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Identification of Sequence Learning in Rhesus Macaques through Infrared Eye Tracking

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Abstract

Identification of Sequence Learning in Rhesus Macaques through Infrared Eye Tracking

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Studies in both humans and monkeys have demonstrated that eye-movements can be useful in the investigation of memory for temporal sequences. For example, a study used non-invasive infrared eye-tracking to observe eye movements while human infants watched videos depicting a sequence of actions. The results suggested that infants made a significant number of anticipatory looks with repeated presentations. The present study aims to replicate this finding in an animal model using Rhesus macaques. In Experiment 1, 3 monkeys were shown repetitions of video sequences while their eye movements were tracked with an infrared camera. Eye-movements that were anticipatory for objects that would move 3 steps in advance and 1 step in advance were examined, along with the number of eye-movements that were directed toward moving objects and distracter (non-moving) objects. A comparison between the initial viewing of the video and subsequent viewings revealed significant differences in the percentages of anticipatory looks for both3- and 1- step intervals, as well as the ratio of moving to distracter objects. In Experiment 2, the effects of familiarizing the monkeys with the video objects on sequence learning were examined. The monkeys performed a delayed match to sample (DMS) task, using the video objects as stimuli, one day prior to being tested on the video task. The results from Experiment 2 revealed that there was no significant effect of the DMS pretreatment on sequence learning. The analyses suggest that, similar to human infants, monkeys demonstrate sequence learning as measured by anticipatory eye-movements. Accordingly, this task could be used, along with neurophysiological techniques, to investigate the neural correlates of sequence learning.

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INTRODUCTION

Medial Temporal Lobe Memory System and Declarative Memory

The medial temporal lobe (MTL) has been recognized as critical for learning and memory since case studies in the late 1950's of patients who suffered from severe memory impairments following bilateral surgical removals of their MTL (Scoville & Milner, 1957). These patients were unable to form new memories (anterograde amnesia) and retrieve certain recent memories (retrograde amnesia), but had no alterations to their personality, general intelligence or technical skills (Scoville & Milner, 1957). Since then, many studies investigating both human amnesic patients and animal models of amnesia have contributed to our understanding of the function and MTL structures, including the hippocampus, entorhinal, perirhinal and parahippocampal cortices, to learning and memory (Squire & Zola-Morgan, 1991).

A specific type of memory that has been linked to MTL function is memory for things of which we are explicitly aware, known as declarative memory (Purves et al., 2008). Declarative memory for events, semantic information, and objects can be expressed and reported verbally (by humans) or non-verbally through responses such as eye movements, gestures and other behavioral responses (by both humans and animal models) (Purves et al., 2008).

Eye Movements as a Measurement of Declarative Memory

Eye movements have been demonstrated to be a useful way to assess memory for images (Ryan et al., 2000; Ryan & Cohen, 2004). Because of an innate preference for novelty, human subjects make fewer eye fixations on objects and regions that they have already previously viewed in comparison to novel items (Althoff & Cohen, 1999). Eye movements have also been utilized in learning and memory studies with monkeys (Jutras & Buffalo, 2009; Jutras, Fries & Buffalo, 2009).

Temporal Sequence Learning and Memory

A more specific subset of declarative memory is memory for a specific sequence of events or temporal order memory. Sequence learning and memory is integral to human behavior and is one of the most common forms of learning in human and animals. Many of the simple daily activities we engage in (speaking, getting dressed, driving etc.) require a memory for and completion of a series of steps in a correct order.

Temporal sequence learning has been investigated on a neuronal level through a dual neuron model that suggests that a certain firing pattern is elicited only when input signals are organized in a specific sequence (Wang & Arbib, 1990). This supports the idea that there are areas and components of the brain dedicated to the receipt and integration of sensory inputs relevant to sequences.

Recall of temporal sequences has been attributed to the hippocampus (e.g. Lehn et al., 2009; Rieckmann, Fischer & Backman., 2010; Gerrad, Burke & McNaughton, 2008; Manns, Howard & Eichenbaum., 2007). The role of the hippocampus in sequence learning has been demonstrated with different techniques including electrophysiology, imaging, and cognitive and behavioral tasks; different forms of sequence learning were characterized in these experiments including motor (such as a running track maze task) (Gerrard et al., 2008), olfactory (Manns et al., 2007), and visual (Pathman, Bauer & Pelphrey, 2008). While much has been done through these studies to delineate when or if sequences have been learnt, there is less research on quantifying the learning process as it is occurring.

Eye Movements as a Measurement of Sequence Learning

It has been observed that human subjects fixate on objects that will be manipulated in the future and can be considered as "look-ahead fixations" or anticipatory eye movements (Mennie, Hayhoe & Sullivan, 2005). Anticipatory eye movements have been studied in both monkeys (Miyashita, Rand, Miyachi & Hikosaka, 1996) and humans (e.g. Mennie et al., 2005; Freyberg & Iig, 2007) and there are many hypotheses regarding their function.

The most common paradigm has revolved around how humans and monkeys use previous information on the probability of an object's directional motion to make predictive eye movements in anticipation of the movement of an object (de Hemptinne et al, 2007; Badler & Heinen, 2006). According to these studies, anticipatory looks are useful in maintaining smooth pursuit eye movements, which are important for accurate and prompt motor responses to environmental stimuli.

Other studies of anticipatory eye movements have focused on their presence in tasks with a temporal sequence or established steps. The most prominent hypothesis suggests that anticipatory looks reveal planning and problem solving for the execution of complex processes. Human subjects undergoing eye tracking with a wearable eye tracker have demonstrated "look ahead fixations" during a sequence based motor task such as hand-washing by making saccades to and fixating on objects that are pertinent only in future actions (Pelz and Canosa 2001).

The same study also rejected the hypothesis that these look ahead or anticipatory fixations were resultant of a general exploratory behavior induced by the conspicuity or salience of objects within a given environment by demonstrating that objects that had already been used in the sequence were rarely fixated on afterwards (Pelz and Canosa, 2001). Pelz and Canosa interpreted these findings to suggest that anticipatory fixations provide a way for the brain to create a seamless continuity of perception and facilitate motor actions.

A non-human primate study similarly discovered that monkeys make anticipatory saccades while learning sequential motor procedures (Miyashita et al., 1991). Japanese macaques were trained to perform the “2x5 Task” while their eye movements were tracked with surgically implanted search coils. The “2x5 Task” involved a panel of 16 light-emitting diode buttons, 2 of which lit up simultaneously during each set. The monkeys had to press the illuminated buttons in a predetermined sequence for reward in each set. Following multiple days of testing on the same predetermined sequence, the likelihood of anticipatory looks towards buttons next in the sequence increased. Because monkeys continued to make anticipatory looks for the sequence that had been previously learnt when introduced to new sequences, it was suggested that the anticipatory looks were an indication of memory for a sequence and not only a result of improved proficiency at the button pressing task with experience (Miyashita et al, 1991).

Eye Movements as a Measure of Declarative Sequence Learning

Although many of these previous studies examined anticipatory eye movements in tasks of procedural or non-declarative memory, anticipatory eye movements have also been demonstrated in a task of declarative memory. Recent work on temporal sequence learning examined infants' eye movements and demonstrated that they made anticipatory eye movements during subsequent viewings of videos of multi-step tasks (Pathman et al., 2008). The study showed repeated viewings of live action videos of goal-orientated tasks made up of distinct steps (such as putting together a toy windmill) to 20-month-old infants. The infants' eye movements were tracked during the showings with a non-invasive infrared eye tracker. The number and duration of eye fixations and the number of anticipatory looks were used as measurements of learning in the task. Importantly, the infants' were able to demonstrate declarative memory for the sequences by physically recreating the steps in the task in a behavioral recall.

The infants were able to demonstrate learning through anticipatory eye movements as well as behavioral recall of the tasks (Pathman et al., 2008). Furthermore, the infants' fixation patterns (number of anticipatory looks) were shown to change with repeated presentation of each video, suggesting that the methodology used in the study could be an indicator of sequence learning. Accordingly, we attempted to use a similar strategy to examine temporal sequence learning in an animal model.

Using Rhesus Macaques as an Animal Model for Temporal Sequence Learning

We selected rhesus macaques (*Macaca mulatta*) as our animal model because they have demonstrated a capacity for completing a task that involves selectively fixating on and remembering complex visual images (Buffalo, Bellgowan & Martin, 2006; Jutras & Buffalo, 2010). Monkeys have also been used effectively in studies that measured memory through other MTL dependent tasks such as object matching, discrimination learning, object discrimination and spatial delayed response (Zola-Morgan & Squire, 1985**).**

Goals of Present Study

Motivated by the findings in Pathman et al. (2008) we chose to use a similar technique and methodology for demonstrating sequence learning with exposure to video stimuli in an animal model. We employed a similar infrared eye tracking system but with different visual stimuli. This study is unique in its attempt to design a task that could quantify temporal sequence learning as it is occurring within an animal model.

In an attempt to enhance the accuracy of the video task, we investigated multiple adaptations of video stimuli (including live action movies and PowerPoint based movies), and their effectiveness in promoting sequence learning in rhesus macaques. We also investigated multiple means of quantifying the data. It was anticipated that the rhesus macaques would show similar capabilities for sequence learning as the infants from the Pathman et al (2008) study.

Because previous research has indicated the importance of the MTL system in sequence learning and memory, we anticipated that the development of a task of temporal sequence learning in macaques would enable future neurophysiologic studies of the neural correlates of sequence learning, with recordings targeted at MTL structures. Additionally because MTL structures are damaged early in Alzheimer's disease, we hoped another future outcome from this study would be the development of an easily accessible, reliable, and valid task for assessing temporal learning and possible cognitive impairment associated with neurodegenerative disease.

