3.3 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 5.4). 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 5.4; Panel A). In contrast, for No-Go stimuli, response latencies appear to diverge into a bimodal distribution during maintenance (Figure 5.4; Panel C). Three outliers who learned extremely slowly were removed for this analysis.

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 5.4). Response latencies during learning were from a significantly 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

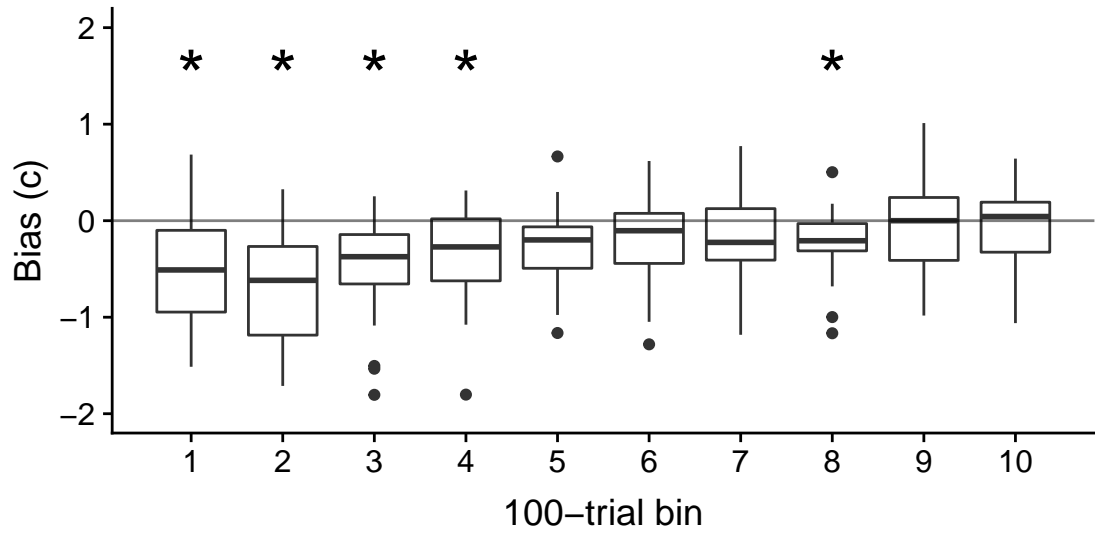


Figure 5.3: Bias (c) for first 10 100-trial bins, where scores  $> 1$  indicate a No-Go bias and scores  $< 1$  indicate a Go bias. Asterisks indicate significance at the 0.05 level (with Bonferroni correction).

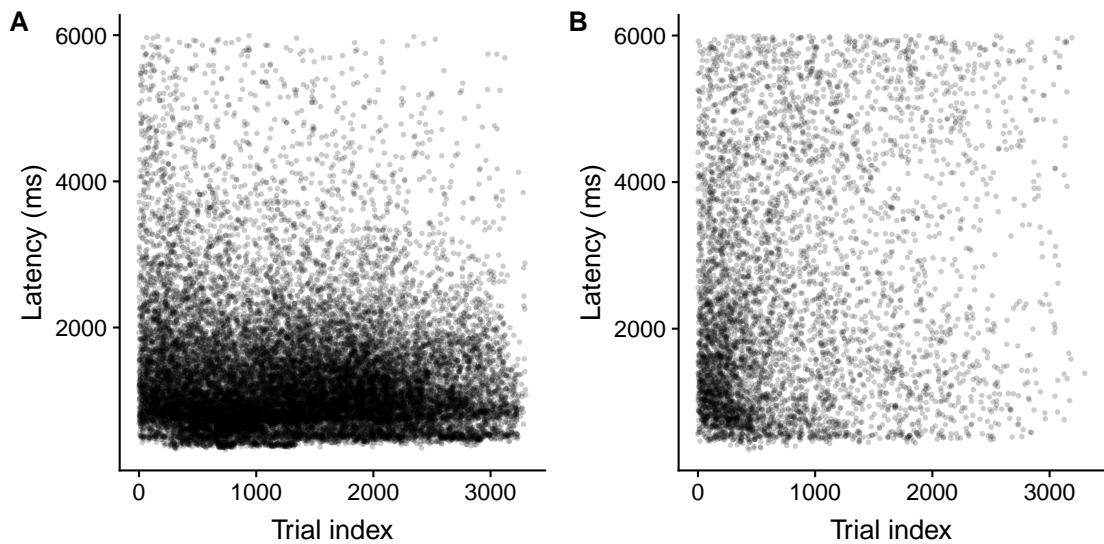


Figure 5.4: Response latencies (in milliseconds) to stimuli throughout learning and maintenance. Panel A is correct responses to Go stimuli; Panel B is incorrect responses to No-Go stimuli.

(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 5.5). 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 5.5; 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 5.5; Panels C & D).

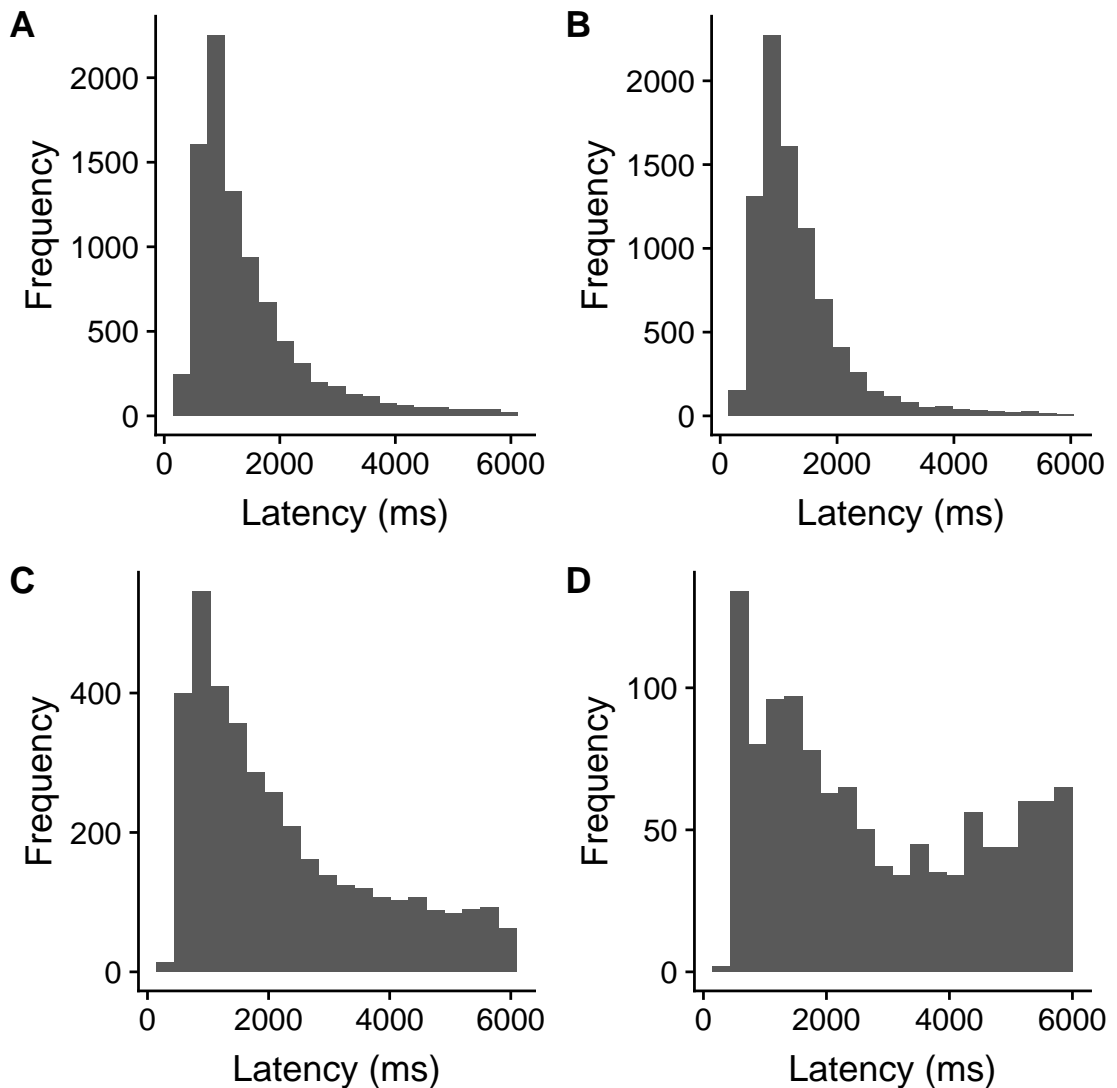


Figure 5.5: Response latencies in milliseconds. A & B) Correct responses to Go stimuli. C & D) Incorrect responses to No-Go stimuli. A & C) During learning (trials 1-1000). B & D) During maintenance (trials 1001-2000).

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 5.6). 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;  $D = 0.43$ ,  $p < 0.0001$ ).

