UNIVERSITY OF CHICAGO

Using epigenetics to identify transcription factors implicated in coordinating the critical period for song learning in juvenile male zebra finches

By

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Abstract

While it has long been observed that zebra finches (*Taeniopygia guttata*) have a critical period for song learning between post-hatch day 30 and post-hatch day 65, it remains an open question as to the underlying neural machinery that coordinates the onset as well as the offset of this learning period. Epigenetic mechanisms of regulation are now thought to play a key role in the transcriptional plasticity of key song-learning structures in the brain and can begin to account for the learning potential of a zebra finch during this period of its development. For example, the addition of an acetyl group to the lysine residue at position 27 on histone H3 as a result of exposure to song was postulated to affect the relationship between the histones and the genomic DNA and alter the probability that one or more transcription factors could bind to their corresponding binding sites. The total sum of these altered probabilities is what then could be a key driver of establishing the critical period for song learning in juvenile male zebra finches. ChIPseq analysis performed here confirmed this hypothesis, identifying a number of transcription factor binding sites differentially expressed in the finches that were exposed to song versus those that were not. Specifically, we identified the binding sites for transcription factors *Zic1*, *Zic2*, and *MEIS3* which all have known functions in nervous system organization and proliferation, as well as *HES6* and *Hic1* which modulate neurogenesis and cell growth. Taken together, these results help to confirm the robust role that the acetylation of H3K27 has in mediating the accessibility of transcription factor binding sites and warrants further investigation into its putative role in the onset and offset of the critical period for song learning in zebra finches.

The sequence of a genome can yield impactful avenues of scientific exploration. From the early detection and intervention for genetic diseases such as cystic fibrosis (Ellsworth et al 1997) to letting drug design itself be guided by our knowledge of gene function and the molecular pathways involved in a particular disease-state (Williams and Hayward 2001), genome sequences are among one of the most powerful tools at scientists' disposal. However, these sequences alone do not necessarily reveal how specific RNAs and proteins are regulated and maintained within a specialized cell. Regulatory mechanisms affecting gene transcription and translation are critically important for viability of the organism, providing a mandate for the study of epigenetics.

Epigenetics' focus is the ways in which modifications are made affecting the functionality of a genome without changing its sequence. Epigenetic mechanisms include--but are not limited to--the addition of methyl chemical groups to sites on a strand of DNA which decrease the likelihood of a proximal gene being transcribed, as well as the addition of acetyl groups to the histone proteins around which the DNA is wound, causing a conformational change in the DNA's structure altering the probability for a gene sequence to be transcribed (Fagliolini et al 2009). More recent scholarship has shown that histones themselves can be methylated as well (Nugent and McCarthy 2015). Common to these mechanisms is the fact they act by increasing or decreasing access to transcription sites along the genome, affecting the likelihood of transcription to be initiated. Keverne and colleagues (2015) noted that much of the work done studying epigenetic mechanisms has focused on non-learned behaviors, such as maternal affect and displays of sexuality. For example, rat dams engaging in more licking and grooming as well as arched-back nursing behaviors was found to be predictive of the kind of parenting that these pups themselves administer in the future when they themselves become

parents (Weaver et al 2004). In seeking the biological mechanism of transmission of this attentive parenting, it was found that receiving these higher quality parental inputs effectively modified the pups' epigenetic profile, increasing glucocorticoid receptor expression in the hippocampus and ultimately conferring upon them a less reactive hypothalamic-pituitary-adrenal axis and higher resiliency to stress. Thus, developing our understanding of epigenetic mechanisms of regulation could deepen our understanding of observed individual differences in behavior, and the confirmation of the involvement of epigenetic mechanisms in non-learned behaviors suggests that they might play a role in learned behaviors as well.

Learned behaviors can further our understanding of how different sensory experiences shape the biology of an individual organism by examining the relationship between experiential input and behavioral output as mediated by the epigenetics of the organism. For example, one could imagine how certain social and cultural environments in infancy and early development could ultimately academic performance in the future and understanding the consequences of each environment is critical for maximizing each student's potential. With regard to learned behaviors, song-learning in zebra finches provides us with an effective model as juvenile males must be taught a song by an adult, male tutor in order to successfully perform one themselves later in adulthood (Eales 1985). Histone acetylation, specifically, is thought to play a role in the interplay between the juvenile male's experience and his neurological development (Kelly et al 2018), but it remains unclear as to which transcription factor binding sites have their accessibility modified.

A zebra finch cannot learn song at any point in its development, however. For male zebra finches, there is a critical period in adolescence where they learn a unique song, essential for future reproduction, although if a song is never learned this critical period will remain open to

allow for song acquisition even after the canonical offset of song-learning acquisition, post-hatch day 65 (Eales 1985). Previous research has already shown that hearing the songs of conspecifics affects the neurogenome of zebra finches as well as their observed behavior (Lin et al 2014, Katsis et al 2018). Importantly for the study at hand, life experiences during the zebra finches' critical period for song learning can be controlled, allowing for key observations as to how these manipulations can affect the birds' epigenome.

Critical periods themselves are a crucial component in the development of learned behaviors and have been observed and studied in the animal kingdom for decades, from higher-order vertebrates down to ants (Scott 1962). Critical periods appear as phases of plasticity in the brain of the animal that are experience-dependent and sensory input during these developmental windows drives brain maturation and function (Berardi et al 2000). Therefore, song-learning in zebra finches could not only shed light on the coordination for the acquisition of learned behaviors, but also speak to critical period onset and offset writ large.

