

The Role of Social Context in Modulating Gene Expression, Neural Activity, and Neuroendocrine Response in Individuals of Varying Social Status

Caitlin Williamson

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## **ABSTRACT**

### The Role of Social Context in Modulating Gene Expression, Neural Activity, and Neuroendocrine Response in Individuals of Varying Social Status

Caitlin Williamson

Social context, which includes both the direct social experience of individuals as well as the characteristics of their social network as a whole, has been shown to be an important modulator of behavior across species. However, relatively little is known about the role of social context in regulating the complex relationships between neurobiology, neuroendocrine response, and behavior in mammals. Historically, the neurobiology of social behavior has been studied at the dyadic level, looking at brief social interactions between pairs of individuals. Given that all social species live in groups, rather than pairs, it is essential that we begin to understand the role social context at the group level plays in regulating physiology. Throughout this thesis, I use a novel behavioral housing system to study how the characteristics of stable social groups and how instances of social opportunity, when individuals are ascending up a social hierarchy, are associated with differential brain gene expression, neuroendocrine output, and behavior. I first extensively analyze the social dynamics of male dominance hierarchies, showing that they are both consistent, in that males reliably form significantly linear dominance hierarchies, and unique, in that the characteristics of these hierarchies vary from group to group. I further prove that mice living in these social hierarchies are extremely socially competent, displaying the ability to respond appropriately to individuals of varying social status. I demonstrate that females are capable of forming dominance hierarchies as well, but that their hierarchies differ from those of males. I then use this foundational knowledge to investigate how these different hierarchy characteristics can lead to differences in physiology, how one's social status is associated with brain gene expression and neuroendocrine response, and how disruption of a hierarchy through removal of the alpha male leads to robust behavioral

as well as physiological consequences. Finally, I use the insights gained from this immediate early gene work to demonstrate the crucial role of the infralimbic/prelimbic region of the medial prefrontal cortex in regulating socially competent response to changing social contexts. Taken together, this work establishes the broad role social context plays in regulating the complex relationships between behavior, brain gene expression, neural activation, and neuroendocrine output.

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## **DEDICATION**

To the 5 Family. I wouldn't be here without your unwavering support and inspiration.



## CHAPTER 1 – General Introduction

Social context, which includes everything from the direct social experience of an individual to the organization, structure, and unique characteristics of their social network, is an important modulator of the relationships between behavior, neuroendocrine output, brain gene expression, and social status. Across species, it has been shown that the social context in which an individual lives can lead to differences in behavior, including aggressive behavior in cichlid fish (Desjardins, Hofmann, & Fernald, 2012) and caching strategies in scrub jays (Emery & Clayton, 2001) and ravens (Bugnyar & Heinrich, 2006). Features of male swallows' bird song changes depending on how many neighbors an individual has, with birds singing longer, more varied songs when they have few individuals around them and shorter songs with more of a rattle when they are in a competitive context (Galeotti et al., 1997). Capuchin monkeys are more likely to eat novel food when they have a social partner than when they are alone (Visalberghi & Fragaszy, 1995). Similarly, social context affects human behavior, from emoticon use on the internet (Derks, Bos, & Grumbkow, 2007) to our response to stress (Kikusui, Winslow, & Mori, 2006) and our recovery from disease (Kroenke, Kubzansky, Schernhammer, Holmes, & Kawachi, 2006). However, while we have a good understanding of the role social context plays in modulating behavior, it has been much more difficult to study its role in modulating neurobiology and neuroendocrine response in mammals in a controlled manner in a laboratory setting.

Due to the constraints of studying large groups of individuals in a laboratory environment, the majority of social neuroscience research has focused on the neurobiology and neuroendocrinology of dyadic relationships (Peters, Pothuizen, & Spruijt, 2015). While this work has been essential in progressing the field up to this point, it is not necessarily ethologically relevant, as all social animals live in groups larger than two and social interactions typically last longer than the 10 minute periods under which individuals

are studied in the lab. In order to come to a holistic understanding of the complexities involved in social behavior, it is crucial that we move towards studying social behavior in a more ethologically relevant manner, as the majority of work in mice has not examined group social behavior. Throughout the following chapters, I have used a novel experimental paradigm to do this. I house mice in groups of 8-30 in a housing structure built to mimic the natural environment of a mouse, and they live together and interact throughout the entire period of the study, up to 8 weeks. This allows me to get a picture of the group as a whole and start to understand not only how overall group dynamics (i.e. the level of aggression of the alpha male, the equitability of the distribution of power, how is the group structure changes throughout the study period, and social network measures) affect the individuals' physiology, but also how an individual's position within the group might be related to his or her behavior, hormonal output, and brain activation and plasticity. The ultimate aim of this work is to study social dominance and complex, contextually appropriate social behavior in a model system (mice) where we can also use advanced techniques to study the brain, as we do not yet have the ability to use advanced neurobiological techniques on the organisms studied in the field.

When mice are housed in social groups in the vivarium, they form significantly linear dominance hierarchies, a highly organized group structure that allows us to investigate the role of social context in affecting behavior and physiology in a controlled manner (Williamson, Lee, & Curley, 2016). Dominance hierarchies emerge when most relationships within a social group are organized such that subordinate individuals consistently yield to those more dominant than them (Chase, 1982), and these hierarchies exist in both males and females (Stockley & Bro-Jørgensen, 2011). Mouse dominance hierarchies are both *consistent*, in that when you put a group together, they will reliably form a hierarchy, and have *distinct characteristics* (as shown in **chapter 2**). Because of these features, dominance hierarchies offer a unique and tractable method for studying social context.

Due to its complexity, a wide range of hormones, genes, and brain regions have been implicated in the control of social dominance behavior (Ely & Henry, 1978; Ervin et al., 2015; Goodson, 2005; Mooney, Peragine, Hathaway, & Holmes, 2014; Selmanoff, Goldman, & Ginsburg, 1977; Wang, Kessels, & Hu, 2014; Zhou, Sandi, & Hu, 2018). There is an extensive literature on the role of HPG axis activity in mediating social dominance behavior in mice (Ely & Henry, 1978; Muller & Wrangham, 2004; Oyegbile & Marler, 2005; Selmanoff et al., 1977), however, the results are mixed, with some studies showing testosterone is higher in dominant individuals and others showing there is no difference in testosterone levels between dominant and subordinate individuals. Further, changes along the HPG axis have been associated with ascent to dominance status (Maruska, Zhang, Neboori, & Fernald, 2013; Maruska & Fernald, 2011, 2013). In the corticosterone literature there is similar disagreement, with some studies claiming dominant individuals have lower levels of corticosterone (i.e. Bronson, 1973; Louch & Higginbotham, 1967; Merlot, Moze, Bartolomucci, Dantzer, & Neveu, 2004) and others suggesting there is no relationship between dominance status and corticosterone (i.e. Barnard, Behnke, & Sewell, 1996; Benton, Goldsmith, Gamal-El-Din, Brain, & Hucklebridge, 1978; Ely & Henry, 1978). In females, the literature is more limited, but estrogen and the genes it modulates have been heavily implicated in a wide range of female social behaviors (Duque-Wilckens & Trainor, 2017; Ervin et al., 2015; Pfaff et al., 2000). Plasticity-related gene expression, specifically that of DNA methyltransferases 1 (DNMT1) and 3a (DNMT3a), has been shown to play a role in modulating neural plasticity, learning, and memory (Champagne, 2010; Feng et al., 2010; Jensen Peña, Monk, & Champagne, 2012; Miller & Sweatt, 2007; Yu, Baek, & Kaang, 2011), which are important moderators of social competence. Further, these genes are involved in regulating social status (Kucharski, Maleszka, Foret, & Maleszka, 2008; Lenkov, Lee, Lenkov, Swafford, & Fernald, 2015), with interesting findings in honeybees demonstrating that silencing DNMT3a during development leads to the preferential development of queens over workers (Kucharski et al., 2008).

The Social Behavior Network (SBN) is a bidirectional circuit of brain regions associated with multiple forms of social behavior (i.e. aggression, communication, social recognition, affiliation and bonding, parental behavior, and social stress response), and it has been found to be evolutionarily conserved across species (Goodson, 2005; Newman, 1999). This network has been the foundation for understanding what parts of the brain are involved in regulating different aspects of social behavior, however, in recent years, brain regions associated with executive functioning (i.e. the prefrontal cortex) (Wang et al., 2011; Zink et al., 2008) and memory (i.e. the hippocampus) (Noonan et al., 2014) have been demonstrated to be essential to proper social hierarchy behaviors, which involve displaying aggression towards those you are dominant to and subordinate behaviors towards those you are subordinate to. Given the complexity of social dominance behaviors, the nodes of the SBN, plus the PFC and hippocampus are all believed to be of importance to proper social hierarchy formation and maintenance, as well as to proper response to changing social contexts.

In this thesis, I take a two-pronged approach to the study of how social context is related to social behavior, neuroendocrine output, and neurobiology. First, I seek to understand the stable behavioral dynamics of this type of social group in both males (**chapter 2**) and females (**chapter 4**), using an innovative vivarium housing system where I can analyze the group social dynamics of 12 mice. I additionally explore these dynamics in larger social groups using social network analysis (**chapter 5**). This foundational understanding of stable social hierarchy dynamics enables me to compare how each group's characteristics are related to their group members' behavior and physiology, as well as how one's position in the hierarchy (i.e. their direct social experience) is related to their neuroendocrine output and brain gene expression (as I do in **Chapters 3-5**). I then expand this understanding of stable social dynamics into an investigation of dynamically changing groups and study how disrupting a group can lead to changes in behavior, physiology, and neural activation (as I do in **Chapters 6-8**). In these chapters, I use a novel social

opportunity paradigm, similar to that previously used to study social ascent up a hierarchy in African cichlid fish (Maruska et al., 2013; Maruska & Fernald, 2010) to investigate both the behavioral and physiological response to changes in one's social context.

Given the wide array of hormones, genes, and brain regions discussed above that have been implicated in social dominance behavior, I choose here to explore various facets of the neuroendocrinology and neurobiology of social behavior. The complicated literature on HPG and HPA mechanisms involved in regulating social status motivated me to study the role social context might play in modulating this relationship between social rank and testosterone and corticosterone (**Chapter 3**), as well as the role the HPG axis plays in transitions from subordinate to dominant status (**Chapter 6**). I further investigate the relationships between HPG and HPA activity and female social status (**Chapter 4**). In my study of the social dynamics and social network structure of a large social group, I examine the role plasticity-related genes might play in the formation and maintenance of a stable social hierarchy (**Chapter 5**). In an attempt to understand the neural network that responds to changes in one's social context, I conduct a whole brain immediate early gene analysis of how individuals respond to changes in social context (**Chapter 7**). This whole brain data then leads to a study of the specific role of prefrontal cortex activity in regulating proper response to changes in social context (**Chapter 8**).

Taken together, the aims of this dissertation are to fully understand the behavioral dynamics of both stable and dynamically changing social hierarchies in an effort to investigate the role of social context in modulating behavior and physiology. Chapter 2 lays the groundwork by thoroughly investigating the behavioral dynamics of stable social hierarchies in males, and each of the following chapters strives to

unite both advanced behavioral and neurobiological approaches to study the role of social context in modulating both the brain and behavior.

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## CHAPTER 2 – Temporal dynamics of social hierarchy formation and maintenance in male mice

Cait M. Williamson, Won Lee, James P. Curley

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## **ABSTRACT**

Dominance hierarchies emerge when individuals must compete for access to resources such as food, territory, or mates. Here, using traditional and network social hierarchy analysis, we show that ten groups of twelve male laboratory CD1 mice living in large vivaria consistently form extremely linear dominance hierarchies. Within each hierarchy we determine that every individual mouse has a unique social rank and behaves with a high degree of consistency in their agonistic behaviour towards other individuals. Using temporal pairwise comparison Glicko ratings and social network Triangle Transitivity measures, we demonstrate that these hierarchies emerge rapidly, and that initial aggression is not predictive of later dominance. We also show that groups vary in how unequally power is distributed over time as social networks stabilize. Our results demonstrate that an ethologically relevant housing paradigm coupled with extensive behavioural observations provides a strong framework for investigating the temporal patterning of mouse dominance hierarchies and complex social dynamics. Furthermore, the statistical methods described establish a strong basis for the study of temporal dynamics of social hierarchies across species.

## INTRODUCTION

Social dominance occurs when one individual repeatedly and consistently yields towards another individual's agonistic behaviour, leading to a de-escalation rather than escalation of future aggression within that relationship (Drews, 1993). A dominance hierarchy emerges when most relationships within a social group are organized such that more dominant individuals consistently induce yielding responses in more subordinate individuals (Chase, 1982b). Hierarchies form when there is competition for resources such as access to mates, food, or territory. Recognizing and adhering to a social rank may be beneficial by preventing the need for constant conflict and risk of injury (Chase & Seitz, 2011). First described by Schjelderup-Ebbe (1922) based upon his observations of domestic fowl forming a 'pecking order', dominance hierarchies are now one of the most well studied forms of social organization, occurring naturally in diverse species, including fish, reptiles, birds, mammals, primates, and humans (Chase & Seitz, 2011). Dominance hierarchies also emerge readily in species studied in the laboratory such as cichlids (Fernald & Maruska, 2012; Oliveira & Almada, 1996), crayfish (Issa, Adamson, & Edwards, 1999), and chickens (Chase, 1982a).

Traditionally, the study of social behaviour in laboratory mice has been limited to brief dyadic interactions occurring in a context separate from the home-cage environment (Brodkin, 2007; Crawley, 2007; Kas et al., 2014). Although these tests reveal behaviour characteristics of individual mice and the relationship between two individuals at a given point in time, they do not provide information about how relationships develop over time or how relationships are adjusted within a large social network. Dominance in pairs of mice is usually assessed with dyadic tube-tests (Curley, 2011; van den Berg, Lamballais, & Kushner, 2015; Wang et al., 2011), food, sex or other reward competition tests (Benner, Endo, Endo, Kakeyama, & Tohyama, 2014; Jupp et al., 2015; Nelson, Cunningham, Ruff, & Potts, 2015), and aggression tests (Branchi et al., 2013; Ginsburg & Allee, 1942). Problematically, results in these social contexts do not necessarily

relate to overall social dominance within a larger group context where relationships are embedded (Chase, 1982b). Studies that have examined social dominance in groups of male laboratory mice have limited their scope to the emergence of an alpha male rather than determining finer details regarding the rank order of all individuals (Ely & Henry, 1978; Lewejohann et al., 2009). Moreover, previous studies of social dominance in the laboratory have limitations such as small group sizes, short duration of observations, and few replicated groups (Arakawa, Blanchard, & Blanchard, 2007; Ely & Henry, 1978; Lewejohann et al., 2009; So, Franks, Lim, & Curley, 2015).

Our lab has developed a novel paradigm for the study of the social behaviour of group-living laboratory mice which addresses these shortcomings. We house groups of mice for several weeks in a large vivarium that mimics the natural burrow system of the ancestral species to laboratory mice *Mus musculus* (Berry, 1970). The environment is comprised of a below ground level of inter-connected nestboxes and above ground levels that contain food, water and environmental enrichment (So et al., 2015; S.Fig1). Since *Mus musculus* are characterized by high male reproductive skew with high inter-male competition (Crowcroft, 1973), in the current study we chose to use all male groups. By collecting live observational data from ten separate social groups and using advanced statistical techniques, in the current study we investigated whether male outbred laboratory mice consistently form linear dominance hierarchies. We then examined the temporal dynamics of mouse social hierarchies, determining how hierarchies are established, how inequitable the distribution of power within the dominance network is, and how stable hierarchies are over time. We believe that this work provides a strong conceptual framework for the study of complex social dynamics within the laboratory that has implications for our understanding of behavioural parameters relevant to social relationships in natural contexts.



## METHODS

### *Animals and Housing*

A total of 120 male outbred CD1 mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 in standard sized cages (27cm x 17cm x 12cm) with pine shaving bedding. All mice were assigned individual IDs and marked accordingly by uniquely dyeing their fur with a blue, nontoxic, non-hazardous marker (Stoelting Co.). These marks last for up to 12 weeks so one application enables unique individual identification throughout the study. At the age of 9 weeks, mice were randomly assigned to social groups (cohorts) consisting of 12 males. In each cohort, six males had no previous experience of any other male in the cohort and six males had previously been housed with only one other male who was in that cohort. Each individual was weighed and placed into a large custom built mouse vivarium (length 150cm, height 80cm, width 80cm; Mid-Atlantic; see **Suppl. Figure S2.1**). Vivaria were constructed as described in So et al. (2015), consisting of multiple shelves, nest boxes, and a metal backboard containing multiple holes for air circulation. Mice could explore and access each shelf and cage via ramps and tunnels. Standard chow and water were provided ad libitum at the top of the vivarium. Multiple enrichment objects such as plastic igloos and round tubes were also provided. Pine shaving bedding was used to cover the shelves and nestboxes in each vivarium. Animals were put into the vivarium just prior to the onset of the dark light cycle on Day 1 of the study and were not disturbed for the duration of their housing in the vivarium (21-23 days). All subjects were housed in the Department of Psychology at Columbia University, with constant temperature (21–24°C) and humidity (30-50%), and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red lights (dark cycle) on at 1200 hours. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol No: AC-AAAG0054) and are in concordance with the guidelines of both ASAB and ABS. At the end of the experiment all animals were euthanized via decapitation with each individual's brain and blood being stored for future analyses.

