ABSTRACT

There is an increasing need to move to automated, computer-driven detection software to measure mouse behavior. In a prior study, we identified a method for marking and identifying multiple mice for tracking in the Reciprocal Social Interaction (RSI) task by placing a powder-based pigment (i.e. Hair Chalk) on the backs of each mouse. While this approach successfully maintained tracking using Ethovision XT software, it remains unknown whether this method interferes with species-typical behaviors. Specifically, the application method, sensation of the powder, and scent of the Hair Chalk may be variables disrupting social or anxiety-like behaviors. To test this, mice were placed through three behavioral tasks to measure scent motivation, grooming behavior and anxiety levels in response to the presence of Hair Chalk. Results indicate no differences in all behaviors measured, suggesting that this marking system is not interfering with species typical behavior.

Validation of a Novel Marking System to

Enhance Automated Behavioral Phenotyping in Mice

By

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INTRODUCTION

Social Behavior

Overview. There is a growing need to better understand social behavior and its underlying processes. This need is met with considerable challenge as the complex psychological and biological factors that influence social behaviors are difficult to tease apart in humans especially when one considers its interactions between our cognitive, emotional and motivational processes. Moreover, genetic and environmental factors can further influence these processes and either enhance or decrease overall function**.** Examples of this are seen in many disorders such as, Autism Spectrum Disorder (ASD) and Attention Deficit/Hyperactivity Disorder (ADHD), which are often associated with altered cognitive processes. Children diagnosed with ASD have reduced social communication which is hypothesized to result from deficits in social motivation and/or face processing networks. Conversely, children diagnosed with ADHD may also have difficulties with social communication but these deficits are due to hyperactivity and inattentiveness. While ASD and ADHD have some symptomatic similarity, their underlying pathologies are due to different neural processes, creating difficulties in understanding the underlying mechanisms that contribute to these behavioral differences.

Recently, the National Institute of Mental Health established the Research Domain Criteria (RDoc) with the goal to integrate levels of information ranging from biological and genetic factors to behavioral symptoms and environmental history. The aim of the RDoC is to revise how individuals are diagnosed with a mental illness under

five proposed domains; negative valence systems (fear, anxiety loss), positive valence systems (reward learning, reward valuation), cognitive systems (attention, perception, working memory, cognitive control), systems for social processes (social communication, attachment formation) and arousal/modulatory systems (arousals, sleep and wakefulness) (Cuthbert & Insel, 2013). The RDoc highlights the major underlying neural processes that are present across the span of neuropsychiatric disorders and note the complexity of accurately diagnosing human disorders. Notably, social processes were defined as a single domain whereas other common behavioral states, such as anxiety and depression, were combined under a broader domain (e.g negative valence).

Our social processing and defining the, "social brain" is difficult to understand due to the unknown interactions between our environment and genetics. Moreover, when studying and dissecting social behavior, limitations are encountered in human subject research for ethical reasons. To this end, mouse models provide ease and insight into measuring social behaviors and are the ideal surrogate to study the complexities of social behaviors found in humans. Due to their relatively short developmental period and ease of gene transfer, which has led to over 9,000 genetically manipulated lines, many manipulations have led to the development of numerous translational mouse-models with observation of behavioral and molecular differences following single gene mutations. While animal models do not provide a direct link to humans, mice afford researchers the unique ability to explore the underlying causes and mechanism of these complexities.

Genetic variability in social behavior. For several decades, mice have been the primary mammalian organisms used to study and understand genetics. The relatively short developmental period and ease of gene transfer has led to over 9,000 genetically

manipulated lines. These manipulations have led to the development of numerous translational mouse-models with observation of behavioral and molecular differences following single gene mutations.

Controlled breeding experiments have led to numerous mouse strains. These strains, analogous to dog breeds, represent genetically homogenous mouse populations that have been characterized by unique behavioral and biological phenotypes. One particularly relevant strain is the C57BL6/J (C57) mouse, which has been known to exhibit high levels of social behaviors (Moy et al., 2007). Due to its highly social tendencies, the C57 mouse is often used as a control mouse in social tasks. For example, in a social approach task, C57 mice spend significantly more time approaching and engaging with a mouse stimulus over novel objects, suggesting its desire to engage in social stimuli (Crawley, 2004; Nadler et al., 2003; Wöhr & Scattoni, 2013). Another highly social mouse, which exhibits hyperactivity in locomotor tasks, is the inbred FVB/NJ (FVB) mouse. Past research has primarily used the FVB mouse to study behaviors of learning and memory processes. However, due to the lack of proper vision in this strain and high locomotor phenotype, the FVB mice are less usefulness in social behavior and visual processing tasks (Errijgers et al., 2007; Taketo et al., 1991). Specifically, in the social approach task, increased locomotor can be a result of greater social motivation or a product of hyperactivity. That is, a hyperactive mouse may make more approaches towards a novel mouse, but may not necessarily be engaged with the mouse socially. While a hyperactive mouse may "pass" assessments of social behavior because of the high number of approaches made, the measure is confounded by the hyperactivity because it is unknown if the mouse is truly engaged with the novel mouse

more or if its measures of increased social approach are just due to elevated locomotor activity.

Conversely, the BTBR T^{+} $Itpr3^{tf}$/J (BTBR) mouse strain shows low sociability and fails tasks related to social preference and motivation by spending equal time in the social approach chambers (McFarlane, Kusek, Yang, Phoenix, Bolivar & Crawley, 2008; Moy et al., 2004; Silverman, Yang, Lord & Crawley, 2010). This low social motivation and social approach behavior characteristic of the BTBR mouse has led to its popularity in modeling behavioral traits of Autism Spectrum Disorder (ASD) due to its inability to form a preference in the social approach task and its reduced social interaction (McFarlane et al., 2008). The C57 and BTBR mice represent inbred strains with conserved genetic dispositions and while more costly, offer a defined genetic background, as compared to outbred mice.

Outbred mice provide the most realistic translation to human disorders as the diverse genetic background of the mice model the heterogeneity of genetics observed in humans. Unlike inbred mice with well controlled genetics, outbred strains complicate experimental designs as the genetic diversity introduces greater variability across conditions. Vaickus, Bouchard, Kim, Natarajan & Remick (2010) conducted a study observing the inflammatory response difference between the inbred mouse, BALB/cJ (BALB) and an outbred mouse, HSD-ICR[CD1] (ICR). Using a model of cockroach allergen (CRA)- induced inflammatory airway disease, Vaickus et al. found no significant difference between inflammatory response in BALB and ICR mice, suggesting that both inbred and outbred strains can produce similar physiological response (Vaickus et al., 2010). While current research suggests outbred mice are better

used for toxicology research and inbred mice are better for behavioral research, Vaickus et al. questioned the validity of that statement. However, while outbred may be more cost efficient, inbred mice provide a better controlled model as reactions or behaviors resulting from a manipulation of the environment or genetics would not be confounded with other variables, such as a mixed genetic. Using a model that provides a conserved genetic pool allows for concise interpretation of data to understanding reactions or manipulations in mouse models.

In addition to inbred and outbred mice, transgenic mice (knockin or knockout) are also used to model humans conditions. While inbred and outbred mice provide a strong translation to human disorders, transgenic mice also allow for a direct measure of the effects of a single gene mutation on biological and behavioral processes. Studying specific human disease can be done in vivo or ex vivo, meaning manipulating genetics in non-human vertebrate species or done through cell culture (Doyle, McGarry, Lee & Lee, 2012). An example of in vivo research can been seen in the knock out of *Shank3*. The deletion of the *Shank3* gene is hypothesized to be linked to deficits in social interactions and self-injurious repetitive grooming. In a study by Peça et al. (2011), the *Shank3* gene was targeted and mutated to identify its function in the central nervous system. Peça et al. studied the effects of the deletion of the *Shank3* gene in the transgenic knockout mice $Shank3B^{-/-}$. $Shank3B^{-/-}$ mice were observed to have pronounced skin lesions by 3-6 months, while in the social approach task, $Shank3B^{-/-}$ mice displayed a typical initiation of social interaction but a perturbed recognition of social novelty (Peça et al., 2011). Compared to wildtype mice, the $Shank3B^{-/-}$ mice displayed deficits when the *Shank3* gene was removed, such as reduced social interactions and increased self-injurious

repetitive grooming (Peça et al., 2011). The ability to knockin or knockout genes affords researchers the opportunity to study how specific genes modulate social behavior, affording researchers the ability to target and study specific genes and disorders that have been linked to mutated or abnormal genetics.

The improvements in gene knockin/knockout technology has led to the use of transgenic models to correct specific gene mutations observed in inbred strains. For example, by genetically modifying the FVB/NJ mouse, a mouse typically predisposed to poor vision, the FVB/Ant, a transgenic mouse, was bred to be free of the rd1 mutation, giving the mouse vision (Errijgers et al, 2007). This has led to a new mouse strain that possesses the behavioral characteristics of the FVB/NJ mouse but with restoration of the visual system. To date, it is unknown how this new transgenic mouse strain behaves in social behavior tasks with the presence of visual processing. Therefore, in this study, we hope to gain insight into this new strain and further understand its social behavior in addition to validating our marking system.

Environmental factors in social behavior. Aside from genetics, behavioral responses are highly sensitive to environmental factors. For example, the behavioral outcomes of offspring are dependent on changes in maternal care. Poor maternal care during the neonatal period can result in reduced social behavior, increased aggression and an increase in immobility (Haug & Pallaud, 1981; Lerch, Brandwein, Dormann, Gass & Chourbaji, 2014). Cross-fostering studies are particularly beneficial for measuring the specific role maternal care plays in mouse social behavior development. For example, when BTBR mice with low sociability are weaned from home litters and reared with B6 mice, they are observed to have an increased sociability score, underscoring the important

role the environment plays in influencing social behaviors (Babineau, Yang & Crawley, 2012). Conversely, mice raised in isolation two weeks after weaning showed deficits in the prefrontal cortex and myelination. Moreover, mice raised in an environment of isolation showed a reduced social interaction and exploration outlining the critical influence environment has on mouse development (Makinodan, Rosen, Ito & Corfas, 2012).

Both studies elegantly outline the benefits and detriments of the environment in mouse development raising the question of how manipulating the environment during the neonatal stage influence pup development. Cross-fostering experiments allow researchers to measure how maternal care specifically influences development. Cross-frosting experiments involve exchanging the pups between two dams of different strains within the first few days post-partum. This environmental manipulation of maternal experience addresses the interaction between genetics and the environment during critical windows of neurodevelopment (Lerch et al., 2014). To study the effects of cross-fostering on the emotional outcome of C57 mice, three groups were compared. The first group comprised of C57 mice raised with their biological mother, the second comprised of neonates fostered a surrogate C57 mother, and the last group consisted of C57 mice cross-fostered with an NMRI mother. Significant differences were observed between groups in the forced swim task, where mice cross-fostered with NMRI or surrogate mothers showed faster immobility times, suggesting a greater emotional deficit compared to the mice raised with their biological mother. That is, mice cross-fostered with a different mother showed a greater emotional and social distress (Lerch et al., 2014). These studies shed light on the critical influence the environment and maternal care has

on offspring. Specifically, mice raised with biological mothers expressing species-typical social behavior are more likely to learn positive emotional and behavioral strategies compares to pups raised with surrogate mothers.

The role environmental factors play in social behavior development is further supported by stimulus partner effects. Bolivar, Walters and Pheonix (2007) observed the difference between seven inbred strains in intra-strain social interactions following a 15-minute acclimation period. Mice in all intra-strains dyads were novel to the environment. BTBR dyads spent significantly less time engaged in social behavior, in comparison to the FVB strain, representing the two ends of the social spectrum. Interestingly, BTBR mice, who typically show low sociability in social dyad parings, spent significantly more time engaged in a social interaction with the FVB mouse, a highly social mouse. This study suggests that the sociability of the stimulus mice can influence levels of social interaction. Importantly, FVB mice were observed to engage in negative social behaviors, such as biting or wrestling, when continual social contacts were made and rejected by the BTBR, highlighting that not all increases in social interactions are positive. While research suggests that the BTBR showed an increase in sociability, the increase in interactions may be due to a, "sensory overload" for the BTBR mouse resulting in the negative behavior engagement (Bolivar et al., 2007). Although aggression is not a typical characterisitc of the BTBR, it has been observed that BTBR mice are more likely to exhibit aggressive behavior when forced to engage in social interactions (Powers, 2000).