The goal of this study was to ascertain which binding sites were made more or less accessible through epigenetic mechanisms to try to infer which transcription factors might be coordinating the song-learning critical period. Past research has shown that when the lysine residue at position 27 on histone 3 is acetylated (H3K27ac) [see Figure 1], corresponding gene regulatory sequences are made more accessible (Barski et al 2007). This is not the only modification that can occur at H3K27, however; Barski and colleagues (2007) also noted that trimethylation of this lysine residue has the opposite effect in that it will induce a conformational change that makes regulatory sites less accessible. One way to probe transcription factor binding site accessibility is through ChIPseq, a chromatin immunoprecipitation DNA sequencing technique (Zhang et al 2008). This technique will sequence sites that are differentially accessible

due to the acetylation of H3K27, and by comparing these sequences to a database of known transcription factor binding site sequences, the factors potentially coordinating song-learning can be identified. Thus, by modulating experiences and comparing the acetylation status at this previously identified site, these animals' epigenetic profiles can help us better understand how the individual experiences of any organism can be encoded into that animal's biology. Understanding this interplay of experience (in this context, the song learning a juvenile male did or did not experience) and the resulting epigenetic profile of the animal will be critical to establishing a more general understanding of how early experiences can determine the observable behavior of an organism in adulthood.

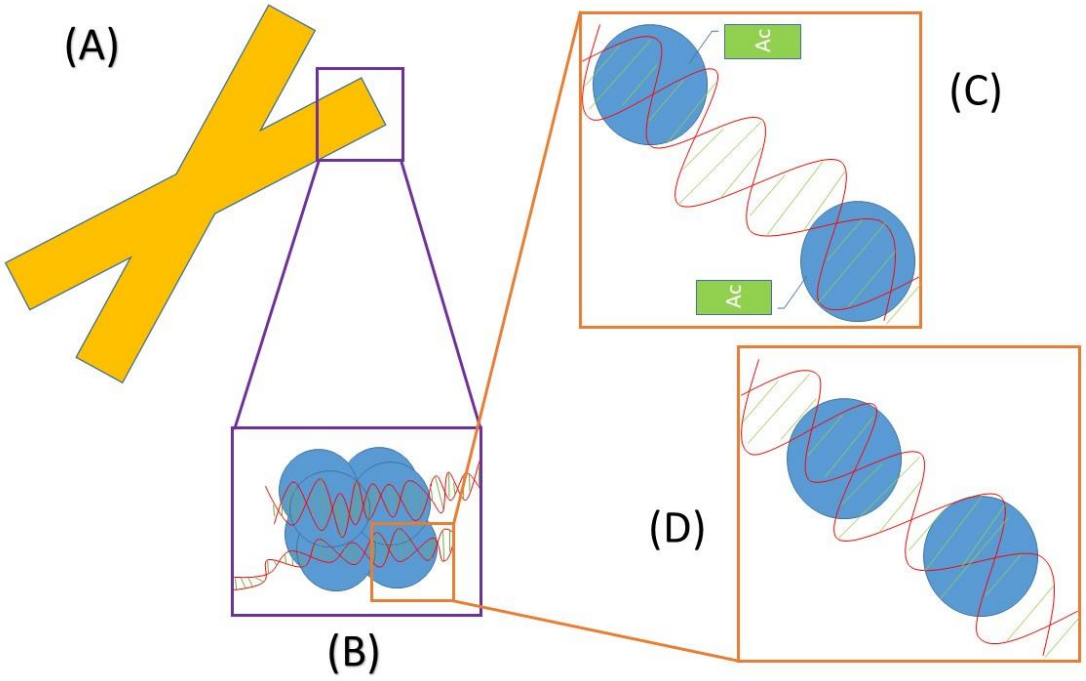


Figure 1 – *Illustration of the structural and spatial relationship of chromatin, nucleosomes, genomic DNA, and acetyl groups. (A) General structure of a generic autosome, Z. (B) View of a nucleosome, comprised of eight histone proteins (represented by the blue spheres) as well as associated genomic DNA. (C) Zoomed-in view of genomic DNA made accessible by the acetylation of its bound histone H3 proteins at Lysine 27 as compared to (D) showing relatively less accessible genomic DNA due to the fact its bound histone H3 Lysine 27 positions are not acetylated.*

The critical period for song learning in zebra finches has been studied from a number of different angles: consistent changes in the levels of specific RNAs during the song learning critical period have been observed, as for the synelfin protein (George et al 1995), and the progression of vocalization from sounds to syllables to song mapped out (Tchernichovski et al 2001). Less scholarship has been devoted to studying the changes in the epigenome that accompany the critical period for song learning, although initial results seem promising: other ChIPseq experiments have demonstrated that tutor song can sculp the epigenome of a male zebra finch and mediate its song-learning potential (Kelly et al 2018). Thus, this current study aims to continue this line of work and further elucidate the neural mechanisms of song acquisition in zebra finches. Ultimately this could serve as a model demonstrating the effects of an organism receiving particular sensory and cognitive inputs during a specified time in development relative to that organism's epigenome. I hypothesize that there will be transcription factor binding sites that are differentially accessible between the male zebra finches who had no tutor song experience as compared to the males who were taught song structure by an adult, male tutor. More specifically, that there will be a larger number of differentially accessible transcription factor binding sties in the males who have not learned a song, indicating a more 'receptive state' for these males who have not yet exited their critical period of song learning.