### ***Behavioural Observations***

Live behavioural observations commenced on the first day of group housing in the vivarium and continued for between 21-23 days per vivarium (see **Table 2.1**). Observations were conducted for between 1 to 3 hours per day during the dark cycle, with the majority of observations occurring in the first four hours after the onset of the dark cycle. The mean  $\pm$  standard deviation of total observations conducted per vivarium was  $43.05\text{h} \pm 6.29\text{h}$ , ranging from 34 to 52 hours. Using all-occurrence sampling, trained observers recorded all occurrences of the behaviours listed in the ethogram in **Suppl. Table S2.1** that occurred between two animals within each one-hour observation period. Often several behaviours co-occur within each aggressive contest. Each contest lasts between 1s-20s (typically 5s-10s). Behaviours were recorded with the following priority: Fighting, Chasing, Mounting, Subordinate Posture, Induced-Flee. For instance, if one animal fought another animal who responded by fleeing, this would be recorded as a 'Fighting' event only, as 'Fighting' takes priority to the co-occurring 'Induced-Flee'. If an animal fled when approached but was not attacked by another animal, then this would be recorded as "Induced-Flee". For each behavioural event, the subject directing the behaviour, the recipient of the behaviour, the time and the location within the vivarium was recorded. Individuals that directed fighting, chasing or mounting were considered winners of each interaction. Individuals that exhibited subordinate posture or induced-flee towards another subject were considered losers of each interaction. If there was no clear winner, then the event was recorded as a tie. Each subject would only receive one win (or one loss) per aggressive interaction even if several behaviours (e.g. chase, fight, subordinate posture) co-occurred during that interaction. This was done as to not inflate the total number of wins and losses per individual. Aggressive interactions were considered to have ended when each individual separated and engaged in different behaviours such as self-grooming, social investigation of other animals, nest-building, feeding, etc. All data were documented live via Google survey on Android devices. All observers were trained to >90% reliability.

### ***Statistical Analysis***

All statistical analyses were undertaken in R version 3.2.2 (R Core Team, 2015). The total frequency of wins and losses accrued by each individual was aggregated into separate frequency win/loss sociomatrices for each cohort with winners in rows and losers in columns. A binarised 1/0 win/loss sociomatrix was derived from each frequency win/loss matrix. Following the methods of (Appleby, 1983) for each cell of the frequency win/loss matrix we assign a 1 to individuals in rows who win absolutely more often against individuals in columns and a 0 to individuals in rows who lose absolutely more often to individuals in columns. If individuals are tied both individuals receive a 0.

Using the frequency win/loss sociomatrices, we calculated the following measures of the strength of the social hierarchy: **i) Landau's Modified  $h'$**  evaluates the extent to which individuals in a hierarchy can be linearly ordered (De Vries, 1995). It ranges from 0 (no linearity) to 1 (completely linear) with the significance of  $h'$  determined by performing 10,000 two-step randomizations of the win/loss frequency sociomatrix and comparing the observed  $h'$  against a simulated distribution of  $h'$ . **ii) Directional Consistency (DC)** assesses the degree to which all agonistic interactions in a group occur in the direction from more dominant individual to more subordinate individual within each relationship. It is equal to  $(H-L)/(H+L)$  where H is the frequency of behaviours occurring in the most frequent direction and L is the frequency of behaviours occurring in the least frequent direction within each relationship. We tested the significance of DC using the randomization test proposed by Leiva, Solanas, & Salafranca (2008). **iii) Steepness** measures the unevenness of relative individual dominance within the hierarchy. It ranges from 0 (differences in dominance ratings between adjacently ranked individuals are minimal) to 1 (differences in dominance ratings between adjacently ranked individuals are maximal). In brief, a cardinal score of the overall success of each individual at winning contests relative to the success of all other individuals is calculated (normalized David Scores – DS) [see De Vries (1995) for more details]. This is derived from a dyadic dominance index ( $D_{ij}$ ) which is the proportion of wins and losses of each individual corrected for

the frequency of interactions). Steepness is then derived by regressing the normalized DS against the rank order of individuals. 10,000 randomizations of the sociomatrix are then performed to calculate the significance of the observed steepness.

Using the binary win/loss sociomatrices, we calculated: **i) Inconsistencies and Strength of Inconsistencies (I&SI) ranking** – the rank order of individuals in each social group (De Vries, 1995; Schmid & de Vries, 2013). This linear ordering algorithm determines the row and column order of each binarised sociomatrix such that as many 1's as possible appear above the diagonal (minimizing inconsistencies) and that those 1's that do appear beneath the diagonal are as close to the diagonal as possible (minimizing the strength of inconsistencies). A perfect linear hierarchy would possess all 1's above the matrix diagonal and all 0's beneath it. If more than one solution is found then the matrix whose rank order correlates highest with the normalized DS is returned as the solution. **ii) Triangle transitivity** measures the proportion (Pt) of relations between all triads (subgroup of three individuals) in a network that are transitive (i.e. if individual A dominates individual B and individual B dominates individual C, then if individual A also dominates individual C the triad is transitive) (Shizuka & McDonald, 2012). Triangle transitivity (t.tri) is scaled between 0 (the number of transitive triadic relations are not higher than random expectation) and 1 (all triadic relations are transitive). The advantage of t.tri is its effectiveness in dealing with unknown relationships (i.e. structural zeros in the sociomatrix). We tested for the significance of t.tri using a Monte-Carlo randomization of 1,000 generated random graphs using the method outlined by Shizuka & McDonald (2012). To determine how t.tri changes over time, we repeated this analysis for each group using subsetting data from the beginning of observations up to the end of each successive day. We repeated this analysis but further subsetting the data to only include up to the last five interactions between any pair of individuals. This was done to more rapidly detect any potential changes to t.tri that would not be picked up if the entire history of all relationships was used. Triangle transitivity was assessed using the R code provided by Shizuka & McDonald (2012).

The temporal changes in individual dominance ratings of each subject in each cohort was calculated using **Glicko Ratings** (Glickman, 1999; So et al., 2015). Glicko ratings are an extension of the ELO dynamic paired comparison models (Neumann et al., 2011), whereby a cardinal dominance score for each individual is derived based on the temporal sequence of wins and losses. Briefly, all individuals begin with the same initial rating (2200) and rating deviation (300). Ratings points increase or decrease for each individual determined by a function accounting for the ratings difference between opponents as well as the measure of certainty of each opponent's rating (their ratings deviation) (See Glickman, 1999; and So et al., 2015 for more details). The Glicko ratings formula uses a constant 'c' that adjusts the rate at which ratings can be modified. Here  $c=3$  based on previous work demonstrating that it is theoretically sound value for mouse agonistic interactions (So et al., 2015).

We calculated the **Gini coefficients** for each cohort using the total number of wins and losses accrued by each individual within each group. The Gini coefficient is a commonly utilized method for assessing the inequality in a distribution and has previously been used to determine inequity in power within dominance networks (McDonald & Shizuka, 2012). It ranges from 0 (no inequity) to 1 (complete inequity). Gini coefficients derived from wins and losses were compared using Wilcoxon Signed Rank Tests. Since the Gini coefficient does not detail whether more dominant or more subordinate individuals are responsible for any inequity, we also calculated the **Lorenz Asymmetry coefficient** (Damgaard & Weiner, 2000). Values of this coefficient that are  $<1$  indicate that inequity is due to individuals with lower scores (e.g. fewer wins or fewer losses) and coefficients  $>1$  indicate that inequity is due to individuals with higher scores (e.g. more wins or more losses). Testing whether the distribution of Lorenz Asymmetry coefficients differed from 1 was undertaken using a Wilcoxon Signed Rank Test. We calculated both Gini and Lorenz Asymmetry coefficients for the whole observation period of each cohort and repeated this analysis using subsetting data from the beginning of observations up to the end of each successive day to assess temporal changes. We further repeated this analysis using only data from the top four most dominant individuals per group

to assess inequality even among more powerful individuals, which has previously been suggested to be an important feature of dominance networks (McDonald & Shizuka, 2012). We also calculated the **proportion of all wins** that each individual accrued within their social group. We then compared the total win proportion by the final alpha and beta males (i.e. those who finished in the first and second rank based on their Glicko rating), as well as computing the absolute difference between these win proportions. This was done for the whole period as well as with data from the beginning of observations to the end of each day to assess temporal change. We then repeated this analysis but redefined alpha and beta males as those who were in first and second rank based on Glicko rating at the end of each successive day.

For each cohort, we also calculated the directional consistency of every relationship within a group. After ordering each directional consistency matrix in I&SI rank order, we derived the median directional consistency of each relationship across all cohorts. We also determined the interaction probability for every relationship in every cohort and likewise generated the matrix of median interaction probabilities by rank. To examine whether early dominance was predictive of final dominance, we correlated Glicko Ratings and total wins accrued by each individual up to the end of each day with final scores using Spearman rank correlations.

Landau's modified  $h'$ , DC and I&SI were calculated using the R package *compete* v0.1 (Curley, Shen, & Huang, 2015). Steepness was calculated using the R package *steepness* v0.2.2 (Leiva & de Vries, 2014), Glicko ratings were calculated using the *PlayerRatings* package v1.0 in R (Stephenson & Sonas, 2012) and Gini coefficients and Lorenz Asymmetry coefficients were calculated using the *ineq* package (Zeileis, 2014).

## RESULTS

The win/loss frequency and binarized sociomatrices are shown in **Suppl. Figure S2.2**.

### *Do Male Mice form a Linear Social Dominance Hierarchy?*

All ten cohorts of vivaria-housed mice (labelled A through J) formed a significantly linear social dominance hierarchy (all  $P = 0$ ). The average modified Landau's  $h'$  value was  $0.86 \pm 0.08$  (mean  $\pm$  standard deviation) and ranged between 0.71 – 0.93. The mean directional consistency was  $0.91 \pm 0.04$  (range 0.84 – 0.99) and was significantly above chance for all groups (all  $P = 0$ ). The mean triangle transitivity was  $0.91 \pm 0.03$  (range 0.86 – 0.95), with all being significantly higher than chance (all  $P = 0$ ). All hierarchies were also significantly steep, with a mean of  $0.63 \pm 0.11$  ranging from 0.42 – 0.76. Out of the 66 unique relationships within each group, the average number of unknown relationships (no observations of any agonistic interaction occurring between two individuals) was only  $6.1 \pm 5.1$  relationships. Landau's  $h'$ , steepness, directional consistency and total number of unknown relationships are all highly correlated with one another (Pearson's Correlation: all  $r > 0.7$ ,  $N = 10$ , all  $P < 0.05$ ). These variables were not significantly correlated with triangle transitivity, a network measure which is much more robust to the presence of unknown relationships in frequency sociomatrices.

**Table 2.1 Group Characteristics and Hierarchy Measurements**

Cohort	Days	Unknown Relationships	Number of Observations	Landau's Modified h'	Directional Consistency	Triangle Transitivity	Steepness Dij
A	21	3	1093	0.83***	0.87***	0.93***	0.67***
B	22	7	1042	0.78***	0.94***	0.94***	0.61***
C	23	0	1345	0.93***	0.84***	0.93***	0.76***
D	21	8	911	0.72***	0.95***	0.86***	0.61***
E	23	11	933	0.71***	0.90***	0.92***	0.50***
F	21	2	1221	0.88***	0.87***	0.88***	0.75***
G	22	8	1050	0.87***	0.94***	0.95***	0.62***
H	23	16	584	0.74***	0.99***	0.91***	0.42***
I	22	0	790	0.91***	0.88***	0.90***	0.70***
J	22	6	892	0.85***	0.89***	0.93***	0.63***
Mean	22	6.1	986	0.82	0.91	0.91	0.63
SD	0.8	5.1	216	0.08	0.04	0.03	0.11

\*\*\* P < 0.001

***Do Individual Mice Occupy Unique Ranks within a Hierarchy?***

The rank order of each cohort was calculated using the I&SI method. 8 out of 10 cohorts had one optimal solution that identified a unique rank of all twelve individuals. Two cohorts had two solutions that were equally optimal. For both of these cohorts, these solutions only differed with respect to which individuals were rank 11 and rank 12 in the hierarchy. Animals are ordered by I&SI rank order in the sociomatrices in **Suppl. Figure S2.2**. In all cohorts, body weight was not related to dominance rank (Spearman Rank Correlations, all P = NS). We then calculated the median directional consistency of agonistic interactions for every relationship within each cohort (i.e. the DC's for rank 1 versus rank 2, rank 1 versus rank 3, etc. through to rank 11 versus rank 12). We found that the median directional consistency of relationships within cohorts was remarkably high (**Figure 2.1A**). 71% of relationships had a median directional consistency of 0.99 or higher, and 86% of relationships had a median directional consistency of 0.9 or higher. Those relationships that were not characterized by a directional consistency of 0.9 or higher were



almost exclusively individuals of rank four or lower that only differed by one, two or three ranks from the other individual (**Figure 2.1B**). Such extraordinarily high directional consistency is indicative of a social system in which individuals have unique ranks and are showing social context-appropriate behaviour to animals of relatively higher or lower status.

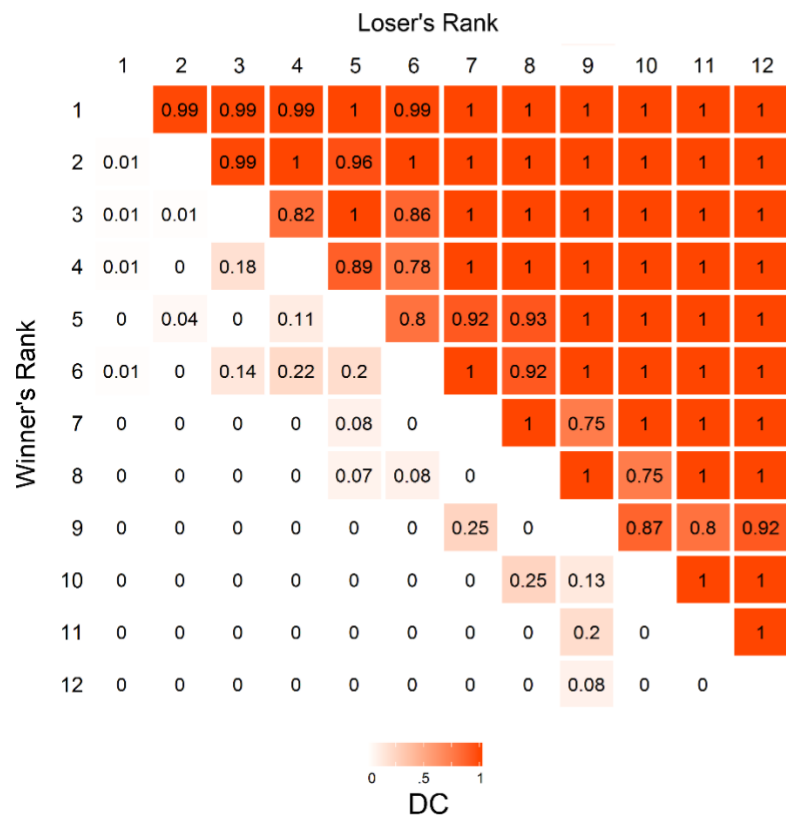
We also examined the median interaction probabilities for each relationship across all cohorts (**Suppl. Figure S2.3**). The most frequent interaction observed is between the alpha and beta males (7.7%) followed by interactions between the alpha male and all other males (all 3% - 6.1%). The most likely interaction between animals other than the alpha male occurs between the beta and gamma male (2.7%). The least common agonistic interactions occur between animals of the lowest ranks, which likely explains the slightly more inconsistent directional consistencies among lower ranks.

**Figure 2.1. Median Directional Consistencies by Relationship across Cohorts**

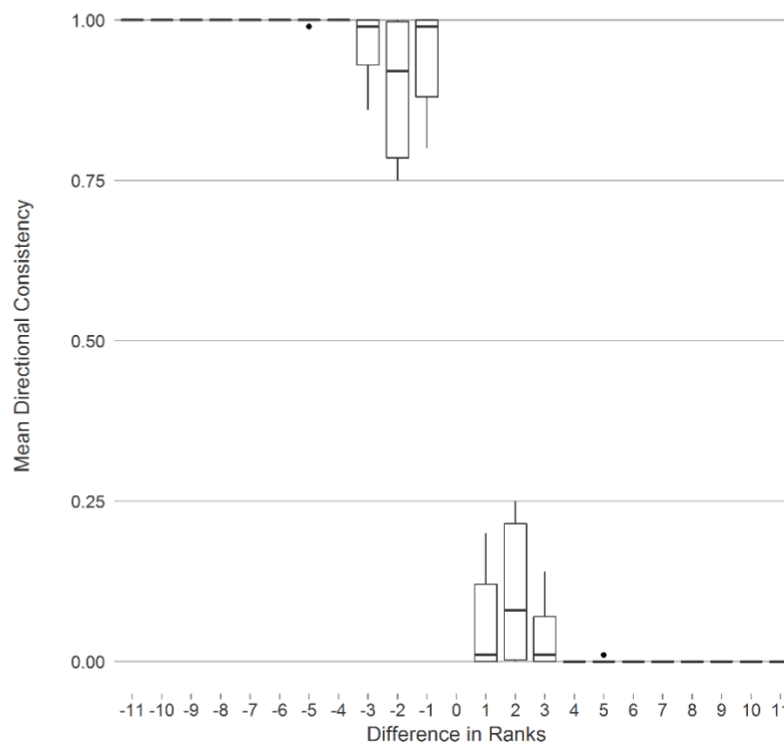
**A.** The median directional consistency matrix for all relationships organized by winner and loser rank across cohorts (A-J). Cells are coloured from white (DC = 0) to red (DC = 1). **B.** Boxplots showing the distribution of directional consistency values from the median directional consistency matrix ordered by absolute difference in winner versus loser ranks.