Measuring Social Behavior. Multiple mouse behavior tasks have been developed to measure different discrete components of social behaviors, which include social interest/motivation. The Social Approach task, automated by Jacqueline N

Crawley, allows researchers to measure social interest in mice (Crawley, 2004). Mice are placed in a three-chambered box and measured for the amount of time interacting with a novel mouse under a cup or exploring an empty novel object (Crawley, 2004; Nadler et al.,2003) (See Figure 1). Highly social mice spend significantly more time exploring the cup with the novel mouse as compared to the novel cup, indicating a preference for social stimuli over novel objects, while mice with low social motivation or interest may spent equal time engaged in both chambers (Crawley, 2004; Nadler et al., 2003; Wöhr & Scattoni, 2013). Use of a photo beam sensor above each chamber and/or visual tracking software have resulted in rapid and unbiased tracking of the mouse's movements into and out of each chamber. That is, resulting behavioral data can be collected in real time and analyzed immediately after data collection. The Social Approach task provides researchers with the ease to measure behaviors through an automated computer analysis program that tracks a mouse's movement, however, this task limits the study of social behaviors as it is only sensitive to a discrete behavioral response from one mouse. That is, social behavior measures are limited to approach/avoidance behavior and are unable to determine the quality of the social interaction upon approach. While the social approach task is popular because of it's efficiency and high-throughput approach to data collection, it is limited in only measuring a subset of social behavior processes. The stimulus mouse is placed in a small cage with its movements confined to a small surface area. This prevents the experimental mouse from eliciting the species-typical social posturing that is fundamental to measuring complex social behaviors in animals. A more robust behavioral task requires multiple mice to be allowed free access to social exploration. However, once multiple mice are introduced into a task, automation to track mice becomes difficult.

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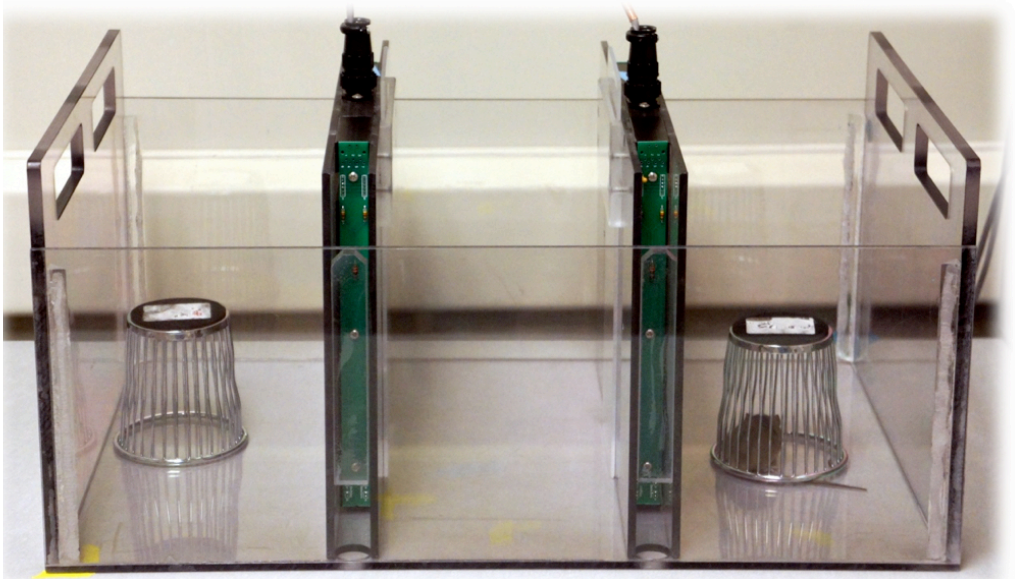

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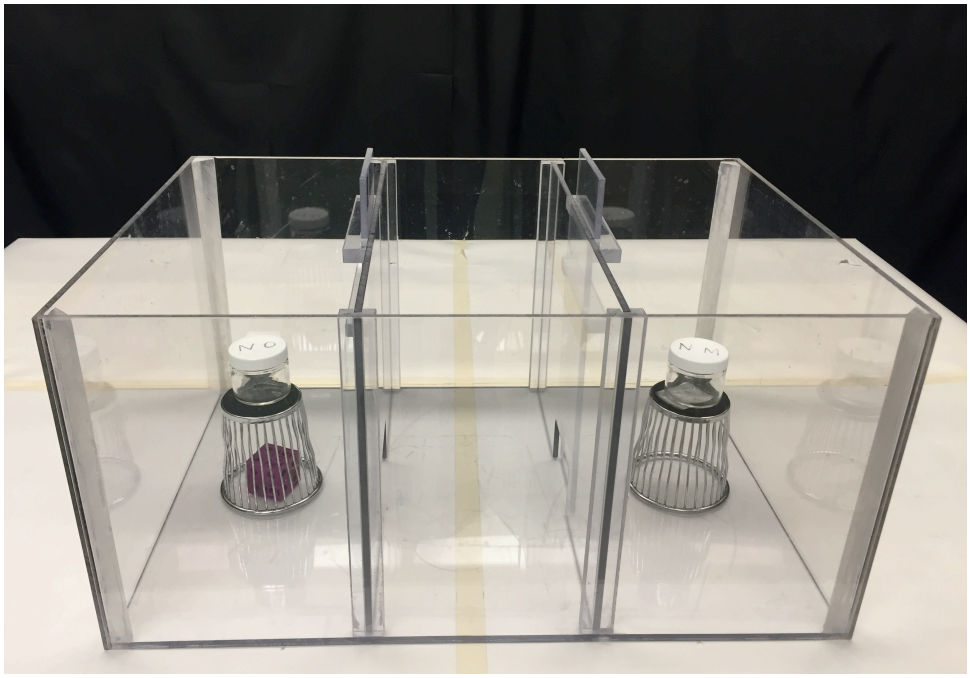
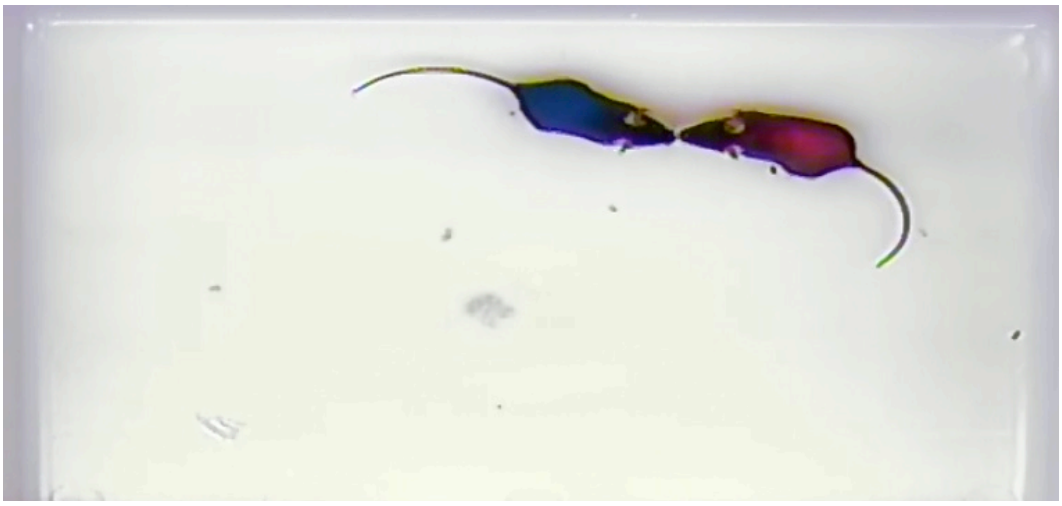


Figure 1. (A) Photo Beam Social Approach Apparatus (B) Automated Tracking Social Approach Apparatus

Mice have a complex and well-defined social ethogram, which includes reciprocal responses to the social behavior of the conspecific (partner). The social approach task is limited to only measuring the movement of a single mouse and fails to detect specific social behavior postures or reciprocal response. This limitation warrants the need for behavioral tasks that are sensitive to the full range of behaviors in the social ethogram and underscores the need to measure multiple mice in freely moving interactions. While simpler tasks are better compatible with computers, other tasks, that show interactions between multiple mice, still require a trained eye.

The Reciprocal Social Interaction (RSI) task allows researchers to study a multitude of social interactions. RSI studies the interaction between two mice by placing mice in an opaque rat box for 20 minutes to study social behaviors, such as nose-to-nose, nose-to-body, or nose-to-genital behavioral postures (See Figure 2). These interactions are then compared to non-social behaviors (Giancardo et al., 2013; Wöhr & Scattoni, 2013). Non-social behaviors are observed to be behaviors of standing alone or walking alone without interaction of another mouse (Giancardo et al., 2013). It has been observed that species typical mice will spend significantly more time engaged in social behaviors and the pattern of social interactions varies across genetic strains. For example, BTBR tend to show a reduced time engaged in social interactions, as compared to the highly social C57 mouse (McFarlane et al., 2008). While RSI allows researchers to observe a greater spectrum of behaviors, this task to date, requires human observers to meticulously score each sequential behavioral posture, resulting in errors and inaccurate assessments of social behaviors. Moreover, hand-scoring videos is not only difficult, but requires several hours of offline coding by a well-trained eye. In order to attain this skill,

A)



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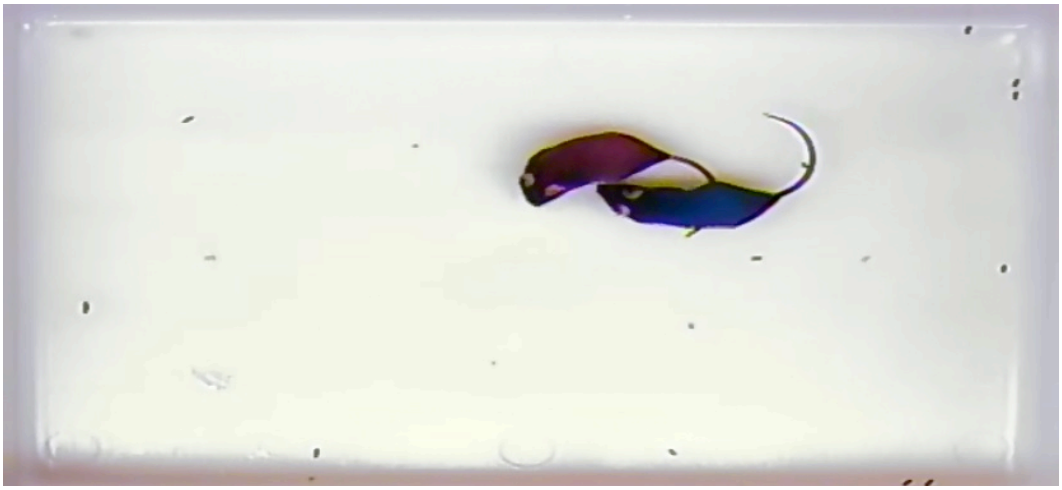

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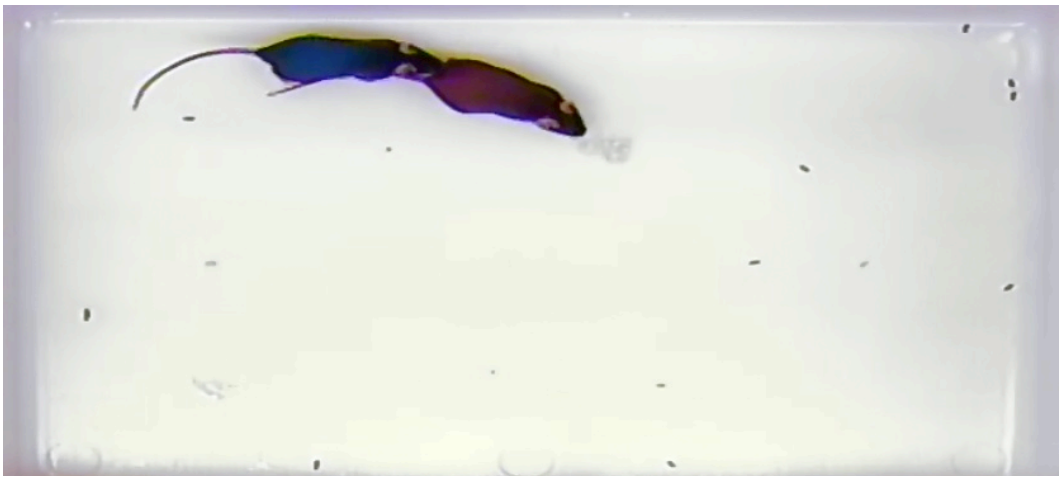


Figure 2. (A) Nose-to-Nose Interaction (B) Nose-to-Center Interaction (C) Nose-to-Tail Interactions in Reciprocal Social Interaction

observers need to be rigorously trained and matched with other trained raters to ensure high levels of inter-rater reliability. Furthermore, the human eye is often not trained well enough to make note of the robust difference in mice and their behaviors and can be subjected to various biases (Ohayon, Avni, Taylor, Perona & Roian Egnor 2013; Chaumont et al., 2012; Giancardo et al., 2013).