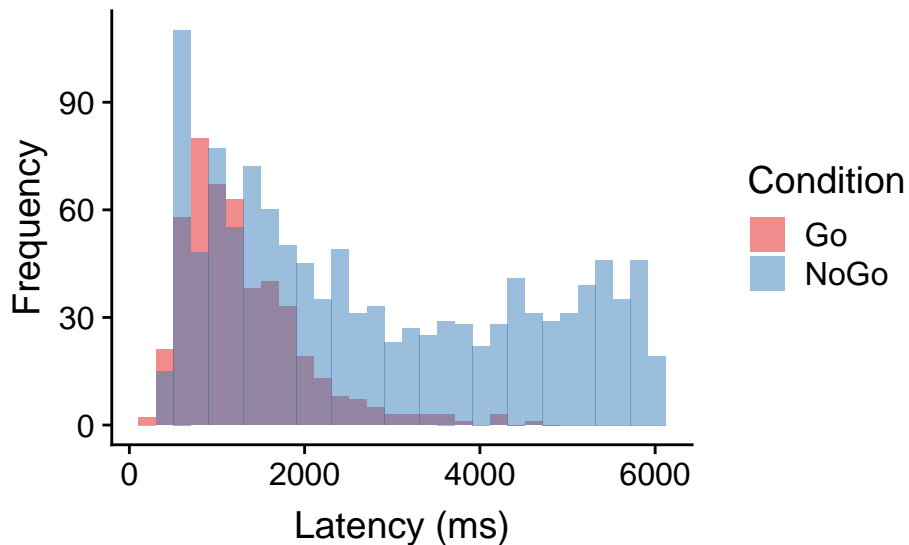


Figure 5.6: Histogram of Go and No-Go response latencies during maintenance. Red bars indicate a generated normal distribution that describes Go response latencies. Blue bars indicate raw No-Go latencies. The purple region is where Go and No-Go response latencies overlap.

#### 5.3.4 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 5.7). 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).

To determine if birds' motivation varied through the day, a number of metrics were calculated for each bird during the maintenance phase. Figure 5.8 shows

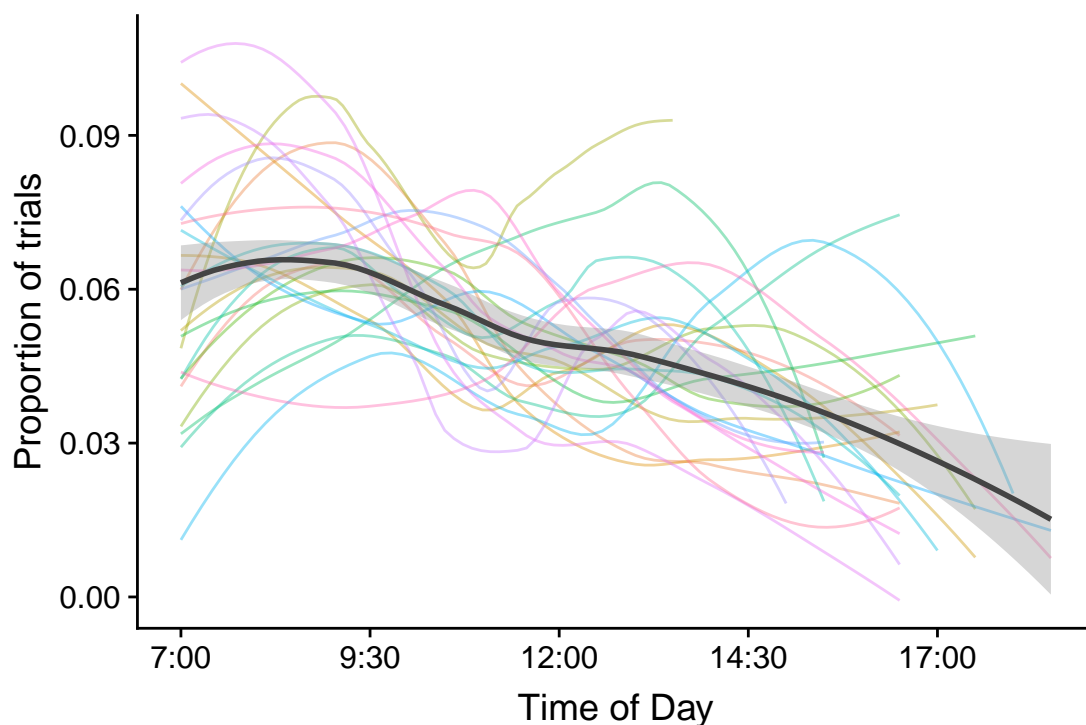


Figure 5.7: Activity levels for individual birds throughout the day, in half hour bins, during the maintenance stage. Lines of best fit are loess regression lines fit to the mean proportion of trials during half hour bins for each individual bird, across all days of maintenance.

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.

### 5.3.5 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 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.

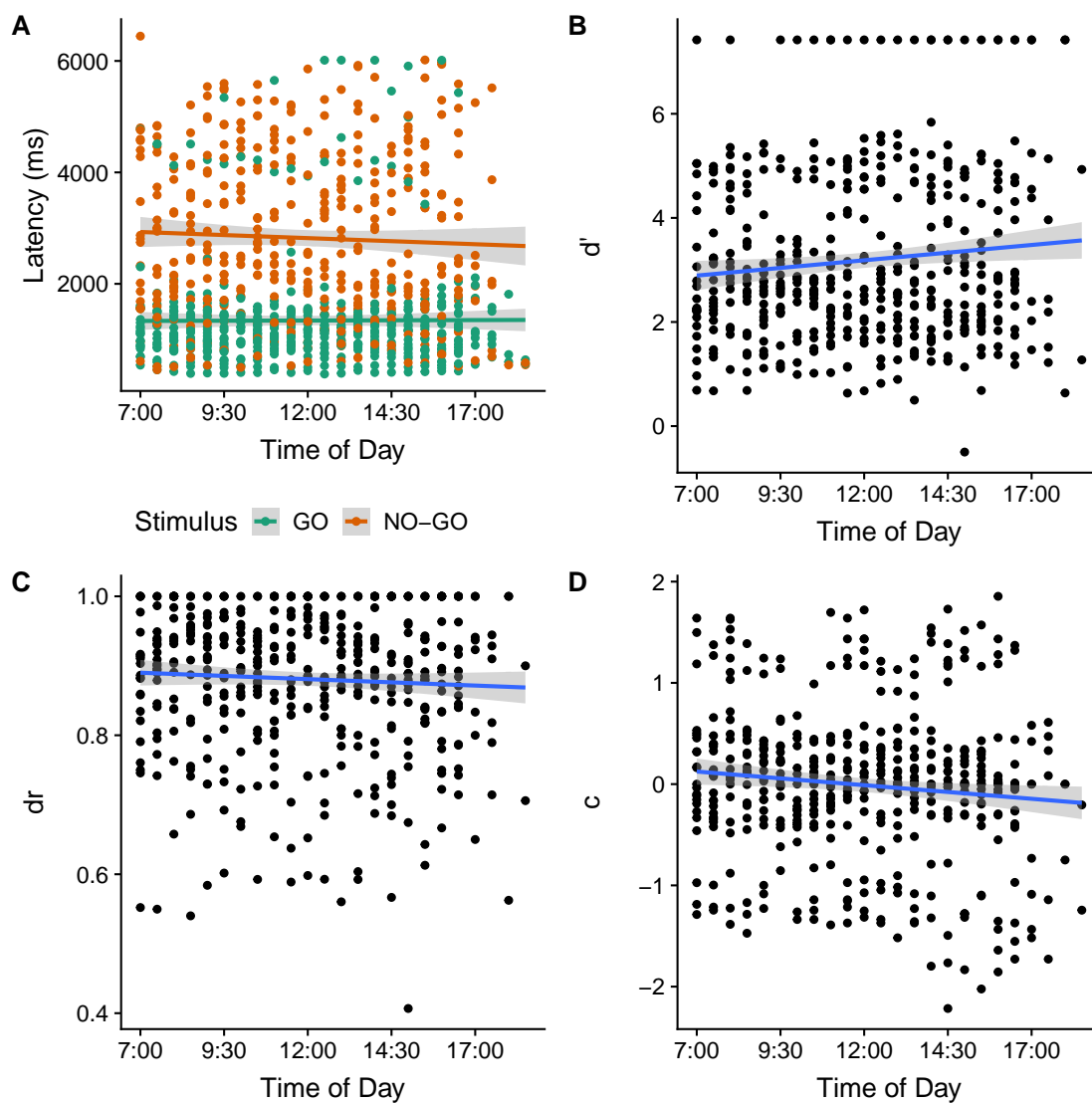


Figure 5.8: Four metrics of behaviour through the day. A) Response latencies to Go and No-Go stimuli. B) Accuracy ( $d'$ ). C) Accuracy (discrimination ratio). D) Bias ( $c$ ). All lines of best fit are linear regressions with standard error shading.



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 5.9, 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 5.9, Panel B;  $\rho = -0.02$ ,  $p = 0.92$ ) were significantly correlated with learning rate.

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 5.9, Panel C;  $\rho = -0.34$ ,  $p = 0.12$ ), but median activity was moderately significantly negatively correlated with learning rate (Figure 5.9, 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.