METHODS

Subjects

The subjects (Dy8, Tt9, Iw8) were three male rhesus macaques (*Macaca mulatta*), between 7 and 8 years old, with experience and training in various other memory tasks (such as Visual Preferential Looking and Delayed Match to Sample) that involved digital photographic images presented on a computer screen. The monkeys each lived individually in protected contact housing that permitted social contact with adjacent cage mates through a partition while physically separating them to maintain the integrity of their surgically implanted head posts and chambers.

All monkeys were on a controlled food diet under guidelines from the Emory University Institutional Animal Care and use Committee and Yerkes National Primate Research Center SOP5.8 Version 1 to provide motivation for food rewards. The food reward consisted of a special chow mixture made from 9 *Lab Diet*® monkey biscuits, one banana, 100 milliliters of Mott's applesauce and a liter of water blended to a smooth consistency. The chow mixture was administered via a tubing system connected to a metal sipper tube fixed to the chair and easily accessible to the monkey's mouth. The chow mix was dispensed by a *Masterflex*® pump which was connected to a computerized reward system. The monkeys performed other tasks, in addition to our task, daily and each ate approximately 750 milliliters of the chow mix every weekday. On weekends they did not perform any tasks and were fed 16 biscuits and one half of an orange on Saturday and 12 biscuits and one half of an orange on Sunday in their home cages.

Monkeys were weighed daily and weekly weight charts were plotted to ensure that they maintained mean weight and standard deviation for monkeys of comparable age

(Van Magenen and Catchpole, 1956). When weight gain became an issue with one monkey, a tablespoon of *Ensure*® supplement powder was added to his chow mixture until he regained a normal weight. Veterinary and Animal Care staff were informed on the progress and health of all monkeys.

Experimental Stimuli

The monkeys were shown videos in avi format created from PowerPoint presentations using *Windows Media Encoder 9.0* and *Adobe Premier Pro*. Each video contained 6 digital photographic images approximately 80 by 80 pixels arranged randomly around a white area of 480 by 720 pixels The photographs were digital pictures taken from Flickr's® free online database (www.flickr.com/explore) and ranged in subject matter from landscapes to animals and everyday objects. The images were comparable to the stimuli used in the other tasks that the monkeys were used to performing (e.g. Delayed Match to Sample and Visual Preferential Looking Task).

During each video, 3 of the 6 photographic "objects" were involved in some form of movement (e.g. moving across the screen to another location, spinning in place or changing in size) which created a sequence of three steps per video. The videos ranged from 15 to 19 seconds in length and had a frame rate of 29 frames per second. Thirteen unique videos (each with 6 unique photographs) were created for Experiment 1: Dy8 only saw video 8, Tt9 saw videos 1 to 4, and Iw8 saw videos 3 – 13. 6 unique videos (each with 6 unique photographs) were created for Experiment 2: Dy8 only saw video 2, Tt9 saw videos 5 and 6, and Iw8 saw videos 1-6.

Experimental Procedure

Each monkey had been trained to sit in a primate chair with an internal touch-sensitive metal bar in a closed room with minimal lighting. Eye movements were captured via an infrared eye tracking system (*ISCAN® Primate Eye Tracking Laboratory, Model RK-826 PCI Pupil/Corneal Reflection Tracking System*) and monkeys were head-posted in order to assure a steady eye scan. The experimental stimuli were presented using a data acquisition and experimental control program (*NIMH CORTEX*, http://www.cortex.salk.edu/) on a 19 inch computer screen positioned 60 cm in front of the monkey.

Experiment 1: Video Task

Each data collection session lasted approximately 15 minutes and consisted of 25 trials of a calibration task followed by 8 trials showing the same experimental video stimulus interleaved with 5 trials of the same calibration task. The calibration task was a simple response task (color change), in which a 5 by 5 millimeter grey square appeared on the black screen and after a randomized time between 500 and 1500 milliseconds, the square changed colors to an isoluminant yellow. The monkeys were rewarded when they responded to the color change by releasing their hold on the touch bar.

The color change task was used prior to the initial video trial to calibrate the eye scan and then to maintain motivation throughout the rest of each session. Monkeys were not rewarded during the video trials. The monkeys had to successfully complete 5 color change trials in between video trials order to move on to the next trial. Unlike the color change calibration trials, the monkeys did not have to respond during the video trials and were free to view the stimuli as they wanted to but had to make an initial fixation for 1 second on a small white crossbar that appeared onscreen before the video stimuli would appear (See Figure 15). To prevent a misinterpretation of this fixation after the video started, no “object” was placed in that center area in any of the video stimuli.

Experiment 2: Video Task with Habituation

For this task, the experimental procedure remained the same as in Experiment 1, but monkeys performed a delayed-match-to-sample (DMS) task with the same 6 pictures used as objects in the video stimuli 24 hours prior to the actual video task. In the DMS task (see Figure 1), monkeys held the internal touch bar and fixated on a small cross-bar (which remained throughout the task) to begin the trial. The first picture shown out of the 6 different pictures in each set became the target picture. This target picture would be followed by a variable number of test pictures from the set (in a randomized order that could involve repetition of certain ones). When the test picture presented matched the target picture, the monkeys released their hold on the touch bar to be rewarded. The second presentation of the target picture always completed each trial.

If the monkeys failed to respond to the matching target picture or if they released the bar prior to the match target picture, they would not be rewarded and the task would continue to the next trial beginning with the initial fixation on the cross-bar. This was a task that each monkey already had previous experience with and they were able to perform it consistently for at least 2 hours on the day before each respective video task.

This supplemental task was used in order to decrease the novelty factor in the initial trial of each video session and reduce the disparity between the overall fixations made in the initial trial versus subsequent trials. By doing so, we hoped the monkeys would focus more on the sequential component of the steps within the video stimuli rather than the visual components of each object.

Experimental Analysis

Eye scans from the video component of both Experiment 1 and 2 were superimposed in real time onto the video stimuli with a script in *MatLab* and all fixations were counted by hand by one researcher for all sessions. A breakdown of the 3 steps of sequence in each video was created by specifying the objects that would be considered an anticipatory object of interest (an object that would move in the next step), as well as defining each step (See Table 1).

Anticipatory looks were defined as a fixation on an object that would move in the future. This operationalization was refined to distinguish between anticipatory looks for objects that would move within 3 steps and anticipatory looks for objects that would move in the next step, the former being an indication of a general anticipation of movement of objects that were part of the sequence and the latter being an indication of a more direct and immediate anticipation of the movement of the object in the very next step of the sequence.

Fixations on “moving” objects, fixation on “distracter” objects, and total number of fixations for each of the 8 trials in each session were also counted. A fixation on a “moving” object was operationalized as any fixation on an object that would move in the future or had moved prior to the fixation, whereas a fixation on a “distracter” object was operationalized as any fixation on an object that did not move at all during the sequence. Fixations on the object that was moving *during* its own step in the sequence were not counted to dismiss incorporating fixations that were elicited by movement itself.

From these raw data we calculated the percentage of anticipatory looks (for 3 steps in advance and 1 step in advance) and the ratio of moving to distracter objects for each trial of each session. Repeated measures ANOVA was used to compare the number of anticipatory looks made across trials. A paired two tailed t-test was used to compare the average percentage of anticipatory looks and the average moving to distracter object ratios of the subsequent peak trials to those from the initial trials.

$$\text{Percentage of Anticipatory Looks} = \frac{\text{\# of Anticipatory Looks Made}}{\text{Total Number of Fixations Made}} \times 100$$

$$\text{Ratio of Moving to Distracter Objects} = \frac{\text{\# of Fixations on Moving Objects}}{\text{\# of Fixations on Distracter Objects}}$$

RESULTS

We examined the monkeys' eye fixations during each video trial to observe sequence learning as it occurred, and we employed four separate measures to ensure internal validity. Three of the four measures involved comparisons between the first trials of each video to its corresponding subsequent peak trial (percentage of 3 step anticipatory looks made, percentage of 1 step anticipatory looks made, and ratio of moving to distracter objects), while the fourth measure compared the average percentage of anticipatory looks made across each of the 8 trials.

Experiment 1

Initial analysis of the raw numbers of fixations in each trial throughout a session within Experiment 1 demonstrated that the monkeys consistently made more fixations overall during the very first trial (when the video stimulus was novel) relative to subsequent trials. This may have been due to an initial interest in the novel pictures which decreased with further exposure, making it difficult to identify any learning that may have occurred solely based on the number of anticipatory fixations made. Accordingly, we instead examined the percentage of anticipatory fixations made relative to the total number of fixations.

Three Step Anticipatory Looks

Consistent with Pathman et al (2008), we found that the highest quantification of anticipatory looks (a percentage in our task versus a raw score in their study) generally did not occur in the very first trial. Out of 15 sessions analyzed, only 1 session had the maximum or "peak" percentage of anticipatory looks for the session in the very first trial. Across sessions, trial 6 occurred as the peak trial for the largest number of sessions (4 sessions, see Figure 2).

The trial in which the peak percentage of anticipatory looks occurred was not always consistent across all the sessions for any monkey, thus, like Pathman et al (2008) we chose the subsequent trial (any trial 2 – 8) that had the highest percentage of anticipatory looks for 3 steps in advance for each session in all further comparisons and analyses. The monkeys had on average a higher percentage of general anticipatory looks in their subsequent peak trials (*M=50.75, SD=15.51*) than in their initial trial (*M=36.11, SD=11.13*) (*two-sample paired t-test, t(14)=3.90, p=0.0016*) (Figure 3). This indicates that monkeys may have acquired a memory for the sequence or learnt the sequence after seeing it at least once.