While both the social approach and reciprocal social interaction task have their strengths, the purpose of this study is to combine the benefits of these two behavioral tasks to optimize the measurement of mouse behavior. We propose using a computer tracking system for high-throughput results and optimizing the reciprocal social interaction task to explore multiple species-typical behaviors. Through the use of a novel marking system, we hope to successfully track two mice in RSI using an image tracking software.

Automated Tracking

There is an increasing need to move to automated tracking for its reliability and high throughput potential. Hand scoring is not only tedious and requires a trained eye, results are often hard to replicate and can sometimes be unreliable due to observer bias. While past studies have been able to successfully track black mice through the manipulation of a computer program, others have found use of marking distinct patterns on mice with bleach or making use of two mouse strains with different coat colors in the arena (Chaumont et al., 2012; Giancardo et al., 2013; Ohayon et al., 2013; Hong et al., 2015). Although effective, many struggle to track two mice of the same strain at the same time. In particular, computer programs are unable to differentiate mice of the same coat color, especially when they are in close proximity. When behaviors or huddling or

crawling over each other appear, computer programs often fail to correctly identify each individual mouse following separation. This arbitrary swapping of mice requires human intervention to correct miss-scored data and detracts from the theoretical benefits of computer automation (Chaumont et al., 2012; Giancardo et al., 2013; Ohayon et al., 2013). One method for overcoming this limitation is by individually marking each animal with unique identifying color or shape. The aim of this project is to develop and validate an effective method to track and distinguish black mice with the sole use of a computer program by incorporating a novel color marking system.

A study first conducted by Chaumont et al. (2012) made use of a computerized learning program which used an algorithm to identify mice based on geometric primitives. Geometric primitives are rectangles, circles and other polygons that are drawn over certain body parts (i.e., head, body, tail) to provide a distinct shape of the mouse (Chaumont et al., 2012). Based on this, mice were automatically tracked through movement and body contusions. While Chaumont et al. found success in first automating the tracking of two mice, a downfall of the study was the computer's inability to fully distinguish the differences between the two mice in the chamber. When mice were in close proximity (i.e., huddling) or partly overlapped (i.e., mouse climbing on top of another), geometric tags were switched or behavior analysis was cut short (Chaumont et al., 2012). As a result, researchers were required to go back and re-score the sections of error, adding significant time to data acquisition of each video. While Chaumont et al. found success in automatically tracking, one downfall of the study was the need for human rescore for missed data.

Giancarlo et al. (2013) took inspiration from Chaumont's computerized program, but made modifications to the tracking of mice. While Chaumont based his mouse tracking on geometric primitives, Giancarlo implanted radio transmitters under the skin (Giancarlo et al., 2013). While successful, imposing such an invasive procedure on mice is invasive and stressful, as it requires the mouse to be anesthetized. Despite not have any problems tracking two mice, even when in close proximity, implanting a radio transmitter in each mouse of interest is not only invasive and stressful for the mouse, it is not a plausible solution long term due to its expense and required recovery period in mice.

In search of a lower cost marking system, Ohayon et al. (2013) proceeded with a similar approach as Giancarlo et al. but distinguished individual mice by bleaching ten difference patterns onto the fur of each mouse. Mice were then observed, under video recording, for five days, to analyze how behavior evolved over the course of the trial (Ohayon et al., 2013). In addition to observing and measuring behavior, the computer program was used to measure the success in identifying the different mouse patterns. While Ohayon found success, the computer was only able to correctly recognize the identity patterns of the mouse 58% of the time correctly. Once again, human intervention was introduced to rescore and reassign identities to increase accuracy to a 97.3% success (Ohayon et al., 2013). While the social behaviors of the bleached mice observed did not show any significant irregularities, bleaching a mouse's fur is still extremely invasive as it requires the mouse to be anesthetized. Additionally, another downfall of Ohayon's study was the misidentification of mice when in close proximity, a limitation similar to Chaumont's original study (Ohayon et al., 2013; Chaumont et al., 2012).

In a more recent study, Hong et al. (2015) took inspiration from these three studies and introduced a 3D tracking and machine learning system to track mouse social behavior. Hong et al. used a black mouse and a white mouse to successfully track two mice at the same time, while manipulating a behavior learning algorithm to identify social behaviors (Hong et al., 2015). While Hong et al. found success in tracking two mice with minimal human intervention, their study is unable to track mice of the same strain and coat color (Hong et al., 2015). In an ideal study, it would be beneficial to make use of mice that share similar coat colors with different social phenotypes. An example would be making use of an asocial mouse, such as the BTBR and social mouse, such as the C57, both, which share a black coat color, to observe differences that the human eye may not be picking up with great accuracy.

Considering the increasing need for accurate, automated detection of complex social behaviors in behavioral assays, it is important to develop and validate a paradigm that allows for an objective measurement of mouse social behaviors. Specifically, while differences in social interactions have been reported across mouse strains, such as the BTBR and C57 mice, these data, to date, have been quantified subjectively, leaving to question whether these differences are present when measured through unbiased computer analyses. While BTBR mice have been known to exhibit reduced social interest, it remains unknown to what extend these data represent true (McFarlane et al., 2008; Silverman et al., 2010). That is, it remains unknown how human bias may influence past research and results as, existing tools are limited to objectively measure social behaviors in freely moving mice. Current approaches make use of invasive marking systems or unreliable tracking software. We propose using a loose, pigment

based powder which would be applied to the back of the mouse with little restraint in their home cage. While less invasive, the effects of the hair chalk on mouse behavior remains unknown.

To this end, the purpose of this study is to test a novel color marking system for tracking multiple mice and to ensure our novel marking system is not interfering with mouse typical behavior. Therefore, the goals of this project will be addressed by two aims; aim one will identify if our color marking system is interfering with species typical behaviors, such as anxiety, social interaction or grooming. Then, aim two will test whether the computer tracked behavioral measures corroborate with previously observed strain difference in mice. More specifically, aim two will test if we are able to replicate past observed behaviors in different mouse strains.

Pitfalls of a Novel Marking System

A major limitation to computer algorithms developed to track multiple animals are their inability to maintain mouse identities when the animals are in close proximity or overlapping. That is, when two mice overlap or are in close proximity (i.e., huddling), the occlusions result in an inability to differentiate each animal and, once separated, the algorithm may misidentify each mouse and begin to track inaccurately. One approach to circumvent this limitation is to place unique identifiers on the coats of each mouse that can be differentiated by the algorithm. We have identified a powder-based pigment (i.e. Hair Chalk) that can be easily applied on the backs of each mouse without physical restraint and low-stress. While this strategy maintains identification of each mouse during periods of occlusions, it poses novel variables that could adversely influence social behavior such as, increasing anxiety and novelty seeking behaviors.

Anxiety can greatly affect mouse social behavior as it may cause an increase in locomotor behavior, social anxiety, aversion and avoidance (Walf & Frye, 2007). Use of traditional identifiers including radio frequency transmitters and shaving or bleaching patterns in the fur coat, all which often require anesthesia and/or acute restraint, techniques that are known to increase arousal and anxiety-like behaviors (Giancardo et al., 2013; Ohayon et al., 2013). Such restraints and invasive procedures not only cause unnecessary stress but often result in an increased state of anxiety which disrupts social behavior processes (Walf &Frye, 2007). Therefore, it is important to ensure that the application of hair chalk we propose to use will not increasing anxiety or arousal. Compared to past application methods of identify mice, the hair chalk is applied with minimal handling and without restraint. However, it remains unknown whether the acute handling and application of the hair chalk result in elevated anxiety-like behaviors.

The Elevated Plus Maze (EPM) is a well-validated and highly reliable test of anxiety states in mice. The task takes advantage of a mouse's preference for enclosed areas, unconditional fear of heights and innate motivation to explore novel environments. Comprised of two open arms without walls and two closed arms with walls a meter off the ground, mice are given the option to explore each arm (See Figure 3). Levels of anxiety can be measure by the ratio of time spent on the open arm and closed arm (Walf &Frye, 2007). It has been observed that species-typical mice will spend significantly more time on the closed arm as compared to the open arm (Moy et al., 2007; Walf & Frye, 2007). Prior stressors, such as restraining, tail suspension or exposure to a novel environment have been observed to decrease motor activity and entries into the open arm (Walf &Frye, 2007). To ensure that the application or scent of the hair chalk is not

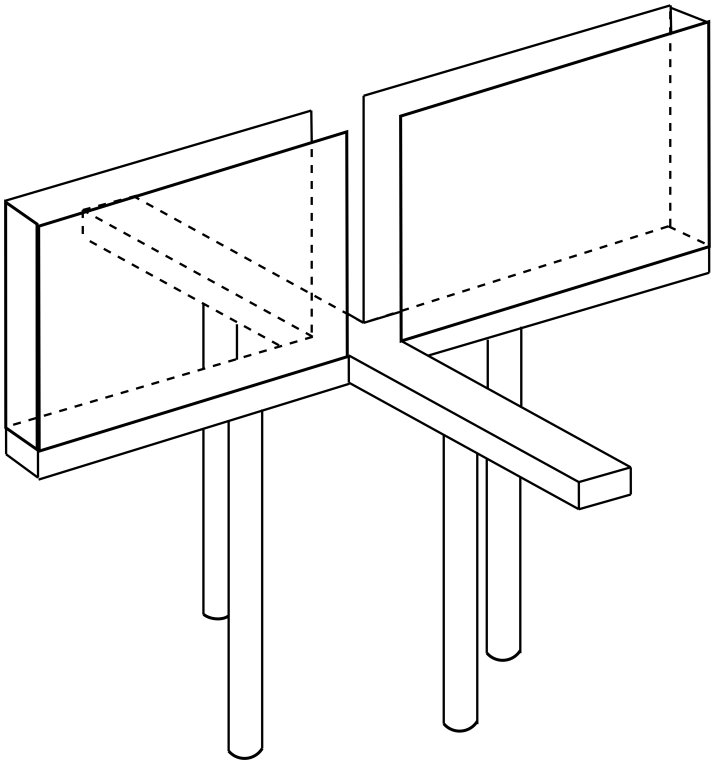


Figure 3. Elevated Plus Maze Apparatus

causing an increased level of anxiety, mice with hair chalk will be compared to mice that received the stimulation of the paintbrush. If hair chalk application increases anxiety response or arousal state, one might expect to see increased time spent on the closed arm in the elevated plus maze. Alternatively, if the hair chalk does not result in elevated arousal or anxiety-like behaviors, we would predict no change in open arm exploration or overall motor activity.