### 5.3.6 Response accuracy during maintenance is affected by time of day and recent preceding behaviour

One of the findings described in Chapter 4 was that the Leiden birds were more accurate during maintenance than London birds. One potential explanation for this effect might be that the Leiden birds interacted with the operant conditioning software throughout the photoperiod, whereas London birds interacted with the operant conditioning software while an experimenter was on site. In Section 5.3.4 I demonstrated that activity levels vary by individual throughout the photoperiod, though I found no linear effect of this on accuracy as calculated using  $d'$ . However, modelling trial-by-trial accuracy, rather than  $d'$  across bins, may provide deeper insights into factors affecting a bird's performance. A series of binomial generalised linear mixed models was run to determine if response accuracy is affected by the inter-trial interval (ITI) from the preceding trial (ITI: log-transformed milliseconds), stimulus type (Stimulus: Go/No-Go), accuracy on the preceding trial (PreAcc: correct/incorrect), stimulus type of the preceding trial (PreType: Go/No-Go), response latency (Latency: milliseconds) and the time of day (TimeOfDay: log-transformed milliseconds from the start of the photoperiod)

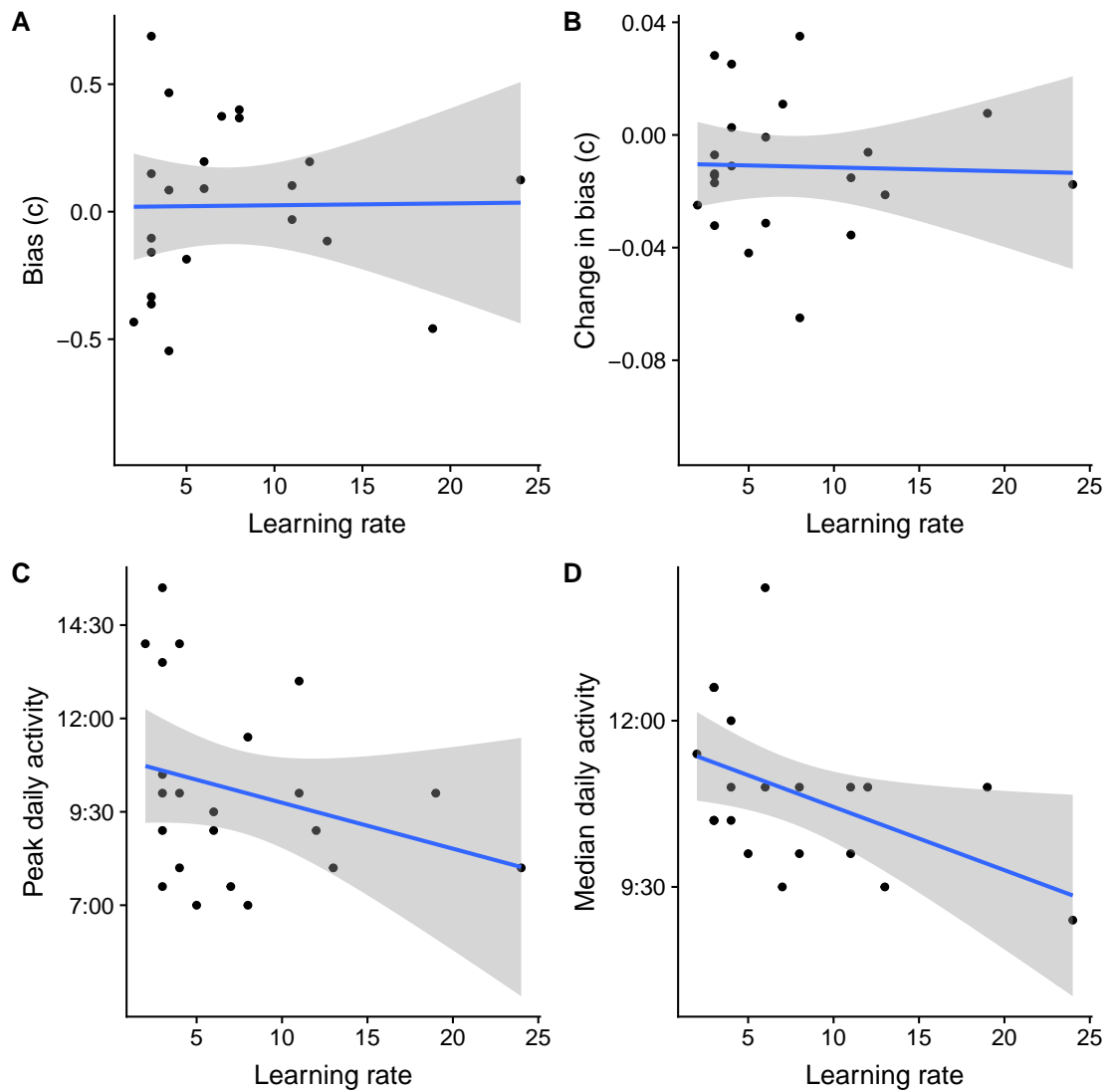


Figure 5.9: Relationship between learning rate, where larger values indicate slower learners, and possible predictors. A) Bias. B) Change in bias through the day. C) Peak activity half-hour time bin. D) Median activity half-hour time bin. Lines of best fit are all linear models with standard error shading.

Table 5.1: GLMMs for modelling accuracy of response during maintenance trials.

Model	Factors	df	AIC	Log-likelihood	Comparator	$\chi^2$ test	P ( $> \chi^2$ )
NULL	(1   BirdID) + Index	3	68103	-34048			
1.1	NULL + Stimulus	4	67845	-33918	NULL	259.6	<2.2e-16
1.2	NULL + TimeLag	4	67918	-33955	NULL	186.5	<2.2e-16
1.3	NULL + PreAcc	4	67759	-33876	NULL	345.4	<2.2e-16
1.4	NULL + PreType	4	68103	-34048	NULL	1.1	0.301
1.5	NULL + Latency	4	68104	-34048	NULL	0.4	0.538
2	Model 1.1 + ITI	5	67655	-33823	Model 1.1	191.6	<2.2e-16
3	Model 2 + PreAcc	6	67331	-33660	Model 2	325.8	<2.2e-16
4	Model 3 + PreType	7	67331	-33659	Model 3	2.0	0.156
5	Model 4 + Stimulus:PreType	8	67330	-33657	Model 4	3.2	0.076
6	Model 3 + PreAcc:ITI	7	67316	-33651	Model 3	17.0	3.7e-05
7	Model 6 + PreAcc:Stimulus	8	67290	-33637	Model 6	28.2	1.1e-07
8	Model 7 + Stimulus:ITI	9	67129	-33556	Model 7	163.2	<2.2e-16
9	Model 8 + PreAcc:ITI:Stimulus	10	67105	-33542	Model 8	26.5	2.6e-07
10	Model 9 + TimeOfDay	12	67091	-33533	Model 9	17.9	0.00012
11	Model 10 + TimeOfDay:Stimulus	14	66984	-33478	Model 10	110.4	<2.2e-16
12	Model 11 + TimeOfDay:PreAcc	16	66980	-33474	Model 11	8.67	0.013
13	Model 12 + TimeOfDay:ITI	18	66978	-33471	Model 12	5.2	0.073

(R package: lme4).

An initial null model with bird ID as a random effect and the index number (by bird; divided by 1000 for scaling purposes) was built. Bird ID controls for within-bird effects, and inclusion of the index number controls for learning effects that may occur even during maintenance trials. Models were incrementally built by adding one factor or interaction and testing the model’s relative goodness-of-fit using Aikake information criterion (AIC) based comparisons. A table of nested models and comparisons documents this process (Table 5.1). Models 6 and above did not converge, but as  $\beta$  estimates did not change, this was deemed to not require further exploration.

Time of day was modelled using splines (R package: splines) because a simple linear model of time of day would not capture the variations in activity seen in Figure 5.7. A series of linear models was designed to test how many splines best fit the data. Incrementally increasing numbers of splines describing time of day were added to a model containing a full interaction between preceding trial accuracy, ITI, and stimulus. The AIC was calculated for each of these models and the number of splines at the “elbow” of the plot was selected as the best fitting model with the fewest necessary degrees of freedom; the model with two splines was used in the remainder of the GLMMs (i.e. Models 10-13) (Figure 5.10).

The best fitting model, Model 12, was selected to include main effects of stimulus type (responses to Go stimuli are more accurate than responses to No-Go stimuli:  $\beta = -0.31$ ,  $p < 2e-16$ ; Model 1.1), ITI (responses are more accurate with a shorter ITI:  $\beta = -0.10$ ,  $p < 2.3e-16$ ; Model 1.2) and accuracy on the preceding

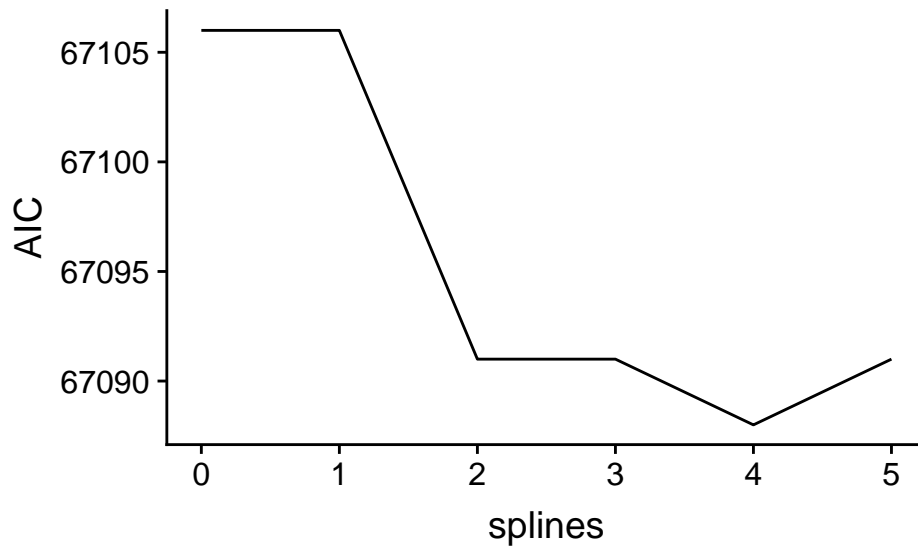


Figure 5.10: Line/elbow graph of AICs by number of splines describing time of day in the nested generalised linear mixed models.

trial (responses were more accurate if the preceding response was accurate:  $\beta = 0.42$ ,  $p < 2e-16$ ; Model 1.3). There was no main effect of preceding stimulus type ( $\beta = -0.02$ ,  $p = 0.30$ ; Model 1.4) or latency ( $\beta < 1.7e-7$ ,  $p = 0.99$ ; Model 1.5). Models 1.4, 4 and 5 all tested the effect of preceding stimulus type, but inclusion of preceding trial type did not significantly improve the model fit in any of these cases (see Table 5.1). Specifically, there is no evidence for an interaction between present stimulus type and preceding stimulus type, indicating that, for example, birds did not receive an accuracy boost from being presented with the same stimulus type as they previously received.