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**A**



**B**

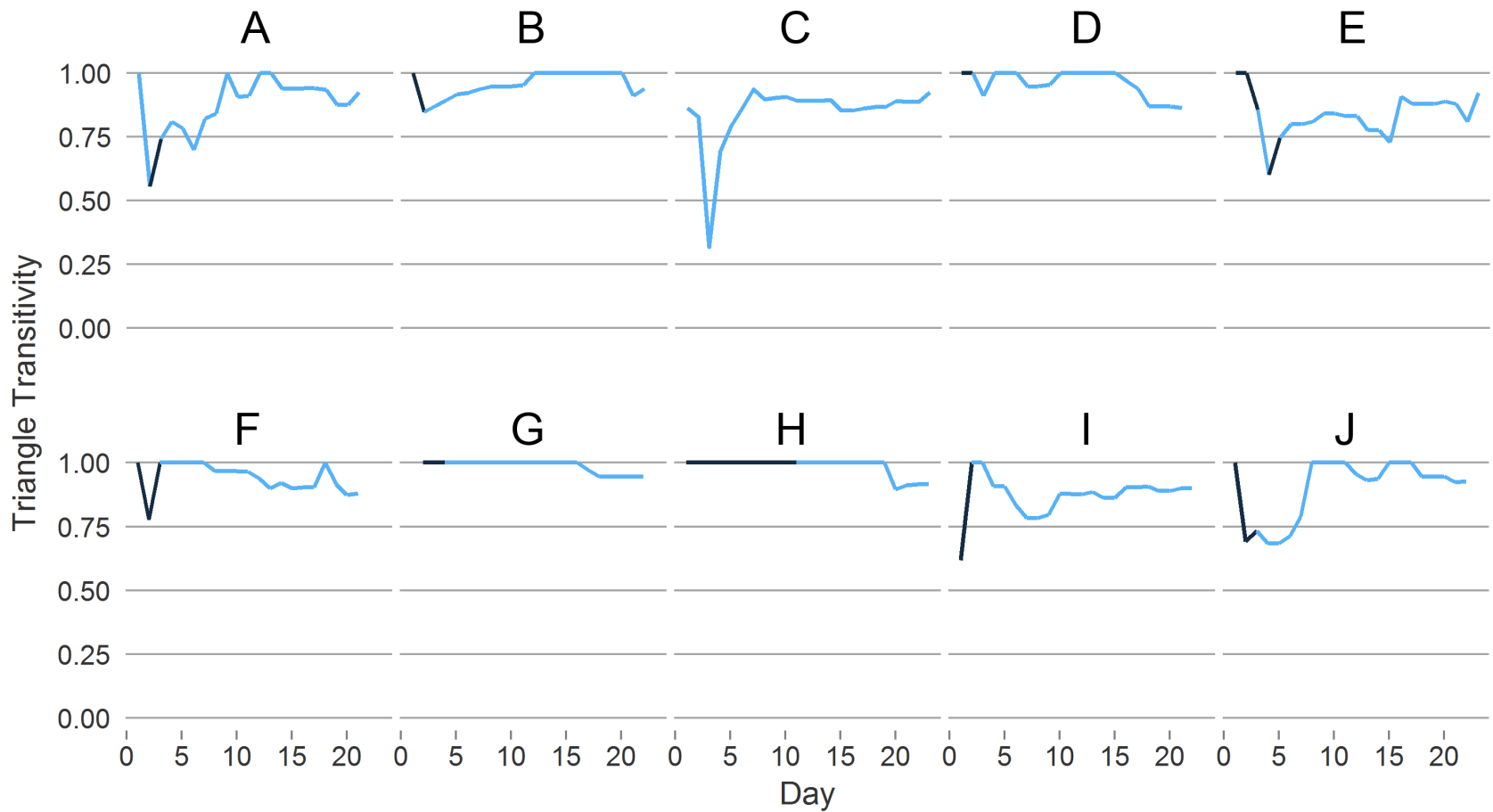


### ***How Quickly Formed and how Stable are Dominance Hierarchies?***

Changes over time in the overall degree of dominance hierarchy were examined by calculating the triangle transitivity by day for each cohort. All cohorts rapidly formed dominance networks with high transitivity (see **Figure 2.2**). Specifically, from the end of Day 2 up to the end of observations, seven of the ten cohorts had continuously significant transitive dominance networks. By the end of day four, nine of the ten had continuously significant transitive dominance networks. The final cohort (H) had a triangle transitivity of 1.0 from Day 1 to Day 19 but it was only significantly transitive from Day 11 onwards likely because this cohort had fewer agonistic interactions and more unknown relationships overall (**Table 2.1**). This consistency in dominance network structure is not due to the exaggerated influence of multiple early interactions, as the pattern of triangle transitivity by day is highly similar when using only the most recent last five observations per relationship (**Suppl. Figure S2.4**).

**Figure 2.2. Triangle Transitivity by Day.**

Each line represents the triangle transitivity based upon the cumulative observations from the beginning of group formation to the end of each successive day for each cohort (A-J). Light blue colours indicate triangle transitivity values that are significantly above chance, dark blue colours indicate triangle transitivity values that are not significantly above chance.

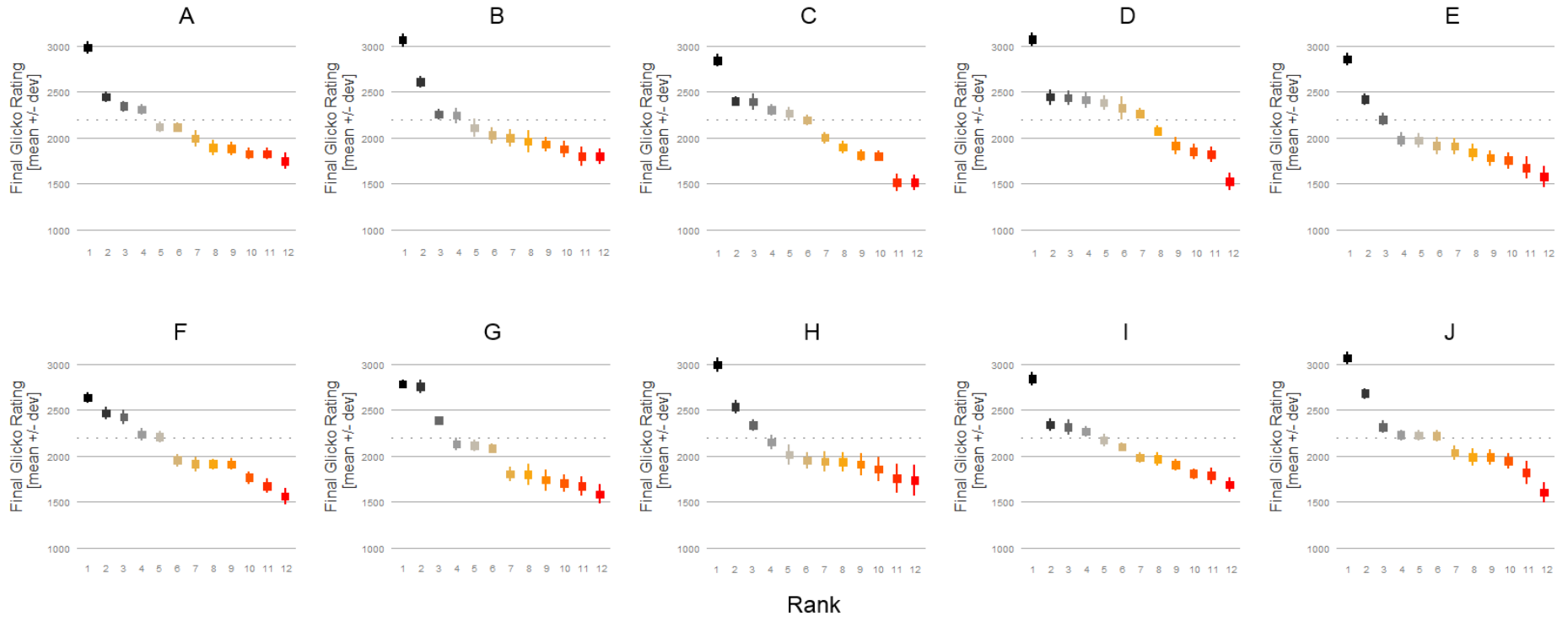


Temporal changes in the formation and maintenance of hierarchies were also assessed using Glicko ratings. The final Glicko ratings for each cohort are shown in **Figure 2.3**. The average Glicko ratings by rank are shown in **Suppl. Figure S2.5**. Each group followed a similar pattern with dominant individuals having disproportionately higher ratings than sub-dominant and subordinate individuals. The median number of individuals that finished above the initial Glicko rating was 4 per cohort (min = 2, max = 7).

The change in individual Glicko rating over time is plotted in **Figure 2.4**. Each plot shows the individual Glicko rating of each individual after each observed agonistic interaction. As cohorts vary in the number of agonistic interactions that occur, vertical dotted lines indicate the beginning of each new week of observations. In 6 of 10 cohorts (A, B, D, H, I, J), the individual who was the most dominant alpha male at the end of observations had already clearly emerged as the most dominant individual by the end of Week 1. In two cohorts (C & E), the eventual most dominant alpha male did not reach this rank until halfway between Week 1 and 2. In the remaining two cohorts (F & G), the eventual dominant alpha males took until near the end of observations (in the third Week). Prior to their ascendancy, other individuals had been clear dominant males. Most notably, in cohort F, the initial alpha male lost a fight to the initial beta male on Day 15 and did not win another fight in the remaining six days of observations. On Day 16, the initial beta male then lost a fight to the original gamma male and he also then failed to win another fight in the remaining five days. The original gamma male thus took over as the alpha male. The rank reversal in cohort G was simply the result of the original beta male defeating the original alpha male three days prior to the end of observations and the directional consistency of this relationship being stable thereafter. Taken together, these data suggest that dominant alpha males readily and rapidly emerge in each hierarchy and are generally stable. However, in a minority of social groups, the original alpha males can lose this position if a challenge successfully defeats them.

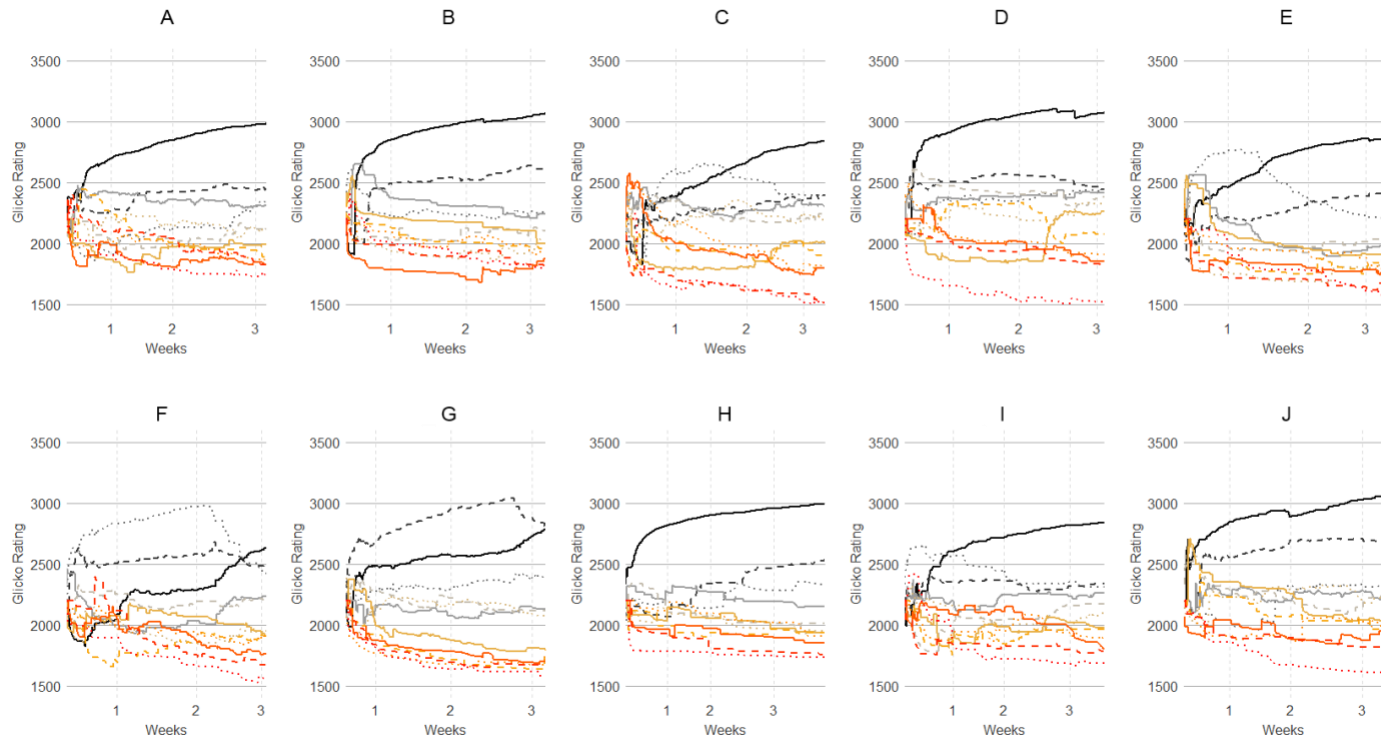
**Figure 2.3 – Final Glicko Ratings by Cohort**

The distribution of final Glicko ratings  $\pm$  deviation in ratings by final rank order for all cohorts (A-J). Colours range from black (rank = 1, most dominant) to red (rank = 12, most subordinate). The horizontal dotted line represents the starting Glicko rating of all individuals.



**Figure 2.4. Temporal Dynamics of Individual Glicko Ratings by Cohort**

The change in individual Glicko Ratings over time for all cohorts (A-J). Each line represents the ratings of one individual with colours ranging from black (more dominant at end of observations) to red (more subordinate at end of observations). The solid black line represents the final alpha male and the dashed black line represents the final beta male. Ratings are recalculated for every individual after each agonistic interaction and are plotted on the y-axis against interaction number on the x-axis. Because each cohort has a varying number of interactions, vertical dashed lines represent the end of each week of observations.

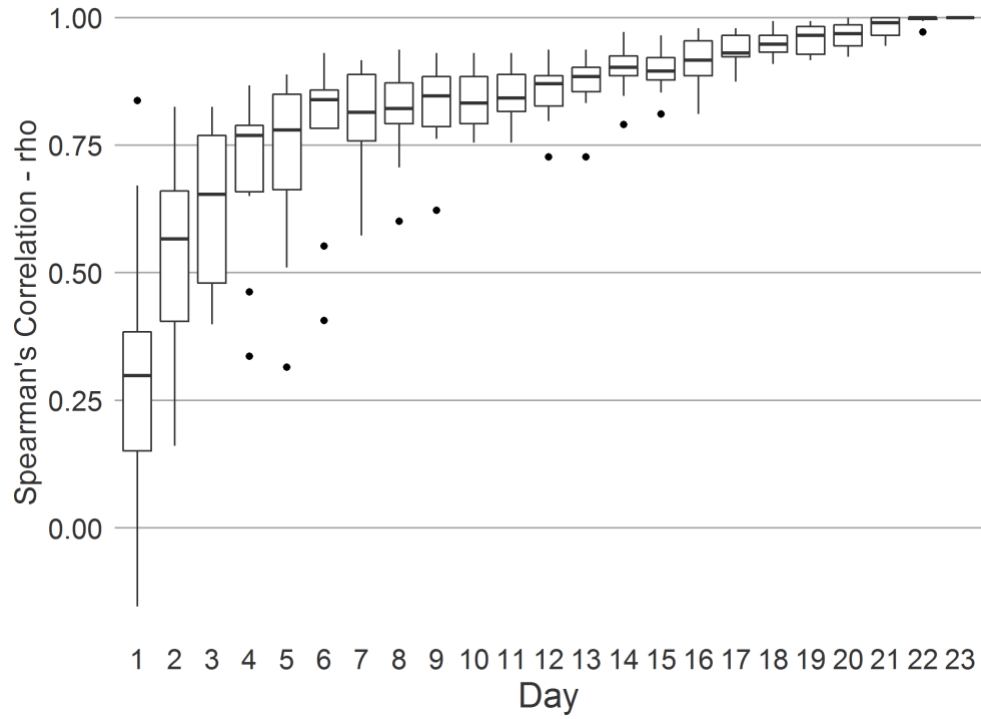


Notably, among the most stable social hierarchies, the initial aggressive behaviour of males was not predictive of their final Glicko ratings and dominance ranks (see **Figure 2.5**). Glicko ratings on Day 1 were correlated with final Glicko ranks in only 2/10 cohorts, which increased to 4/10 cohorts using Day 2 Glicko ratings. Total fights won on Day 1 were correlated with final Glicko ranks in 4/10 cohorts, which increased to 5/10 groups using Day 2 total fights won. By Day 4, both the Glicko ratings and total fights won are significantly correlated with final Glicko ratings in 8/10 cohorts. This increases to 9/10 cohorts on Day 7 and 5 for Glicko ratings and total fights won respectively and to all groups on Days 8 and 6 respectively.



**Figure 2.5. Correlation between Glicko Ratings after Each Day and Final Glicko Ratings**

Boxplots representing the distribution of Spearman's ranks correlation rho values across all cohorts calculated from correlating the Glicko rating of individuals up to the end of each day against final Glicko Ratings.



### ***How Unequally Distributed is Power within Hierarchies?***

Dominance inequality was analysed using the Gini coefficient and Lorenz Asymmetry (**Table 2.2**).