Methods

(A) Birds - For this study, zebra finches were bred in laboratory aviaries with similarly controlled living conditions across the different groups. There were three groups total. For two of those groups, at post-hatch day 23, juvenile males were removed from the aviaries and placed into sound-attenuating chambers with 1-3 of their peers and two adult, female foster birds. At post-hatch day 30, each individual male was then placed into a different sound-attenuating chamber with two adult zebra finches. For one group of juvenile males, both cohabiting adults

were female zebra finches who make vocalizations but do not sing. This condition we labeled as "Isolate" to designate that they did not hear song during the critical period for tutor song memorization. The biology of these birds would hopefully give us insight as to the impact of not hearing tutor song during the critical period for song learning acquisition. The second group of juvenile males were placed with one adult female and one adult male, the latter of whom was able to tutor the juvenile male throughout their critical period for song-learning [see Figure 2]. This condition we labeled as "Tutored" to designate that they did hear song during the critical period for tutor song memorization. The biology of these birds would hopefully give us insight as to the impact of hearing tutor song during the critical period for song learning acquisition while having a similar social environment as the Isolate group (i.e. comparable living conditions with the same number of adult conspecifics). Juvenile males who were never removed from the home aviary constituted a third group which we labeled as "Normal." The biology of these birds would hopefully give us insight as to whether or not there is a quantifiable impact of the social environment (i.e. the number of cohabitating conspecifics including singing males) on transcription factor binding site accessibility while being exposed to tutor song during the critical period for tutor song acquisition just as the Tutored birds were. All birds had free access to seed and water at all times and were kept on a 14H-10H light-dark cycle.

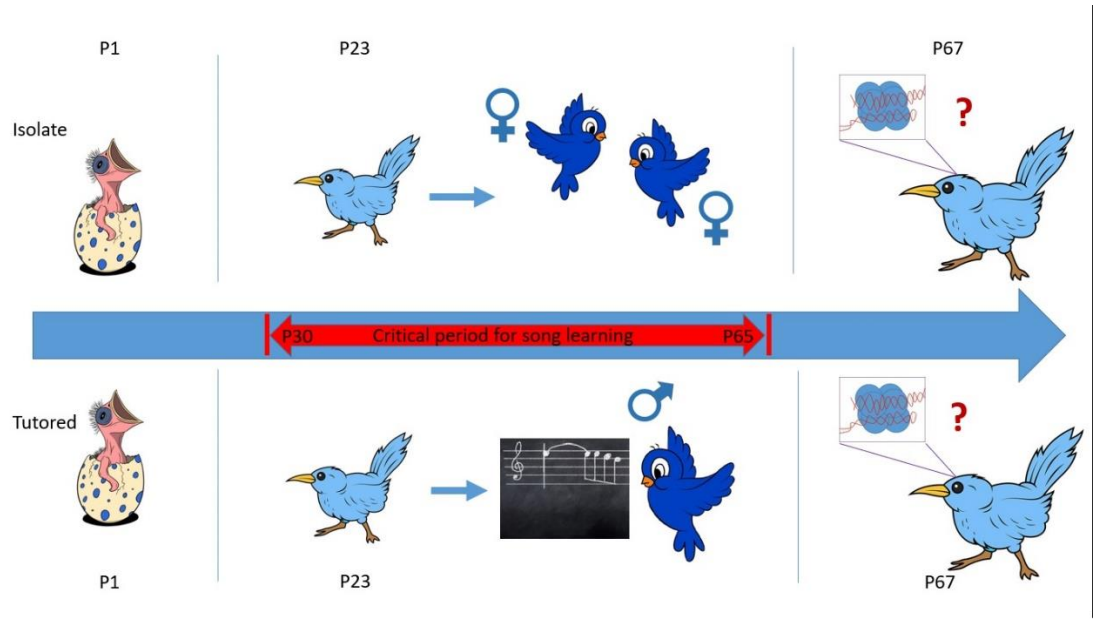


Figure 2 – *Illustration depicting the two parallel developmental timelines for the Isolate and Tutored conditions of the study. After hatching, birds are removed from the aviary at day 23 ~1 week prior to the onset of the song learning critical period. As shown, one group is placed into isolation with two adult females, while another group is placed with one adult female and one adult male, the latter of whom serves as a song tutor for the developing juvenile. After canonical critical period offset at post-hatch day 65, tissue is collected from these two different groups and assessed for acetylation status at histone H3, K27.*

(B) Tissue - Auditory forebrain tissue samples were collected from these birds at post-hatch day 67 and stored at -80^{O} Celsius until processing.

(C) ChIPseq – The Active Motif laboratory conducted the ChIPseq experiments on the collected tissue. Briefly, their methodology is as follows: the auditory forebrain tissue was treated with antibodies specific for acetyl groups at H3K27. These strands of genomic DNA/histone-DNA complexes were broken apart into smaller fragments via sonication, and these fragments were precipitated out of the solution. The regions of genomic DNA made accessible via the acetylation of H3K27 were then sequenced during a polymerase chain reaction (PCR) [see Figure 3].