Tests of nested models demonstrated that there were a number of significant interactions between variables predicting response accuracy. In Model 6, a significant interaction between preceding accuracy and ITI ( $\beta = -0.065$ ,  $p = 3.9e-5$ ) indicates that if a bird was accurate on the preceding trial, increasing ITIs decrease the likelihood of an accurate response on the present trial. In Model 7, a significant interaction between stimulus type and previous accuracy ( $\beta = -0.23$ ,  $p = 1.1e-7$ ) indicates that if the bird was accurate on the preceding trial, presentation of a No-Go stimulus type decreases the probability of an accurate response relative to the presentation of a Go stimulus type. In Model 8, a significant interaction between stimulus type and ITI ( $\beta = 0.18$ ,  $p < 2e-16$ ) indicates that, for Go stimuli, a longer ITI decreases the probability of a correct response, but ITI does not appear to have an effect on the accuracy to No-Go stimuli. Model 9 demonstrates a significant three-way interaction between stimulus type, ITI, and preceding trial accuracy ( $\beta = 0.16$ ,  $p = 2.5e-7$ ). One example of how this manifests is the combination of a No-Go stimulus with a long ITI between the previously accurate

trial results in a higher probability of an accurate response to the present trial (Figure 5.11).

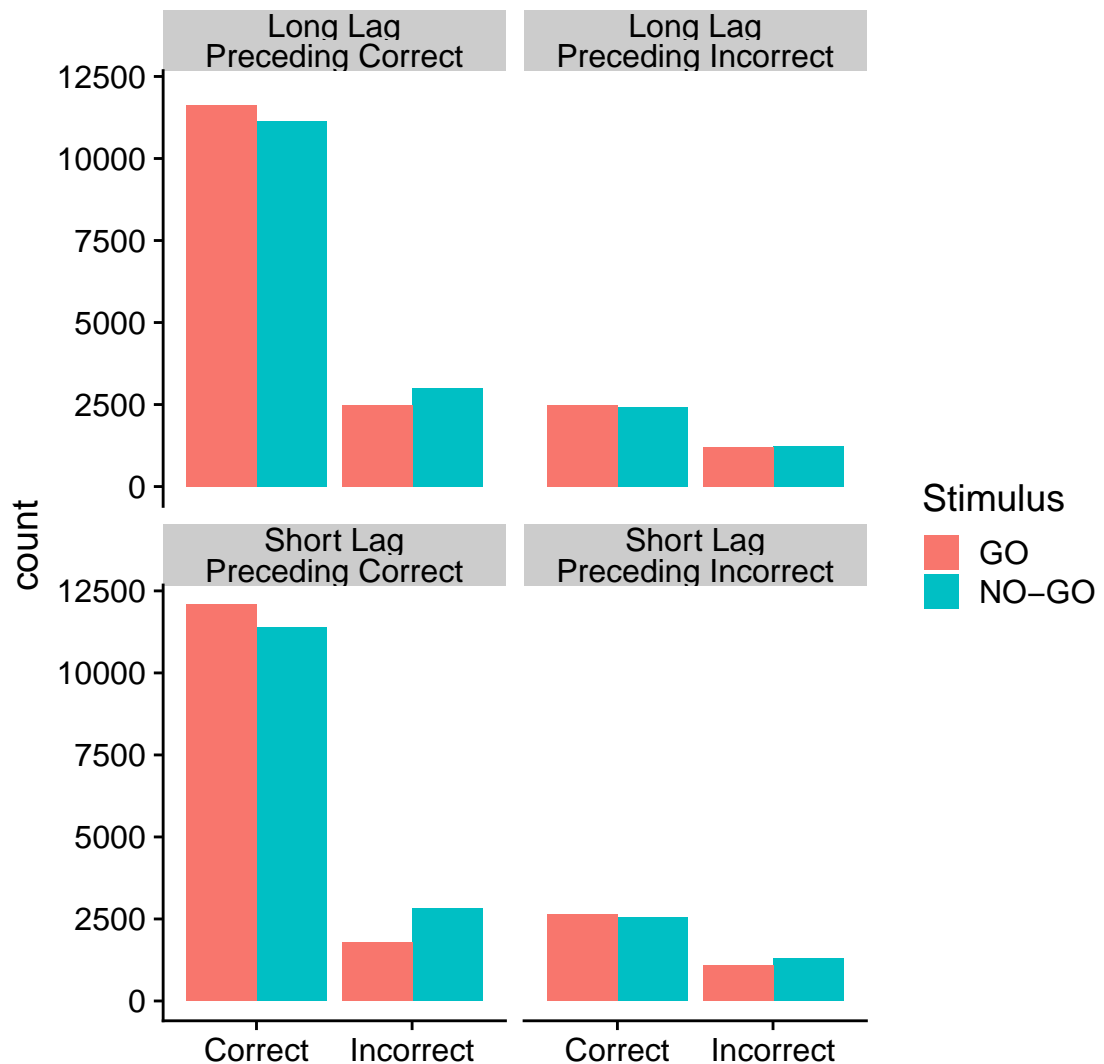


Figure 5.11: Visualisation of the three-way interaction between stimulus type, preceding trial accuracy, and lag. For this figure, lags have been grouped into long or short based on the median lag duration; lags were modelled as continuous data in the GLMMs.

The two-spline time of day variable was added to Model 9, which contains the three-way interaction. Within Model 10, both splines had significant  $\beta$  estimates (spline 1:  $\beta = 0.014$ ,  $p = 0.13$ ; spline 2:  $\beta = -0.31$ ,  $p = 0.001$ ). This indicates that the first spline of time of day has a positive relationship with accuracy whereas the second spline has a negative relationship. Once stimulus type, ITI, preceding accuracy, bird ID and index throughout the trial have all been controlled, a bird is more likely to respond accurately in the first part of the day than the second part of the day. This, therefore, is a refinement of the results described in Section 5.3.4.

In Model 11, a significant interaction between time of day and stimulus is found for spline 1 ( $\beta = -1.15$ ,  $p < 2e-16$ ) but not for spline 2 ( $\beta = -0.21$ ,  $p = 0.27$ ). This indicates that for the early part of the day birds are less likely to respond accurately to a Go stimulus than in the late part of the day, but that this is not true for responses to No-Go stimuli (Figure 5.12). In Model 12, an interaction between time of day and preceding trial accuracy was found for spline 1 ( $\beta = 0.35$ ,  $p = 0.004$ ) but not for spline 2 ( $\beta = 0.16$ ,  $p = 0.46$ ). That is, there is a slight trend for birds to respond accurately if the preceding response was accurate in the later part of the day (Figure 5.12). The addition of an interaction between time of day and ITI did not significantly improve the model (Model 13, Table 5.1) and therefore no attempts were made to fit additional interaction terms between time of day and other predictor variables.

## 5.4 Discussion

I 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. I 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. I 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. I 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.

To integrate many of these findings I ran a series of nested GLMMs on response accuracy and found a series of main effects: responses to Go stimuli are more accurate than responses to No-Go stimuli, shorter ITIs from the preceding trial increase the likelihood of a correct response, and responses are more accurate if the preceding trial response was accurate. I also found no main effect of preceding trial type or latency on the present trial. Additionally, a series of complex interaction effects culminated with a three-way interaction between stimulus type, ITI and preceding trial accuracy. That is, predictions of response accuracy are best made