**Table 2.2. Gini Coefficients and Lorenz Asymmetries (LA) of Total Fights Won and Lost across Groups**

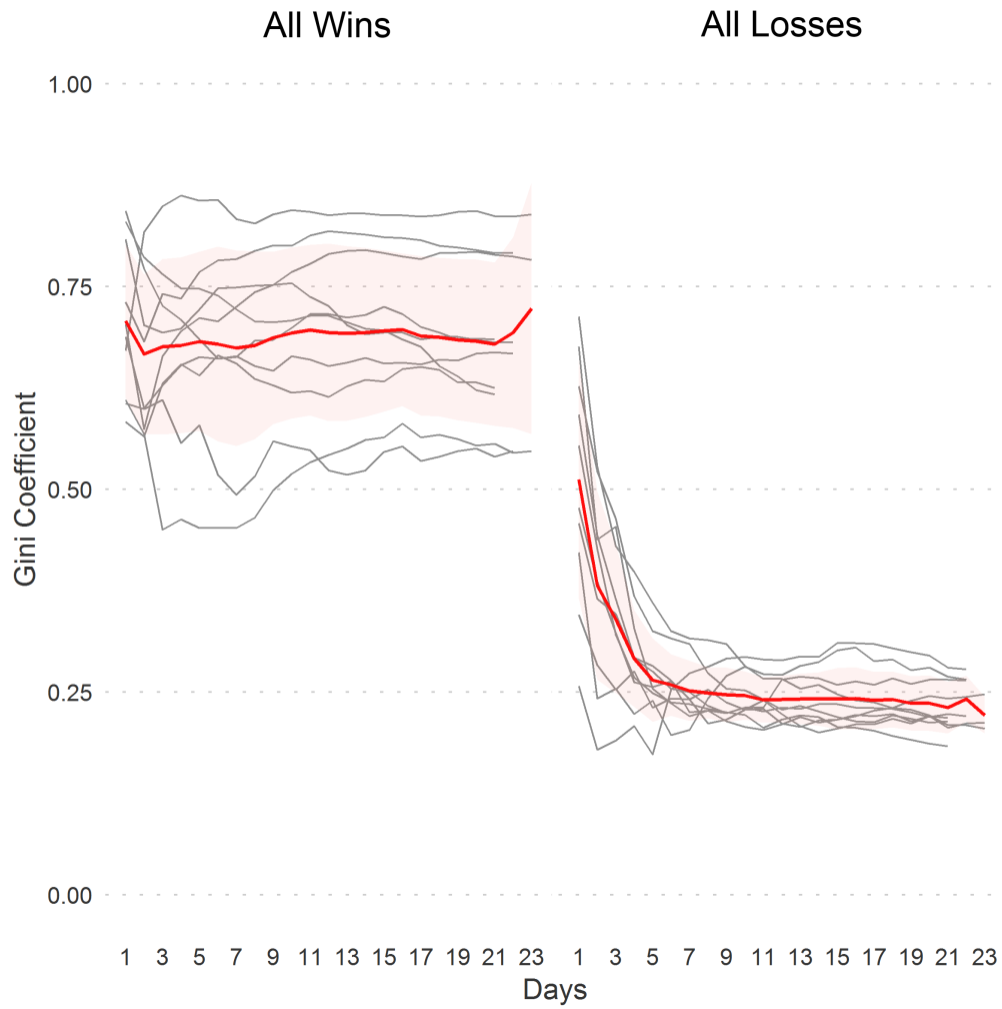
	Gini – Win	Gini – Loss	Gini – Win (top 4)	Gini – Loss (top 4)	LA – Win	LA – Loss	LA – Win (top 4)	LA – Loss (top 4)
Mean	0.68	0.23	0.42	0.29	1.03	0.93	1.14	0.88
Standard Deviation	0.10	0.03	0.13	0.05	0.10	0.16	0.17	0.17
Min	0.55	0.18	0.24	0.22	0.87	0.76	0.78	0.71
Max	0.84	0.28	0.61	0.36	1.25	1.17	1.32	1.15

The Gini coefficients of total fights won were significantly larger than the Gini coefficients of total fights lost when including all animals (Wilcoxon Signed Rank Test:  $V = 55$ ,  $N = 10$ ,  $P = 0.006$ ) or just the top four most dominant individuals (Wilcoxon Signed Rank Test:  $V = 52$ ,  $N = 10$ ,  $P = 0.010$ ). Total fights lost was therefore relatively evenly distributed among group members, whereas the total fights won was very unequally distributed even when considering differences just between the top four individuals of each cohort. Across groups, the Gini coefficients of winning and losing were not correlated with one another. When including all animals, the Lorenz Asymmetries for both winning and losing did not differ significantly from 1, indicating that inequality in winning and losing was equally due to increased dominance of more dominant individuals and decreased dominance of less dominant individuals. However, when considering only the top four individuals, the Lorenz Asymmetry of winning was higher than 1 (Wilcoxon Signed Rank Test:  $V = 46$ ,  $N = 10$ ,  $P = 0.065$ ). It was absolutely greater than 1 in eight out of ten cohorts, with a ninth group having an asymmetry of 0.996. The cohort with the lowest Lorenz Asymmetry for total wins by top four animals (0.78) was the one cohort (F) where the eventual dominant alpha male was the third ranked gamma male for much of the observation period. The Lorenz Asymmetry of losing among just the top four animals was significantly less than 1 (Wilcoxon Signed Rank Test:  $V = 7$ ,  $N = 10$ ,  $P = 0.037$ ). Thus, especially

among the most dominant top four individuals, there is a very uneven distribution of power, with the most dominant animals having a disproportionately higher number of wins to losses compared to sub-dominant individuals. The change in Gini Coefficients across days for total wins and losses by all animals is shown in **Figure 2.6**.

**Figure 2.6. Changes in Gini Coefficient in Winning and Losing by Day**

Each grey line represents the Gini Coefficient of total wins or losses accrued against all other opponents based upon cumulative observations from the beginning of group formation to the end of each successive day for each cohort (A-J). The red line indicates the mean value of all cohorts and the shaded area is  $\pm 1$  standard deviation of the mean.

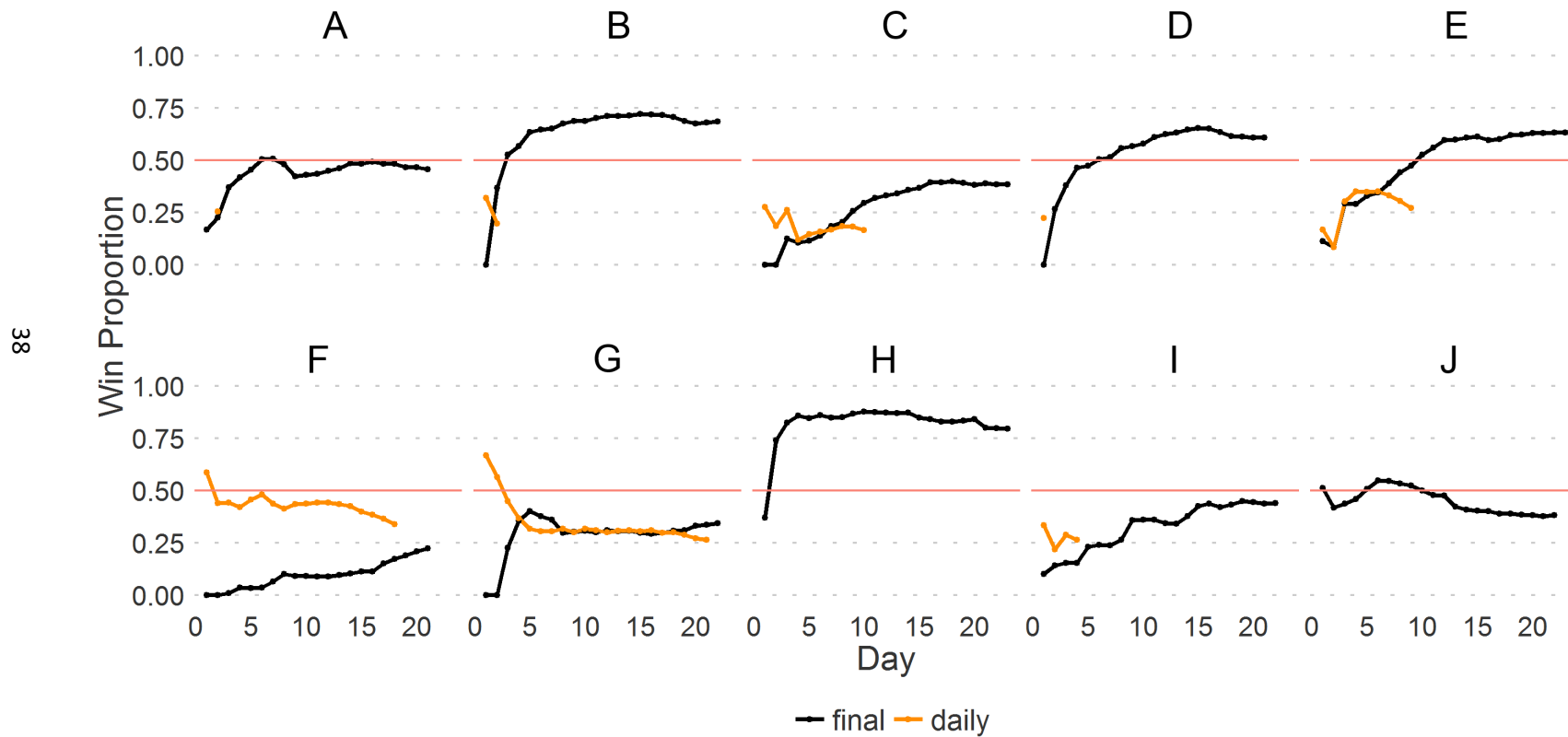


The Gini Coefficient of winning remains consistently high throughout the observation period, although there is some between group variability in the overall patterning. The Gini Coefficient of losing drops dramatically from Day 1 to Day 2 (Wilcoxon Signed Rank Test:  $V = 45$ ,  $N = 10$ ,  $P = 0.004$ ), before asymptoting by Day 5. Again, there is some inter-cohort variability with some cohorts having a more precipitous and earlier decline. The changes in Gini coefficient for the top four most dominant animals are shown in **Suppl. Figure S2.5**. Similar to when considering all individuals, there is a sharp decline in the Gini coefficient of losing fights from group formation onwards. Using a mixed-effects model with each cohort having its own random slope we found a significant effect of day on the Gini coefficient of winning fights ( $\beta = 0.004 \pm 0.001$ ,  $df = 209$ ,  $t = 4.03$ ,  $P < 0.001$ ), with Gini coefficients between top four winners increasing over days. This indicates that the inequity in power between the most dominant individuals within each hierarchy gradually increases over time.

We also examined how despotic alpha males across groups were by evaluating how each alpha male monopolized agonistic interactions within their social group. **Figure 2.7** shows the cumulative win proportions of each alpha male as recorded at the end of observations. In 4 of 10 cohorts (B, D, E, H) the alpha male won over 50% of all agonistic interactions that occurred. Each of these interactions was characterized by a sharp increase in the win proportion of the alpha male shortly after group formation. In two further cohorts the alpha male was the winner of over 50% of all interactions at least at some point during the observation period. In the remaining four cohorts, the win proportion of alpha males was always less than 50%.

**Figure 2.7. Win proportions by Alpha Males across Cohorts**

Each line represents the proportions of all wins accrued by alpha males based upon cumulative observations from the beginning of group formation to the end of each successive day for each cohort (A-J). The black lines represent the win proportions by the individual who was the final alpha male. The orange lines represent the win proportions by the individual who was determined to be the alpha male at each successive day. If the final alpha and by day alpha male are the same individual then only the black line is shown for clarity.



The despotism of alpha males was also assessed by determining the absolute difference in win proportions between alpha and beta males (**Suppl. Figure S2.6**). The final win proportions of the eventual alpha and beta male of each group are given in **Table 2.3**. The most despotic cohort was H where the alpha male consistently won around 87% of all interactions and the beta male only 5-7% through the majority of the observation period, meaning that the absolute win proportion difference was consistently around 0.8 or 80%. The groups that had alpha and beta males with the closest win proportions were the two groups where the alpha male was displaced (F & G) and group J. The remaining six groups had alpha males that consistently exhibited win proportions that ranged between 0.23 - 0.55 higher than the win proportions of beta males.

**Table 2.3. Win Proportions of Final Alpha and Beta Males**

<b>Cohort</b>	<b>Alpha Win Proportion (%)</b>	<b>Beta Win Proportion (%)</b>	<b>Difference (%)</b>
<b>A</b>	46	14	32
<b>B</b>	68	17	51
<b>C</b>	38	15	23
<b>D</b>	61	6	55
<b>E</b>	63	19	44
<b>F</b>	27	22	5
<b>G</b>	34	25	9
<b>H</b>	80	8	72
<b>I</b>	44	11	33
<b>J</b>	38	32	6
<b>Mean</b>	49.8	16.9	32.9
<b>SD</b>	17.2	8.0	22.7

### ***Where Do Agonistic Interactions Occur?***

The distribution of agonistic interactions by location across all cohorts are shown in **Suppl. Figure S2.7**. The most common location for fights was in the top section of vivaria with 80.1% of contests occurring

there, which is significantly higher than the proportion of fights that occurred in nestboxes (Wilcoxon Rank Sum Test:  $W = 100$ ,  $N = 10$ ,  $P < 0.001$ ). We also found that the total frequency of contests significantly differed between locations within the top section of the vivaria (Friedman's Rank Sum Test:  $\chi^2 = 8.6$ ,  $df = 2$ ,  $N = 10$ ,  $P = 0.014$ ). Post-hoc t-tests revealed that across all cohorts significantly more fights took place by the food hoppers on the top shelves compared to the middle shelf ( $P < 0.001$ ) or to the bottom shelf by the tube entrances ( $P = 0.035$ ). Total fights did not differ in frequency between the middle and bottom shelves of the top part of the vivarium.



## **DISCUSSION**

Our analyses of multiple cohorts of group-housed adult male mice reveal their ability to self-organize into highly linear, long-lasting and stable social hierarchies. The frequency and binary sociomatrices of winners and losers for all ten groups resulted in significant values of all three measures of hierarchical organization - Landau's  $h'$  value, directional consistency and triangle transitivity. Within each of these groups we were also able to identify the distinct rank order of all twelve individuals. Further, by examining the temporal variation in both individual dominance ratings and overall dominance network measures, we found that each social group varied in overall stability, time taken to establish a linear hierarchy, and degree of despotism of the alpha male.

### ***Formation and Maintenance of Social Hierarchies***

Our data suggest the rapid formation of highly organized linear social hierarchies in mice occurs within 48-96 hours. Typically an alpha male emerges within two days while the rank order of mid-ranking and lower-ranking individuals are resolved shortly thereafter, consistent with Chase's jigsaw model of hierarchy formation (Chase, 1982b, 1985). In some cohorts we found that the Gini coefficients of winning increase in the first few days post group-formation whereas in others these coefficients decreased. This suggests that the patterns of aggression undertaken by more dominant individuals to achieve their rank order may differ between groups likely related to their particular social context. Previous studies using male mice have focused on the social dominance within dyads or small groups of mice (3-5 animals) living in standard housing. These studies have found that one animal will rapidly (within 1-2 days) and reliably become the alpha dominant male and that sub-dominant males occasionally are also discernible (Mondragón, Mayagoitia, López-Luján, & Diaz, 1987; Poshivalov, 1980; Rodriguiz, Chu, Caron, & Wetsel, 2004; Ulrich, 1938). Studies of larger groups have also shown that dominant and sub-dominant alpha

males will emerge if mice are given more space to establish relationships (Ely & Henry, 1978; Lewejohann et al., 2009; Poole & Morgan, 1975; Poshivalov, 1980; Weissbrod et al., 2013). Indeed, from our location data and behavioural observations, it appears that dominant alpha males typically patrol the top half of the vivarium, forming a territory surrounding the location of food. Access to this area appears to be the primary cause of the conflict leading to groups of mice organizing themselves into a linear hierarchy, with each subject being able to determine their own unique social rank.

Although linear hierarchies are established quickly, many individuals are willing to engage in agonistic interactions in the first few days and many mice that eventually become very subordinate may even win several contests (**Figure 2.4**). Our finding that Glicko ratings and total wins in this time period immediately post-group formation are not predictive of final ratings or wins raises two important issues. First, individual differences in aggression are not the sole mediator of social dominance in mouse hierarchies. Other individual characteristics that support fitness and health or promote social dominance (e.g. personality variables like risk-taking or boldness, or social competence) may be just as or more important than aggression in determining social status in mice (David, Auclair, & Cézilly, 2011; Fox, Ladage, Roth II, & Pravosudov, 2009; Hsu, Earley, & Wolf, 2006; Taborsky & Oliveira, 2012). Secondly, this finding suggests that standard laboratory tests of social dominance using animals tested in pairs in tasks such as the tube-test (van den Berg et al., 2015; Wang et al., 2011), food-competition (Benner et al., 2014; de Jong, Korosi, Harris, Perea-Rodriguez, & Saltzman, 2012; Timmer, Cordero, Sevelinges, & Sandi, 2011) or aggression (Bales & Carter, 2003; Branchi et al., 2013) tests, are not necessarily robust indicators of an individual's ability to ascend a social hierarchy when living within a large social group comprised of a number of complex social relationships.

Another notable feature of our social hierarchies was the displacement of stable alpha males in two of the cohorts (F & G) during the third week of observations. Following the loss of alpha status, displaced males were much less interactive with other mice consistent with the social withdrawal observed in deposed alpha males in many species (Price, Sloman, Gardner Jr., Gilbert, & Rohde, 1994; Setchell, Wickings, & Knapp, 2006; Uehara, Hiraiwa-Hasegawa, Hosaka, & Hamai, 1994) as well as in chronically socially defeated males in rodent models of depression (Berton et al., 2006). Previous long-term observations of laboratory mouse social groups (3-5 per group) have anecdotally reported that males who had been the most dominant alpha for several weeks in groups may lose this ranking abruptly (Haemisch, Voss, & Gärtner, 1994; Ulrich, 1938). Studies of alpha male descent in natural populations of primates have found that it occurs for many possible reasons, including the alpha male being no longer physically capable of staving off challenger males, alpha males losing coalitionary support, the immigration of more-dominant individuals into the social group, or the sexual maturation of younger, more dominant individuals (O'Shea, 1976; Perry, 1998; Uehara et al., 1994). It is highly metabolically costly for alpha males to consistently defend their dominance status and territory through physical fighting (Briffa & Sneddon, 2007; Castro, Ros, Becker, & Oliveira, 2006; Rohwer & Ewald, 1981) and other behaviours such as scent marking (Gosling, Roberts, Thornton, & Andrew, 2000). Dominant alpha males of many species also have higher levels of testosterone and cortisol that may be physiologically damaging (Gesquiere et al., 2011; Higham, Heistermann, & Maestriperi, 2012; Mendonça-Furtado et al., 2014; Sapolsky, 2005). There is some evidence that more dominant mice may have elevated testosterone and corticosterone, though these findings vary depending upon social context, how dominance was assessed and other paradigmatic features (Bronson, 1973; Ely & Henry, 1978; Haemisch et al., 1994; Hiadlovská et al., 2015; Oyegbile & Marler, 2005; Selmanoff, Goldman, & Ginsburg, 1977; Zielinski & Vandenberg, 1993). We propose that in our study, the mice who lost alpha status were physiologically no longer capable of maintaining their social position, although this hypothesis remains to be tested.

### ***Variation in Dominance Inequality***

In the current study, almost all animals exhibited willingness to contest agonistic interactions (only four out of the 120 males used in 10 cohorts failed to win any fights, and only one male never lost any fight). Unsurprisingly, we found that there was a large discrepancy in the distribution of total wins and losses within each cohort, suggesting the formation of a variety of social structures within the hierarchical framework. Few previous studies have rigorously addressed the degree of despotism in male mice living in large groups ( $\geq 12$  individuals). Two report that alpha males are highly despotic, winning fights almost to the exclusion of all other individuals (Lewejohann et al., 2009; Poshivalov, 1980). One other study suggests that alpha males are unlikely to be despotic in large spaces (Poole & Morgan, 1975). By studying ten separate cohorts of twelve male mice, our data suggest that none of the alpha males in this study could be considered truly despotic in the sense that they prevent any other individual from winning any agonistic interactions. Rather, there exists a range of how unequally distributed power is within each social hierarchy. It remains to be determined what combination of characteristics of alpha males and other males within each group are associated with how despotic each alpha male becomes. It is possible that social groups characterized by an extremely dominant alpha male only occur when there is an individual of high aggression or fighting ability in conjunction with a lack of challenger sub-dominant males, or, it may be sufficient to only have one of these. From our data, it seems that every social group does have enough animals that attempt to rise up the social hierarchy, therefore we suggest that high inequality in the distribution of dominance power is more related to the hyper-aggressive characteristics of individual alpha males.