One Step Anticipatory Looks

We performed the same comparison between the first trial and the same subsequent peak trail (the trial with the peak percentage when considering 3 steps of anticipatory looks) but only counting anticipatory looks that were 1 step in advance (fixations on objects that moved in the very next step of the sequence). Figure 4 shows that this analysis similarly provides significant evidence for learning: monkeys averaged higher on immediate anticipatory looks in their subsequent peak trials (*M=29.94, SD=12.62*) than in initial trials (*M=17.41, SD=6.36*) (*two-sample t-test, t(14)=4.25, p<.001*). Trial 5 had the highest number of peak percentages when considering only 1 step anticipatory looks (see Figure 2).

Ratio of Moving to Distracter Objects

We compared the ratio of moving to distracter objects during the subsequent peak trial to the ratio during the initial trial. Across all sessions, the monkeys demonstrated a higher average ratio of fixations on moving objects to fixation on distracter objects in the subsequent peak trial (*M=2.22, SE=0.382*) than the initial trial (*M=1.039, SE=0.143*) (*two-sample paired t-test, t(14)=3.18, p=0.0147*) (Figure 5). This indicates that the monkeys may remember the objects that are pertinent to the sequence after at least one presentation of the video.

Anticipatory Looks Across Trials

In order to supplement the analyses done by looking at only “peak” trial data, we also evaluated the average percentage of anticipatory looks across the 8 trials of all videos. A one-way repeated-measures ANOVA was used to assess possible differences in the percentage of anticipatory looks across the trials. While the comparison for 3 step anticipatory looks was not significant (see Figure 6), the comparison for 1 step anticipatory looks (Figure 7) revealed that the percentage of anticipatory looks across trials were different, (F(7,98) =3.75, p=.001). Repeated measures t-tests revealed that monkeys made a significantly higher percentage of anticipatory looks in trial 5 than all other trials except trial 3 (*trial 5 vs trial 1: t(14)= 3.222, p=.006; trial 5 vs trial 2: t(14)=3.298, p=.006; trial 5 vs trial 4: t(14)=3.427, p=.004; trial 5 vs trial 6: t(14)=2.841, p=.013; trial 5 vs trial 7: t(14)=4.029, p=.001; trial 5 vs trial 8: t(14)=3.218, p=.006).* This is consistent with our finding from Figure 2, where trial 5 was the peak trial for the most number of videos for 1-step anticipatory looks.

Experiment 2

The same analysis as in the previous experiment was performed on the data from Experiment 2. Like the first experiment, most of the peak percentages of anticipatory looks for each video occurred in a subsequent trial: only 1 video had its peak presentation in the first trial. The trial with the highest number of peak percentages was trial 6 with 3 videos for both analyses of 3- and 1-step anticipatory looks (See Figure 8).

As in Experiment 1, monkeys still made a higher percentage of 3-step anticipatory looks (Figure 9) in subsequent peak trials (*M=46.20, SD=17.48*) than in the first trial (*M=28.96, SD=5.66*) (*two-sample paired t test*, *t(8)=3.06, p=.016*), and in 1-step anticipatory looks (Figure 10) made in subsequent peak trials (*M=25.06, SD=13.39*) compared to the first trial (*M=11.33, SD=3.24*) (*two-sample paired t-test, t(8)=3.18, p=.013*). However, the ratios of moving to distracter objects between the initial and subsequent peak trials were only marginally significantly different (Figure 11), and the analysis of the number of anticipatory looks made across trials was not significant (Figure 12 & 13).

Finally, we compared the raw number of total looks made across trials during Experiment 1 and Experiment 2 (See Figure 14). Surprisingly, the pretreatment did not reduce the initial interest in the pictures during the first trial. Repeated measures analysis indicated no effect of the DMS habituation on the number looks made across trials. Future studies are needed to determine whether pre-treatment within the same day of testing on the video task would have an effect.

DISCUSSION

The objective of this study was to modify the video task coupled with the eye tracking methodology introduced by Pathman et al (2008) in order to examine temporal sequence learning in rhesus macaques. We hypothesized that rhesus macaques would demonstrate sequence learning through anticipatory eye movements, similar to human infants (Pathman et al., 2008). The results of the current study supported this hypothesis by demonstrating that repeated presentations of video sequences resulted in a change in the percentage of anticipatory looks made across trials.

For both Experiment 1 and Experiment 2, the monkeys generally made a higher percentage of looks in anticipation of objects that were going to move immediately in the next step (1 step anticipatory) as well as those which were going to move at any point during the entire sequence (3 step anticipatory) after at least one presentation of a video. Despite the differences in methodology, these findings were consistent with the findings in human infants from Pathman et al (2008) in terms of 3 steps anticipatory looks and the findings in human adults from Pelz and Canosa (2001) in terms of immediate anticipatory looks.

In addition, the ratio between moving and distracter objects was also higher in the subsequent trial than in initial trials indicating that the monkeys made more fixations on objects that had moved or would move after they had already seen the stimuli at least once. This latter finding in particular suggests that the video task may be useful as a non-verbal indicator of declarative memory.

The differences in percentage of anticipatory looks made across the trials in Experiment 1 (Figure 7) may be explained by an initial learning curve that is followed by a gradual disinterest in the stimuli. This method of analysis may be of significant interest in the quantification of sequence learning because it appeared to be sensitive to only immediate anticipatory looks (objects directly involved in the next step of the sequence).

We hypothesized that the DMS task would habituate the monkeys to the stimuli and reduce the initial disproportionately high number of fixations made. A repeated measures two-way ANOVA also showed that there was no significant effect of DMS habituation prior to the tests on the total number of fixations made across trials. The similarities in findings from Experiment 1 and 2 also suggest that the delayed match to sample (DMS) task may not have an effect on the sequence learning. The comparisons from Experiment 2 had lower significance than Experiment 1, but still represented the same relationships. These findings suggest that the pre-trial habituation of the monkeys to the stimuli has no effect on their initial interest in the stimuli once presented in the sequence. However, the the small sample size necessitates that these negative results be interpreted carefully.

In addition to these limitations there are some other improvements that could be made on the task and method of analysis. Due to the sample size limitation, all three monkeys’ data were collated and analyzed together. For future replication on a larger scale, it may be interesting to see if separation of the trials by monkey demonstrates stronger relationships than what these data demonstrate because each monkey may have different set, minimum and maximum points in terms of their ability to learn and remember.

Another variable that was not considered in this modification of the video task is the actual time spent during each fixation. Each single fixation was recorded as one fixation whether it lasted for only a few milliseconds or for more than one entire step. Similarly, each anticipatory saccade was recorded as one anticipatory look regardless of how long it occurred before the object actually moved provided it was within the defined parameters of the 1 step and 3 steps of anticipatory looks. This method of quantification of sequence learning and declarative memory would benefit from more details concerning how long monkeys spend looking at different objects and how closely do they make anticipatory looks before the movement.

These kinds of analyses would require some form of independent and automatic computerized program, which would further improve the accuracy and reliability of the data. Additionally, an automated procedure would essentially “blind” the rater in the scoring of the videos. While the use of only one scorer for all trials increased the consistency of scoring across the videos, it was much more time consuming and lent itself to expectancy effects. Automating the analysis task would streamline and improve the accuracy of the task in preparation for future studies.

Most detections and investigations of anticipatory looks have been in non-inasive tasks like ones mentioned previously and there is less known about their use and the neuronal circuitry underlying them. Future studies could include neurophysiology techniques and recordings from the structures within the medial temporal lobe to determine which areas support performance on this task.

Declarative memory impairments concurrent with atrophy or damage to structures within the MTL have been associated with mild cognitive impairment as well as degenerative cognitive diseases such as Alzheimer's (e.g. Gold and Budson, 2008; Clifford et al., 1997; Nestor, Fryer & Hodges, (2005); Jobst et al., (1992)). Current tests for dementia and mild impairment including the Mini-Mental State Examination, Clock Drawing Test (CDT), Mattis Dementia Rating Scale, and Informant Questionnaire on Cognitive Decline in the Elderly (IQCODE) have been deemed useful in assessing the degree of cognitive impairment (Petersen et al., 2001). However, none of these tests assess temporal memory or temporal learning specifically. This video task could allow for a unique approach to quantifying declarative memory.

The memory and learning of temporal sequences will continue to be of interest in the study of the brain as more is revealed about the underlying neurological mechanisms. Our results indicate that non-invasive techniques such as eye-tracking can contribute to the quantification of memory. With further research and investigation, tests such as the video task from our study may become useful in the early detection of cognitive illnesses and abnormalities.

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Table 1: Example of a Description of a Loop from PowerPoint Movie "Natty002". Shows the breakdown of the video, general area of interests, and area of interests that are considered anticipatory in each step.

Details of Video		Areas of Interests	
Sequence Step	Video Action	General AOI	Anticipatory Look AOI
-	-	Beads Grapes Lock Tree Towel Burger	**Burger** Grapes Lock
Burger	Burger moves from bottom right corner to top middle	Beads Grapes Lock Tree Towel Burger	**Grapes** Lock
Grapes	Grapes move from middle left side in up and down zig zag to middle of screen	Beads Grapes Lock Tree Towel Burger	Lock
Lock	Lock turns clockwise and changes colors in bottom right corner	Beads Grapes Lock Tree Towel Burger	-

Figure 1: Delayed Match to Sample (DMS) task design. Task begins with a 1 sec fixation on a white crossbar in the middle of screen. The first picture shown becomes the target and the monkey is rewarded if he responds correctly when the same picture reappears.