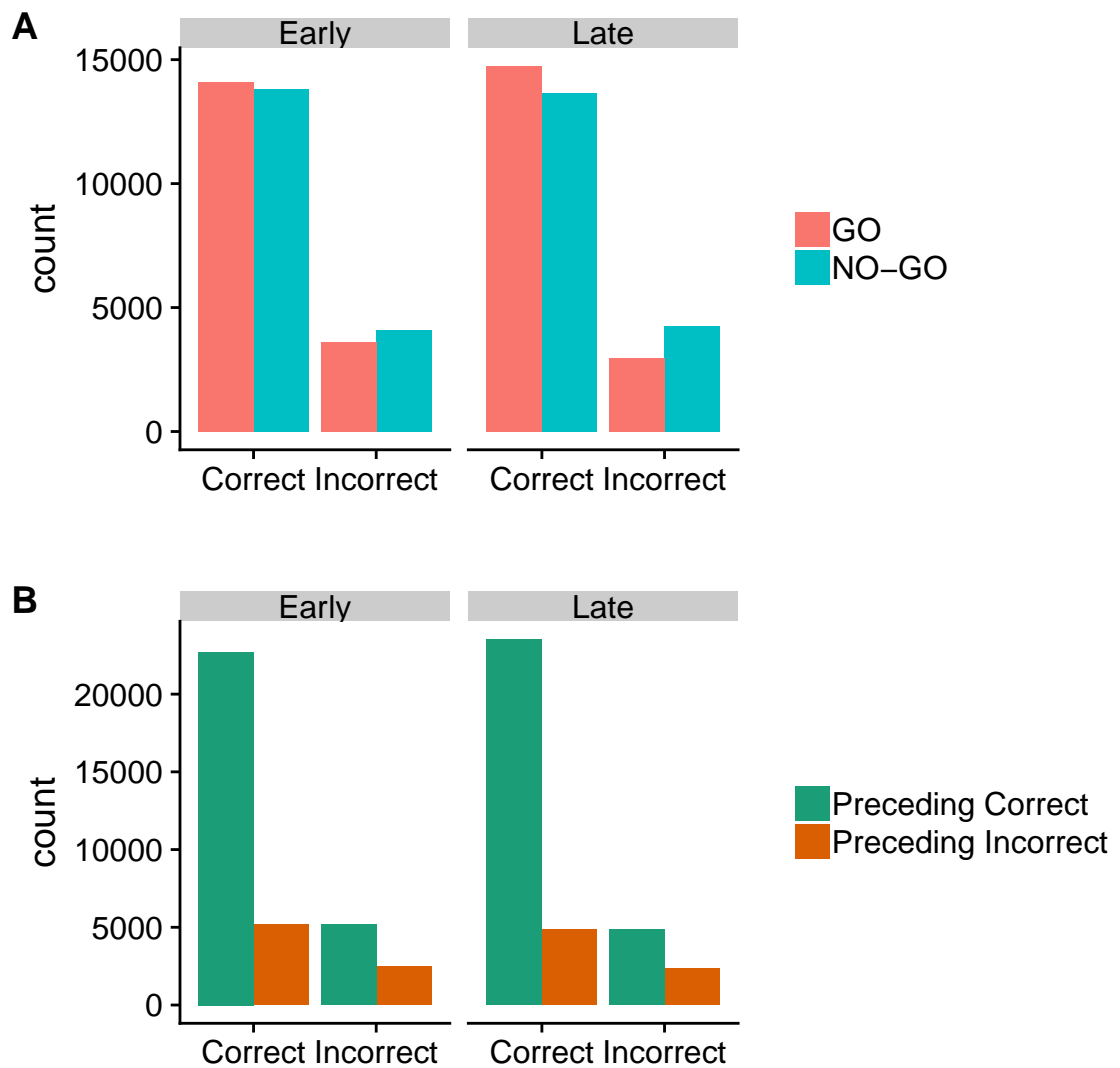


Figure 5.12: Bar chart of correct responses during early and late parts of the day to A) Go and No-Go stimuli and B) stimuli to which the preceding responses were either accurate or inaccurate. The times of day have been divided by a median split for this visualisation, but the GLMMs model time of day using two automated splines, which are unlikely to be knotted at the median time of day.

by modelling not only the two-way interactions between stimulus type and ITI, stimulus type and preceding accuracy, and ITI and preceding accuracy, but also the three-way interaction between the three predictor variables. Further, the time of day significantly affected response accuracy, with response accuracy higher during the first part of the day than during the second part of the day. Finally, interactions between time of day and stimulus type as well as time of day and preceding accuracy improved the model fit.

### 5.4.1 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, I cannot distinguish between these possibilities. However, our behavioural response data, along with others (Gess et al., 2011), do suggest that the learning of Go and No-Go stimuli is not performed at the same rate. I 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.

### 5.4.2 Response latencies

Further evidence for the dissociation of Go and No-Go learning is found in our response latency results. I 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 ~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, I 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.

### 5.4.3 Time of day

I analysed the patterns of trial initiation throughout the day to inform the improvement of our protocol for animal welfare purposes. During maintenance, I 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. I 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. I believe that my 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 this bias is not seen 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.

I 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 I wanted to characterise our own birds' learning rates for gene expression analyses. I 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 I found a negative correlation between learning rate and time of day activity. I 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 I 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.

#### 5.4.4 Complex interactions predict response accuracy during maintenance

Having examined the relationship between learning rate and time of day activity, I sought to understand whether time of day has an effect on response accuracy during operant conditioning maintenance. In order to do so, I modelled response accuracy as a binomial variable using a series of nested generalised linear mixed models. The significant main effect of stimulus type, whereby responses to Go stimuli are overall more accurate than responses to No-Go stimuli, simply indicates that birds produce the Go behaviour in response to the Go stimuli more than they produce the No-Go behaviour in response to the No-Go stimuli; this can also be interpreted as an overall slight bias towards the Go response to both stimulus types, which could be explained by either the birds preferentially learning the Go behaviour (in contrast to the Bengalese finches in Morisaka & Okanoya, 2009) or by the birds struggling to inhibit the No-Go behaviour (as in Gao & Mingming, 2017). This finding contrasts with the analysis shown in Figure 5.3, where by the end of learning, the Go bias that was present during learning attenuated; this may be because the modelling of response accuracy here is conducted after controlling for bird ID and for any possible effects of ongoing learning, even during maintenance. Additionally, as trials were not binned to calculate bias scores, the GLMM analysis has higher sensitivity than the analysis of binned bias scores using Bonferroni correction.

Previous literature has investigated the duration of inter-trial intervals in both classical and operant conditioning, with some describing a benefit of massing trials and others describing a benefit of spacing trials (Gibbon et al., 1977; Roberts, 1972; Spence & Norris, 1950). Here there was a significant main effect of ITI, where longer ITIs were associated with a less accurate response. This broadly supports the notion that massed trials improve response accuracy relative to spaced trials. However, it must be noted that inter-trial interval durations have not been experimentally controlled here as they have in previous literature; instead, inter-trial interval durations were determined by the initiation of trials by the birds. The motivation of birds to initiate a trial, which cannot be explicitly measured and which may therefore effect a bird's likelihood to produce a Go behaviour, may be non-independent of inter-trial interval durations.

A main effect of previous response accuracy was also found; the  $\beta$  for this effect had the greatest absolute value of all main effects, indicating that it has the greatest effect on response accuracy. This would be expected during learning, where

response accuracy increases and response accuracy for each trial could be expected to be predicted, in part, by response accuracy for the preceding trial(s). However, during maintenance, this suggests that response accuracy remains autocorrelated; that is, birds could be said to get stuck in “good periods” and “bad periods” throughout maintenance. Further work to assess the duration of these periods of relative accuracy and inaccuracy may aid in the understanding of avian operant response behaviours.

Perhaps as interesting as the significant main effects are the predictor variables for which there was no main effect. First, response latency did not improve the model describing response accuracy. Although response latency was shown to be related to stimulus type (Figure 5.5) and to response accuracy, in the GLMM containing bird ID and the by-bird index control variables, response latency did not significantly affect response accuracy. Second, preceding trial stimulus type did not affect response accuracy. A lack of effect of preceding stimulus type indicates that the birds did not gain an advantage, during maintenance, from the preceding trial being either Go or No-Go. Additionally, preceding stimulus type did not interact with present stimulus. This indicates that, for instance, birds gained no advantage on a Go trial if the preceding trial was a Go trial. Preceding stimulus type was expected to interact with present stimulus to influence response accuracy, as the birds might be expected to hold the preceding stimulus type, their response and the outcome in short-term memory, and use that to improve the likelihood of correct response on the next trial. Given that being accurate on the preceding trial significantly improves the likelihood of accuracy on the present trial, this was a surprise. Though not explicitly tested, it could indicate that, for example, the zebra finches are able to use the outcome from their response to a preceding Go stimulus to determine their response to either a Go or a No-Go stimulus, which would require complex working memory. This would be particularly surprising as zebra finches struggle to recall information from two categories of information in combination (K. Sanford & Clayton, 2008)

The series of interactions between stimulus type, ITI and preceding trial accuracy all demonstrate the richness and complexity of the birds’ decision making during maintenance of this operantly learned discrimination. Most intriguing is the interaction between stimulus type and ITI, whereby longer ITIs decrease the likelihood of a correct response for Go stimuli but not for No-Go stimuli; that is, after a long ITI, a bird is generally more likely to make a No-Go response to both Go and No-Go stimuli. This could be explained by either motivational or bias factors. From a motivational perspective, the birds might be initiating a trial after a long ITI for purposes of environmental enrichment (as in Holveck & Riebel,

2014); the depressed Go response might be due to the bird not requiring the food reinforcement. This interaction could also be explained by a bias perspective; a bird's decision criterion may be set with a No-Go bias in order to decrease the potential for receiving a lights-out punishment. This bias may be stronger when the birds have not recently received reinforcement or punishment with which to update their decision criterion, as in the case with longer ITIs.

As previously discussed, preferential time of day activity has a negative relationship with learning rate, with birds active earlier in the day learning the discrimination slower than birds active later in the day. In the GLMMs, time of day has a main effect on response accuracy during maintenance, with birds more likely to respond accurately during the first part of the day than the second part of the day. The contrast between this finding and that in Panel B of Figure 5.8 (where time of day did not have a significant effect on accuracy) can be explained by three differences in the analyses: 1) time of day was modelled using a linear model (effectively one spline) in the analysis shown in Figure 5.8 whereas it was modelled using a two-spline model in the analysis described in Table 5.1; 2) in Figure 5.8, the response variable was  $d'$ , a summary statistic created by binning multiple trials together whereas the response variable in the GLMM-based analysis was raw accuracy on a trial-by-trial basis; 3) time of day in the first simple linear model analysis was modelled without controlling for any other variables, whereas time of day in the GLMM analysis was modelled after controlling for bird ID and the three-way interaction between preceding response accuracy, ITI and preceding accuracy.

In addition to the main effect, time of day also interacted with stimulus type. Specifically, during the early part of the day, birds are less likely to respond accurately to Go stimuli but this is not true for No-Go stimuli. This finding can be rephrased as birds are more likely to have a No-Go bias during the morning than during the afternoon, which causes reduced accuracy to Go stimuli but not to No-Go stimuli. This reflects the slight negative slope of the linear regression line in Panel D of Figure 5.8. This finding supports my hypothesis that the depressed asymptotic response accuracy demonstrated in Chapter 4 may be due to the difference in experimental design, whereby London birds were given *ad libitum* access to food when the experimenter was not on site whereas Leiden birds interacted with the operant conditioning apparatus through the entire photoperiod.