### ***Individuals Behave Consistently and Appropriately According to their Social Rank***

Dominance hierarchies are characterized by social relationships that show consistently high asymmetries of behaviour. Importantly, these asymmetrical relationships when considered together are ordered such that dominance networks have low levels of intransitivity. Theoretical and empirical work has shown that such orderliness may emerge given sufficient differences in prevailing attributes (e.g. fighting ability) or through individuals having the ability to infer relative rank via experiential effects such as winner, loser and bystander effects (Chase & Seitz, 2011). Across all of our cohorts, we find that individual animals exhibit extremely high directional consistency in their own individual relationships with each other (see **Figure 2.1**). These data demonstrate that all individual mice in these social systems are able to recognize their relative status to all other animals in the group and behave appropriately to those who are ranked above and below them in the hierarchy. This high degree of social competence that we observe is not simply a function of every mouse only responding appropriately to the alpha male, as even mid- and lower-ranking individuals respond correctly during agonistic interactions towards those ranked above them (i.e. show subordinate behaviour) and below them (i.e. show agonistic behaviour). Moreover, social competence can be achieved through even very limited social interaction. For example, though only 1.1% of fights occur between ranks 3 and 4 and 0.1% between ranks 11 and 12, there are still a sufficient number of interactions to reliably generate a social hierarchy with high directional consistency within these relationships. Individuals also appear to update this information rapidly as social status changes, as demonstrated by the fact that when there is a sudden change in the social hierarchy, such as the alpha male being displaced by a sub-dominant, the directional consistency continues to be remarkably high albeit in the opposite direction.

An important outstanding question is through what mechanism these mice are able to recognize their relative social status to other mice and how this recognition facilitates hierarchy formation and

maintenance. It is well established that social recognition via olfactory cues is fundamental to mice being able to recognize their own social status. Compared to subordinate animals, dominant males have higher levels of major urinary proteins (MUPs) that bind to signalling volatile compounds (e.g. 2-sec-butyl-4,5-dihydrothiazole and 3,4-dehydro-exo-brevicommin) (Apps, Rasa, & Viljoen, 1988; Guo, Fang, Huo, Zhang, & Zhang, 2015; Harvey, Jemiolo, & Novotny, 1989; Humphries, Robertson, Beynon, & Hurst, 1999; Kaur et al., 2014; Nelson et al., 2015; Stowers & Kuo, 2015). Some of these (e.g. MUP3, MUP20) are known to promote or inhibit the aggressive behaviour of males that receive these signals dependent upon their own social status. Other volatiles such as  $\alpha$ - and  $\beta$ -farnesene produced in the preputial gland are also excreted in urine and are higher in dominant males compared to subordinate males (Harvey et al., 1989; Novotny, Harvey, & Jemiolo, 1990). Such olfactory cues may certainly be sufficient for learning about the most dominant alpha male in a social group, but it is not yet clear whether such markers allow mice to reliably discriminate between individuals of mid and lower rank and whether these cues could be utilized for discriminating subtle rank differences. A further issue is that these chemosensory differences appear to emerge over time and therefore may be used to identify social dominance in established groups but are not necessarily utilizable by individuals for learning about initial group formation (Harvey et al., 1989).

Another potential mechanism is individual recognition (Barnard & Burk, 1979). Mice are able to use a number of volatile and non-volatile chemosignals (e.g. MHC class I peptides) to discriminate between and recognize individuals (Brennan, 2009; Hurst et al., 2001). Individual males may couple olfactory cues related to each opponent after initial agonistic contests and continue to update this information through repeated interaction. For instance, a mid-ranking individual must learn the individual odours of all animals that he has previously and recently lost to and beaten and then use that information to guide future interactions. In our vivarium, almost every agonistic interaction is preceded by direct chemosensory investigation, suggesting that individuals are using this information to update their relative social status

to each other (So et al., 2015). Although the most likely sensory system is olfaction, we do not preclude the possibility that such learning may also occur through auditory or visual cues, both of which have previously been suggested to mediate some dominance interactions in rodents (Assini, Sirotin, & Laplagne, 2013; Wesson, 2013).

A limitation of individual recognition is that this is a very energetically costly method of forming a social hierarchy. It is also therefore likely that mice use socio-cognitive mechanisms to guide their agonistic interactions. A number of species including cichlids, corvids, and primates use third-party observational learning and transitive inference to learn about which animals in a social group are more dominant to which other animals (Bond, Kamil, & Balda, 2003; D'Amato & Colombo, 1988; Grosenick, Clement, & Fernald, 2007; Hogue, Beaugrand, & Laguë, 1996; Kumaran, Melo, & Duzel, 2012; Paz-y-Miño C, Bond, Kamil, & Balda, 2004). Individuals may also determine their social status through winner and loser effects (Chase, Bartolomeo, & Dugatkin, 1994; Dugatkin, 1997). Winner effects are short-term boosts to the likelihood of winning future encounters that individuals gain following winning a conflict. Loser effects are the increased likelihood of losing subsequent encounters following a loss (Barnard & Burk, 1979; Frey & Miller, 1972). Both expedite social hierarchy formation (Chase 1982). Empirical support for the presence of these experiential effects exist in numerous taxa including some mouse species (e.g. *Peromyscus californicus*) (Oyegbile & Marler, 2005). In this study, we do not appear to have strong evidence for winner effects as those individuals that won contests on Day 1 were not necessarily continuing to win contests thereafter. However, it does seem that we do see loser effects. Individuals that suffered a significant loss appear to become much less likely to engage in future contests. This is true not only for individuals that lose fights early on in group formation, but also for displaced alpha males.

## CONCLUSION

The organization of social groups into dominance hierarchies is a phenomenon that has been investigated thoroughly across taxa, both in the lab and the field. Here, we have shown that laboratory mice reliably form linear and stable dominance hierarchies after being put together within 48-96 hours. Importantly, each mouse within a hierarchy has a unique and distinct social rank and responds consistently to more and less dominant members of their network with appropriate behaviour indicative of high socio-cognitive competence (Branchi et al., 2013; Taborsky & Oliveira, 2012). There also exists variability between groups in how unevenly power is distributed within the hierarchy. In the extreme, despotic dominant alpha males may monopolize up to 80% of all fights, but in other groups there is much more extended competition as to which males become alpha or beta males. In some groups this competition leads to the original alpha male being unable to maintain their position at the top of the hierarchy. We believe that studying the temporal dynamics of mouse social hierarchy formation in such an ethologically relevant manner will provide an insightful basis for the future genetic and neurobiological investigation of complex social dynamics in mice and provide insights into the behavioural and biological dynamics critical for characterizing social groups in general. Finally, the statistical methods described here for identifying temporal stability and instability in dominance hierarchies provide a framework for the study of temporal dynamics of social hierarchies across species.



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## SUPPLEMENTAL MATERIAL

### Supplemental Tables

**Supplemental Table S2.1. Mouse Social Behaviour Ethogram**

Priority	Behaviour	Description
1	Fighting	Individual lunges at and/or bites the other individual.
2	Chasing	Individual follows the target individual rapidly and aggressively while the other individual attempts to flee.
3	Mounting	Individual mounts another individual from behind with the recipient attempting to flee or otherwise being pinned to the floor.
4=	Subordinate posture	Individual responds to the approach from another individual by remaining motionless and/or exposing their nape.
4=	Induced-Flee	Individual flees without any aggression shown by another individual.

**Supplemental Table S2.2. Proportion of Agonistic Interactions by Location across All Cohorts**

	Nestboxes	Bottom Shelf	Middle Shelf	Top Shelf
<b>Min</b>	0.03	0.09	0.07	0.25
<b>Max</b>	0.49	0.34	0.29	0.59
<b>Mean</b>	0.20	0.23	0.19	0.38
<b>SD</b>	0.13	0.10	0.08	0.10



Supplemental Figures

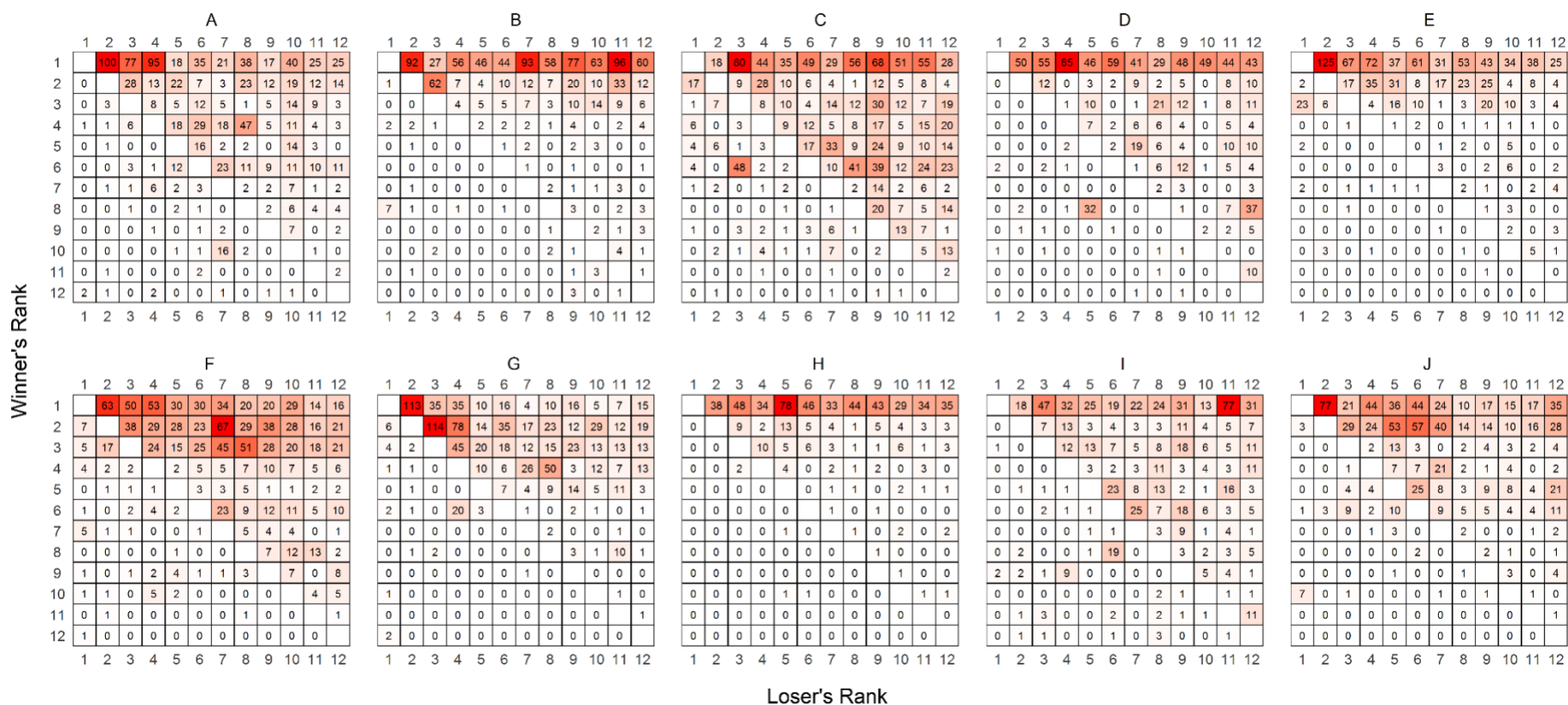
Supplemental Figure S2.1 – Housing Vivarium



### Supplemental Figure S2.2a. Frequency Win-Loss Sociomatrices

Total frequency of agonistic interactions that occurred between all pairs of individuals across all cohorts (A-J) over the entire observation period. Winners of each contest are listed in rows and losers are listed in columns. Ranks were calculated using the I&S1 method (see methods). Cells of each matrix are coloured on a gradient from white (lowest value in each matrix) to red (highest value in each matrix).

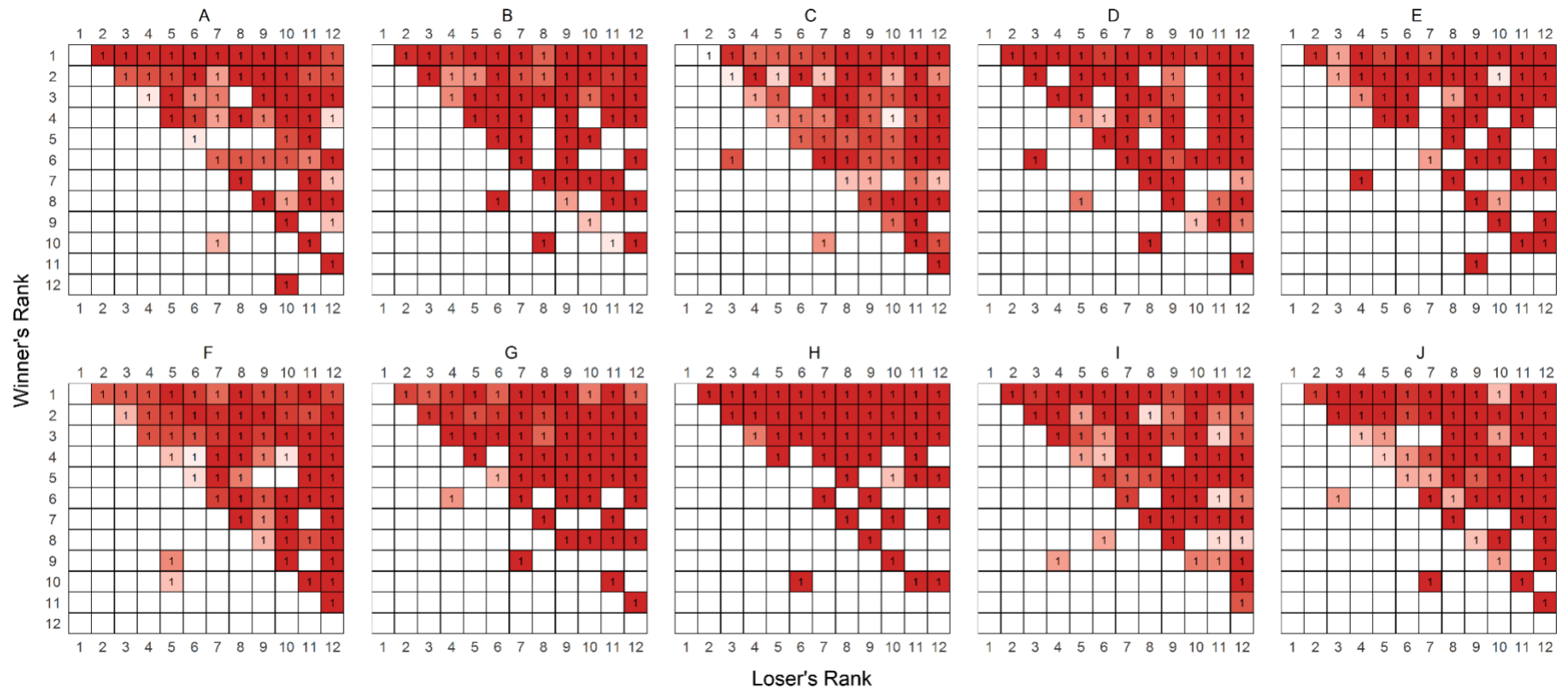
85



### Supplemental Figure S2.2b. Binarized Win-Loss Sociomatrices

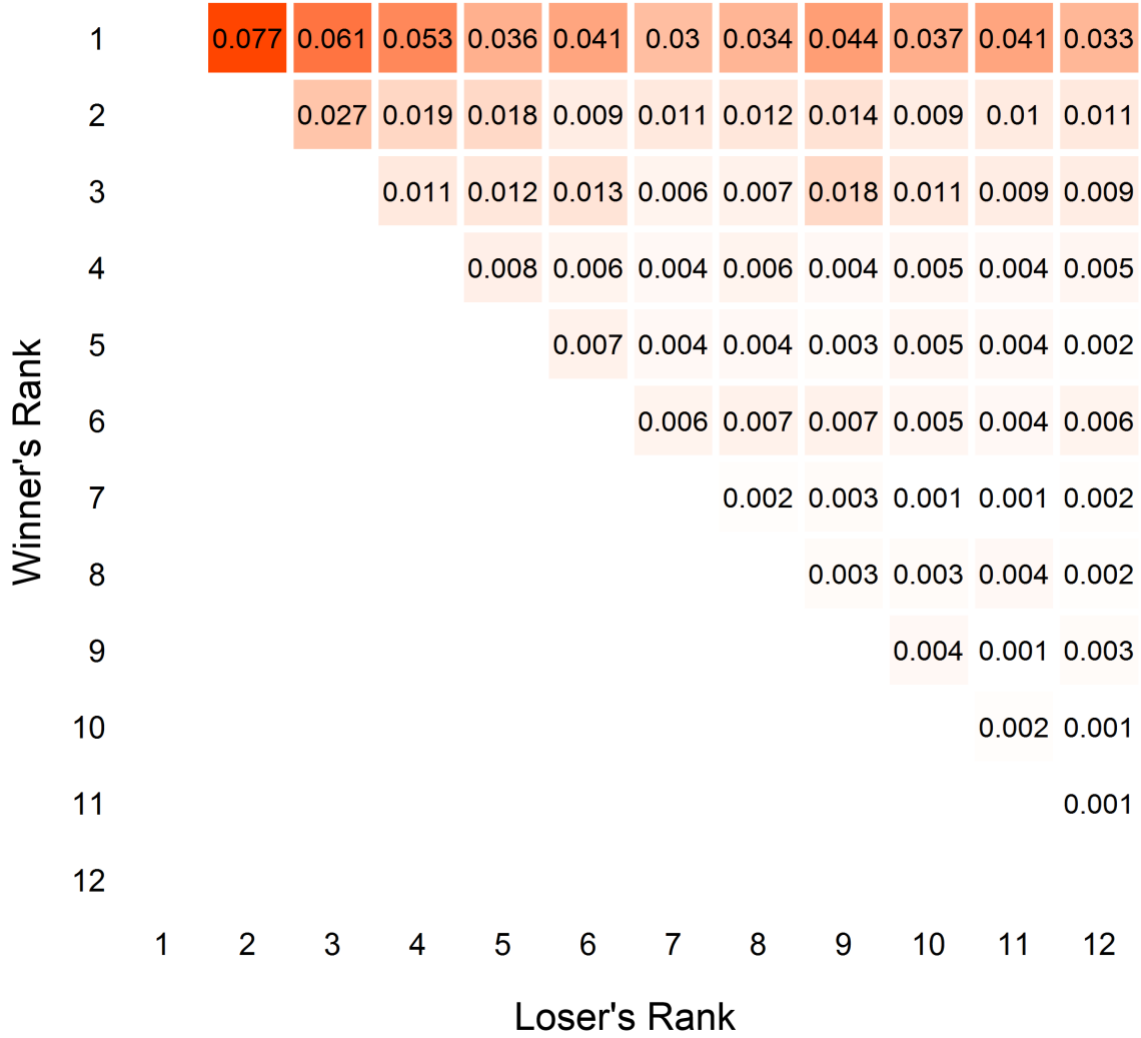
For each relationship within each cohort the winner and loser was calculated by determine which individual had the most wins over all observations (see methods). Winners of each contest (A-J) are listed in rows and assigned a value of 1. Losers are listed in columns. Ranks were calculated using the I&SI method (see methods). Cells of each matrix are coloured on a gradient from white to red with the redness being directly related to the directional consistency of each relationship.