Aside from anxiety, scent cues are an important factor that must be considered when developing a novel identification system. Mice have an innate curiosity to explore novelty, with social novelty having greater valence over object novelty. This phenomenon is easily observed in the social approach task where social mice spend significantly more time engaged with a novel mouse as compared to a novel object (Crawley, 2004; Nadler et al., 2003; Wöhr & Scattoni, 2013). When introducing hair chalk to this task, one must account for odor, as odor or scent influences approach, reproduction, communication and avoidance (Arakawa, Blanchard, Arakawa, Dunlap & Blanchard, 2008). Arakawa et al. looked at scent marking in mice to observe the interactions of behavior between responsiveness to predator and conspecific odors in mice. Odors influence mouse behavior specifically in reproduction, aggression, and social avoidance. Olfactory detection of mice provides information of a mouse's sex, species and individual identity as well as social characteristics, such as reproduction and dominance (Arakawa et al., 2008). Scent influences the behavior of mice as, for example, males in a subordinate position are more likely to reduce urine marking to minimize detection and attack from dominant mice (Desjardins, Maruniak & Bronson,1973). Scent/odor is socially engaging as well as arousing. The influence of

scent raises a concern with regards to hair chalk, as it is important to ensure that the hair chalk is not increasing or decreasing motivation to approach a novel mouse.

To test whether odors from the powder-based hair chalk emits an appetitive or aversive odor, mice will be given the option of exploring a novel mouse with hair chalk on its back and a novel mouse without. It is hypothesized that mice will spend equal time with both novel mice if the odor emitted from the hair chalk is neutral compared to social cues. However, if significantly more time is spent with the mouse with hair chalk, it would indicate preference for the hair chalk odor and artificially increase social engagement as mice are being motivated by scent. Conversely, if mice spend significantly more time with the mouse without hair chalk on its back, it can be suggested that the hair chalk has an aversive characteristic. An aversive scent can be an example of a predator-prey scent leading mice to exhibit behaviors of avoidance, which if observed in RSI, could result in artificially decreased social interaction.

Lastly, mice engage in regular and stereotypical grooming behavior to maintain a healthy and clean coat. Disturbances to the coat, such as water, result in increase grooming behavior. Given that hair chalk is a fine powder that is applied to the coats of the mouse, it is important to ensure the application and sensation of the hair chalk is not interfering with grooming behavior. Self-grooming is an innate behavior for the cleaning of a mouse or rat's skin and fur that often occurs in a sequential, organized pattern. A pattern of grooming often starts at the face and moves to the body. This pattern of grooming is observed as the syntactic chain pattern (Kalueff, Stewart, Song, Berridge, Graybiel & Fentress, 2016). Typically, this behavior is observed under normal or healthy

conditions, however abnormal grooming can be seen in situations of stress or disruptions to the mouse's coat (Shiota, Narikiyo, Masuda & Aou 2015).

Shiota et al. (2015) studied the effects of a water spray-induced grooming in rats to understand its association with depressive-like behaviors. Mice were observed for grooming behavior after being sprayed with a water mister and compared to grooming behavior following, Open-field test (OFT), Elevated Plus Maze (EPM) and the Forced swimming test (FST). Grooming was defined as continuous self-grooming without interruption, specifically when grooming occurred around the nose, the head and neck or the body area including the tail and genital area. Shiota et al. found that once sprayed, rats spent significantly more time grooming their body as compared to their face. Conversely, mice placed in the Open Field Task and Elevated Plus Maze spent more time engaged in face-dominate grooming and grooming in an interrupted and disorganized pattern. These findings highlight the changes in grooming patterns elicited by stress. Typically, rats and mice groom in a cephalo-caudal (head to tail) pattern but this pattern becomes irregular during periods of arousal and distress when grooming becomes irregular, this can be a sign of anxiety and/or arousal (Shiota et al., 2015; Spruijt, Van Hooff & Gispen, 1992).

Therefore, aim one of this study will be focused on ensuring the effects of hair chalk are not significantly influencing mouse behavior. If no significance is found, this loose pigment can be further used to automate behavior tasks that provide optimal insight to mouse behavior. Moreover, the hair chalk will aid in automatically tracking mouse behavior and removing human bias that may have influenced previous data.

Method

Mice:

Adult C57BL6/J (C57), FVB/Ant (FVB) and BTBR T^{+} *Itpr3*^{tf}/J (BTBR) mice were used for this study. Mice were be bred at Mount Holyoke College and kept on a 12h-12h light-dark cycle (lights on from 0700 hr to 1900hr). Mice ranged from 8-10 weeks of age. Food and water were available *ad libitum* and experiments ran between the hours of 0700 hr and 1200hr. All procedures were under the conditions and regulations of Mount Holyoke College's Institutional Animal Care and Use Committee. Additionally, animal care guidelines for ethical treatment were followed in accordance with the National Institutes of Health Office of Laboratory Animal Welfare.

Experiment 1: Determining whether the hair chalk application influences behavior

Elevated Plus-Maze. The Elevated Plus Maze (EPM) was used as the apparatus to test risk-taking/ anxiety-like behaviors. The apparatus consists of 4 arms in total (2 open arms and 2 closed arms), with open and closed arms extending perpendicular from a central platform. The EPM is made of a white opaque plexiglas with each arm extending 50x10cm. A total of 32 adult C57 mice were used for this task; 16 mice received hair chalk on their back while the other 16 received the stimulation from the paintbrush in absence of hair chalk. Mice were video recorded for five minutes while on the EPM under bright white lights. Recorded videos were analyzed using EthoVision XT to track time spent on the open arm, closed arm and central platform. Additionally, percent time spent on the open arm will be calculated by dividing the time in open by the time in open

and closed arm and the number of entries into each arm was quantified to assess locomotor differences. The maze was cleaned with 70% ethanol between each trial.

Social Approach. The Social Approach task was used to measure the mouse's preference for a mouse with hair chalk compared to a mouse without hair chalk. The Social Approach task had a sample size of 37 adult C57 mice. Under dim illumination, mice were placed in the center of a three-chamber box made of a clear polycarbonate with retractable sleeves, which allow entrance into the outer chambers. For the first ten minutes, mice habituated in the middle chamber. After, mice were given the opportunity to explore the entire three-chamber box. Following this, mice were returned to the center chamber and a mouse with hair chalk and a mouse without hair chalk were placed in each of the outer chambers. For the remaining ten minutes, the experimental mouse was video recorded and scored on the amount of time spent interacting (i.e., sniffing, circling around the mouse with hair chalk and without) with either the novel mouse without hair chalk or the novel mouse with hair chalk on its back through EthoVision XT. Between each trial, the three-chamber box was be cleaned with 70% ethanol.

Grooming. Grooming behaviors were measured to see if any significant difference in grooming persists between mice with hair chalk and without. In the grooming task, a sample size of 24 adult C57 mice were used. 12 mice received hair chalk, while the remaining 12 did not receive hair chalk or the stimulation of the paintbrush. Mice were placed in a clear Plexiglas container and given 10 minutes to acclimate. After acclimation, mice were video recorded for ten minutes. Grooming behaviors were measured for frequency and duration of paw licking, head washing, body grooming, leg licking and tail/genital grooming (Pearson et al., 2011). Videos were

hand-scored between two researchers blind to treatment conditions. Inter-reliability was verified using intra-class correlation coefficient for a .90 or higher to ensure accuracy.

Experiment 2: Automated detection of stain-specific difference in social interactions

Reciprocal Social Interaction. Reciprocal Social Interaction (RSI) was used to measure species-typical social behaviors (ie. nose-to-nose, nose-to-tail, and nose-to-center), while also measuring the total time mice spent in social behaviors. In the first experiment, a sample size of 14 was used to analyze difference between BTBR and C57 mice, while the second experiment had a sample size of 64; 51 C57 and 13 FVB/Ant. Prior to behavioral testing, mice were moved into the behavior room and allowed to acclimate for 30 minutes. Following acclimation, mice were placed in a clean, empty, opaque box with the measurements of 42.5 x 38.8cm and given an additional 15 minutes to further habituate to the behavior box. Then, the backs of experimental and stimulus mice were marked with hair chalk by applying liberal amounts (enough to cover the mouse's body) of powder using a standard paintbrush. Mice were labeled with either blue (experimental) or pink (stimulus) colors, and the dyad was returned to the behavior box and video recorded for 20 minutes under bright lighting. Frequency and duration of interaction (ie. nose-to-nose, nose-to-tail, and nose-to-center) were measured through EthoVision XT's Three-Point Module and Social interaction Module. Between each trial, the arenas were cleaned with 70% ethanol.

Ethovision XT11.5:

Behaviors were video recorded and scored through EthoVision XT11.5 software (Noldus) using a Three-Point module and Arena Tracking settings. Regions of interest were drawn over discrete areas of each video to indicate chamber (social approach) or

open or closed arms (elevated plus maze). Social Approach and RSI were analyzed for species-specific social behavior and locomotor activity. Social Approach was analyzed for the amount of time spent interacting with the mouse (hair chalk, or no hair chalk), total time and amount of entries into each chamber, as well as total time spent engaged in a sniffing behavior around the cup. Reciprocal Social Interaction made use of the Three-point Body module and a Social Interaction module to study the frequency of species-specific behaviors. A social interaction was defined as all instances when the nose point of the experimental mouse is within 2.5cm of the nose (ie. nose-to-nose) center point (ie. body sniff) or tail-point (nose-to-tail) of the stimulus mouse. Lastly, Elevated Plus Maze behavior was calculated as a percentage of open arm explorations by calculating the total time in the open arm divided by the total time spent on either the open or closed arm of the arena.

Statistics

Behavioral data will be analyzed using SPSS version 21. For both rounds of RSI task, an independent samples t-test was run to determine strain differences between C57-C57 and BTBR-BTBR dyads, as well as difference between the FVB/Ant and C57 mice. The duration and frequency of nose-tail, nose-center and nose-nose interactions were both measured as the dependent variable. For the EPM, an Independent Samples t-test was run with treatment (hair chalk or no hair chalk) as the between subjects variable and the amount of time spent on the open and closed arm as the dependent variable. For the Social Approach task a pair-samples t-test was run to determine whether there is a significant preference for the chamber with the hair chalk stimulus mouse or no hair chalk stimulus mouse within each experimental animal. Time in each chamber and the

time spent sniffing/exploring the novel mouse with hair chalk or without hair chalk will be the dependent variable. For grooming, an independent samples t-test was run with treatment (hair chalk or no hair chalk) as the between subjects variable and the amount of time spent grooming as the dependent variable. A Bonferroni Post Hoc was run when applicable. All statistical tests were two-tailed with the alpha set a 0.05

RESULTS

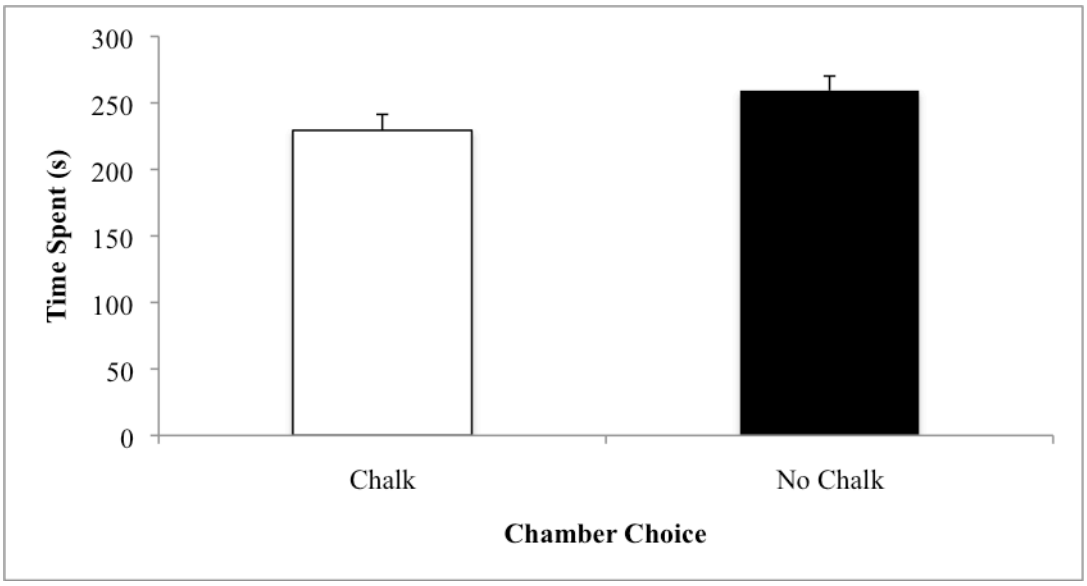
Three-Chamber Social Approach

A paired-samples t-test was run to determine if there was a significant preference between two stimulus mice with either hair chalk on their back and mice without hair chalk on their back. There was no significant difference in the total time spent in either chamber, t(23) =-1.374, p=0.183 (See Figure 4a), as well as the number of entries into either chamber, t(23) 0.540, p=0.594 (See Figure 4b). Moreover, sniff time was analyzed to further explore interaction between the experimental mouse and cups, as well as the amount of time mice spent engaged sniffing both cups in the respective chambers. No significant difference were observed between both cups, t(37) = .269, p=0.789 (See Figure 5). The lack of difference between mice with hair chalk and without suggests that social approach behavior is not altered in response to changes in odor from stimulus mice wearing hair chalk.