Finally, time of day interacted with whether the bird was accurate on the preceding trial, reflecting a slight effect for birds to be more likely to respond accurately during the later part of the day if they were accurate on the preceding trial. This

may indicate that birds are more likely to refer to the preceding trial during the later part of the day than the earlier part of the day. The reason for this remains unexplored, but could be related to complex non-monotonic decreases in working memory function throughout the circadian rhythm (as reviewed in Smarr, Jennings, Driscoll, & Kriegsfeld, 2014).

### 5.4.5 Conclusion

Here I found differential learning of the Go and No-Go stimuli, which I 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, I 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. I 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. I further demonstrated that inter-trial interval duration, preceding trial accuracy, present trial stimulus type, and time of day all affect the likelihood of a bird responding accurately during maintenance trials. Together, these findings suggest that response accuracy during maintenance may be depressed by our experimental design, which involves giving the subjects free access to food during late afternoon and early evening.

## Chapter 6

# 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 I train 10 female zebra finches on a Go/No-Go task; after training and four days of maintenance of the Go/No-Go discrimination, I expose the birds to 10 minutes of acute song playback of either the reinforced or the punished stimulus. During this song playback, I video record the birds' behaviours, and analyse these using an array of statistical techniques. I 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. I 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.

## 6.1 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 acute stress, such as puffing and flying towards the wall (Olson et al., 2014), 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 emotional state of the subject, albeit with consideration that this approach has its limitations, which include the possibility that attentional, memory and/or judgement biases may co-vary with emotional state (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 5, 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, I am 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 in response to acute unreinforced playback would provide support for persistence of operant performance even following devaluation of the stimuli (Kirsch et al., 2004).

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 food stimuli with innate low 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 for which innate preference can be measured, have not, to our knowledge, been demonstrated in adult female zebra finches. The use of sexual stimuli as the conditioned stimulus in the experiments described in Chapters 2, 4 and 5 complicates interpretation of response behaviour, as birds may respond on the basis of either innate preference for the sexual stimulus or preference learned through operant conditioning. Investigations of flavour preference, for which organisms have an innate preference, have demonstrated that it is possible to dissociate innate preference from learned preference (Rozin & Zellner, 1985). Here, behavioural evidence that females respond differentially to the Go and No-Go stimuli in the absence of reinforcement and punishment might reflect a change in learned preference through the Go/No-Go conditioning.

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.

### 6.1.1 Aims and predictions

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, as demonstrated in Chapter 5. Here I aim to characterise the response to acute unsolicited playback of these stimuli after training occurs. First, I predicted that acute playback of the trained stimuli would result in different activity levels to silence. Second, I predicted that there would be more than one pattern of behaviours, with, for example, a positive correlation between alarm calls and puffing. Finally, I predicted that these patterns of behaviours, or behavioural states, would be related to whether the bird heard the Go or the No-Go stimulus.

## 6.2 Methods

### 6.2.1 Animals

10 female zebra finches (*Taeniopygia guttata*) originally from a breeding line at the University of Glasgow were bred at Queen Mary University of London in a large free breeding aviary (20-80 individuals, 3.9 m x 4.3 m). It is unknown which, if any, of the females here had previously been involved in breeding. Prior to the initiation of the experiment, they were then housed in a single sex aviary with 4-10 females at any given time (1.9 m x 2.0 m x 2.0 m high) 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 the same 16:8 light cycle (7:00 to 23:00) in the free breeding aviary, single sex aviary and in the sound attenuation chamber. 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.

### 6.2.2 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. Further details of the chamber design can be found in Chapter 3. Please refer to Figure 5.1 for a diagram of the chamber apparatus.

Table 6.1: 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

### 6.2.3 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 6.1). 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 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.

### 6.2.4 Operant conditioning

Please refer to Section 5.2.4 for details of the operant conditioning protocol.

### 6.2.5 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.

### 6.2.6 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).

### 6.2.7 Statistics

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

## 6.3 Results

### 6.3.1 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 6.1).

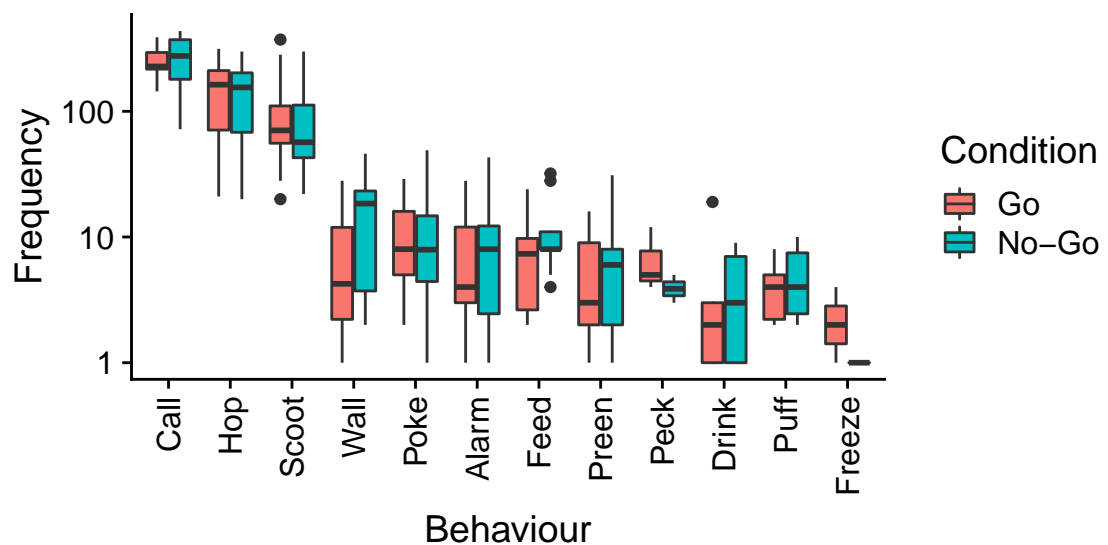


Figure 6.1: Number of times each behaviour was performed during and after playback, by condition.

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 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

Table 6.2: GLMMs for total incidences of all behaviours.

Model	Factors	df	AIC	Log-likelihood	Comparator	$\chi^2$ 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

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 6.2). Therefore, there are no significant main effects of condition or period, nor is there an interaction between condition and period on behaviour counts.

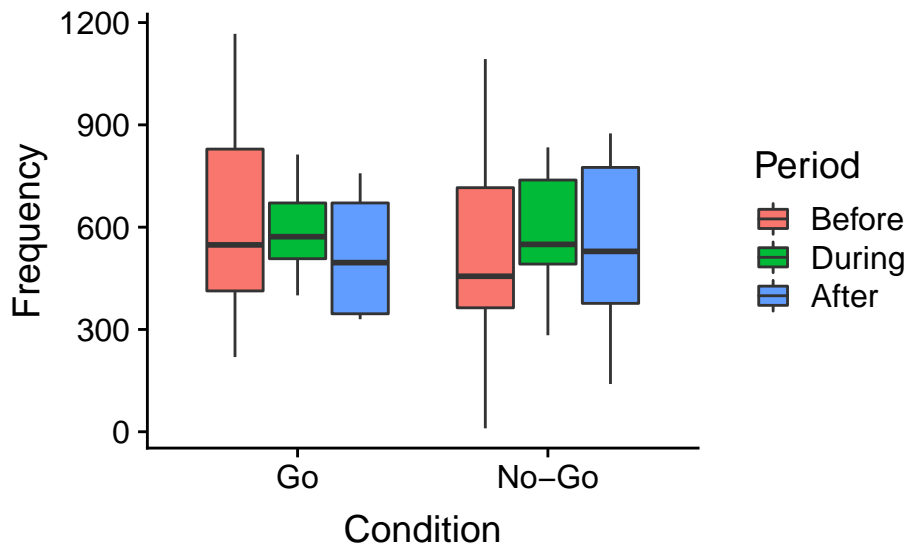


Figure 6.2: Total activity level by condition and period.

A Levene's test on the log-transformed data did not support the visual suggestion (see Figure 6.2) of less variance during the playback period than before or after playback ( $F(2, 42) = 1.81, p = 0.18$ ; car package, R).

### 6.3.2 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.

### 6.3.3 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 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 6.3). 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).

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 6.4; Panel A) and of PC1 versus PC3 (Panel B) demonstrates that there is no clear separation between conditions.

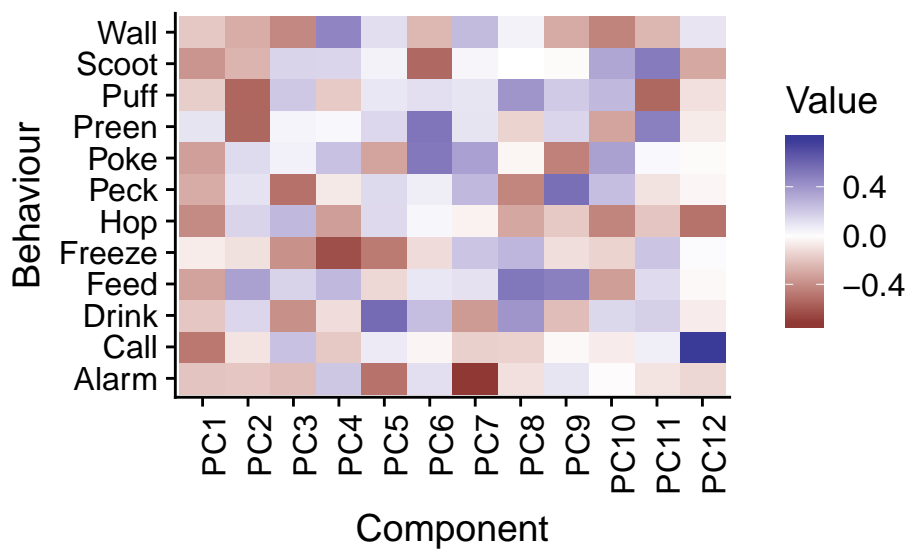


Figure 6.3: Loadings for the PCA.