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**Supplemental Figure S2.3. Interaction Probabilities by Rank**

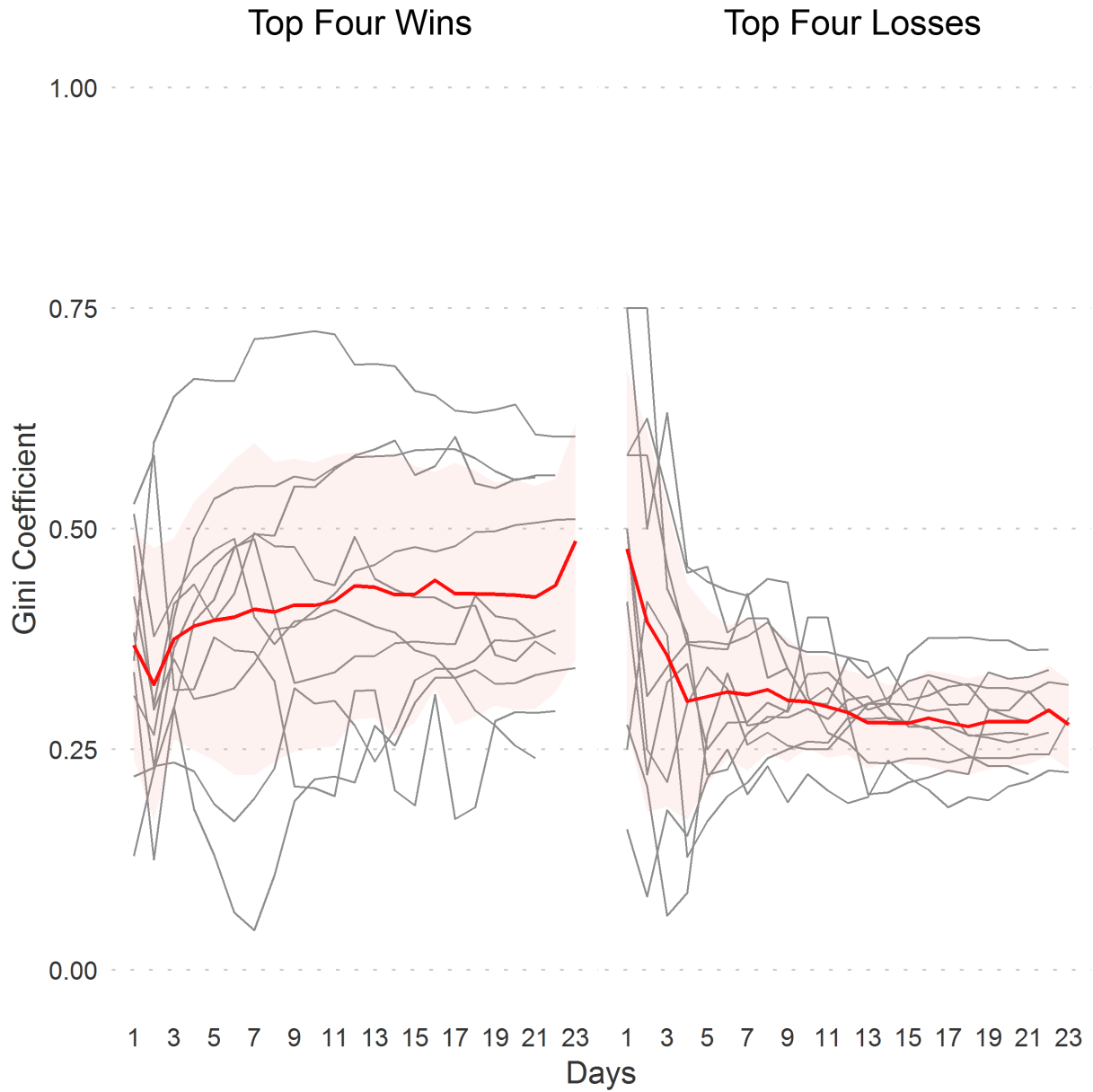
Each cell represents the median antagonistic interaction probability across all cohorts between winners and losers of each social rank.





### Supplemental Figure S2.5. Gini Coefficients of Wins and Losses against Top Four Most Dominant Individuals

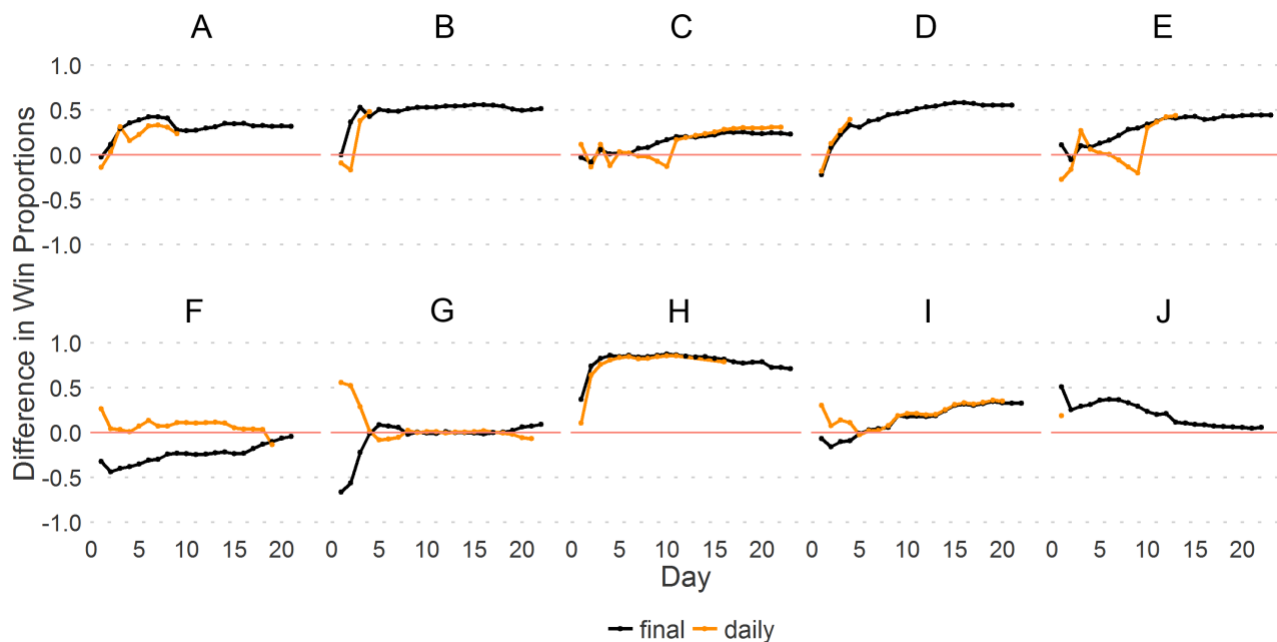
Each grey line represents the Gini Coefficient of total wins or losses accrued against the top four most dominant opponents based upon cumulative observations from the beginning of group formation to the end of each successive day for each cohort (A-J). The red line indicates the mean value of all cohorts and the shaded area is  $\pm 1$  standard deviation of the mean.



### Supplemental Figure S2.6. Difference in Win Proportions between Alpha and Beta Males across Cohorts

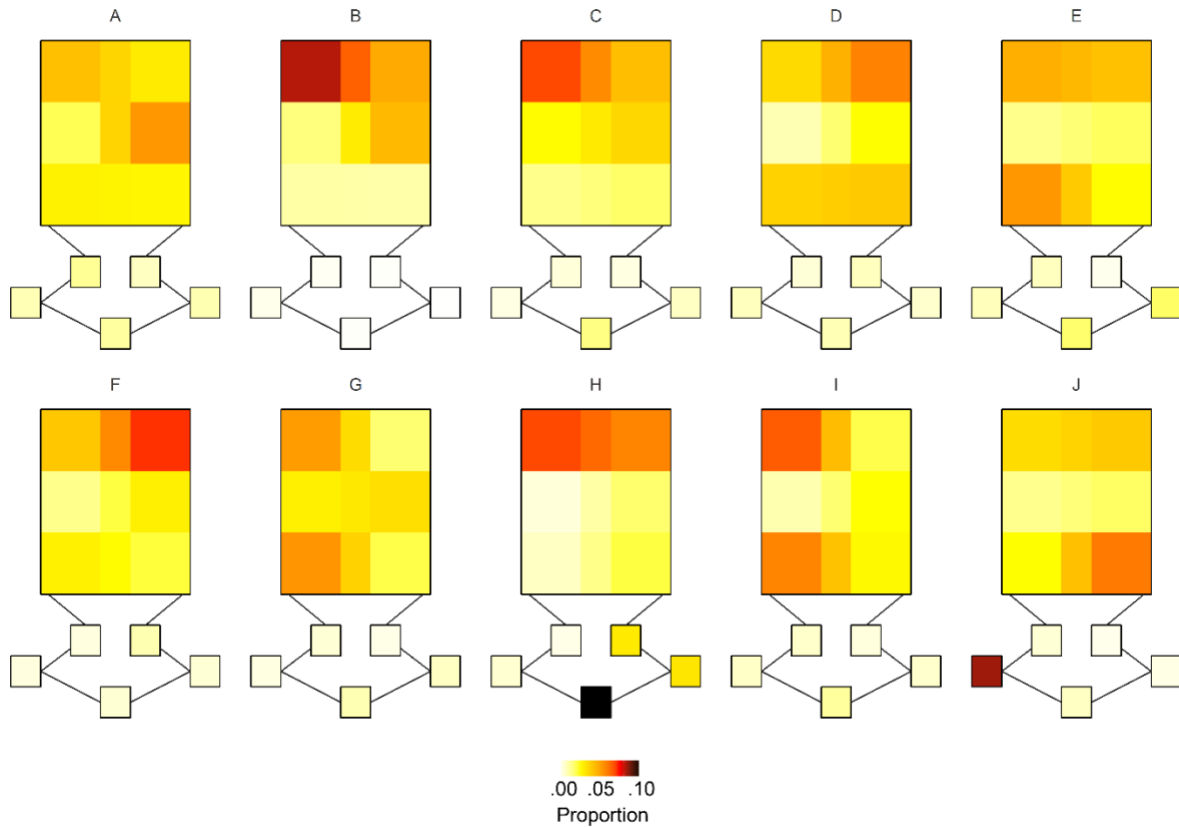
Each line represents the absolute difference in win proportions between alpha and beta males based upon cumulative observations from the beginning of group formation to the end of each successive day for each cohort (A-J). The black lines represent the difference between the two individuals that were determined to be the final alpha and beta males. The orange lines represent the difference between the two individuals that were determined to be the alpha and beta male up to each successive day. If the final alpha and beta male are equivalent to the alpha and beta male up to each day then only the black line is shown for clarity.

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### Supplemental Figure S2.7. Location of Agonistic Interactions by Cohort

Schematic showing the proportion of aggressive contests that occurred in the vivarium of each cohort (A-J). The largest square represents the top section of each vivarium. Each row of the largest square represents the three shelves (top, middle, bottom) of the top section of a vivarium. The five smallest squares represent the five nextboxes in the bottom section of the vivarium. Lines represent the tubes connecting nestboxes and the top section. Colours range from white (0% of all contests occurred in location) to black (10% of all contests occurred in location).





## CHAPTER 3 – Social context-dependent relationships between mouse dominance rank and plasma hormone levels

Cait M. Williamson, Won Lee, Russell D. Romeo, James P. Curley

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## ABSTRACT

The associations between social status and endogenous testosterone and corticosterone have been well-studied across taxa, including rodents. Dominant social status is typically associated with higher levels of circulating testosterone and lower levels of circulating corticosterone but findings are mixed and depend upon numerous contextual factors. Here, we determine that the social environment is a key modulator of these relationships in *Mus musculus*. In groups of outbred CD-1 mice living in stable dominance hierarchies, we found no evidence of simple linear associations between social rank and corticosterone or testosterone plasma levels. However, in social hierarchies with highly despotic alpha males that socially suppress other group members, testosterone levels in subordinate males were significantly lower than in alpha males. In less despotic hierarchies, where all animals engage in high rates of competitive interactions, subordinate males had significantly elevated testosterone compared to agonistically inhibited subordinates from despotic hierarchies. Subordinate males from highly despotic hierarchies also had elevated levels of corticosterone compared to alpha males. In pair-housed animals, the relationship was the opposite, with alpha males exhibiting elevated levels of corticosterone compared to subordinate males. Notably, subordinate males living in social hierarchies had significantly higher levels of plasma corticosterone than pair-housed subordinate males, suggesting that living in a large group is a more socially stressful experience for less dominant individuals. Our findings demonstrate the importance of considering social context when analyzing physiological data related to social behavior and using ethologically relevant behavioral paradigms to study the complex relationship between hormones and social behavior.

## INTRODUCTION

Across species, elevated endogenous plasma testosterone is positively associated with dominant behaviors (e.g. fighting, biting and chasing) that enable individuals to attain and maintain high social status within social hierarchies. The majority of these findings come from studies of male non-human primates such as chimpanzees (Muller and Wrangham, 2004), baboons (Beehner et al., 2005; Sapolsky, 1993) and lemurs (Cavigelli and Pereira, 2000; Engelhard et al., 2000), though associations have also been observed in cichlid fish (Oliveira et al., 1996), reptiles (Greenberg and Crews, 1990), rats (Monder et al., 1994), and guinea pigs (Sachser, 1987; Sachser and Pröve, 1986). High levels of testosterone among dominants are presumed to facilitate the formation of male dominance relationships and maintain ongoing dominance behavior (Luttge, 1972; van den Berg et al., 2015). High testosterone has also been found to be associated with female dominance. In lemurs, dominant females have high androstenedione concentrations than subordinates suggesting a pathway for masculinization of features underlying their aggressive behavior (Drea, 2007). Additionally, socially dominant female breeding mole rats exhibit higher levels of testosterone than non-breeding female mole rats (Lutermann et al., 2013).

Conversely, dominant individuals living in social hierarchies have been found to have significantly lower basal endogenous glucocorticoid levels than subordinate individuals, the latter of whom presumably experience higher levels of social stress, in species such as non-human primates (Sapolsky, 2005, 1982), rats (McEwen et al., 2015; Monder et al., 1994) and guinea-pigs (Sachser and Lick, 1989). Importantly, there are many exceptions to these general findings, with studies identifying either no relationship between these hormones and social rank or, in the case of glucocorticoids, finding the opposite association, with dominant males exhibiting higher levels of glucocorticoids than subordinate males (Creel et al., 1992; Schoech et al., 1991). In mice there is no clear consensus regarding the relationship between dominance rank and basal plasma testosterone or glucocorticoid levels (summarized in **Table 3.1** and **Table 3.2**).

Social context, which includes both the direct social experience of an individual as well as the organization, structure, and unique characteristics of the social network as a whole, may be a key modulator of the relationship between hormones and social status. The role of social context in regulating the endocrine system may account for the variability in findings relating social status to hormone levels across species (Almeida et al., 2014; Cavigelli and Pereira, 2000; Greenberg and Crews, 1990). A well-established example of this is the challenge hypothesis which proposes that testosterone will be more highly correlated with dominance status and agonistic behavior during times of instability and increased competition (Wingfield et al., 1990). Evidence supporting the challenge hypothesis has been found across species including birds (Wingfield et al., 1990), cichlid fish (Oliveira et al., 1996) and non-human primates (Sapolsky, 1982). For example, in male baboons, testosterone is highly correlated to the expression of dominance behaviors when there is a power struggle for the alpha position but not when social groups are stable and there is no competition for social rank (Sapolsky, 1982). Rank instability has similarly been shown to result in elevated basal cortisol concentrations in all individuals in unstable relationships (Sapolsky, 1992).

Previously, we have demonstrated that housing groups of 12 male outbred CD-1 mice in large, complex environments leads to the rapid establishment of linear stable dominance hierarchies, where each mouse has a unique rank and behaves in a socially appropriate manner to individuals of relatively higher and lower social status (Williamson et al., 2016). Additionally, we have shown that each social hierarchy possesses unique social dynamic characteristics. In particular, we have demonstrated that alpha males vary in their ability to inhibit the aggression of other males in their group, an ability referred to as despotism. In hierarchies with highly despotic alpha males, other males are much less likely to express aggressive behaviors towards each other, whereas in hierarchies with less despotic alpha males, power is more equally distributed among sub-dominant mice (Curley, 2016a; Williamson et al., 2017, 2016). In the present study, we sought to determine the role of despotic social context in modulating the relationship between testosterone, corticosterone and social rank. We examined the relationship between

endogenous plasma testosterone and corticosterone with social status within social hierarchies that were characterized either by high or low alpha male despotism. Additionally, we compared endogenous levels of testosterone and corticosterone in males of dominant and subordinate social status living in group social hierarchies, where individuals flexibly express both aggressive and subordinate behaviors, to those males living in stable dyadic social relationships where individuals almost only ever express either aggressive or subordinate behavior once their relative social status has been determined.