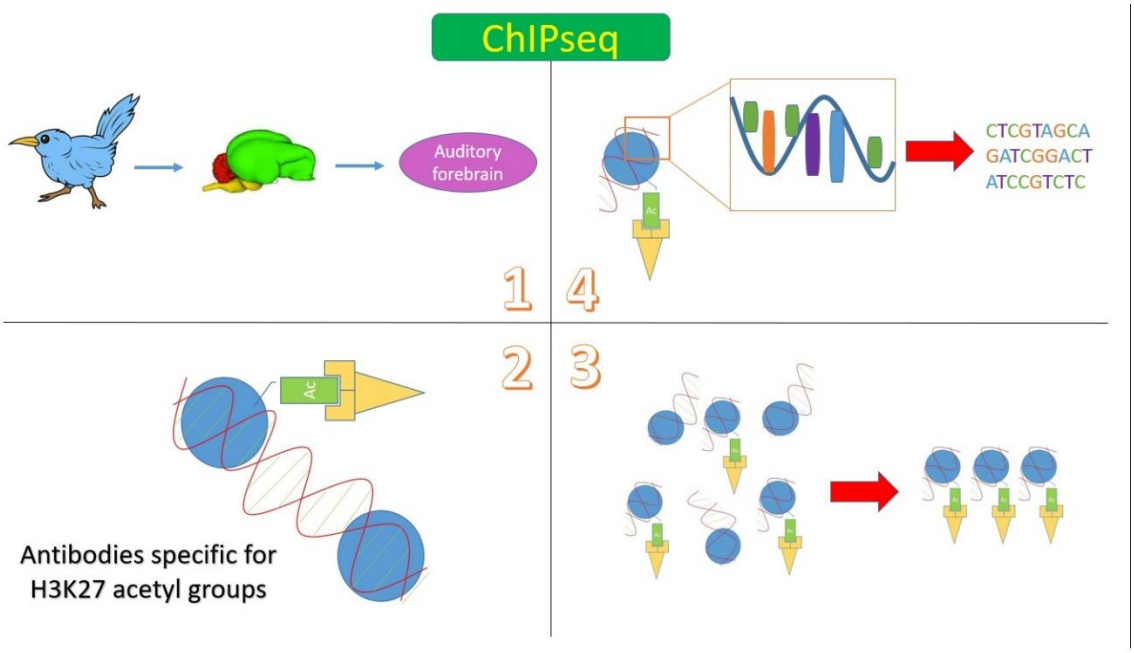


Figure 3 – *Chromatin immunoprecipitation - DNA sequencing (ChIP-seq).. (1) Auditory forebrain is extracted from each animal for processing. (2) Antibodies specific for acetyl groups at H3K27 are introduced to the extracted tissue samples. (3) After sonication to break the DNA/DNA-histone complexes into smaller fragments, they are precipitated out and collected. (4) Accessible regions of genomic DNA are sequenced during polymerase chain reaction (PCR) and this sequencing information is then the launching point for the processing pipeline as depicted in Figure 4.*

(D) ChIPseq Analysis – Unless otherwise noted, all of the bioinformatics processes described hereafter were executed using the tools offered by The Galaxy Project (www.usegalaxy.org) and data was uploaded and processed on their servers. Furthermore, all of the files involved in this pipeline use single-ended libraries which is reflected in the settings selected for the following bioinformatics processes. The ChIPseq analysis begins with a quality control check via a process called FastQC (Andrews, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Key metrics provided by FastQC are a basic statistics report that provides read length and total number of reads, as well as what quality-encoding format was used in the .fasta file. The ‘per base sequence quality’ is provided,

giving a sense of the quality and reliability of the returned sequencing output. Finally, FastQC provides 'sequence duplication levels,' indicating the number of unique sequence reads among all total reads for the sample which serves as a general indicator of the success of the experiment. These metrics provided confidence as to the accuracy and reliability of the raw read data used for the ChIPseq pipeline. In order to decompress the .gz files outputted by the ChIPseq process into their corresponding .fasta format, they were submitted to FastQ Groomer (Blankenberg et al 2010). Next, Trim Galore! (Krueger, https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) is used to trim out the adapter sequences used to bind the primers at the start of PCR; these adapter sequences are captured using the automatic detection setting of Trim Galore!. I decided not to remove any nucleotide reads from the 3' end of the sequence due to the high-quality of the reads as indicated by the initial FastQC process. Next, Bowtie2 (Langmead et al 2009, Langmead and Salzberg 2012) was used to align the reads from each processed sequencing file to the NCBI zebra finch genome assembly (specifically version bTaeGut1_v1.p). The genome used is the most updated assembly provided by NCBI, which was downloaded directly from NCBI's website (https://www.ncbi.nlm.nih.gov/assembly/GCF_003957565.1/) and uploaded directly to Galaxy as a reference file. Before peaks are called, the Filter SAM/BAM function (Li et al 2009) was used to filter out any portions of the sequencing files that were not able to be mapped to the genome. The resulting .bam file is what was used in the next process, MACS2 (Zhang et al 2008, Feng et al 2012), which calls genomic peaks. The configuration for this process included setting the reference genome length as roughly 1.06E9 with a q-value of 0.05, meaning for each individual sample file uploaded for the Isolate, Tutored and Normal conditions, only peaks that were enriched past this moderately stringent threshold would be included in the output .bed file.

This .bed file was then generated for each of the samples indicating the peaks identified by the MACS2 processing. In order to avoid having to manually modify each individual .bed file to switch away from the NCBI's custom naming conventions, a reference genome using standard chromosome positions (e.g. "chr1: 1-100) was created. After the individual chromosome files were downloaded, a simple text editor (in this case, Notepad) was used to edit the headers inside of the files to a more readily-digestible chromosome name ("chr1," "chr1A," "chr2," etc.) and saved. A Unix concatenate function executed from the command line then combined these chromosome assemblies into a single genome assembly, which was then uploaded to Galaxy. Thus, when a sample's sequencing file is run through the pipeline, the chromosome nomenclature is palatable to all downstream processes without having to manually modify the .bed file [see Figure 4].