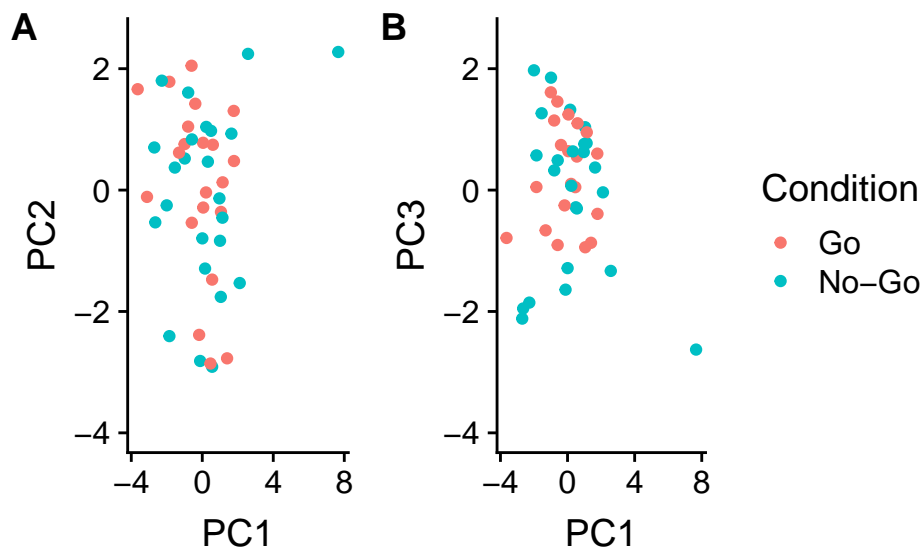


Figure 6.4: Principal components plotted against each other. A) PC1 plotted against PC2. B) PC1 plotted against PC3.



Table 6.3: GLMMs for individual behaviour types.

Model	Factors	df	AIC	Log-likelihood	Comparator	$\chi^2$ 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

### 6.3.4 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 6.3). I was 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.

### 6.3.5 Individual differences in behavioural responses

Although I did not find any significant differences in patterns of behaviour between conditions, I did find individual differences in behavioural responses to the song playback (Figure 6.5). 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.

### 6.3.6 Power analysis

In order to determine how much power our experiment had to find differences between conditions, I ran a power analysis on Model 1 from Table 6.2 ( $\alpha = 0.05$ , 1000 iterations; simr package, R). With our sample size there was 78% power to detect a medium effect size (0.5) of condition and 99% power to detect a large

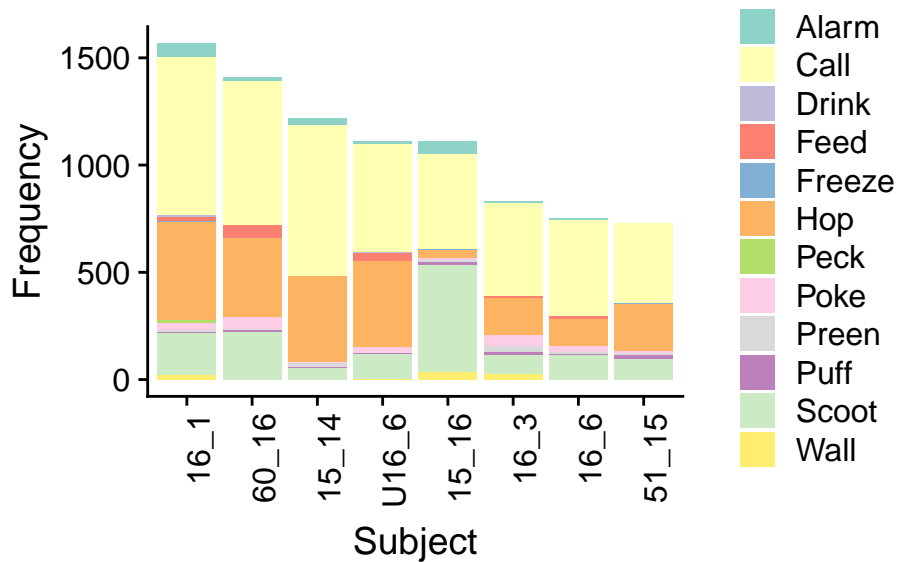


Figure 6.5: Individual differences in behavioural response to song playback.

effect size (0.8) of condition. Therefore, there was not enough power to detect small effects of the total number of behaviours.

## 6.4 Discussion

Here I 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, I 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.

### 6.4.1 No evidence for an acute response to song presentation

The most fundamental finding is that there is no overall change in activity levels during presentation of a conspecific song from the previous silence. This is surprising given that previous literature has found marked behavioural responses to acute passive song playback (see Dong & Clayton, 2009 for a review; Verner & Milligan, 1971). 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. I therefore propose that the birds in the present study were more behaviourally habituated to the song presentation than the 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).

#### 6.4.2 No evidence for clusters of behaviours

There was also 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, I found that after being socially isolated in a sound attenuation chamber for a few weeks, patterns of behaviour do not vary consistently between individuals.

Additionally, I found no evidence that the presentation of a song associated with reinforcement elicits a different pattern of behaviours than a song associated with punishment. This contrasts with flavour preference studies on rodents and humans where innate flavour preferences can be altered by conditioning (Rozin & Zellner, 1985; Schonberg et al., 2014). Though I 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 preference for conspecific songs for female zebra finches. Further, I 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, despite the learning of a stimulus-response-outcome association (Kirsch et al., 2004).

### 6.4.3 Implications for interpretation of gene expression studies

I suggest that these findings indicate that our operant conditioning training and maintenance experimental design, followed by passive exposure to a trained conspecific song, does not drive overt 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.

### 6.4.4 Conclusion

Here I found no evidence for discrete behavioural states among female zebra finches exposed to previously learned conspecific songs. I also found no evidence for an acute response to song playback. I suggest that the birds experienced the passive song playback passively, with no large shifts in behaviour during or after the song presentation. Further, I found that behaviour did not depend on the the previously learned association (i.e. the reinforcement or punishment) of the song. I 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 7

## Discussion

As described in the Introduction, our understanding of the mechanisms of learning, memory and perception requires appropriate model systems. The careful measurement of processes across multiple levels of organisation (from molecular, through circuits, to behaviour) can aid our understanding of these psychological processes (Clayton, Balakrishnan, & London, 2009). I introduced the zebra finch as an attractive model species, especially for auditory learning, memory and perception, in part because the zebra finch has had a particular impact in the study of vocal learning during the juvenile critical period (Bolhuis et al., 2010; Doupe & Kuhl, 1999; Gobes et al., 2017; Marler & Doupe, 2000), and because the neuroanatomy and the genome of the zebra finch are fairly well understood (Reiner, Perkel, Mello, & Jarvis, 2004; Warren et al., 2010). Over the last few decades, investigators have begun to apply operant conditioning paradigms in the zebra finch to study other forms of learning and perception that occur through the lifespan (Beckers et al., 2003; Bregman et al., 2016; Heijningen et al., 2013).

Here I aimed to develop a rigorous base of technique and knowledge for operant conditioning studies of the zebra finch. My first aim was to determine whether auditory memories, after Go/No-Go operant conditioning, might be encoded differently in the auditory forebrain depending on the behaviour that was associated with the song. To do this, I worked in collaboration with the ten Cate laboratory at Leiden University and used a molecular technique for mapping neural activity (Chapter 2). My second aim was to thoroughly characterise the learning process itself, at a behavioural level (Chapter 5). To do this I first developed (Chapter 3) and validated (Chapter 4) a new hardware/software system for zebra finch operant conditioning. My third aim was to investigate whether the experience of hearing learned Go versus No-Go stimuli, in a non-reinforced context, elicits

observable differences in behaviour that might confound interpretation of neural-level responses (Chapter 6).

## 7.1 How are learned auditory associations encoded in the brain?

Previous literature into learning in zebra finches includes investigations of male song learning/copying, a time-limited process (for a review see Gobes et al., 2017). Zebra finches have also been used as a model species for a simple form of learning that continues through the lifespan: auditory habituation (for a review see Dong & Clayton, 2009). For both of these forms of learning, the IEG *ZENK* has been used as a proxy for activity. 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). Here I used *ZENK* as a proxy for activity after operant conditioning, a form of associative learning that continues through the lifespan. There has been previous interest in understanding the neurological response to operant conditioning in songbirds (B. A. Bell et al., 2015; Gentner & Margoliash, 2003; Gentner et al., 2004) and one of these studies similarly used *ZENK* as a proxy for activity in an operant conditioning experiment, though that study was conducted on male starlings and confounded stress and associative learning by testing stimuli during maintenance or reversal learning (Gentner et al., 2004). Here I conducted a more refined experiment, measuring the *ZENK* response to auditory stimuli in an unreinforced context where active learning no longer occurs.