Elevated Plus Maze

An independent samples t-test was run to determine if the application of hair chalk alters levels of anxiety. There was no significant difference in the percentage of time spent in the open arms of the elevated plus maze between groups, t(30) =-0.074, p=0.942, indicating that the application of the hair chalk does not increased anxiety-like behavior (See Figure 6a). Moreover, there was no significant difference between the number of entries made into the open arm, t(30) = -1.367, p=0.182, as well as the closed arm, t(30) = -1.039, p=0.307 between both groups (See Figure 6b).

A)



B)

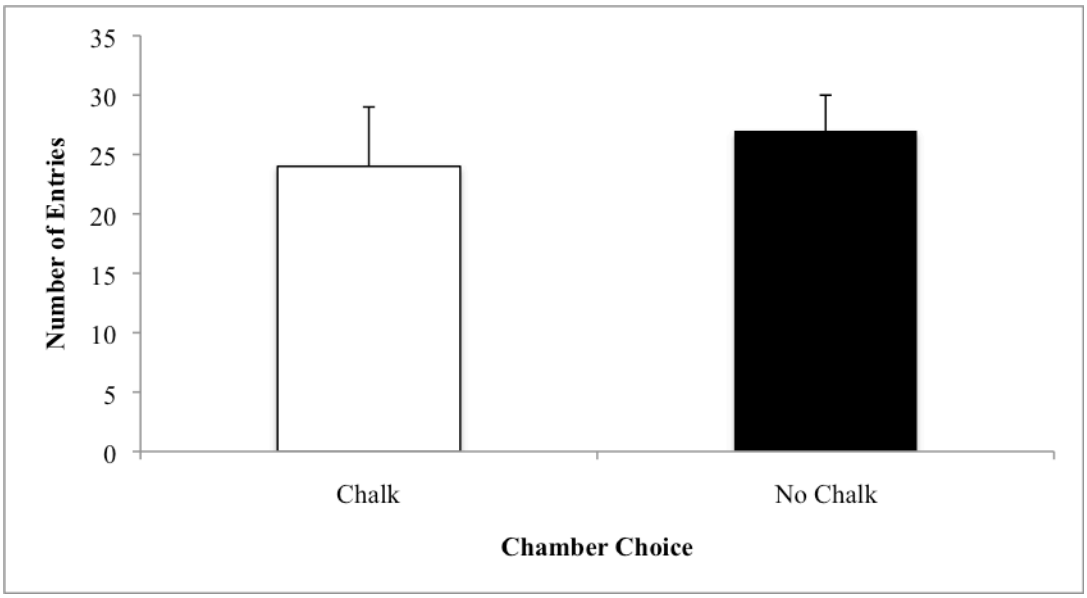


Figure 4. Chamber Preference in Social Approach
(A) Time spent between chambers containing a mouse with hair chalk and the chamber containing a mouse with no hair chalk. (B) Number of entries mice made into the chamber containing a mouse with hair chalk and mouse without hair chalk. Standard errors are represented.

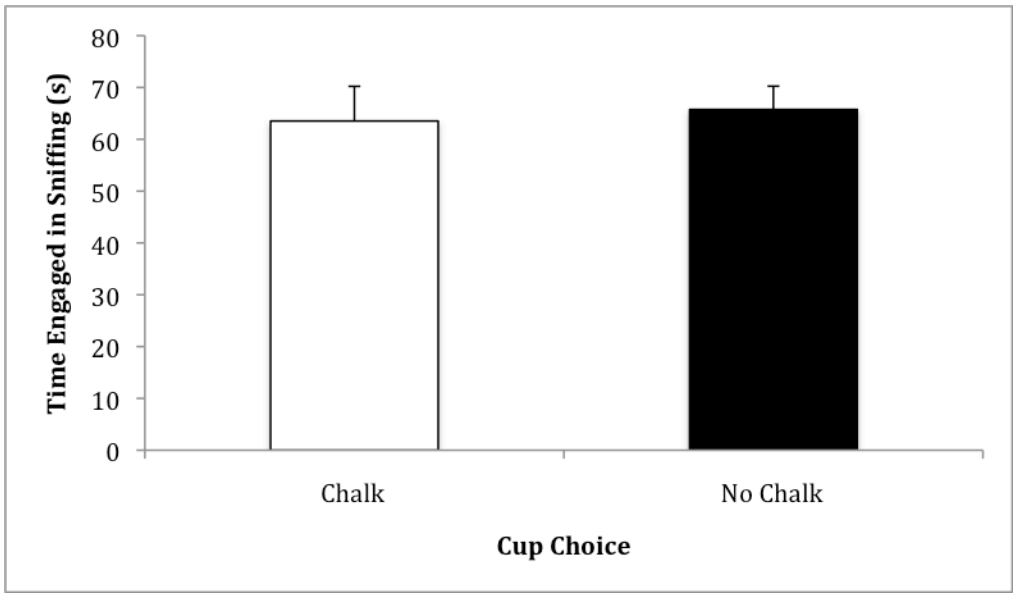
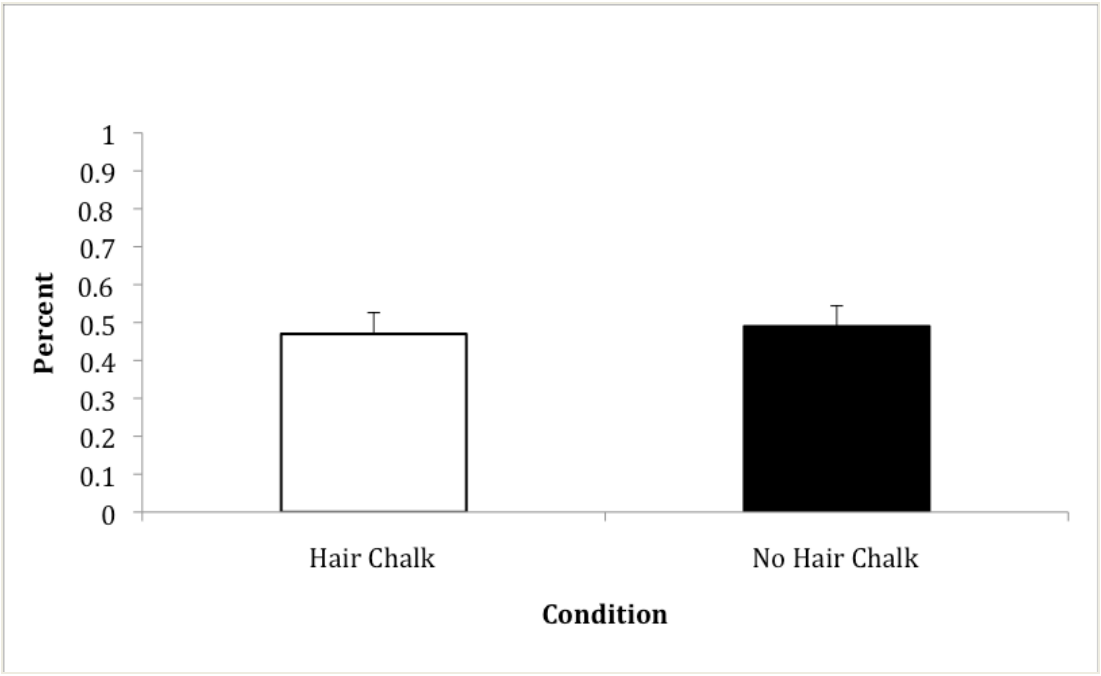


Figure 5. Time engaged in sniffing behavior for chamber cup**.** Standard errors are represented.

A)



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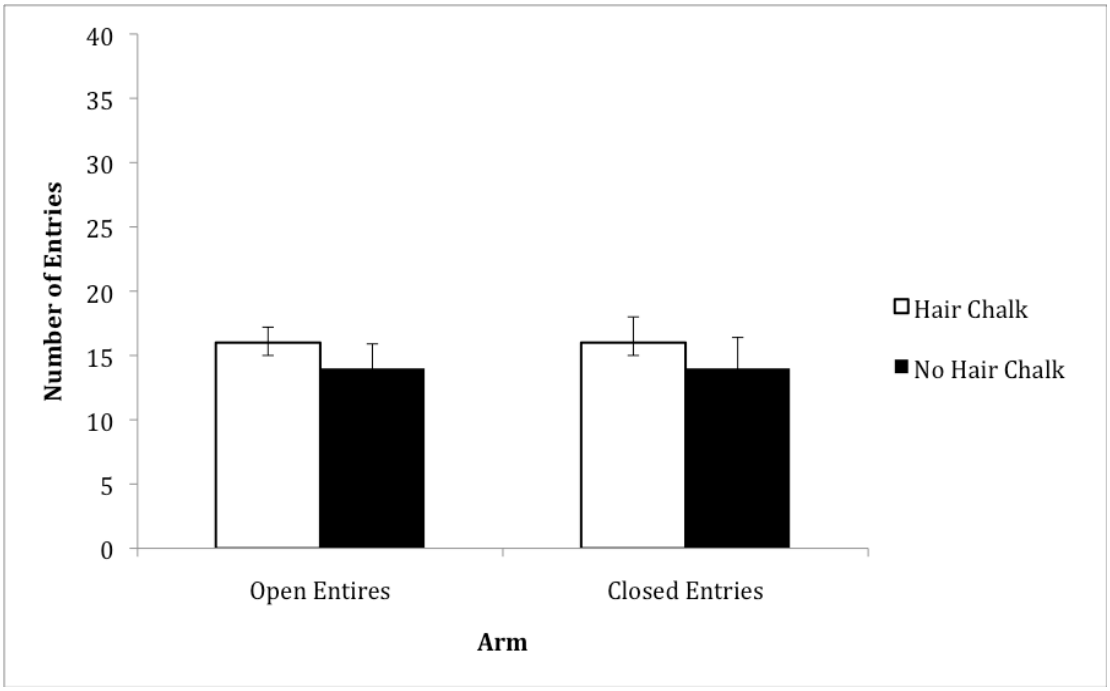


Figure 6. Anxiety Measure on the Elevated Plus Maze
(A) The percent time spent on the open arm comparing mice with hair chalk on their back and mice who soley recived the stimulation of the paintbrush on their back. (B) Number of entries made into the open and closed between mice that received chalk on their back and mice who received the stimulation of the brush. Standard errors are represented.

Grooming Behaviors

In order to determine whether the application of hair chalk to the fur of mice would result in increased grooming behavior, an independent samples t- test was run to determine if there was a significant difference in the total time spent grooming between mice with hair chalk and mice without hair chalk. Moreover, using an using intra-class correlation coefficient, inter-rater reliability was found to be .97, indicating blind raters had similar times (See Figure 7). The total time spent grooming during a 10-minute period showed no significant difference between mice with hair chalk on their back and mice who received the stimulation of the paintbrush, $t(22) = 0.210$, $p=0.825$, indicating that the presence of hair chalk does not stimulate excessive grooming behavior (See Figure 8). Together, these data suggest that, the application, scent and sensation of the hair chalk is not influencing altered behaviors in mice.