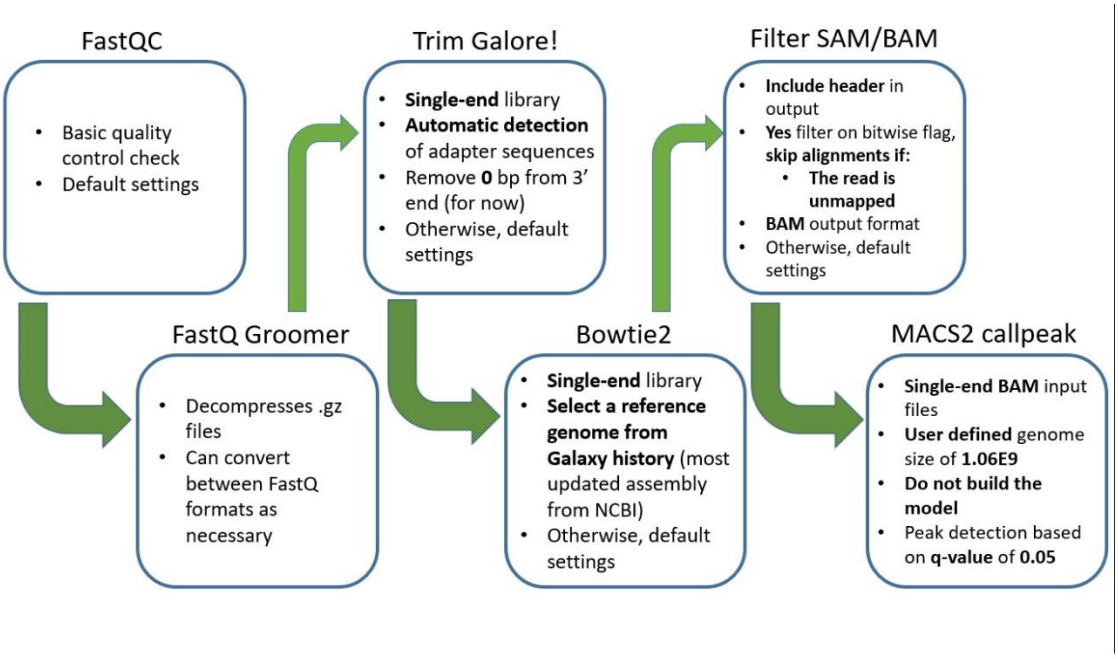


Figure 4 – *Graphical depiction of the bioinformatics pipeline used to process the reads returned from the ChIPseq analysis. All processes were executed through the Galaxy platform available at usegalaxy.org. Items in* ***bold*** *indicate specific user-defined settings.*

Next, a process called DiffBind (Ross-Innes et al 2012) was used to identify the sequences that are differentially accessible between multiple samples from two comparison groups. My main analysis concerned the comparison of the Isolate versus Tutored birds, as these groups had similar social and environmental rearing conditions, but critically one group had the opportunity to learn song from an adult, male tutor and one did not. As its inputs DiffBind takes the .bed files from each group—3 total for each of the Isolate, Tutored, and Normal conditions—which indicate the significant peaks relating to the accessibility of various transcription factor binding sites as well as the corresponding .bam files generated by the MACS2 process. The false detection rate (FDR) was set to 0.05, a moderately strict setting allowing for a high degree of confidence that any output transcription factor binding site is one that is, in fact, differentially accessible between the two groups. The output of DiffBind is a single .bed file containing the absolute chromosomal location of these differentially accessible genomic peaks. A custom script within the Galaxy toolkit is able to extract the actual DNA sequences using this absolute interval information (Ananda and Von Kluster, https://toolshed.g2.bx.psu.edu/repository?repository_id=5e1b70f8c4a31a72), output as a genomic .fasta file. To identify the biological relevance of these DNA sequences, the peak-motifs tool within the greater Regulatory Sequence Analysis Tools (RSAT) suite was utilized (Thomas-Collier et al 2011, Thomas-Collier et al 2012). For this process specifically, the .fasta files were uploaded directly to the RSAT server for processing. The output of this process is twofold: statistically significant motifs are identified, and these motifs are run against the JASPAR known vertebrates database (Fornes et al 2019) in order to identify the transcription factors whose binding sites match the identified motifs. Identifying the transcription factors that

are potentially coordinating the neurobiology of song learning in zebra finches was the overall purpose of this investigation.

Results

Given the three different groups of juvenile males under investigation, two comparisons were examined: 1) the Isolate birds with no song-learning experience versus the Tutored juveniles who were tutored by a single adult male and 2) those Tutored males compared to the Normal males raised in in a larger colony of birds. The former of these two comparisons highlights song-learning as the independent variable, while the latter condition compares what effect—if any—the social environment has on transcription factor binding site accessibility.

(A) Isolates vs. Tutored- The peak-motifs tool of RSAT returned a total of 10 relevant motifs from the input .fasta sequences (Table 1), half of which yielded potential matches in the JASPAR database. As these are highly similar but not identical matches, these results are probabilistic; for the sake of clarity I will include the top three transcription factor binding site matches as determined by RSAT's normalized Pearson correlation. The results of the matches for the Isolate vs. Tutored birds are shown in Table 2.

Table 1 – *All motifs identified by the RSAT tool peak-motifs in the Isolate versus Tutored comparison. The green 'a' represents adenine, the blue 'c' represents cytosine, the yellow 'g' represents guanine, and the red 't' represents thymine. Above each motif is a ID assigned by peak-motifs and below each motif is the number of sites that the motif was found within the .fasta input file. Multiple letters at a given position indicate the relative prevalence of that nucleotide at that position based on the height of the letters.*