**Table 3.1 – Relationship Between Testosterone and Social Rank**

<i>Strain</i>	<i>Group Size</i>	<i>Cage Dimensions</i>	<i>Time Spent Together (Days)</i>	<i>Females Present ?</i>	<i>Relationship Between Rank and Plasma Testosterone</i>	<i>Reference</i>
<b>CD-1</b>	4-6	N/A	N/A	No	Dominant > Subordinate	Marchida, Yonezawa, & Noumura (1981)
<b>CBA/J</b>	17 (5 M, 12 F)	Eight 23 x 11 x 11cm inter-connected cages	28	Yes	Dominant > Subordinate	Ely (1981)
<b>DBA/1/Bg</b>	2	16 x 26.5 x 11.5cm	5	No	Dominant = Subordinate	Selmanoff, Goldman, & Ginsberg (1977)
<b>CFLP</b>	6	30 x 30 x 30cm	5	No	Dominant = Subordinate	Barnard, Behnke, & Sewell (1996)
<b>DBA/1/Bg and DBA/2/Bg</b>	7-8 (2 M, 5-6 F)	16 x 26.5 x 11.5cm	120-180	Yes	Dominant = Subordinate	Selmanoff, Goldman, & Ginsberg (1977)
<b>DBA/1/Bg</b>	8	16 x 26.5 x 11.5cm	150	No	Dominant = Subordinate	Selmanoff, Goldman, & Ginsberg (1977)
<b>Swiss</b>	10	N/A	21	No	Dominant = Subordinate	(Hilakivi et al., 1989)

**Table 3.2 – Relationship Between Corticosterone and Social Rank**

<i>Strain</i>	<i>Group Size</i>	<i>Cage Dimensions</i>	<i>Time Spent Together (Days)</i>	<i>Females Present?</i>	<i>Relationship Between Rank and Plasma Corticosterone</i>	<i>Reference</i>
<b>DBA/2J</b>	3 (siblings)	26.5 x 42.0 x 18.5cm + added 5cm high platform below	56	No	Dominant > Subordinate	Haemisch, Voss, & Gärtner (1994)
<b>BALB/c ByJ</b>	5	23 x 16cm	84	No	Dominant > Subordinate	(Merlot et al., 2004)
<b>CFW</b>	4	36 x 24cm	1	No	Subordinate > Dominant	(Louch and Higginbotham, 1967)
<b>CF-1</b>	4	N/A	3	No	Subordinate > Dominant	(Bronson, 1973)
<b>CBA/J</b>	15 (5 M, 10 F)	Eight 23 x 11 x 11cm inter-connected cages	14	Yes	Subordinate > Dominant	(Ely and Henry, 1978)
<b>CBA/J</b>	15 (5 M, 10 F)	Eight 23 x 11 x 11cm inter-connected cages	42	Yes	Subordinate > Dominant	Ely & Henry (1978)
<b>Albino TO</b>	2	30 x 22 x 11cm	7	No	Dominant = Subordinate	(Benton et al., 1978)
<b>C57BL/6J</b>	2	15 x 15 x 30cm	7	No	Dominant = Subordinate	Chapman, Desjardins, & Bronson (1969)
<b>CD-1</b>	3 (siblings)	45 x 25 x 20cm	22	No	Dominant = Subordinate	(Bartolomucci et al., 2001)
<b>CF-1</b>	4	N/A	1	No	Dominant = Subordinate	(Bronson, 1973)
<b>CF-1</b>	4	N/A	6	No	Dominant = Subordinate	(Bronson, 1973)
<b>CF-1</b>	4	N/A	14	No	Dominant = Subordinate	(Bronson, 1973)
<b>CFW</b>	4	36 x 24cm	0.25	No	Dominant = Subordinate	(Louch and Higginbotham, 1967)
<b>CFLP</b>	6	30 x 30 x 30cm	5	No	Dominant = Subordinate	Barnard, Behnke, & Sewell (1996)
<b>CBA/J</b>	15 (5 M, 10 F)	Eight 23 x 11 x 11cm inter-connected cages	105	Yes	Dominant = Subordinate	Ely & Henry (1978)

## **METHODS**

### ***Literature Search***

We manually collated as many previous studies as possible in the published literature on the relationship between social status and circulating testosterone and corticosterone levels in male mice. We searched Google Scholar, Web of Science and PubMed using a combination of search terms including “plasma testosterone” or “plasma corticosterone”, plus “social rank” or “social status” or “dominance” plus “laboratory mouse” or “*Mus*”. The search returned approximately 1500 matches. Each paper’s abstract and title was checked to identify if the paper would likely contain relevant data. If this condition was satisfied we determined if it contained findings relevant to the relationship between social rank/status and plasma corticosterone and/or testosterone. Additional relevant studies were identified by cross-referencing with citations from each relevant study. Selection criteria were that the study had to be conducted in mice housed together and the hormone assay had to be conducted on blood plasma. For each study we recorded the housing group size, whether groups were mixed sex or male only, how long mice were housed together prior to blood collection, and the type of housing environment (i.e. standard sized cages or more enriched housing systems). The search resulted in 13 studies satisfying these criteria.

### ***Husbandry***

Throughout the study, subjects were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21–24°C), humidity (30-50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red lights (dark cycle) on at 1200 hours. Mice had no visual or olfactory contact with female mice. For the vivarium groups, all mice were uniquely marked by dyeing their fur with a blue, non-toxic animal marker (Stoelting Co.). These marks remain for up to 12 weeks and only require one application, thus enabling each animal to be visually identified throughout



the study. For the dyadic portion of the study, one mouse from each pair was marked with non-toxic permanent marker on the tail in order to distinguish between the two individuals. No open wounds or signs of poor health or welfare due to competition were observed in any individuals. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol Nos: AC-AAAP5405, AC-AAAM1450).

### ***Pair Housing***

Twenty-two male, outbred Crl:CD1(ICR) (CD-1) mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 1 week in standard sized IVC cages (27 × 17 × 12 cm; 1836 cm<sup>3</sup>/animal) with pine-shavings bedding. Mouse weight ranged from 30.5 g to 36.0 g at time of arrival. At 8 weeks of age, each individual was weighed and placed in a new standard sized cage (2754 cm<sup>3</sup>/animal) with a randomly assigned non-sibling unfamiliar partner. To enable comparison with group-housed animals, we similarly paired animals that had no prior social experience with each other. Mice were observed during the dark light phase for a total of 6 hours over the course of the housing period: 1 hour directly following pairing, 1 hour on each of day 2 and 3 of pair-housing, 30 minutes on day 5 and day 12 after cage-cleaning, 1 hour on day 19, and 2 hours directly prior to taking blood (day 22) (see **Figure 3.1**). During these live observations, observers used all occurrence sampling to record the winner and loser in all instances of fighting, chasing, mounting, subordinate posture, and induced-flee behaviors (see **Supplemental Table S3.1** for an ethogram of these behaviors). At the end of the housing period (day 22), individuals were weighed and euthanized via decapitation two hours post lights-off, and trunk blood was collected into heparinized tubes. Blood was immediately placed on ice, centrifuged at 4°C in a refrigerated centrifuge, and plasma separated and frozen at -80°C until analyzed for corticosterone and testosterone levels via radioimmunoassay.

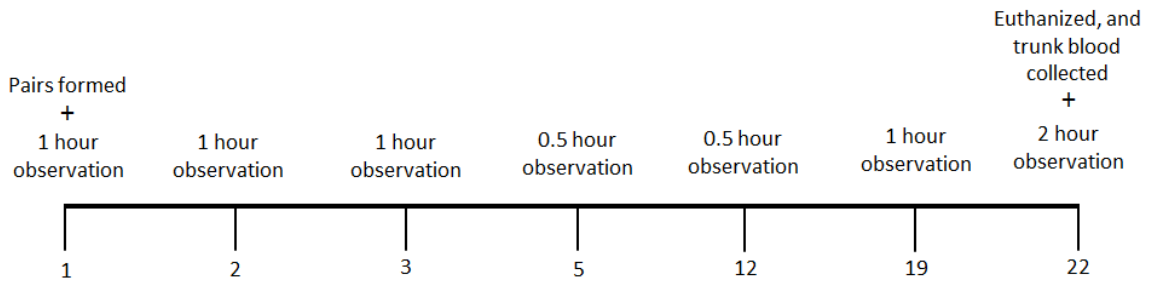
### **Large Group Housing**

A total of 240 (20 groups of 12) male, outbred Crl:CD1(ICR) mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 2 weeks in standard sized cages. Mouse weight ranged from 30.5 g to 36.0 g at time of arrival. At 9 weeks of age, groups of 12 mice were weighed and placed into large, structurally complex vivaria (length 150cm, height 80cm, width 80cm; 80,000 cm<sup>3</sup>/animal; Mid-Atlantic; **Supplemental Figure S3.1**) as described in (Williamson et al., 2016). In each group of 12 males, each male had previous social experience with a maximum of one other male and at least six males per group had no previous experience with any other male in the group. Each vivarium contains an upper level consisting of multiple shelves covered in pine-shavings bedding and a lower level consisting of a series of nestboxes filled with pine-shavings bedding, connected by tubes. Mice can explore all levels of the vivarium via a system of connected ramps. Standard chow and water were provided *ad libitum* at the top of the vivarium, encouraging movement and exploration of all the levels. Animals were placed into the vivarium just before onset of the dark cycle on Day 1 of the experiment and were observed by trained observers for 1-2 hours per day (see **Figure 3.1**). The average number of hours of observation per group over the housing period was 37.5 hours. The total number of observers used in the study was 23, with each cohort observed by between 4-11 unique observers (mean 8.4 unique observers per cohort). Inter-observer reliability was very high ( $\kappa > .99$ ). During these live observations, observers used all occurrence sampling to record the winner and loser in all instances of fighting, chasing, mounting, subordinate posture, and induced-flee behaviors (see **Supplemental Table S3.1** for an ethogram of these behaviors). Winners of each agonistic interaction were considered to be those animals that bit, chased or mounted another individual (the loser) or forced that individual to exhibit a subordinate posture or flee. All observations took place under red light during the dark cycle. At the end of group housing (occurring on average on Day  $22.2 \pm 0.6$  across cohorts) the 2 most dominant and 2 most subordinate individuals from each group were determined using the Glicko Rating System (Glickman, 1999; Williamson et al.,

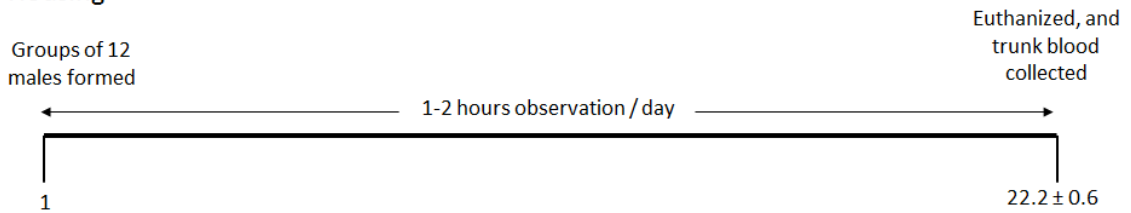
2016) and were weighed and euthanized via decapitation two hours post lights-off. Trunk blood was collected and stored prior to performing radioimmunoassays as described above. All blood was collected within 10 minutes of removing animals from the group.

**Figure 3.1. Schematic of Experimental Timeline.** Pairs of 2 males (N=11) were randomly formed on Day 1 of behavioral observations. Six hours of agonistic interaction observations were conducted as shown between Day 1 and Day 22 when animals were euthanized and trunk blood collected. Groups of 12 males (N=20) were put together on Day 1 and agonistic observations occurred for up to 2 hours per day until animals were euthanized and trunk blood collected which occurred on average on Day  $22.2 \pm 0.6$ . The average total hours of observation per group was 37.5 hours.

**Pair-Housing**



**Group-Housing**



**Hormone Assays**

Plasma testosterone and plasma corticosterone concentrations were measured using commercially available kits (MP Biomedicals) and conducted using the manufacturer’s specifications. For pair-housed animals, the average inter-assay coefficient of variation for the testosterone assay was 5.2%, the lowest

detectable was 0.09 ng/ml, and the highest detectable was 10.19 ng/ml. For the corticosterone assay, the coefficient of variation was 7.3%, the lowest detectable was 23.31 ng/ml, and the highest detectable was 972.06 ng/ml. In the pair-housed animals, one individual from one of the pairs did not yield enough plasma for the corticosterone assay, so this pair was excluded from corticosterone analyses. In one additional pair, it was not possible to determine who was dominant or subordinate and this pair were excluded from both testosterone and corticosterone analyses. For group-housed animals, samples were run in duplicate in 4 separate batches and values were averaged. For the testosterone assays, the average inter-assay coefficient of variations was 12.7%, the average lower limit of detectability for the assays was 0.10 ng/ml, and the average highest detectable was 10.93 ng/ml. For the corticosterone assays, the average inter-assay coefficient of variations was 8.7%, the average lower limits of detectability for the assays was 24.02 ng/ml and the average highest detectable was 971.38 ng/ml. Two subordinate males (one rank 11 and one rank 12) and 1 beta male did not yield enough blood for radioimmunoassay and were therefore eliminated from the analyses. The final group-housed hormone analyses contained 20 alpha males, 19 beta males, and 38 subordinate males (ranks 11 and 12). Sample sizes for hormone analysis were determined a priori based on previous research (Haemisch et al., 1994; Machida et al., 1981).

### ***Statistical Analysis***

All statistical analyses were undertaken in R version 3.3.1 (R Core Team, 2016) in RStudio version 0.99.486 (RStudio Team, 2015).

*Pair Behavioral Analysis:* The dominant and subordinate mouse within each pair was determined based on wins and losses. Dominant mice were those that consistently exhibited wins without losing in the last week of pair housing (during observations conducted on days 19 and 22). Subordinate mice were those

that consistently exhibited losses without winning in the last week of pair housing. Individuals in all pairs except one could be identified as dominant or subordinate.

*Group Behavioral Analysis:* The total number of wins and losses experienced by each individual over the course of the housing period were aggregated into frequency win/loss sociomatrices for each cohort. From these sociomatrices, we calculated the Landau's modified  $h'$  (De Vries, 1995) to confirm the presence of a linear social hierarchy (See Williamson et al., 2016 for a more detailed description). The significance of  $h'$  is determined by performing 10,000 two-step randomizations of the win/loss frequency sociomatrix and comparing the observed  $h'$  value against a simulated distribution of  $h'$ . Significant  $h'$  values indicate a linear social hierarchy. We also calculated the triangle transitivity ( $ttri$ ) of each group as a further characterization of the hierarchical organization of each cohort. In brief, this measure determines the proportion of relationships within all triads (group of three individuals) of the hierarchy that are transitive (i.e. if A is dominant over B who is dominant over C then A also is dominant over C), versus intransitive. We derived a binarized 1/0 win/loss sociomatrix from the frequency sociomatrix and used this binarized matrix to calculate  $ttri$  (see (Williamson et al., 2016). Both  $h'$  and  $ttri$  were calculated using the R package 'compete' (Curley, 2016b). Ranks of each individual in each cohort were calculated using Glicko ratings. Briefly, all individuals in each group start with the same initial rating and gain or lose points following each agonistic interaction based on the rating difference between themselves and the individual they defeat or lose to (Glickman, 1999; Williamson et al., 2016). Individuals with the highest Glicko ratings are considered alpha males, those with the second highest ratings are beta males. To compare alphas, betas, and subordinate individuals across groups, we normalized Glicko scores by dividing each score by the square root of the sum of each score squared. Glicko ratings were calculated using the R package 'PlayerRatings' (Stephenson and Sonas, 2012). Stability of each social group was verified through observation of stabilization of Glicko ratings by the end of the second week. Further, the Stability Index, a metric for the overall stability of a hierarchy during a time period, was calculated using

the R package 'EloRating' (Neumann et al., 2011; Neumann and Kulik, 2014). This index analyzes rank reversals, the closer to 1 the index is, the fewer rank reversals have occurred throughout the time period being analyzed (Neumann and Kulik, 2014). Alpha males, beta males, and the two most subordinate males (ranks 11 and 12) were used in the analyses.

The despotism of each alpha male was calculated by determining the proportion of all wins over the entire observation period attributed to the alpha male. Alpha male despotism was also calculated only over the final two days by calculating the proportion of all wins over the final two days attributed to the alpha male. Social hierarchies with alpha males having despotism scores  $>0.5$  were considered to be highly despotic whereas alpha males with despotism scores  $<0.5$  were considered to have low despotism (See Williamson et al., 2016 for a more detailed description). To compare the frequency of wins/hour and losses/hour between animals of different social ranks in high and low despotism cohorts, we performed unpaired Wilcoxon rank sum tests.

*Hormone Analysis:* To test the relationship between plasma corticosterone or testosterone levels and pair social status we ran generalized linear mixed effect models (GLMM). We specified each hormone level as the outcome variable, social status as a fixed effect and pair ID as a random effect. To examine the relationship between plasma corticosterone or testosterone levels and dominance rank across all social hierarchies, we ran a GLMM with each hormone level as the outcome variable, social status as a fixed effect and cohort and hormone batch as random effects. To examine the relationship between social status and plasma corticosterone or testosterone in high vs. low despotism social hierarchies, we ran the same GLMM as above for each group (high vs. low despotism). To examine the effect of housing condition (pair vs. group) on hormone levels, we ran generalized linear models separately for alpha and subordinate males.

The relationship between social status and body weight or body weight change in paired-housed animals was assessed using a paired Wilcoxon Signed Rank Test. In group housed individuals, social rank effects on body weight were examined by running a GLMM with initial body weight or body weight change as the outcome variable, social status as a fixed effect and cohort as a random effect. To examine the relationship between body weight and circulating hormone levels, we ran GLMMs with hormones as outcome variables and initial body weight or body weight change as predictor variables with pair ID and social status as random factors in pair-housed animals and hormone batch, cohort and social status as random factors in group-housed animals.

Appropriate GLMMs were used for each analysis according to the distribution of both data and residual from fitted models. For models with corticosterone as the outcome variable, we ran a normal GLMM using the R package 'lme4' (Bates et al., 2015). For models with testosterone as the outcome variable we ran a GLMM with multivariate normal random effects using Penalized Quasi-Likelihood with the R package 'MASS' (Ripley, Brian et al., 2016) and specifying the family lognormal. We used the package 'lmeRTest' (Kuznetsova et al., 2015) to derive p-values for GLMMs and assess statistical significance by evaluating beta coefficients and p-values following standard criteria (Bates et al., 2015).