Reciprocal Social Interaction

To determine if strain differences were detected using automatic tracking, BTBR and C57 mice were used. An independent samples t-test was run to determine if there was significant difference in the number and duration of interaction differed between groups (C57-C57, BTBR-BTBR). One outlier was removed from the data set due to its data being three standard deviations away. A significant difference was observed between the pairs in total time engaged in social behavior, $t(12) = 2.496$, $p=0.030$, as well as time engaged in nose-to-nose interactions $t(12) = 3.568$, $p=0.004$. No significant differences were observed in the total time engaged in nose-to-tail interactions, $t(12)= 1.790$, $p=0.103$ and nose-to-center interactions, $t(12)=1.135$, $p=0.279$ (See Figure 9a and 9b).

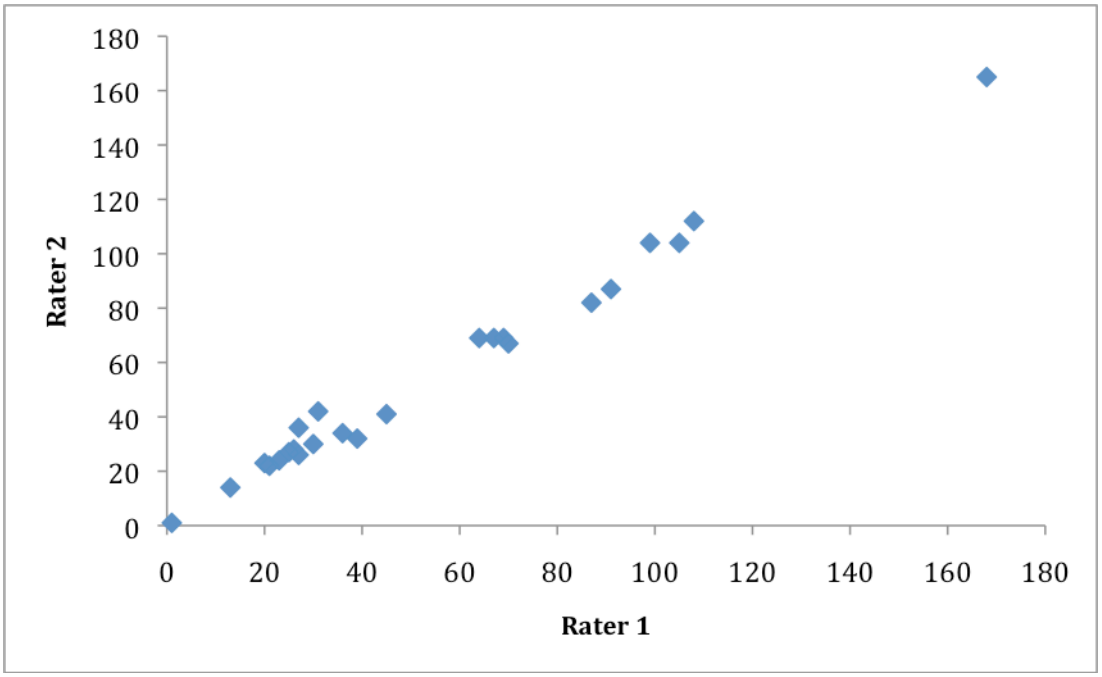


Figure 7. Interrater Relaibilty Graph with time scored and rater

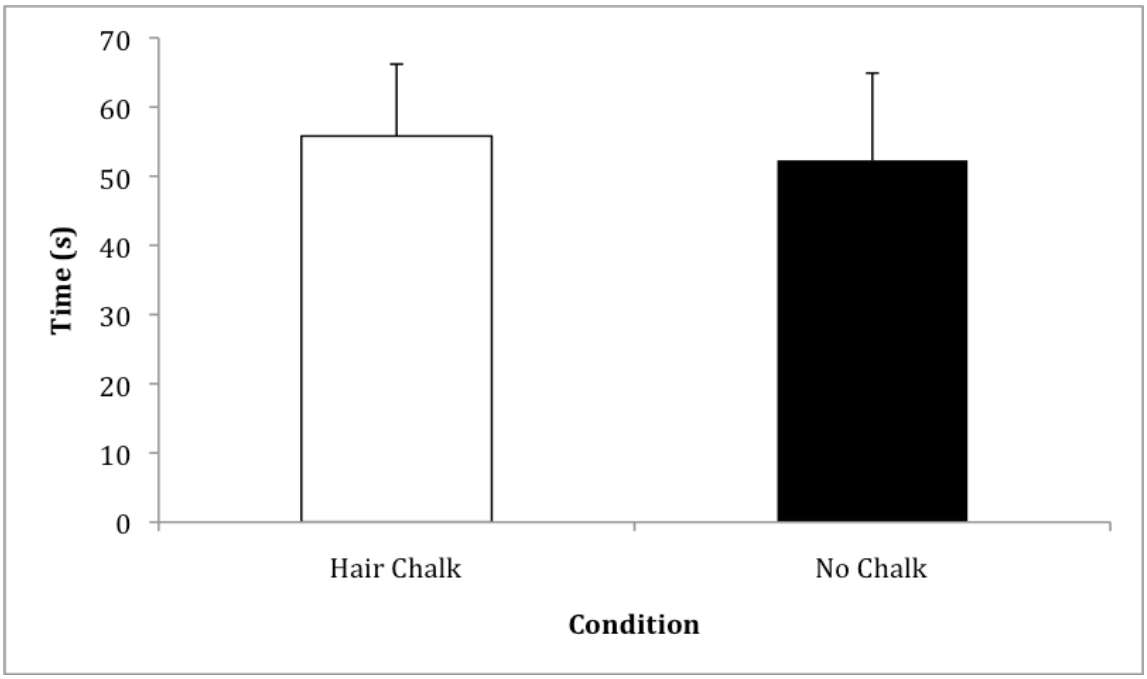
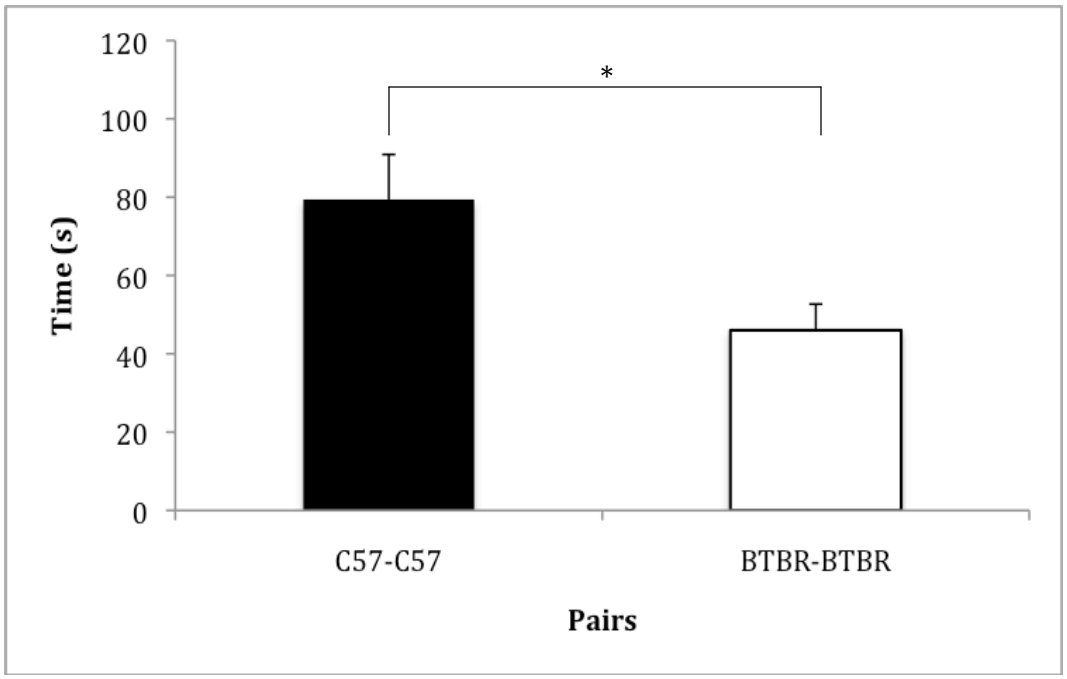


Figure 8. Grooming Behaviors between Groups
The amount of time mice spent grooming depending on their condition (hair chalk on back or no hair chalk on the back). Standard errors are represented.

A)



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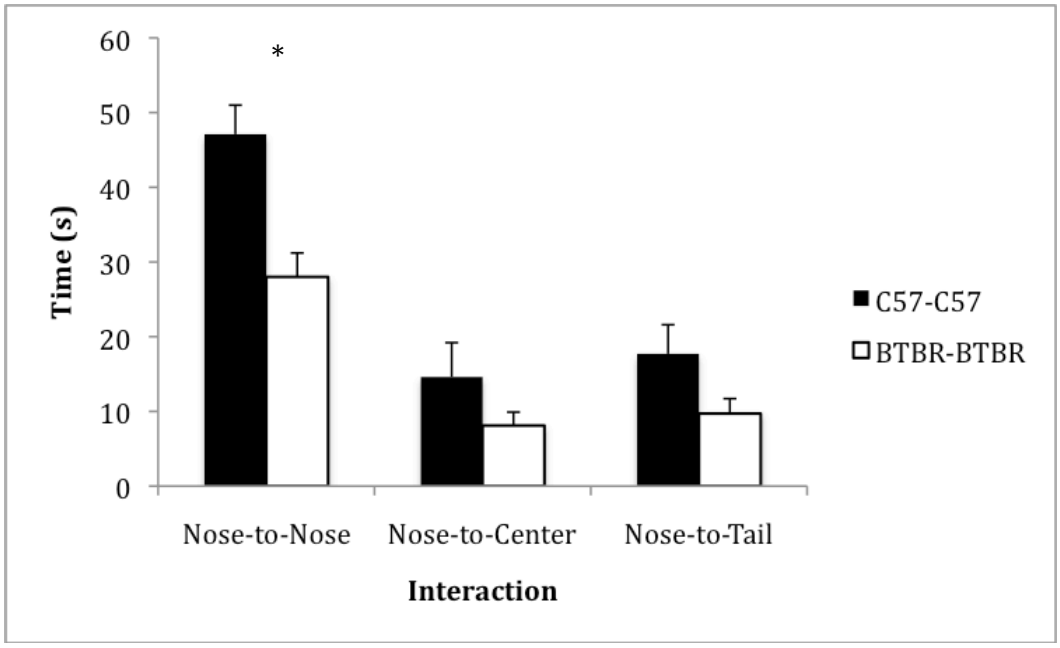


Figure 9. Reciprocal Social Interaction between C57 and BTBR Pairs
(A) Total time engaged in social behavior between C57 and BTBR mice in the Reciprocal Social Interaction Task (B) Total time of Nose-to-Nose, Nose-to-Tail and Nose-to-Center interaction between C57 and BTBR mice. Standard errors are represented.

Additional RSI tasks were run to measure if differences were observed between C57 and FVB mice. Significant differences were observed between mice in the total time spent in a social interaction, $t(62) = 3.862$, $p=0.001$ (See Figure 10). Further exploring these interactions, a significant difference was observed between the total time mice engaged in nose-to-tail interactions, $t(62) = 5.143$, $p= 0.001$ and nose-to-center interactions (See figure 11A). Moreover, there was a significant difference in the number of nose-to-tail interactions $t(62) = 3.265$, $p=0.001$ (See Figure 11B). No significant difference were observed between mice in the total distance traveled through out the task, $t(62)=0.564$, $p=0.575$ (See Figure 12).