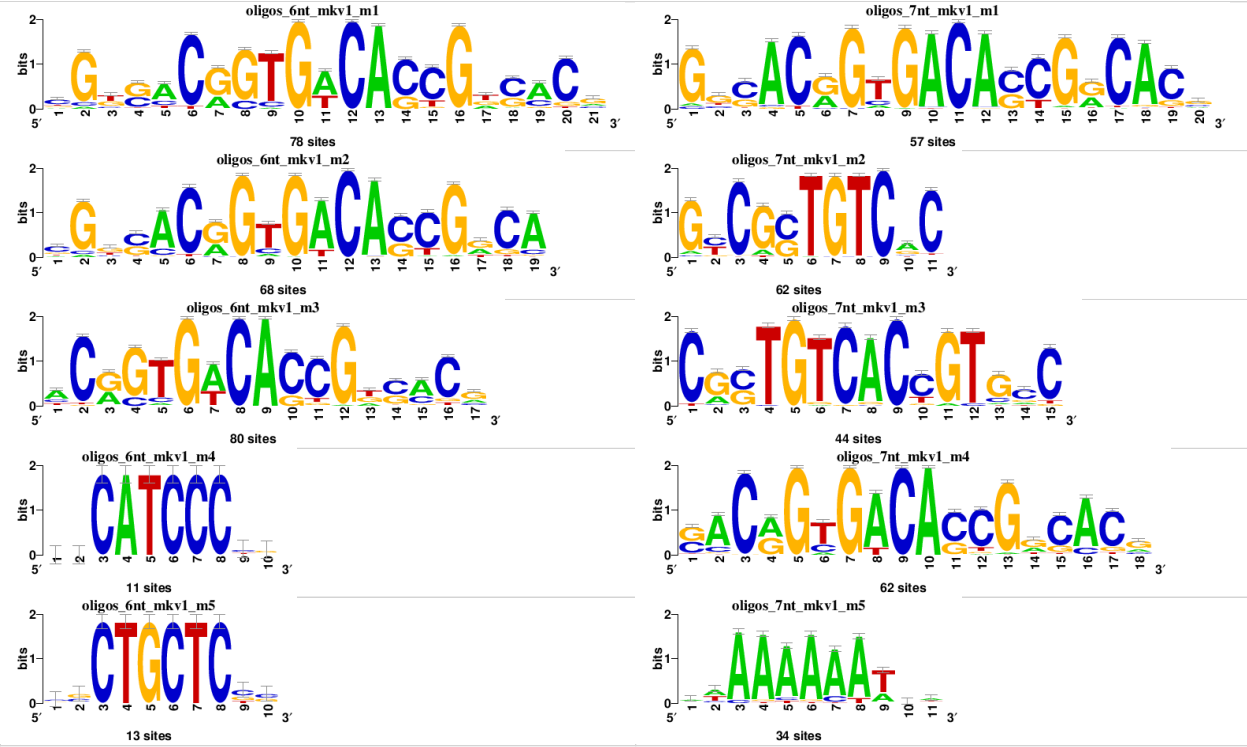
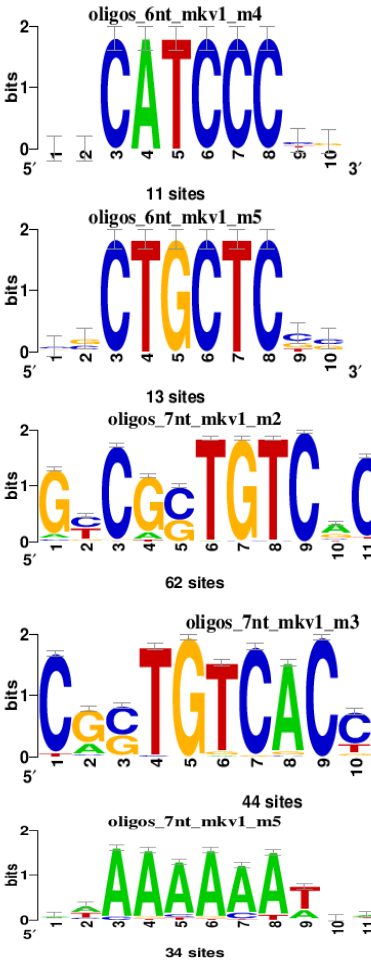


Table 2 – *peak-motifs output from RSAT displaying the statistically significant motifs that returned matches from the JASPAR vertebrate database. The top 3 transcription factor binding sites are shown based on percent alignment between the input sequences and the known database. Blue and orange highlighting was used to separate binding sites identified for different motifs, while the lighter and darker shades aimed to make the table more readable within a given motif.*

Motif	Transcription Factor Matches	Percent Alignment
	ZNF263	0.8333
	TEAD3	0.8
	MZF1	0.7692
	Zic1::Zic2	0.9091
	ZNF341	0.8333
	Ascl2	0.5385
	MEIS3	0.7273
	MEIS2	0.7273
	MEIS1	0.6364
	NFIC::TLX1	0.7059
	MEIS3	0.5333
	MEIS2	0.5333
	ZNF384	0.9167
	Foxd3	0.7692
	ONECUT3	0.5625

(B) Tutored versus Normal- The peak-motifs tool of RSAT again returned a total of 10 relevant motifs from the input .fasta sequences (Table 3), 9 of which yielded potential matches in the JASPAR database. Just as before, these results are probabilistic and for the sake of clarity I will

include only the top three transcription factor binding site matches based on percent alignment.

The results of the Tutored versus Normal birds are shown in Table 4.