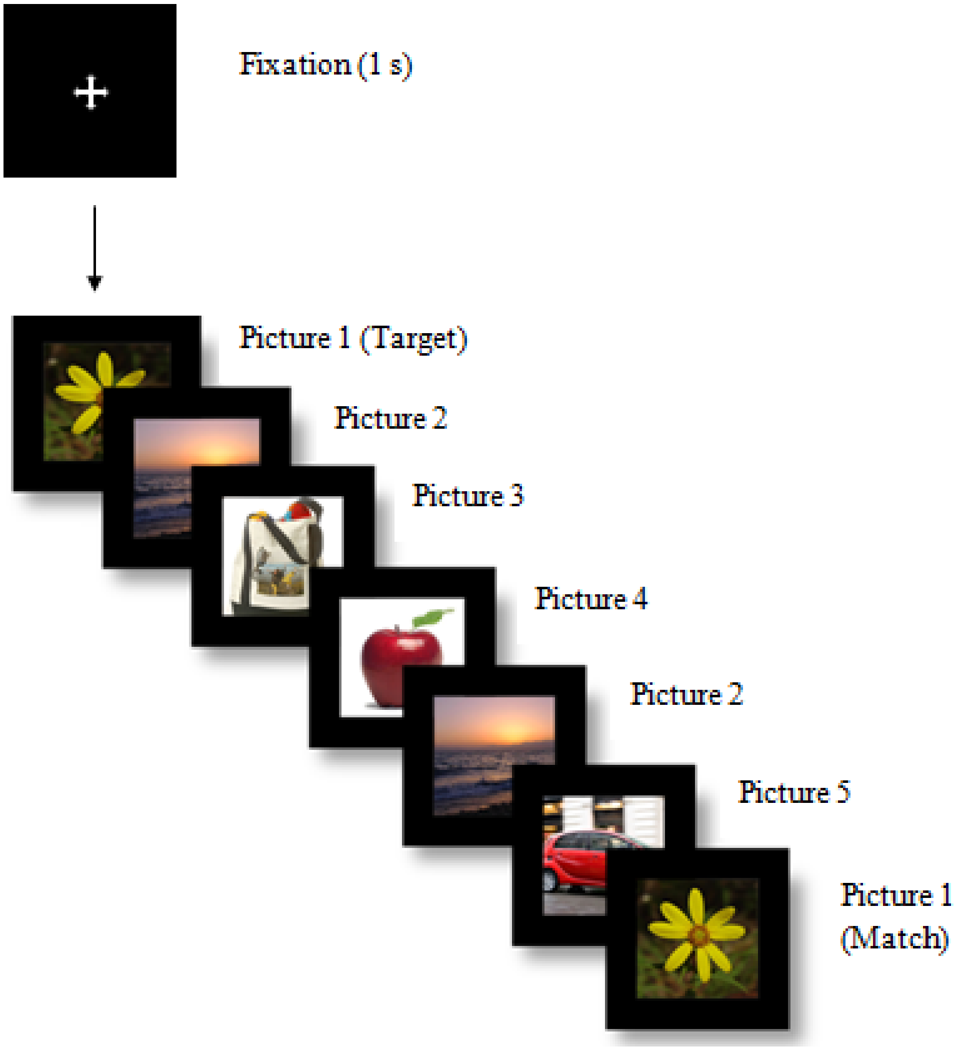

Figure 2: Presentation trial where peak percentage of anticipatory looks occurred for all monkeys in Experiment 1. Monkeys made their peak percentage of anticipatory looks in trial 6 (4 videos) for the 3-step analysis and trial 5 (5 videos) for the 1-step analysis.

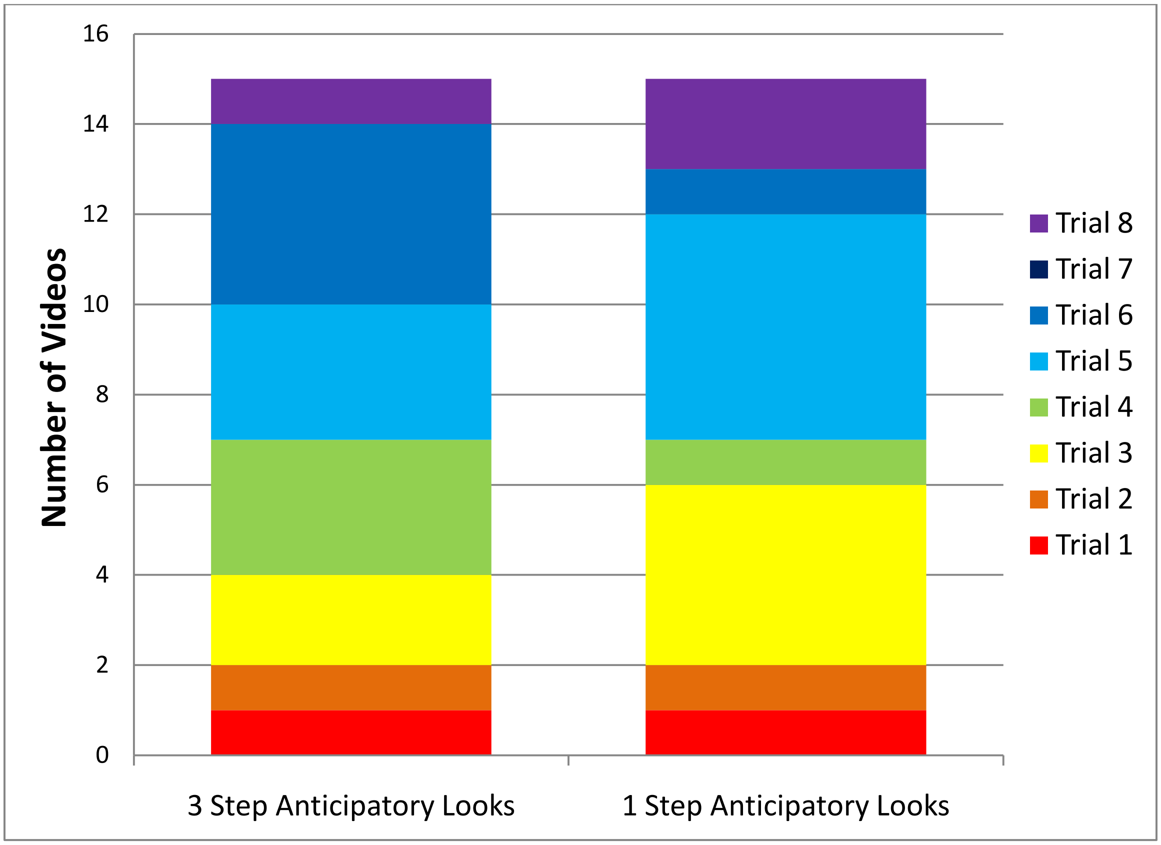

Figure 3: Average percentage of anticipatory looks (3 steps) in first vs subsequent peak trial for all monkeys in Experiment 1. Monkeys made a significantly higher percentage of anticipatory looks during the subsequent peak trial than the initial trial (p=.0016).

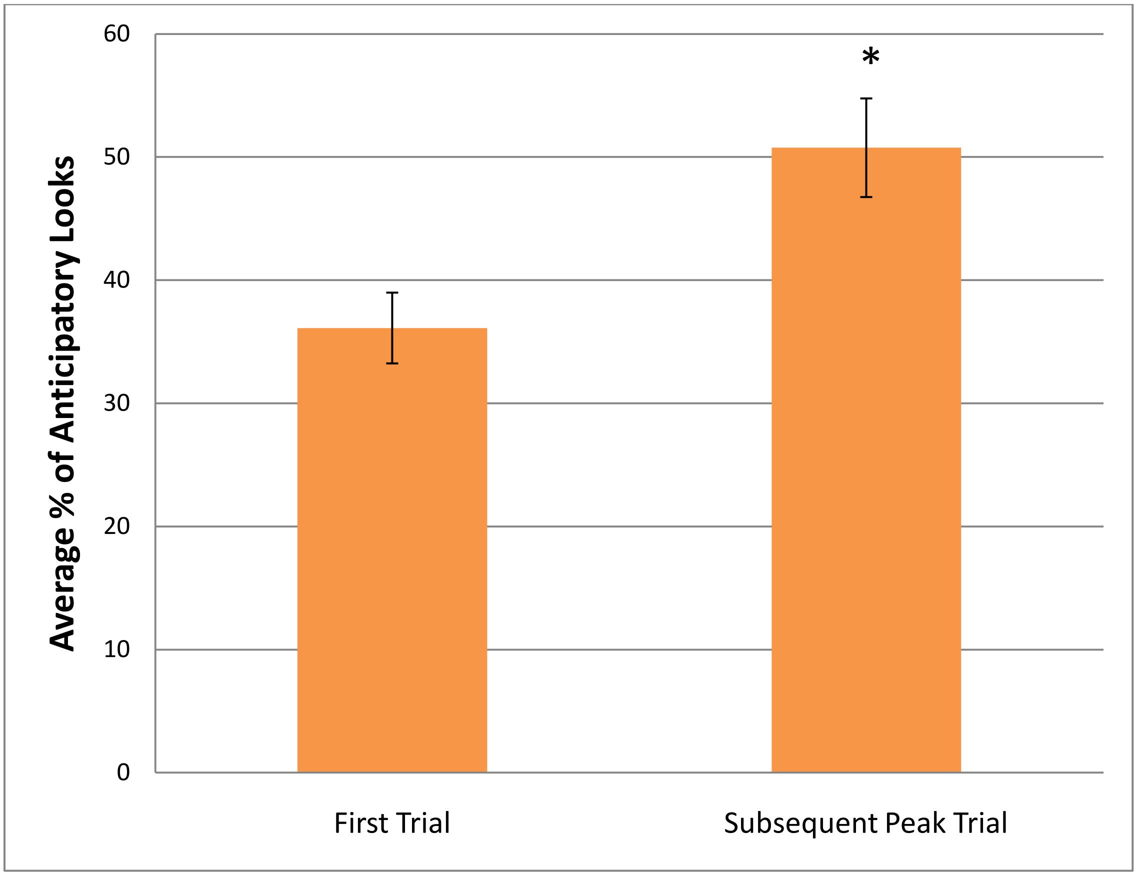

Figure 4: Average percentage of anticipatory looks (1 step) in first vs subsequent peak trial for all monkeys in Experiment 1. Monkeys made a significantly higher percentage of anticipatory looks during the subsequent peak trial than the initial trial (p<.001).