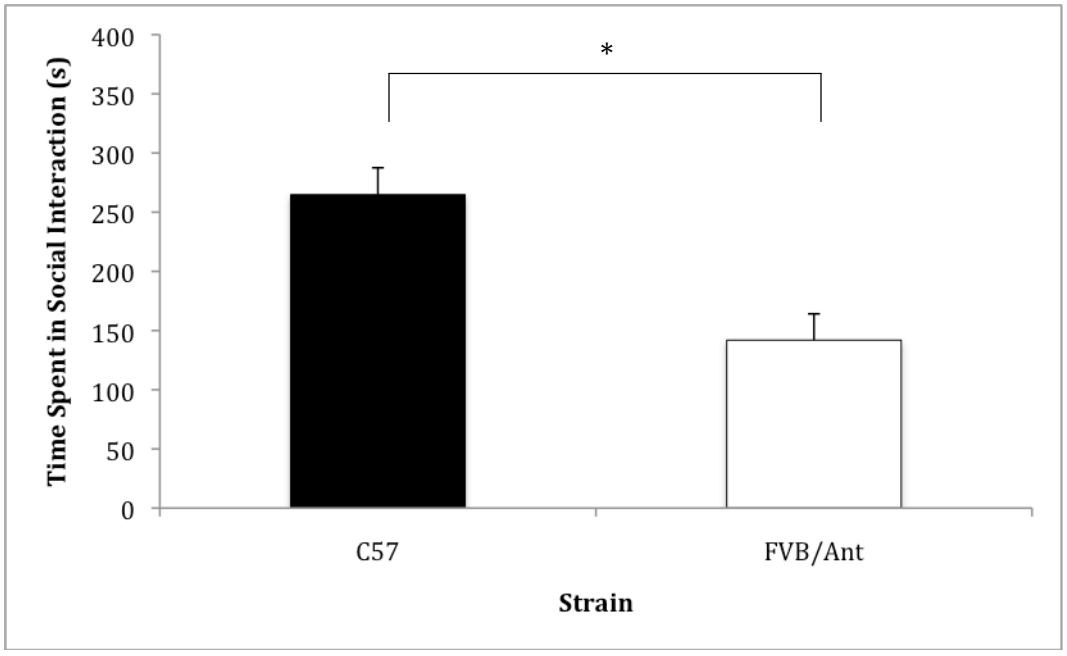
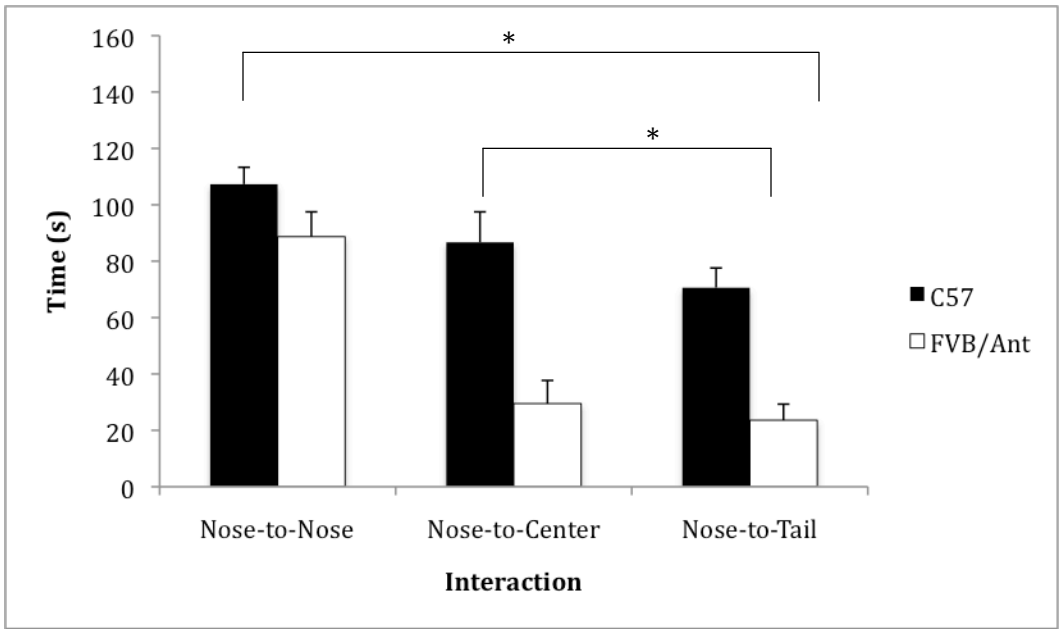


Figure 10. Total time FVB/Ant and C57 engaged in social interactions. Standard errors are represented.

A)



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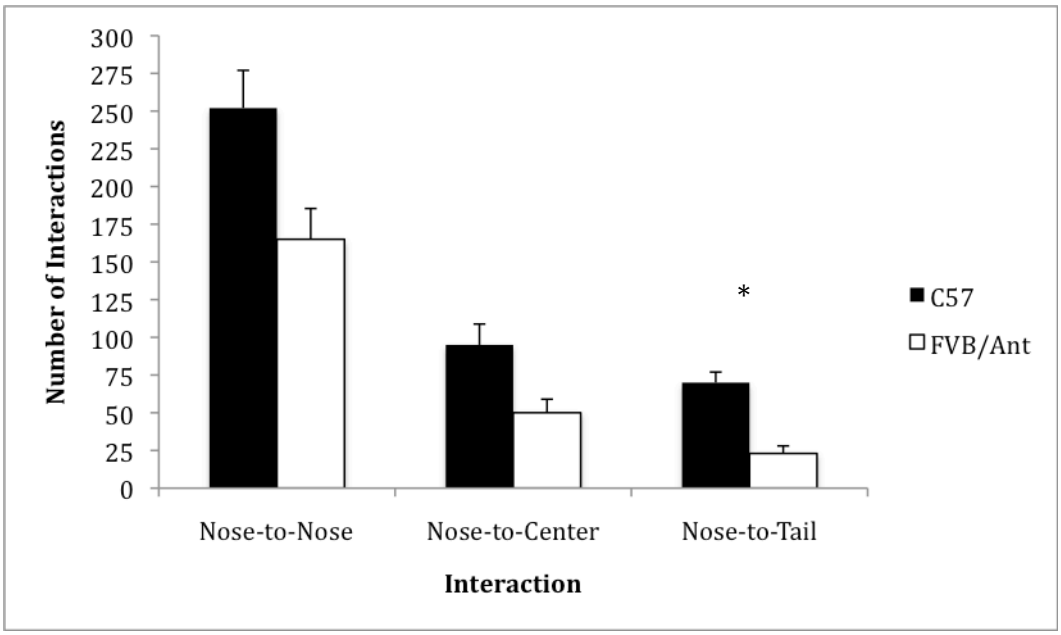

Figure 11. Reciprocal Social Interaction between FVB/Ant and C57 mice
(A) The amount of time FVB/Ant and C57 mice spent engaged in a Nose-to-Nose, Nose-to-Center and Nose-to-Tail Interaction. (B) The number of Nose-to-Nose, Nose-to-Center and Nose-to-Tail interaction FVB/Ant and C57 engaged in the Reciprocal Social interaction Task. Standard errors are represented.

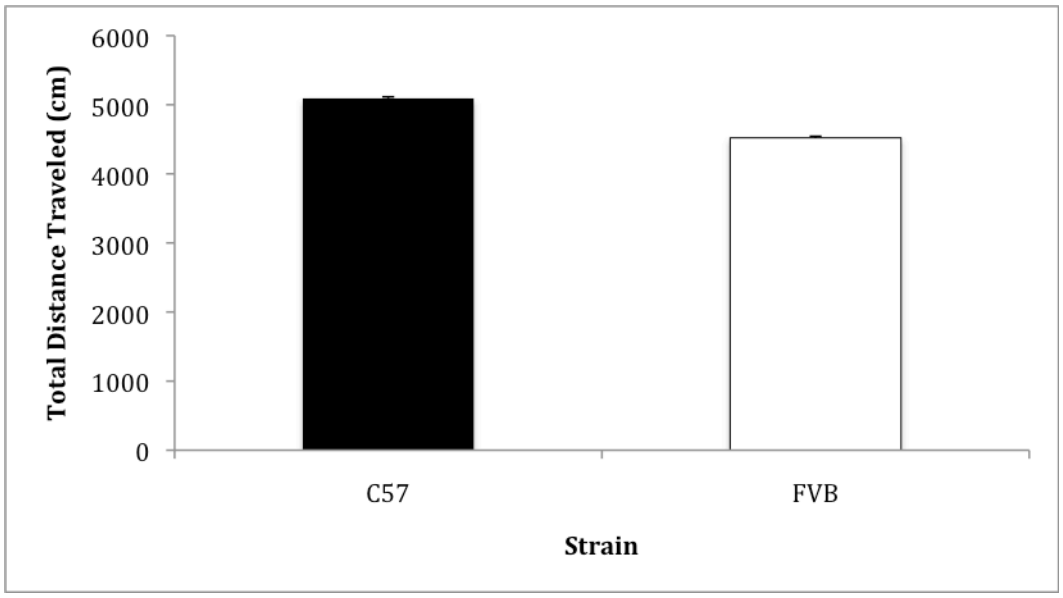

Figure 12. Total distance traveled by FVB/Ant mice and C57 mice in the Reciprocal Social Interaction Task. Standard errors are represented.

DISCUSSION

Overview

To meet the increasing need to understand the behavioral and neurobiological underpinnings of the social brain, researchers are seeking automated and high-throughput strategies for assessing social behavior in preclinical animal models. While current research has found success in tracking mice, many studies have run into similar downfalls including, misidentification of mice when in close proximity or use of invasive marking techniques (Chaumont et al., 2012; Giancardo et al., 2013; Ohayon et al., 2013). This study focused on validating a non-invasive marking system (i.e., hair chalk) to mark and automatically track social behaviors in mouse dyads with the same coat color. To achieve this, this study set two aims. Aim one focused on exploring the effects of hair chalk on mouse behavior by exploring whether the application and use of a powder-based pigment would disrupt anxiety-like and social behaviors. Then, aim two explored whether commercially available animal tracking software, EthoVision, could automatically detect strain-specific differences in social interaction as observed in past resarch. Our results support the use of the reciprocal social interaction task as a high-throughput, reliable, and valid technique for automatically measuring complex social behaviors in mice given that no significant differences in mouse behavior were detected when the hair chalk was applied. Moreover, behavioral differences were automatically detected between mice with the use of the hair chalk, allowing research moving forward to be free of human bias.

Effects of Hair Chalk on Behavioral Measures

Elevated Plus Maze. In the Elevated Plus Maze, mice were measured for changes in risk-taking/risk-avoidance behavior following the application of hair chalk. No significant differences were observed between mice who received hair chalk on their back and mice who solely received the stimulation of the paintbrush. On average mice in both conditions spent 47% of the time on the open arm and showed no significant preference for the open or closed arm. Suggesting that the application of hair chalk is not increasing anxiety or arousal in mice.

While results indicate no change in behavior, it has been observed that C57 and BTBR mice typically spend less than 20% of the time on the open arm (Moy et al., 2007). In this study, it was observed that both groups spent about 50% of time on the arm. It can be suggested that the differences observed in the elevated plus maze may be due to environmental enrichments, experimenter handling and the parameters the EPM was scored under. While results have been conflicting, it has been observed that mice housed with Tapvei stairs (wooden enrichments) have anxiolyic effects when being tested on the elevated plus maze (Õkva, Nevalainen & Pokk, 2013). That is, mice housed with enrichment have been observed to exhibit a decreased level of anxiety when tested on the EPM. Õkva et al. studied the influence of environmental enrichments in home cages of C57 and BLAB/c mice on the elevated plus maze. An environmental enrichment has been loosely defined as a housing condition that offers mice an enhanced sensory, motor and cognitive stimulation or adding biologically relevant features to a cage environment (i.e., litter mates). Moreover, including enrichments for mice to build nests or be among

a social setting (i.e., 2+ cage mates) were also characteristics defined as environmental enrichments (Toth, Kregel, Leon & Musch, 2011).