Table 3 - *All motifs identified by the RSAT tool peak-motifs for the Tutored versus Normal comparison. The green 'a' represents adenine, the blue 'c' represents cytosine, the yellow 'g' represents guanine, and the red 't' represents thymine. Above each motif is a ID assigned by peak-motifs and below each motif is the number of sites that the motif was found within the .fasta input file. Multiple letters at a given position indicate the relative prevalence of that nucleotide at that position based on the height of the letters.*

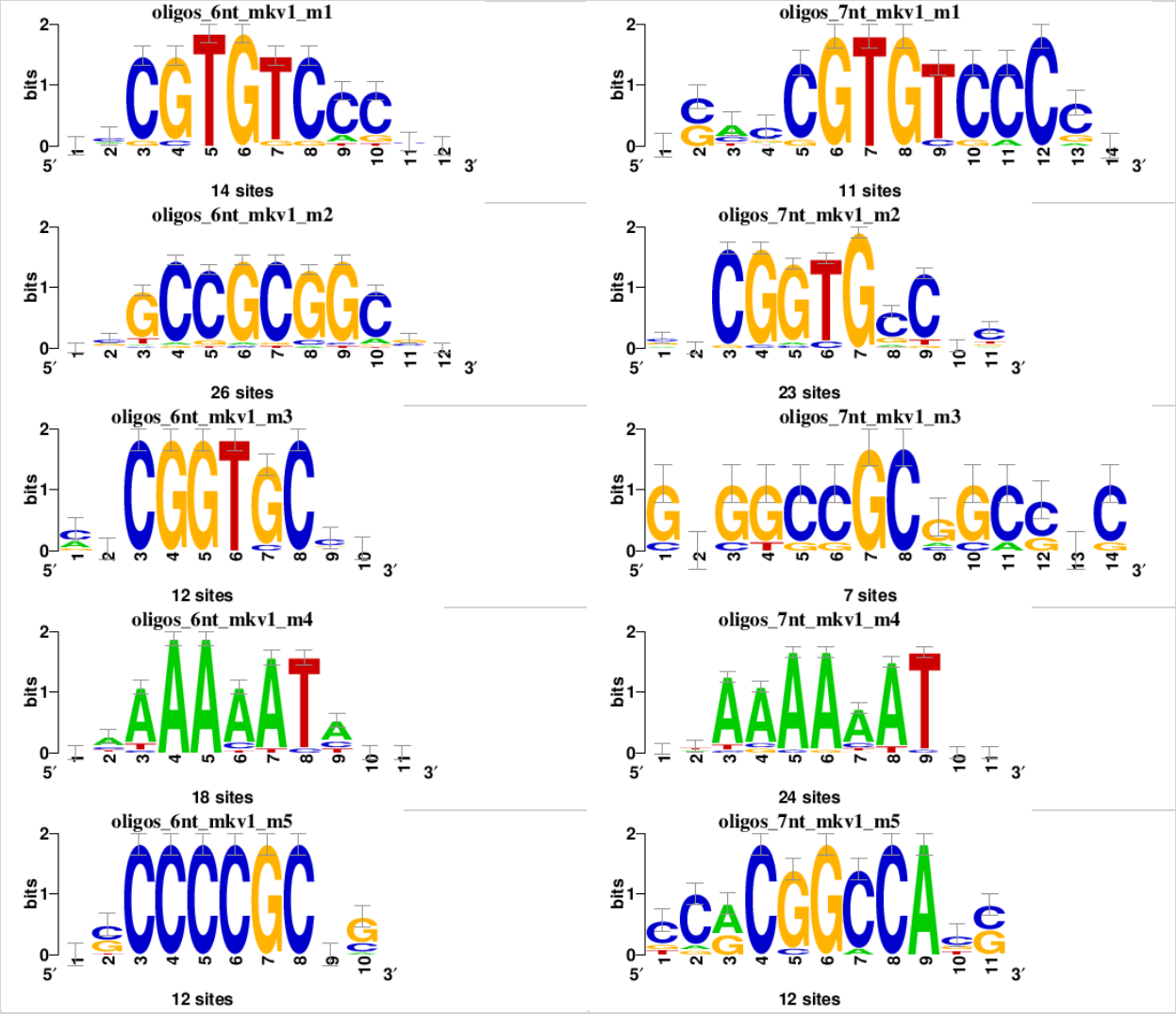
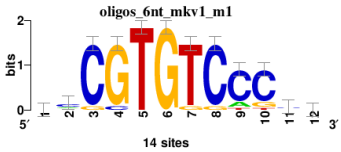
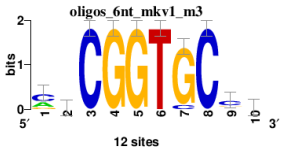
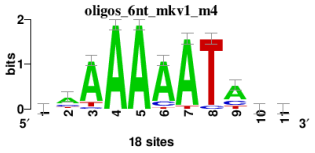
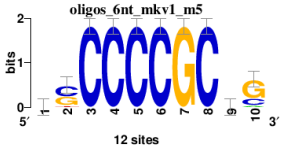
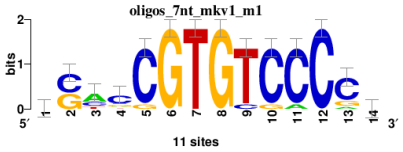
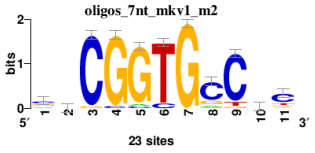
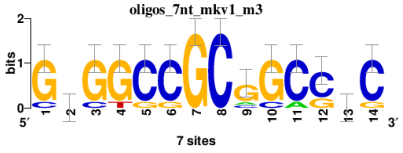
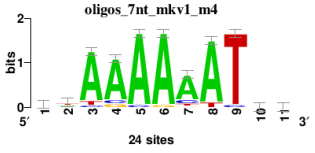
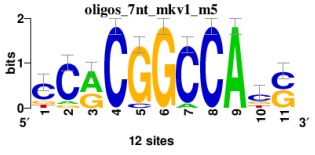


Table 4 – *peak-motifs output from RSAT displaying the statistically significant motifs who returned matches from the JASPAR vertebrate database. The top 3 transcription factor binding sites are shown based on percent alignment between the input sequences and the known database. Blue and orange highlighting was used to separate binding sites identified for different motifs, while the lighter and darker shades aimed to make the table more readable within a given motif.*