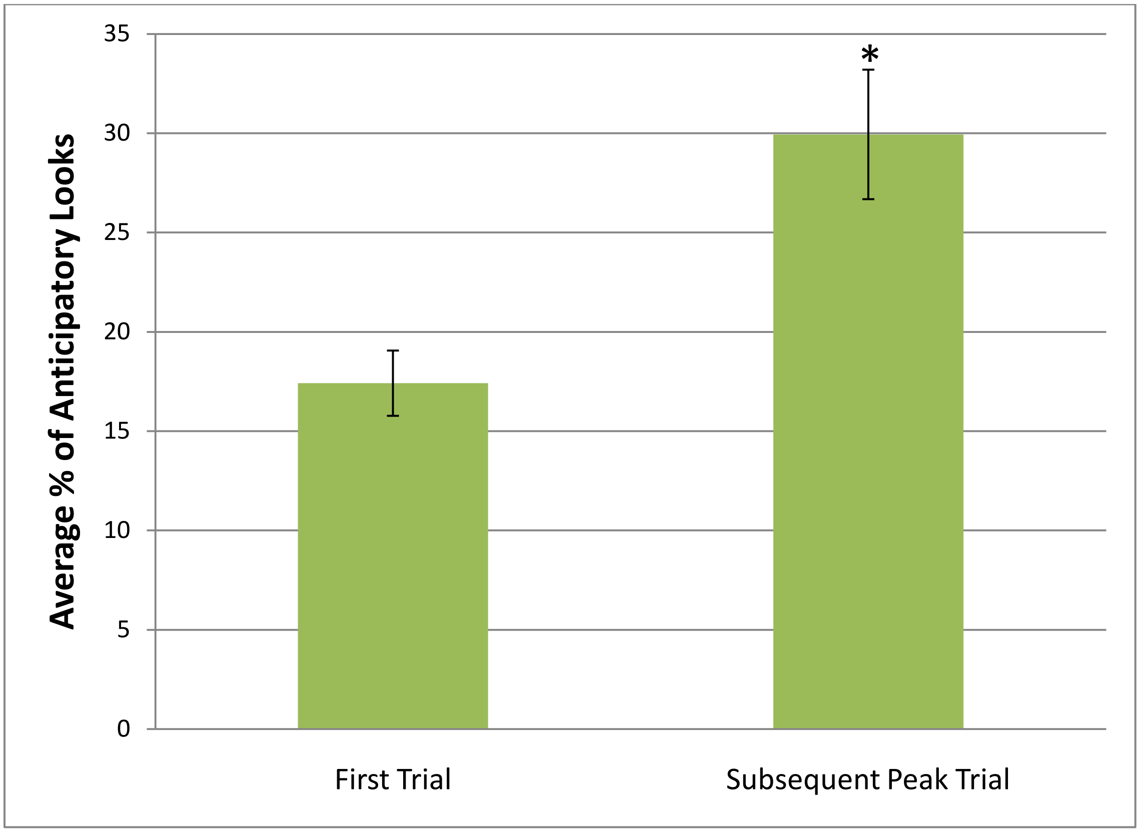

Figure 5: Average ratio of looks to moving compared to distracter objects in first vs subsequent peak trial for all monkeys in Experiment 1. Monkeys had a significantly higher ratio of looks to moving compared to distracter objects during the subsequent peak trial than the initial trial (p=.0147).

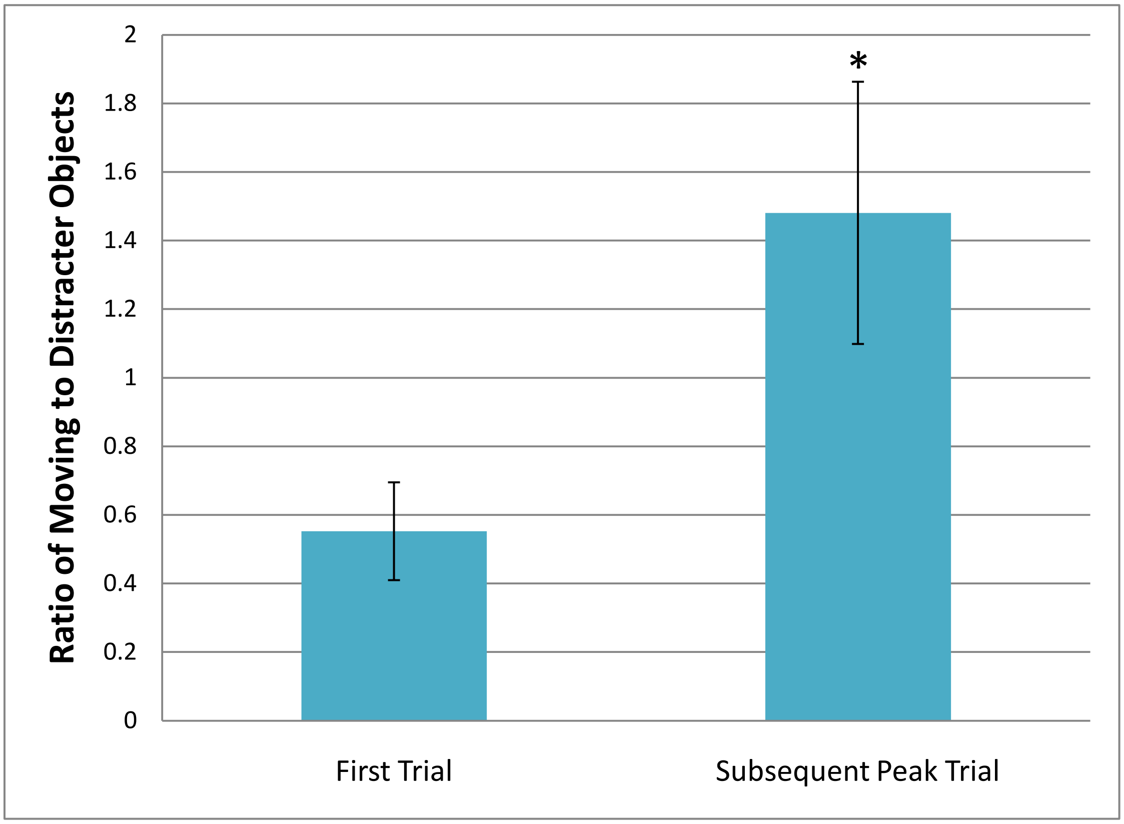

Figure 6: Average percentage of anticipatory looks (3 steps) across trials of videos in Experiment 1. A one-way ANOVA revealed no significant effect of trial (*p*>.10).