In Chapter 2, I trained birds to discriminate between two songs using operant conditioning, 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 found great individual differences in *ZENK* response, but showed that these did not relate to the playback condition. To our knowledge, this is the first evidence that the novel/familiar difference in *ZENK* expression across the auditory forebrain is not absolute. This characterisation of molecular and neuroanatomical responses to operantly conditioned song playbacks enhances the current literature, which has previously focused on active learning rather than on passive exposure to previously learned songs (e.g. Gentner & Margoliash, 2003; Jarvis et al., 1995). It

is possible that there is no differential IEG response across our four conditions once a bird has fully habituated to a song attenuation chamber. However, it is also possible that *ZENK* is not the right gene to detect these differences in this context. To that end, there are findings from an allied RNA-Seq study suggesting that expression levels for some genes (though not *ZENK*) do vary between the Go and the No-Go conditions (George & Clayton, n.d.).

Though I found that the category of playback did not alter the overall levels of *ZENK* expression in the auditory forebrain, I did show that the Go song elicited a more coordinated response in the auditory forebrain than any of the three other conditions. This is an intriguing finding; perhaps there is functional structure within the auditory forebrain that results in different patterns of correlated activity depending on stimulus association. The auditory forebrain is a small part of the songbird brain with high connectivity between regions (Vates et al., 1996), and gaps remain in our understanding of the boundaries between regions (as reviewed in Section 2.1.2).

As such, this was a suitable problem for the application of graph theoretical approaches. The use of graph theory to discover neural patterns of activity is not novel (Tanimizu et al., 2017), though this is believed to be the first use of graph theory to find central vertices, or regions that correlate with many other regions, within the songbird auditory forebrain. I found that for the Go response only, lateral CMM was the most central vertex, indicating that lateral CMM either drives or simply reflects activity in other regions within the auditory forebrain in response to Go songs. Lateral CMM did not have this relationship with other auditory forebrain regions for the other conditions. This finding elicits a clear hypothesis: does lateral CMM drive coordinated activity across the auditory forebrain? Future work could test this through the use of electrophysiology, measuring the timing of neural activity in lateral CMM and other regions.

One 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 reflect 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.

## 7.2 Can behaviour during operant conditioning enhance our understanding of the learning process?

In Chapter 3, I described the building of Operanter, open source hardware and software for avian operant conditioning. Currently available systems for operant conditioning on the market either rely on proprietary software and/or hardware components (Pineno, 2014; Tchernichovski et al., 2000), or are narrowly designed to function for one specific purpose (H. Chen & Wang, n.d.). In collaboration with colleagues with electronics and software programming experience, I developed a suite of Java-based software and non-proprietary hardware designed to function with manufacturer-independent components. This software and hardware is versatile, easily extendable, and inexpensive, and those features will allow researchers with small budgets or specific requirements to build operant conditioning setups. In Chapter 4, I showed that Operanter can be used to successfully train zebra finches to discriminate between two conspecific songs. I compared birds trained using the Operanter system with birds trained using a proprietary system at Leiden University, and demonstrated that the asymptotic learning of Operanter birds was lower than the asymptotic learning of Leiden birds.

For Chapter 5, 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. Finally, I found that the time of day, inter-trial interval duration, accuracy of the preceding trial and stimulus stype all predict the accuracy of response during maintenance of discrimination. 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.

The research described in Chapters 4 and 5 fits in with a wealth of previous literature about the mechanisms underlying operant conditioning (Herrnstein, 1961; Kalenscher et al., 2005; Skinner, 1938; Thomas et al., 2009; Yechiam et al., 2006). Through the training of nearly three dozen female zebra finches, I acquired a large dataset of simple operant conditioning learning and maintenance behaviours. Though the experiments were not explicitly designed to test hypotheses about the psychological mechanisms behind operant conditioning, exploratory interrogation of this behavioural data allowed me to characterise multiple features of operant conditioning learning and behaviour. The findings have multiple implications for experimenters using operant conditioning to test avian perception.

Given the evidence I found for differential learning of Go and No-Go stimuli, I suggest that researchers use conservative metrics for establishing a learning criterion. That is, as birds tend to respond more accurately to Go stimuli than No-Go stimuli earlier in the learning process, if experimenters want to ensure that both the Go and No-Go stimuli have been learned, they should set learning criteria based on either the percentage correct for both Go and No-Go, or bias metrics (Alves-Pinto, Sollini, & Sumner, 2012; Wickens, 2001). Experimenters should also be aware that asymptotic accuracy levels may be depressed by turning off the operant conditioning apparatus in the afternoon, which they may be requested to do for animal welfare purposes. This depression in accuracy is significant, and may cause difficulty in experiments where small differences in responses to probe stimuli are sought (e.g. Chen et al., 2015). This evidence could therefore be used to argue that operant conditioning should occur throughout the entire photoperiod in order to increase the power of the research and reduce the number of animals required to find an effect.

One series of findings directly addresses the impact that time of day has on operant conditioning response behaviours. I found that there was great individual variation in the time of day that birds initiated trials, and that birds that were preferentially active in the morning tended to learn the discrimination more slowly than birds preferentially active later in the day. I also found that birds were more accurate in the early part of the day than the later part of the day, and that time of day also interacted whether the bird was accurate on the preceding trial to determine accuracy. That is, birds are more likely to refer to the preceding trial during the later part of the day than during the early part of the day. When viewed in an attention framework (e.g. Ammons et al., 1995), these findings together suggest that learning, and therefore response accuracy, may be facilitated by birds

being allowed to initiate trials throughout the day for two reasons. First, one explanation for the learning rate effect may be that birds that are preferentially active in the morning are slower at learning the discrimination because they have a longer gap between the bulk of their trials and the next morning. This gap would be attenuated by allowing the birds to initiate trials throughout the photoperiod. Second, if birds refer to the preceding trial during the later part of the day to inform responding but not during the earlier part of the day, response accuracy during maintenance might be improved by increasing the number of trials that birds can initiate during the later part of the day.

Future research should extend the present findings by experimentally manipulating the time of day effect. One potential experimental design involves altering the times of day during which the birds can engage with the operant apparatus. Three groups should be used: morning (e.g. 7:00-13:00), afternoon (e.g. 13:00-19:00) and split (7:00-10:00, 16:00-19:00). If the time of day learning rate effect is caused by the morning-active birds having a long gap between the bulk of their trials and the next morning, the morning and the afternoon groups will learn similarly slowly, with the split group learning more quickly. However, if the time of day effects demonstrated in this thesis are caused or mediated by attentional shifts throughout the circadian rhythm, the morning group will learn the slowest and the afternoon group will learn the fastest, as the afternoon group will be most able to avail of accuracy information from the preceding trial; the split group will learn at a rate between the morning and afternoon groups. In this way, the cause of the learning rate effects can be determined to be attentional or due to the spacing of inter-trial intervals.

I also found a pattern of responses latencies to Go and No-Go stimuli during learning and maintenance that, to my knowledge, has not been previously reported. These differential patterns support models of Go/No-Go discrimination learning that state that Go and No-Go stimuli are learned differentially and that discrimination of Go and No-Go songs is therefore two learning processes, and not one unitary process (Yechiam et al., 2006). On a more practical level, researchers should limit the response window to 3000 ms after the stimulus presentation to avoid the capture of responses to No-Go stimuli that may be due to boredom, impatience or the forgetting of the most recent stimulus. Limiting the response window will ensure that most captured responses will be true “false positives”, whereby a bird produces a genuine incorrect response. By making this change, researchers using probe-trial type studies will more accurately assign responses to probes to either the Go or the No-Go response category.

### 7.3 Do behaviours in non-reinforced contexts correlate with neural processes in the same context?

After describing very subtle differences in *ZENK* expression activity in response to the song playbacks, it was critical to consider factors that may confound the interpretation of the coordinated *ZENK* activity. I therefore sought to characterise the behavioural response to the same unsolicited song playback that was used in the measurement of *ZENK* study. In Chapter 6, 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.

These findings directly contrast with previous published literature, where the birds froze for minutes upon hearing a conspecific song, even following habituation (Stripling et al., 2003). These birds' extensive habituation, which occurred over a period of weeks as described in Chapter 5, may be a sufficiently different process to the habituation described by Stripling et al. (2003). The birds here actively initiated song playbacks, whereas birds in Stripling et al. (2003) were passively exposed for the habituation procedure. Future research could examine whether this could cause a differential response to non-solicited presentation of one of those songs.

### 7.4 Integrating findings across three aims

As a whole, development of the Operanter system provided the opportunity to train multiple female zebra finches in order to conduct behavioural analyses. The findings from Chapter 5, 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 6, 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 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 6 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 6, 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 2 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.

One limitation of the current research is that the birds in Chapters 2, 5 and 6 were trained in 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.

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).

## 7.5 Conclusion

In this thesis, I sought to integrate behavioural, neuroanatomical and neurogenomic data to enable a holistic investigation of operant conditioning, a form of learning available to adult zebra finches. 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, operant conditioning does subtly alter the neurogenomic response to song presentation. I 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, nor does it drive a differential behavioural response.

Though it is frequently used as a simple and clean way to test perceptual abilities (Heijningen et al., 2013; Lohr & Dooling, 1998), operant conditioning is a complex form of learning, and the measures presently used are unlikely to fully capture the experience of the subject. In order to rectify this, I deeply characterised the behaviour involved in learning a discrimination through operant conditioning, demonstrating complex relationships between factors including learning rate, the

time of day, and recent preceding activity. In sum, my results suggest that Go/No-Go operant conditioning may drive two distinct types of learning, which may be reflected in subtle variations in gene expression in the auditory forebrain.

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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 in its entirety is available at <http://github.com/maevemcmahon/thesis>