Motif	Transcription Factor Matches	Percent Alignment
	Creb3l2	0.6154
	HEY2	0.5714
	HES6	0.5714
	NFIC::TLX1	0.7143
		
	MEF2A	0.7333
	MEF2C	0.7333
	ZNF384	0.7692
	VEZF1	1
	MAZ	0.9091
	KLF15	0.75
	HEY1	0.7143
	HEY2	0.7143
	HES2	0.7143
	NFIC::TLX1	0.6667
	Hic1	0.5385
	HIC2	0.5385
	Zfx	1
	HINFP	0.625
	ZNF460	0.5789
	ZNF384	0.9167
	MEF2A	0.7333
	Sox5	0.6364
	YY2	0.8333
	KLF11	0.8333
	KLF3	0.6923

Discussion

In the Isolate versus Tutored comparison, a prevalent theme among the transcription factors implicated in coordinating the critical period for song acquisition is that of nervous system organizational development. *Zic1* and *Zic2* are involved in neural crest determination and cerebellar development, as well as regulating in neuronal differentiation (Aruga et al 1998, Sato et al 2005, Nagai et al 1997, Aruga et al 2002, Nagai et al 2000). *MEIS3* is thought to play a role in hindbrain differentiation and organization of the neural plate (Vlachakis et al 2001, Elkouby et al 2010) while the closely related *MEIS2* plays a role in cranial neural plate development (Machon et al 2015, Capdevila et al 1999). *Foxd3* is involved in neural crest development and the maintenance of pluripotency (Sasai et al 2001, Dottori et al 2001). *TLX1* has been shown to promote differentiation and development in the hindbrain of chicks (Logan et al 1998). Taken together, this would suggest that exposure to tutor song contributes to the accessibility of particular transcription factor binding sites that regulate brain organization.

Still other transcription factors seem to contribute to this period in development among male zebra finches: *TEAD3* affects early maturation (Christensen et al 2017) and *MZF1* controls cell proliferation (Gaboli et al 2001). Critically, though, the gene family for *TORC2*, *mTOR*, has been shown to modulate experience-dependent synaptic plasticity in the auditory forebrain of songbirds and is, indeed, required for tutor song memorization (Ahmadiantehrani and London 2017). Additionally, *mTOR* was shown to enhance non-associative learning in adult songbirds, again in the auditory forebrain (Ahmadiantehrani et al 2018).

In the Tutored versus Normal comparison group, two key themes that emerge from the known function of the 10 most reliable putative transcription factor binding sites are cell

proliferation and regulatory mechanisms of activating or silencing gene expression. *HES6* regulates myogenic differentiation but more importantly promotes cortical neurogenesis (Cossins et al 2002, Gratton et al 2003) and *Hic1* is a tumor-suppressor gene working against cell proliferation (Briggs et al 2008, Fleuriel et al 2009). While its role is less clearly defined than that of other transcription factors discussed here, *YY2* is implicated as having a role in cell proliferation (Wu et al 2017) and *Zfx* plays a role in the proliferation of B lymphocytes and stem cells (Galen-Caridad et al 2007, Arenzana et al 2009). Upregulated cell proliferation and subsequent synapse development could be part of the underlying biology contributing to zebra finch tutor song memorization.

A number of the differentially accessible transcription factor binding sites identified are not associated with cell proliferation, however. *MAZ* plays a potential role in both initiating and terminating transcription through its ability to affect the 3D structure of DNA (Cogoi et al 2014, Bossone et al 1992) and *VEZF1* serves to protect sites on the genome from DNA methylation, although it seems to be most prevalent in endothelial cells (Dickson et al 2010, Xiong et al 1999). *Sox5* downregulates *SPARC* gene expression (Stolt et al 2008), and while now considered non-essential, *KLF11* has been associated with cell growth and gene expression (Song et al 2005). Staying within the Kruppel-like factors family, KLF3 plays a role in transcriptional silencing in addition to the regulation of lipoprotein assembly and secretion (Zhang et al 2013). Owing to its similarity to *Creb3l1*, *Creb3l2* is involved specifically in vasopressin gene expression and inhibiting cell proliferation (Greenwood et al 2014, Denard et al 2011, Panagopoulos et al 2007). The potential involvement of such a diverse range of transcription factors highlights the complexity of this system and highlights the open question of which transcription factors are driving tutor song memorization in zebra finches.

This is not to say that there are no leading candidates, however; the identification of two MEF-family transcription factor binding sites, *MEF2A* and *MEF2C*, across multiple motifs suggests a potentially robust role for these transcription factors: *MEF2* has been shown to play a role in neuronal maturation in mice (Lyons et al 1995) and a role in neuronal cell differentiation in fruit flies (Schulz et al 1996). Lin and colleagues (1996) corroborated the Schulz finding in mice, finding both *MEF2A* and *MEF2C* to be involved in central nervous system differentiation. Furthermore, more recent scholarship has implicated MEF2 family members in the synaptic plasticity underlying memory formation (Rashid et al 2014). Thus, the social environment for a juvenile male zebra finch within the song-learning critical period have a profound effect on tutor song memorization as mediated by nervous system development and differentiation. Additional studies into the effect of having exposure to a single male tutor versus multiple male tutors seems warranted.

In summary, both exposure to tutor song as well as the richness of the social environment during the critical period affected the birds' transcription factor binding site accessibility in quantifiable ways. Many of these transcription factors were related to nervous system organization and cell proliferation, which could be biological underpinnings to tutor song memorization during this key developmental period. Future investigations into the effects of tutor song exposure and conspecific cohabitation could yield additional insights into the biological coordination of this complex phenomenon.

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