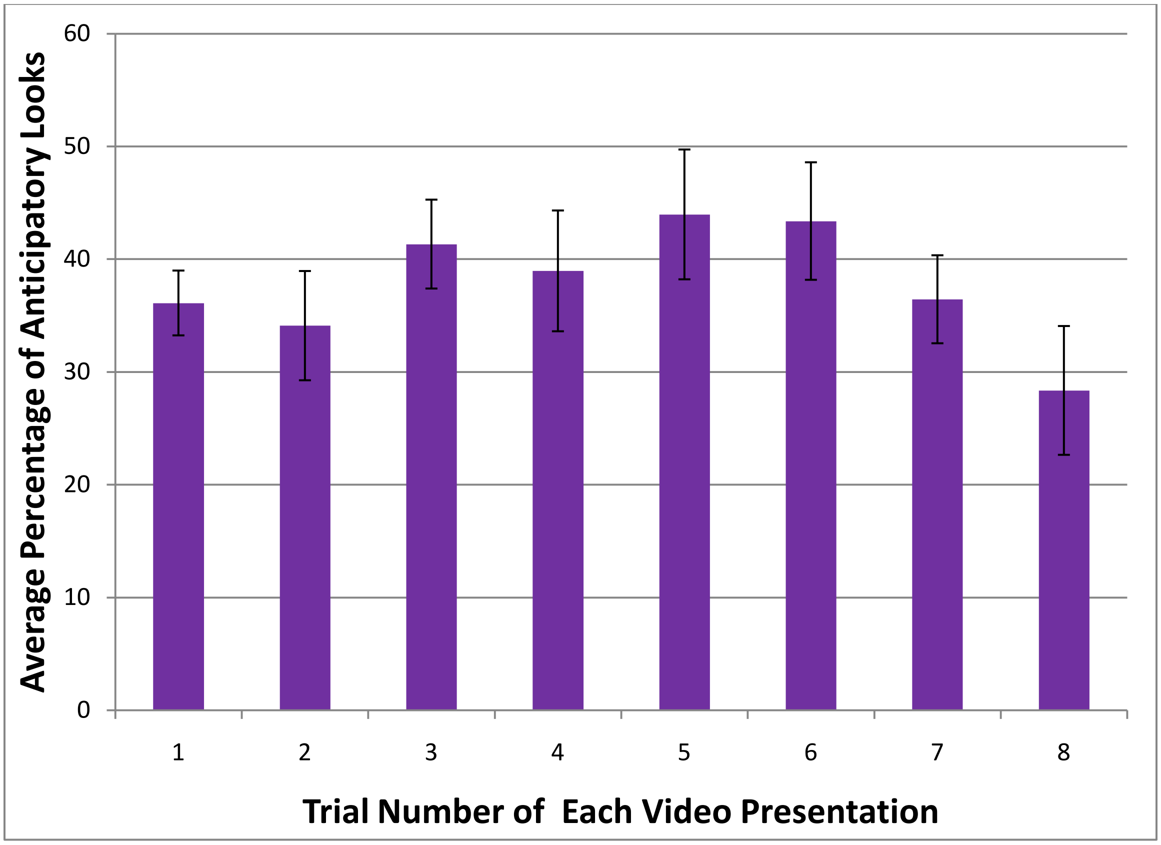

Figure 7: Average percentage of anticipatory looks (one step) across trials of videos in Experiment 1. A one-way ANOVA revealed a significant effect of trial (*p* <.05). Post-hoc *t*-tests revealed that the percentage of anticipatory looks in trial 5 was significantly higher than all other trials excluding trial 3 (all significant *p* values <.05).

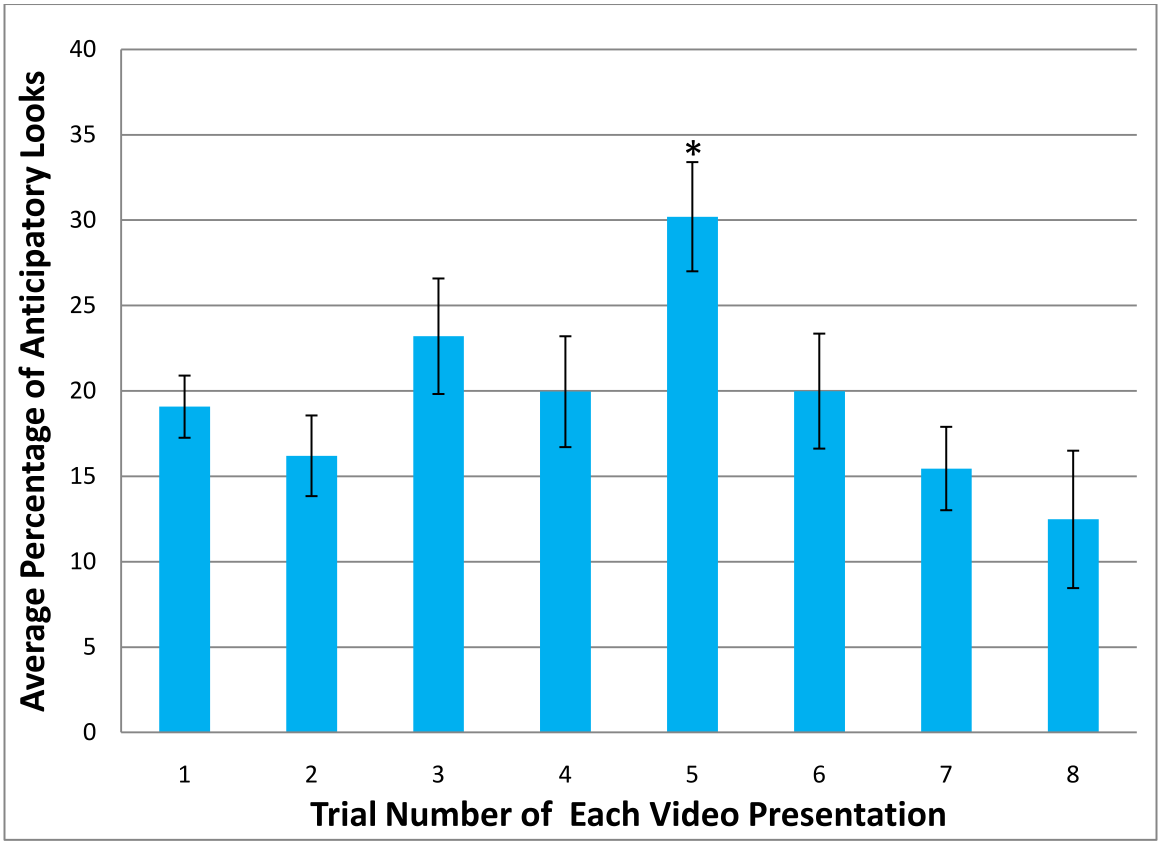

Figure 8: Presentation where peak percentage of anticipatory looks occurred for all monkeys in Experiment 2. Monkeys made their peak percentage of anticipatory looks in trial 6 (4 videos) for both the 3-step and 1-step analyses.

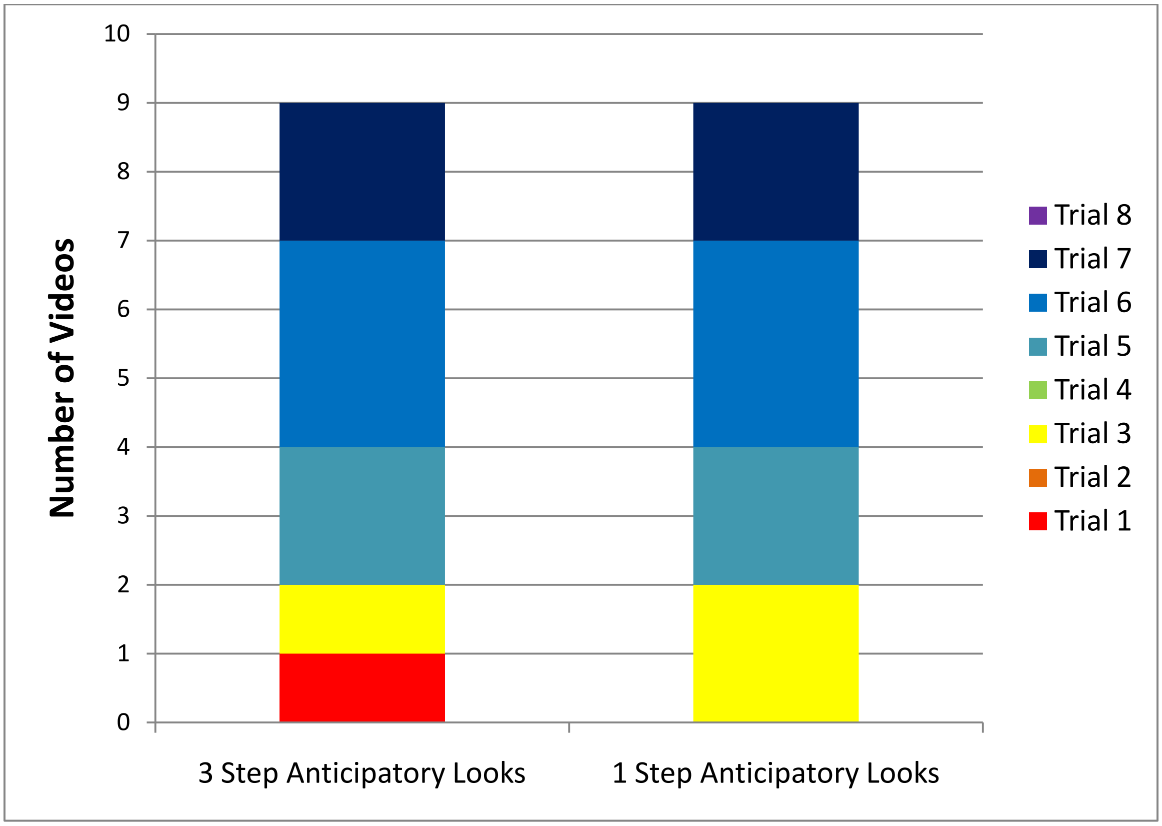

Figure 9: Average percentage of anticipatory looks (3 steps) in first vs subsequent peak Trial for all monkeys in Experiment 2. Monkeys made a significantly higher percentage of anticipatory looks during the subsequent peak trial than the initial trial (p=.016).