Research surrounding the influence of environmental enrichments have been mixed, as some studies suggest that environmental enrichments, such as bedding, litter mates or igloos accentuate strain differences and increase locomotor activity on the elevated plus maze, while others suggest that supplying environmental enrichments such as nestlets, do not influence behavioral outcomes (Abramov, Puussaar, Raud, Kurrikoff & Vasar, 2008; Toth et al., 2011; Haemish, Voss & Gartner, 1994; Gordon, 2004). Abramov et al. explored the behavioral differences observed in the B6 mouse and 129S6/SvEv mouse (129) when housed with environmental enrichments. After examining the difference between the mice housed with environmental enrichments, Abramov et al. found that strain differences were enhanced with number of entries into the arms (Abramov et al., 2008). Conversely, Haemish et al. studied the influence of environmental enrichments and aggressive behavior in male DBA/2J mice (DBA). An increase in aggressive behavior was found when an intruder was introduced into the cage, suggesting that housing mice with environmental rich structures (i.e., PVC pipe) showed an increase in dominance behavior. Haemish study argues the negative effect of environmental enrichments while Abramov argues the positive effects of environmentally rich home cages. While both studies highlight potential influences of an enriched environment, few studies have found a concise conclusion.

Prior to experiments, mice in this study were house in cages with corncob bedding with 2 – 4 littermates (siblings) and were provided with nestlets to tear apart and create nests with. Cages were changed weekly and mice were moved to the behavior room

down the hall. If we were to agree with findings that suggest enriched environment (bedding, nestlets and littermates) decrease anxiety-like behaviors on the elevated plus maze, it can be inferred that the reason why mice spent about 50% of the time on the open arm may be due to the environment the mice were housed in. Such enrichments are hypothesized to decrease anxiety due to the opportunity given to mice to adjust to novel environments. For example, an enriched home cage environment might provide opportunities for burrowing (digging under the bedding) or social interactions, in turn providing mice the opportunity to adjust to novel environments with ease. Then, when placed in the novel environment of the elevated plus-maze, these environmental enriched mice would be more likely to explore open arms due to their ongoing exposure and positive experiences in enriched and novel environments.

An alternative explanation for the increase percent of time on the elevated plus-maze may be due to experimental handling. Mice are able to sense a handler's emotion. If a handler is feeling anxious, a mouse may exhibit more anxious like behaviors in return. Despite inferences of a handler's emotion and its influence on mice, little research has been conducted analyzing the influence human odors have on mouse behavior (Rivard, Moser, Ambrose & Lin, 2013; Sorge et al., 2014). Rivard et al. studied the response of mice when exposed to human urine and 2,4,5-trumethyl-thiazoline (TMT), a scent secreted by foxes and omnivores. TMT is well established and has been known to illicit a fear response in mice. Both odors were compared to a response to DMSO, a diluent used for TMT in a 1:10 ratio. In a two-chamber box, mice were exposed to two scents and scored on behavioral responses. No significant differences were observed in mice in the two-chamber box when exposed to DMSO and human urine

in chambers, however, after being exposed to TMT overtime, a similar fear response occurred when mice were exposed to TMT and human urine. While extreme, this study brings to light the potential influence humans may have on mice. Sorge et al. (2014) further expanded on this study and studied the response of pain and fear in mice depending on olfactory cues of humans (male or female). Results revealed that males give off an olfactory scent that elicits a higher pain response in mice when given an inflammatory agent (sterile zymosan A) (Sorge et al., 2014). When human females were present in the room, pain markers were reduced, suggesting that females give off a less aversive scent to mice. To determine pain, mice were placed in a small box after the injection and facial expressions of pain were recorded in mice. Blind scorers analyzed the videos and measured/ scored levels of pain exhibited. In the current study, experimental handlers were primarily female with prior experience in handling mice. Despite current research, few studies have further investigated the influence of handlers in mice. Therefore, there may be underlying influences of experimental handlers that are altering elevated plus-maze performance in our mice.

Another potential factor that may have influenced the results found on the elevated plus maze include the scoring parameters used to measure behavior in the elevated plus maze. Automatically tracking mice on the elevated plus maze is a relatively new tool in current practices. While past research has primarily hand-scored elevated plus maze videos, the difference in scores found in this study may be explained by the novelty of computer tracking. Rosenthal and Kermit (1963) explored the effects of human bias on results. In Rosenthal and Kermit's study, participants were either primed with information with what behavior to expect from a rat while others were not

primed at all. Interestingly, individuals who were primed with what behavior to expect from the rats exhibited a significant influence on the data found (Rosenthal & Kermit, 1963). Given that much of historical literature on the elevated plus-maze were developed from hand-scoring measures, it might be plausible that the data found in our study did not align to past research because we have removed human bias in scoring. That is, hand scoring may have over attributed time in the closed arm due to the rater's expectation of reduced time in open arms of the maze. Therefore, in the future it would be important to go back and hand-score the videos to ensure that scores measured by humans align with the EthoVision.

Social Approach. In the Social Approach task, mice were given the choice between exploring a novel mouse with hair chalk and a novel mouse without hair chalk. We hypothesized that if the hair chalk produced an aversive odor, our experimental mouse would spend less time exploring the hair chalk chamber indicating a change in social approach behavior due to the presence of hair chalk. However, mice displayed no preference between both chambers in the Social Approach task suggesting that the presence of the hair chalk does not interfere with social approach behavior. Moreover, sniff time was analyzed and used as a more accurate measure of social interaction to identify if our experimental mice were spending equal time engaged with each mouse in additional to overall time in each chamber. That is, if the hair chalk elicits an aversive odor, we might expect that our experimental mouse may spend less time sniffing the hair chalk mouse while still spending equal time in each chamber. However, no significant differences were observed between the amount of time spent between both chambers suggesting that the scent of the hair chalk did not influence an increase or decrease in

interaction. Mice displayed equal preference for both chambers suggesting the novelty of the mouse was more engaging than the scent of the hair chalk. These results further support that the hair chalk is not producing an aversive scent influencing mouse approach.

Past research has observed if given the choice between a novel mouse and familiar mouse, mice spend significantly more time exploring the novel mouse, representing the social motivation and interest to explore a social stimuli (Crawley, 2004; Moy et al., 2007). The social approach task measures a mouse's natural motivation to explore novelty. While traditionally the social approach task has given mice the opportunity to explore a novel mouse or novel object, this study modified the task to explore the interaction between mice when given the choice between two novel mice. It was hypothesized that mice would spend equal amounts of time between both chambers, however, we did not directly measure this assumption prior to conducting our social approach task. Mice were given the choice between approaching a novel mouse with hair chalk and a novel mouse without any hair chalk. We hypothesized that if the hair chalk elicited aversive odors then our experimental mouse would avoid the chamber and spend more time in the chamber with the un-altered stimulus mouse. Even though we observed no differences in chamber time between the two stimulus conditions, our interpretations of this outcome are limited given that we are have not directly tested the assumption that mice do not innately prefer one novel mouse over another. To this end, there is a need to run a future study analyzing a mouse's choice between two novel mice without manipulation.

In addition to measuring chamber time, mice were measured for sniff time by analyzing the total time the nose of the mouse was in close proximity to each of the stimulus mice. Sniff time has been analyzed before and has been identified as a more direct measure of social interaction (Silverman et al., 2010). While mice may make appropriate entries into chambers, a mouse engagement with the novel mouse in the chamber may not be present. That is, a mouse may "pass" the social approach task, but may not be engaged with the mouse specifically. Analyzing sniff time affords researcher the ability to get an accurate representation of how a mouse is interacting in the task. Further investigating the data found in this study, mice spent equal time sniffing cups in both chambers (65 seconds), further suggesting that hair chalk did not influence mouse behavior.

Grooming. In the grooming task, past research has observed mice to spend approximately 50 seconds engaged in stereotypical grooming behavior in a 10-minute period (Yang et al., 2007b). In this study, mice with hair chalk on their back spent about 55 seconds engaged in a grooming behavior while mice who did not receive hair chalk on their back engaged in grooming for 52 seconds out of the ten minutes. No significant differences were observed between the hair chalk and control conditions, suggesting that the sensation of the hair chalk was not influencing or increasing/decreasing grooming-like behaviors. While mice engaged in grooming behaviors within normal range, the pattern in which mice groomed in provide an additional measure as a disrupted grooming pattern may indicate an increase in stress or anxiety.

Grooming is defined as paw licking, head washing, body grooming, leg licking and tail/genital grooming (Pearson et al., 2011). Mice engage in grooming behaviors in a

sequential, organized pattern from head to rump region. We explored whether the presence of hair chalk would interfere with mice's fur coat and elicit excessive grooming. Importantly, if our color markers elicited more grooming, it would reduce time dedicated to social interactions and interfere with our ability to detect differences in social behavior across strains and treatment conditions. While no significant difference was observed in the amount of time mice engaged in a grooming behavior, it will be important in the future to further analyze these grooming behaviors to determine whether the typical head to tail grooming patterns were altered following the application of hair chalk. Abnormal or disrupted grooming can be seen when a mouse's coat is disrupted (i.e., water sprayed on) or in situations of stress or anxiety (Shiota et al., 2015). Instead of grooming cephalo-caudal (head to tail), grooming behaviors would be observed to be irregular and not in a predictable order. Further analyzing the mouse's grooming patterns will not only reaffirm grooming patterns, the results would provide better insight to how the loose pigment is effecting mouse grooming.

Reciprocal Social Interaction. Aim two focused on automatically detecting strain difference between two mouse strains previously shown to differ in social interaction times in the reciprocal social interaction task. A significant difference was observed in the total time BTBR and C57 engaged in social interactions as well as the total time spent in nose-to-nose interactions. Moreover, a significant difference was observed between the FVB/Ant and C57 mouse in the total time mice were engaged in a social interaction, as well as the amount of time mice spent engaged in a nose-to-tail interaction. However, little research is present to compare data found between the FVB/Ant and C57 as little is known and published on this recently developed FVB/Ant

mouse strain. While, the FVB/NJ background used for the FVB/Ant mouse is known to be highly social and hyperactive, it remains unknown what behavioral consequences may have arisen from the genetic manipulation used to create the transgenic FVB/Ant strain.

Differences observed between the C57 and BTBR mouse align with past research as noted in Bolivar et al. (2007). In understanding partner effects, Bolivar explored the differences in social interaction between seven strains. Bolivar et al. studied the sociability levels of BTBR mice when interacting with a novel stimulus mouse of the same (BTBR) or more social strain (FVB). When BTBR mice were paired with a BTBR stimulus, Bolivar found the characteristic of low sociability preciously reported. The BTBR mouse was then compared to the highly social FVB mouse and spent significantly less time engaged in social interactions, representing two ends of the spectrum, with the BTBR consistently exhibiting low social engagement/ interaction between the other strains. However, when a BTBR mouse was paired with the FVB strain, the BTBR mouse showed increased social interaction times. In a future study, it would be important to hand-score videos to measure the accuracy of automated tracking and identify whether our human-scored behaviors match the values acquired by EthoVision.

Conclusion

The use of hair chalk has been demonstrated to not interfere with mice behavioral states and shows promise as a technique for marking mice for video-tracking software as we are also able to automatically detect strain differences between our asocial BTBR mouse and highly social C57 mouse. However, the hair chalk is a fine powder with a short longevity when applied to the fur of mice because it is easily brushed off during bouts of grooming, especially with the BTBR mice. This creates difficulty in sustaining

automated tracking of the black mouse for periods of time longer than 30 minutes without reapplying additional layers of hair chalk. Therefore, while beneficial for short social interaction tasks, our use of hair chalk is not suitable for long-term observation studies. Future developments in animal tracking and marking are warranted to develop a long-term solution to sustaining hair chalk on a mouse's back. However, moving forward, the use of hair chalk to automatically track mice is plausible as no change in behavior in mice were detected. To this end, with the use of hair chalk and the move to automated tracking, we have a found a way to successfully track black mice using a non-invasive marking system while removing human bias in scores.

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