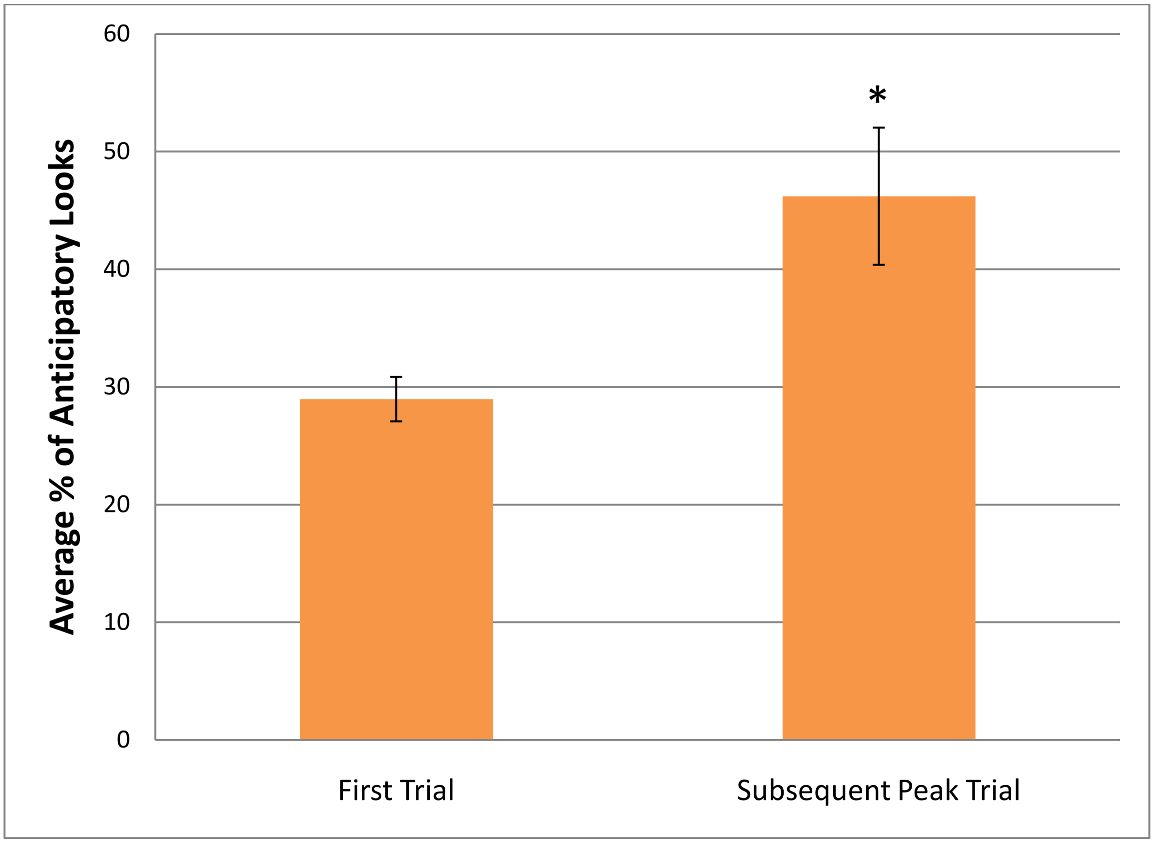

Figure 10: Average percentage of anticipatory looks (1 step) in first vs subsequent peak trial for all monkeys in Experiment 2. Monkeys made a significantly higher percentage of anticipatory looks during the subsequent peak trial than the initial trial (p=.013).

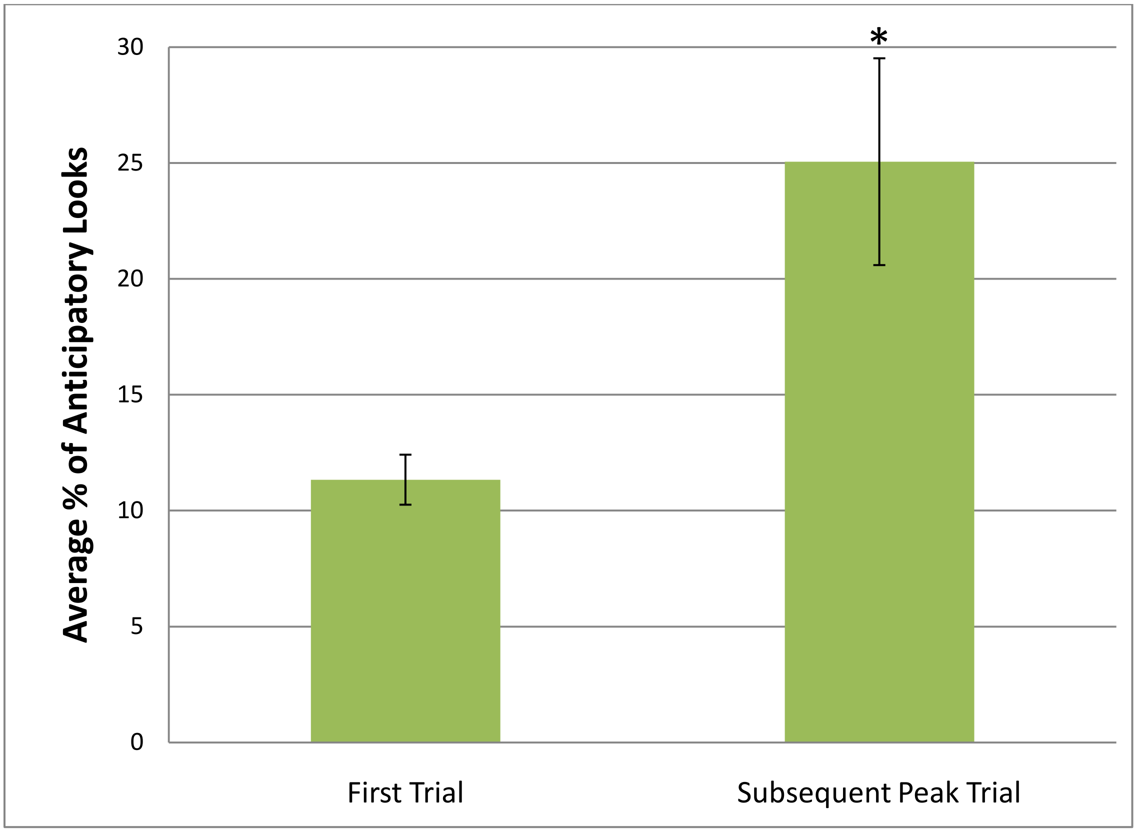

Figure 11: Average ratio of moving to distracter objects in first vs subsequent peak trial for all monkeys in Experiment 2. The difference between the ratios was slightly significant (*p* <.10).

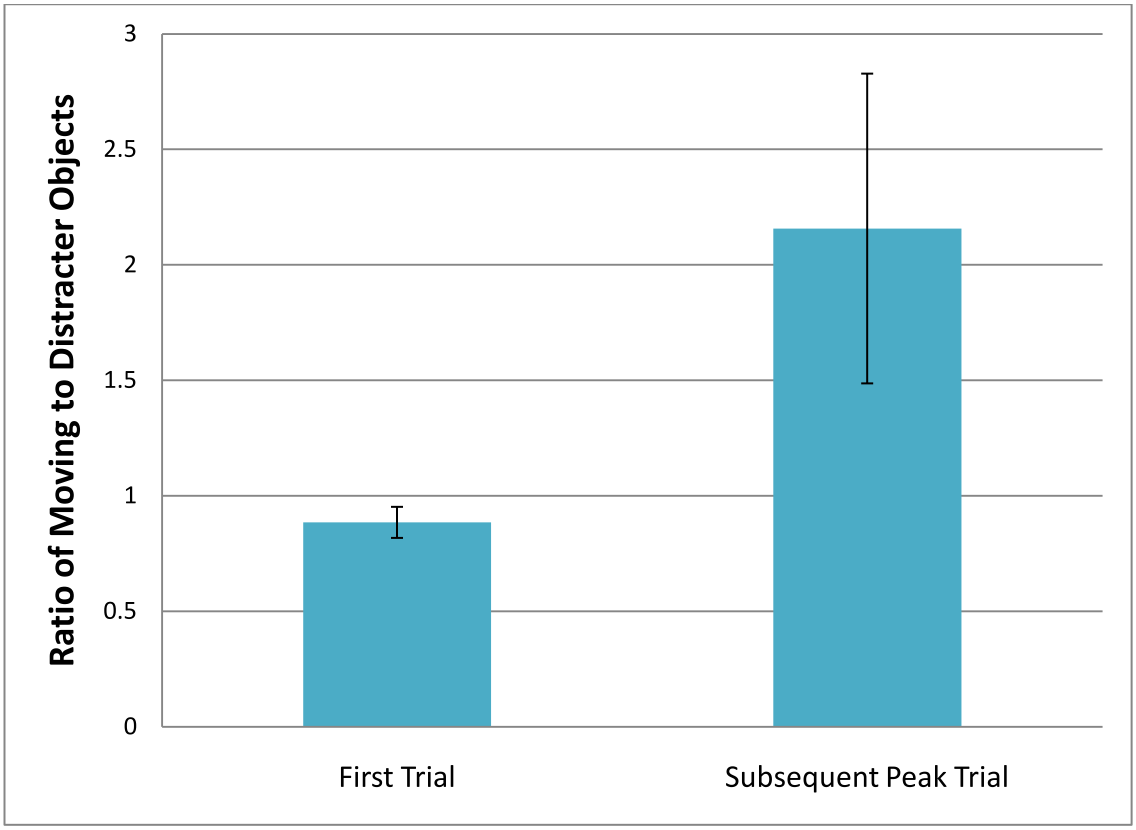

Figure 12: Average percentage of anticipatory looks (3 steps) across trials of videos in Experiment 2. A one-way ANOVA revealed no significant effect of trial (*p* >.10).

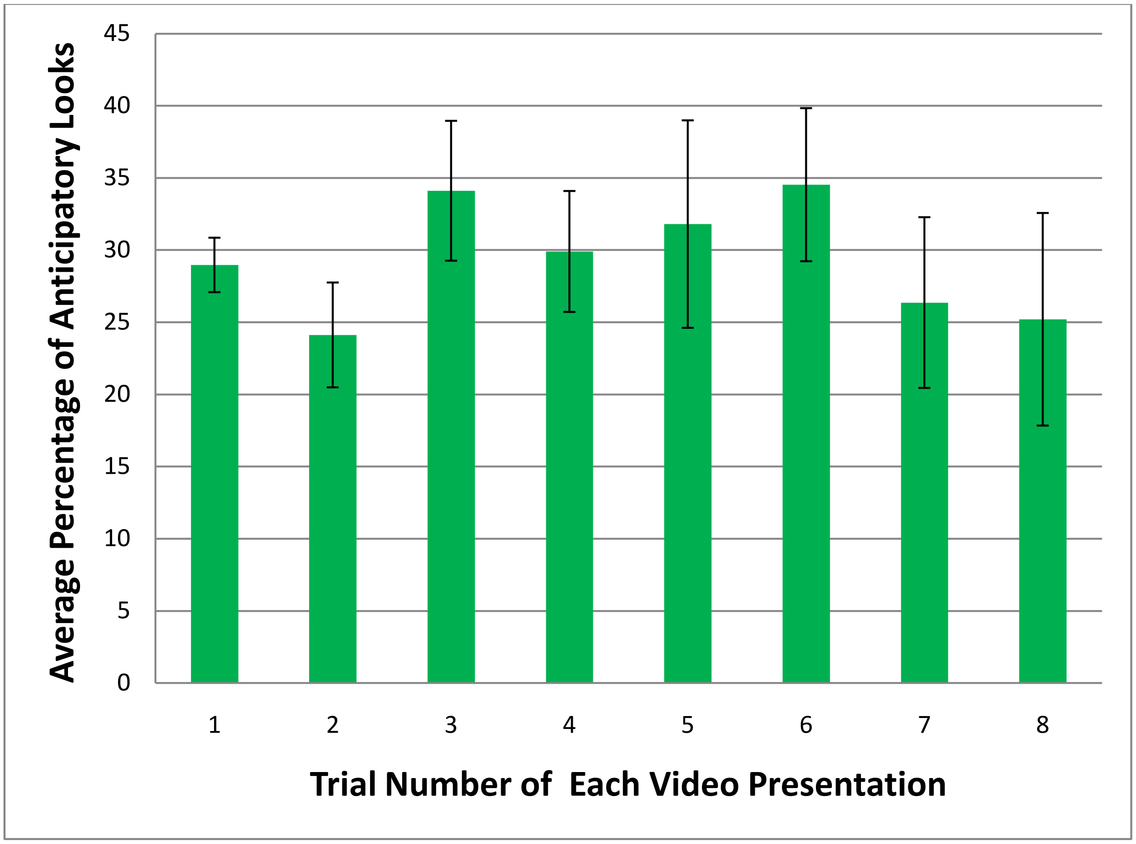

Figure 13: Average percentage of anticipatory looks (1 step) across trials of videos in Experiment 2. A one-way ANOVA revealed no significant effect of trial (*p* >.10).

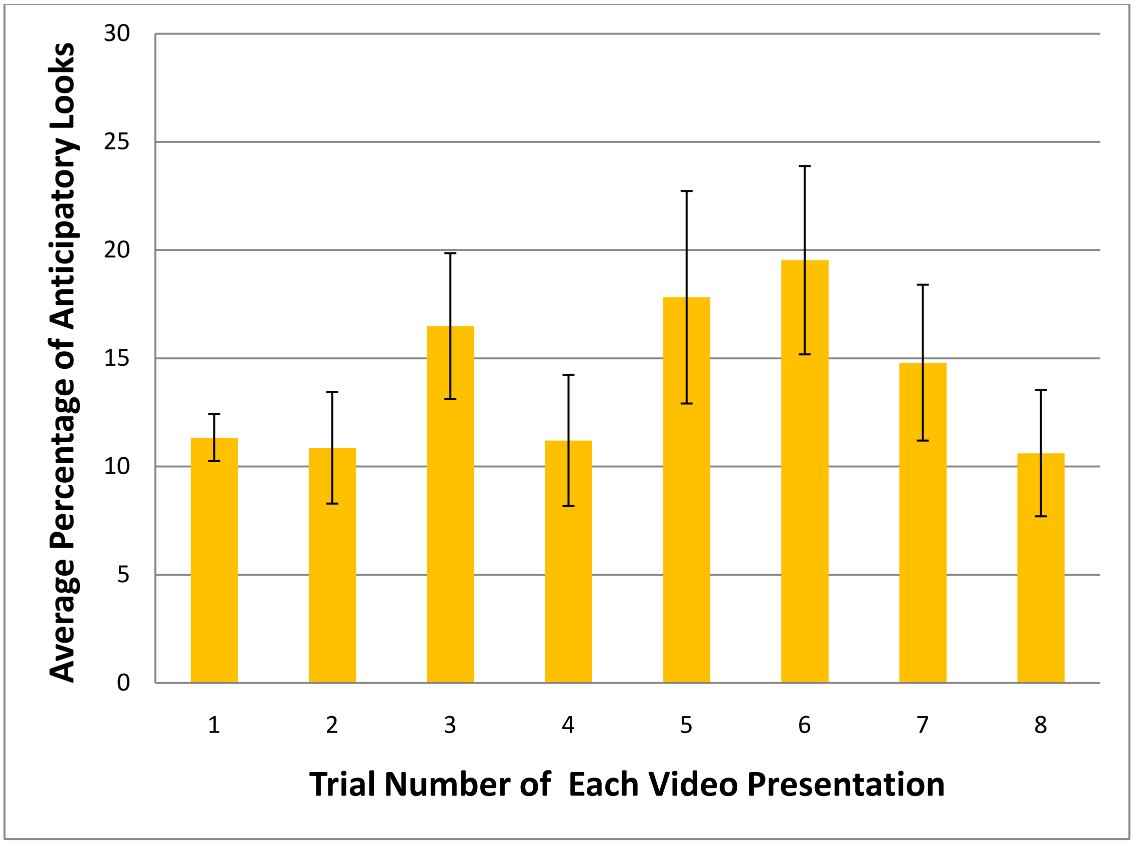

Figure 14: Average number of total fixations made across all trials of videos in Experiments 1 and 2. DMS pretreatment did not decrease the initial interest in the stimuli. Though it appears the DMS pretreated trials had a higher average of total fixations made, a repeated measures ANOVA indicated there was no significant effect of Experiment (*p*>.10).

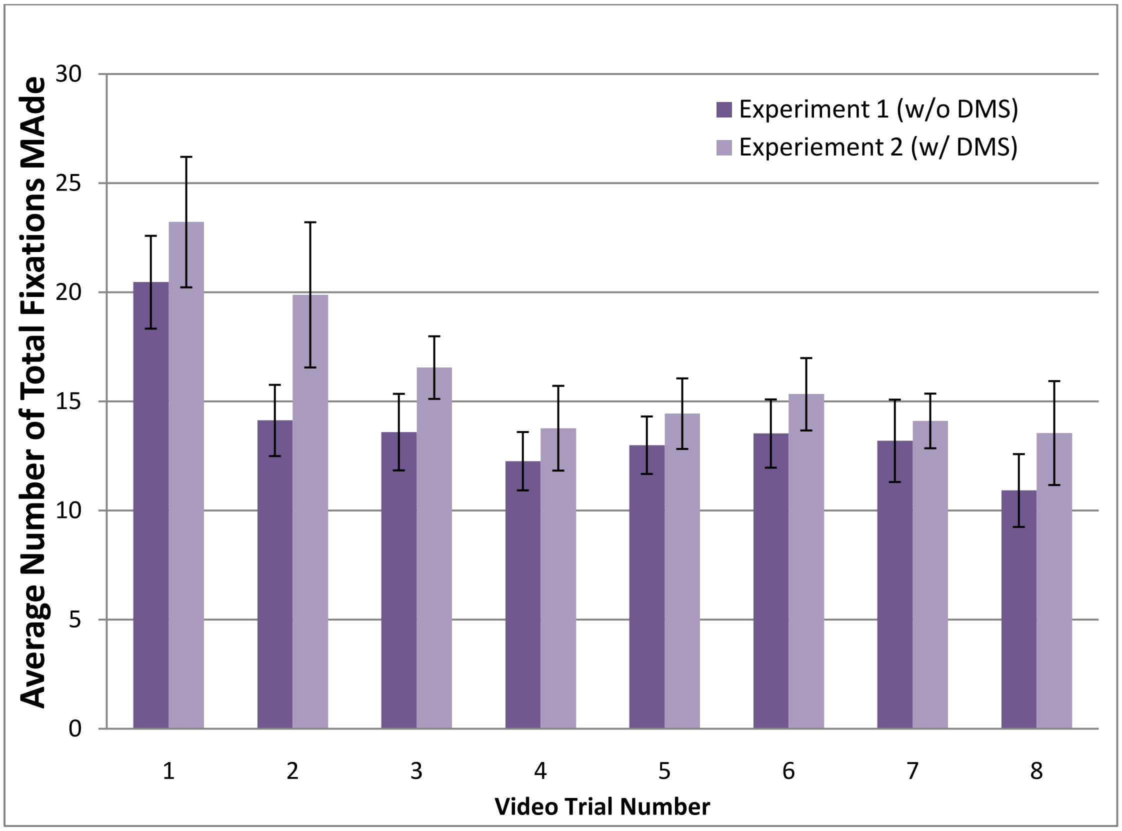

Figure 15: Screen shots of the video task showing the blue eye trace superimposed onto the videos in real time. Monkeys begin with the initial fixation on the white cross-bar (A), they are free to explore the screen with their eyes (B). With repeated viewings, the demonstrated anticipatory eye movements towards objects before they would move (C) and often continued to follow the object as it moved (D).