### *Effect Sizes*

For all Wilcoxon rank sum tests, effect sizes were calculated with the formula  $r = \frac{z}{\sqrt{N}}$ . An  $r$  value below 0.3 indicates a low effect, between 0.3 and 0.5 indicates a moderate effect, between 0.5 and 0.7 indicates a large effect.

## RESULTS

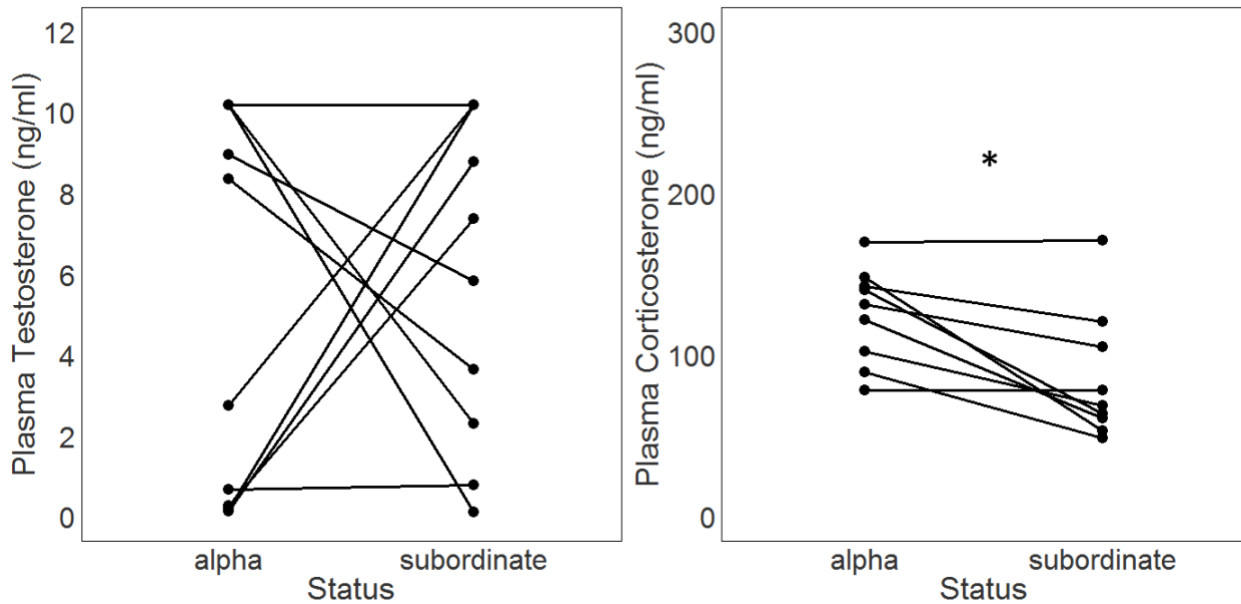
### *Hormone Relationships in Pair-Housed Males:*

After 22 days of paired housing, 10 of 11 pairs of mice formed unambiguous dominant/subordinate relationships, with one individual consistently winning fights and one individual consistently losing fights and demonstrating subordinate postures during the final week of paired housing. The pair that did not form a clear dominant/subordinate relationship was excluded from the analysis. Neither initial body weight (Dominants =  $32.69 \pm 0.43\text{g}$  vs Subordinates =  $31.51 \pm 0.53\text{g}$ ;  $V = 42$ ,  $p = 0.160$ ,  $r = 0.33$ ) nor body weight change over the housing period (Dominants =  $5.50 \pm 0.62\text{g}$  vs Subordinates =  $5.80 \pm 0.60\text{g}$ ;  $V = 23$ ,  $p = 1.000$ ,  $r = 0.02$ ) was associated with social status. Over the course of the 6 hours of observation over the housing period, a mean of  $17.2 \pm 2.5$  fights per pair were observed (range 5-40). Dominant males won an average of  $2.27 \pm 1.84$  wins/hour compared to subordinates winning  $0.35 \pm 0.57$  wins/hour. No clear relationship existed between social status and plasma testosterone levels (**Figure 3.2A**, GLMM:  $\beta = 0.134 \pm 0.353$ ,  $N = 20$ ,  $p = 0.712$ ). There was, however, a significant relationship between plasma corticosterone levels and social status, with dominant individuals in pairs having higher levels of corticosterone than subordinates (**Figure 3.2B**, GLMM:  $\beta = -36.358 \pm 11.137$ ,  $N = 18$ ,  $p = 0.013$ ). Neither initial body weight or body weight change were associated with testosterone or corticosterone levels (GLMMs: all  $p > 0.200$ ).



**Figure 3.2. Testosterone and Corticosterone in Pair-housed Males.** Plasma testosterone (N = 10 pairs) (A) and plasma corticosterone (N = 9 pairs) (B) levels in dominant and subordinate pair-housed males. Lines connect individuals housed in each pair. \* difference between dominant and subordinate males  $p < 0.05$ .

Figure 2



*Social Hierarchy Behavior:*

All 20 cohorts of 12 males formed significantly stable, linear dominance hierarchies, as measured by Landau's  $h'$  value, triangle transitivity values, calculation of Neumann's stability index (**Table 3.3**), and verification of stable Glicko scores across the final three days. Further, no alpha male lost a fight in the final week, verifying the stability of our alpha males. The average number of aggressive interactions per group over the housing period was 993.2, with a standard deviation of 295.5. As each hierarchy was linear, we determined individual ranks and the normalized Glicko scores of each social status group (**Table 3.4**). We determined that 9/20 alpha males had despotism scores  $>0.5$  and were considered as having high

despotism. The remaining 11/20 alpha males had despotism scores  $<0.5$  and were considered as having low despotism. Highly despotic alpha males won significantly more fights per hour than low despotism alpha males (**Figure 3.3A**,  $W = 87.5$ ,  $p = 0.004$ ,  $N = 20$ ,  $r = 0.64$ ). Subordinate males in low despotism groups won significantly more fights per hour than subordinate males in high despotism groups (**Figure 3.3A**,  $W = 114$ ,  $p = 0.028$ ,  $N = 38$ ,  $r = 0.36$ ). There is also a trend towards beta males in low despotism groups winning more fights per hour than beta males in high despotism groups ( $W = 21$ ,  $p = 0.053$ ,  $N = 19$ ,  $r = 0.45$ ). There was no statistically significant difference in the frequency of losses per hour for alpha or beta males between the high and low despotism groups (alphas:  $W = 35$ ,  $p\text{-value} = 0.287$ ,  $N = 20$ ,  $r = 0.25$ ; betas:  $W = 51$ ,  $p\text{-value} = 0.661$ ,  $N = 19$ ,  $r = 0.11$ ). There is a trend for subordinate males in highly despotic groups to experience fewer losses per hour than subordinate males in low despotism groups (**Figure 3.33B**,  $W = 114$ ,  $p\text{-value} = 0.060$ ,  $N = 38$ ,  $r = 0.31$ ). There were no significant differences between social ranks in initial body weight, though subordinate males had a trend towards lower initial body weight than alpha and beta males (Alphas =  $34.06 \pm 0.49\text{g}$ , Betas =  $34.16 \pm 0.46\text{g}$ , Subordinates =  $33.23 \pm 0.30\text{g}$ ; GLMM: alphas vs. betas:  $\beta = 0.105 \pm 0.556$ ,  $N = 80$ ,  $p = 0.851$ ; alphas vs. subordinates:  $\beta = -0.823 \pm 0.481$ ,  $N = 80$ ,  $p = 0.093$ ; betas vs. subordinates:  $\beta = -0.928 \pm 0.481$ ,  $N = 80$ ,  $p = 0.059$ ). Change in body weight over the housing period was not different between ranks (Alphas =  $3.43 \pm 0.39\text{g}$ , Betas =  $3.50 \pm 0.34\text{g}$ , Subordinates =  $2.96 \pm 0.41\text{g}$ ; GLMM: alphas vs. betas:  $\beta = 0.070 \pm 0.634$ ,  $N = 80$ ,  $p = 0.913$ ; alphas vs. subordinates:  $\beta = -0.470 \pm 0.553$ ,  $N = 80$ ,  $p = 0.399$ ; betas vs. subordinates:  $\beta = -0.540 \pm 0.553$ ,  $N = 80$ ,  $p = 0.333$ ).

**Table 3.3 – Variation in Measures of Social Hierarchy Dynamics**

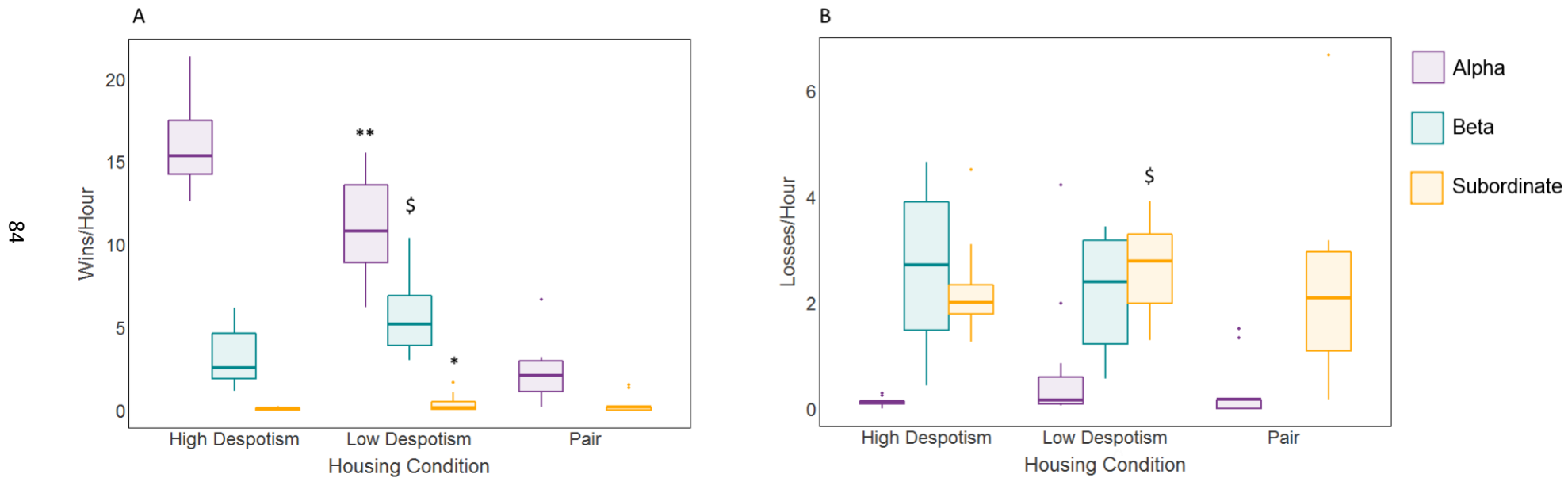
	<i>Linearity (h') (all p = 0)</i>	<i>Triangle Transitivity (all p &lt; 0.001)</i>	<i>Despotism</i>	<i>Stability Index</i>
<b>Median</b>	0.77	0.85	0.48	0.87
<b>Max</b>	0.98	1	0.80	0.94
<b>Min</b>	0.54	0.63	0.23	0.79
<b>Interquartile Range</b>	0.72-0.87	0.79-0.92	0.38-0.66	0.84-0.89

**Table 3.4 – Normalized Glicko scores**

	<i>Alpha (rank 1)</i>	<i>Beta (rank 2)</i>	<i>Sub1 (rank 11)</i>	<i>Sub2 (rank 12)</i>
<b>Median</b>	0.41	0.35	0.23	0.22
<b>Max</b>	0.44	0.36	0.25	0.23
<b>Min</b>	0.38	0.31	0.19	0.18
<b>Interquartile Range</b>	0.40-0.42	0.34-0.35	0.22-0.23	0.20-0.22

**Figure 3.3. Rate of Wins and Losses by Housing Condition and Despotism.** Wins (A) and Losses (B) per hour by social status in high and low despotism hierarchies and pair-housed animals. Boxplots show medians, interquartile ranges and 95% confidence intervals. Purple = alpha males, Green = beta males, Orange = subordinate males. High despotism N = 9 groups (9 alpha, 9 beta, 17 subordinate); low despotism N = 11 groups (11 alpha, 10 beta, 21 subordinate males); pairs N = 11 groups (11 alpha, 11 subordinate males). Significant differences in behavior rates between high despotism and low despotism hierarchies are shown - \*\* p < 0.01, \* p < 0.05, \$ p < 0.10.

Figure 3



### *Hormone Relationships in Group-Housed Males:*

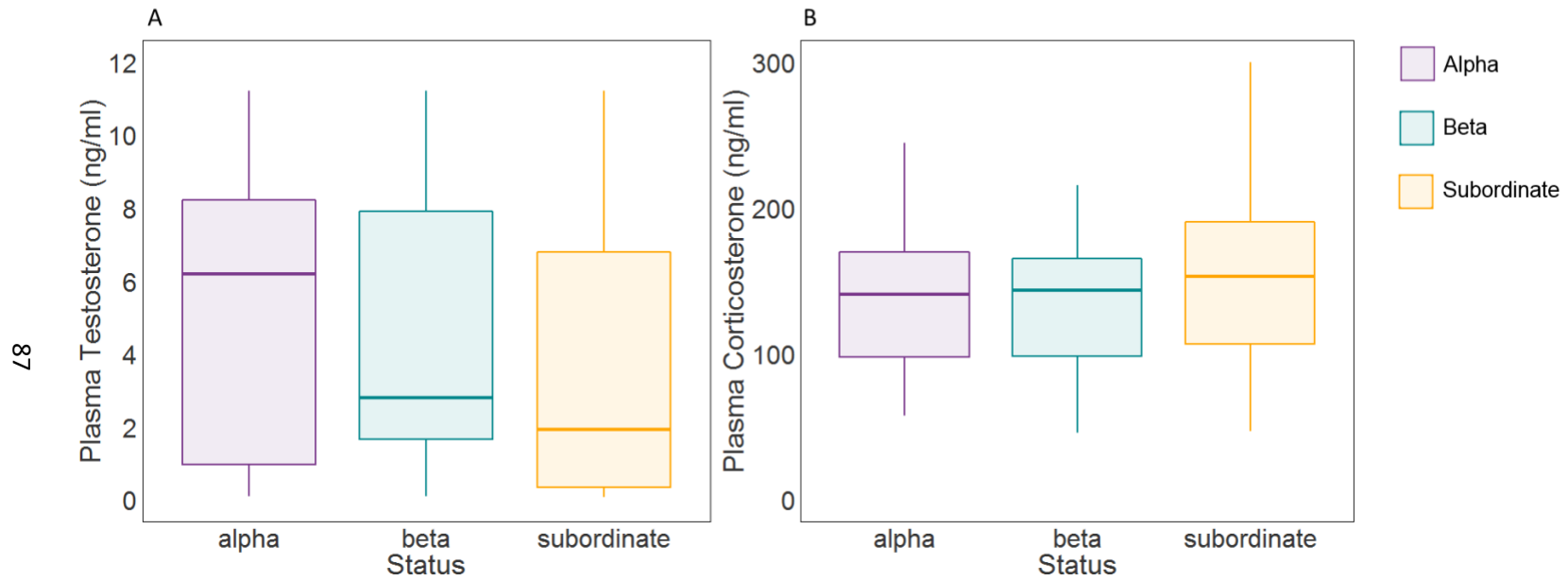
Neither initial body weight (GLMM:  $\beta = -0.491 \pm 0.602$ ,  $N=77$ ,  $p=0.418$ ) or body weight change ( $\beta = 0.140 \pm 0.656$ ,  $N=77$ ,  $p=0.831$ ) were associated with testosterone levels. There was also no difference in plasma testosterone levels between alpha and beta males or between beta and subordinate males across all hierarchies (**Figure 3.4A**, GLMM: alphas vs. betas:  $\beta = -0.182 \pm 0.252$ ,  $N = 77$ ,  $p = 0.473$ ; betas vs. subordinates:  $\beta = -0.134 \pm 0.251$ ,  $N = 77$ ,  $p = 0.594$ ). Alpha males did have higher levels of plasma testosterone than subordinate males (GLMM: alphas vs. subordinates:  $\beta = -0.316 \pm 0.224$ ,  $N = 77$ ,  $p = 0.163$ ), but this was not significant. When considering high vs. low despotism groups separately, there was a strong relationship between dominance rank and testosterone levels in highly despotic groups, with subordinate males showing significantly lower levels of testosterone than alpha males and moderately lower levels of testosterone than beta males (**Figure 3.5A**, GLMM: alphas vs. subordinates:  $\beta = -0.908 \pm 0.383$ ,  $N = 35$ ,  $p = 0.025$ ; betas vs. subordinates:  $\beta = -0.723 \pm 0.403$ ,  $N = 35$ ,  $p = 0.083$ ). In these highly despotic groups, there was no difference between alpha and beta male testosterone levels (GLMM: alphas vs. betas:  $\beta = -0.186 \pm 0.291$ ,  $N = 35$ ,  $p = 0.528$ ). There was no effect of dominance rank on testosterone levels in low despotism groups (GLMM: alphas vs. betas:  $\beta = -0.254 \pm 0.322$ ,  $N = 42$ ,  $p = 0.436$ ; alphas vs. subordinates:  $\beta = 0.161 \pm 0.228$ ,  $N = 42$ ,  $p = 0.486$ ; betas vs. subordinates:  $\beta = 0.415 \pm 0.282$ ,  $N = 42$ ,  $p = 0.150$ ). Subordinate males in the low despotism group showed significantly higher levels of plasma testosterone than subordinate males in the high despotism group (GLMM:  $\beta = 1.372 \pm 0.379$ ,  $N = 38$ ,  $p = 0.001$ ). When only considering despotism over the final two days the same effects were observed (**Supplemental Figure S3.2A**).

Initial body weight was not associated with corticosterone levels (GLMM:  $\beta = 0.001 \pm 0.004$ ,  $N=77$ ,  $p=0.802$ ). However, animals of all ranks that gained less body weight over the housing period had significantly higher corticosterone levels ( $\beta = -0.013 \pm 0.004$ ,  $N=77$ ,  $p=0.003$ ). There was no relationship between plasma corticosterone levels and social rank across all hierarchies (**Figure 3.4B**, GLMM: alphas

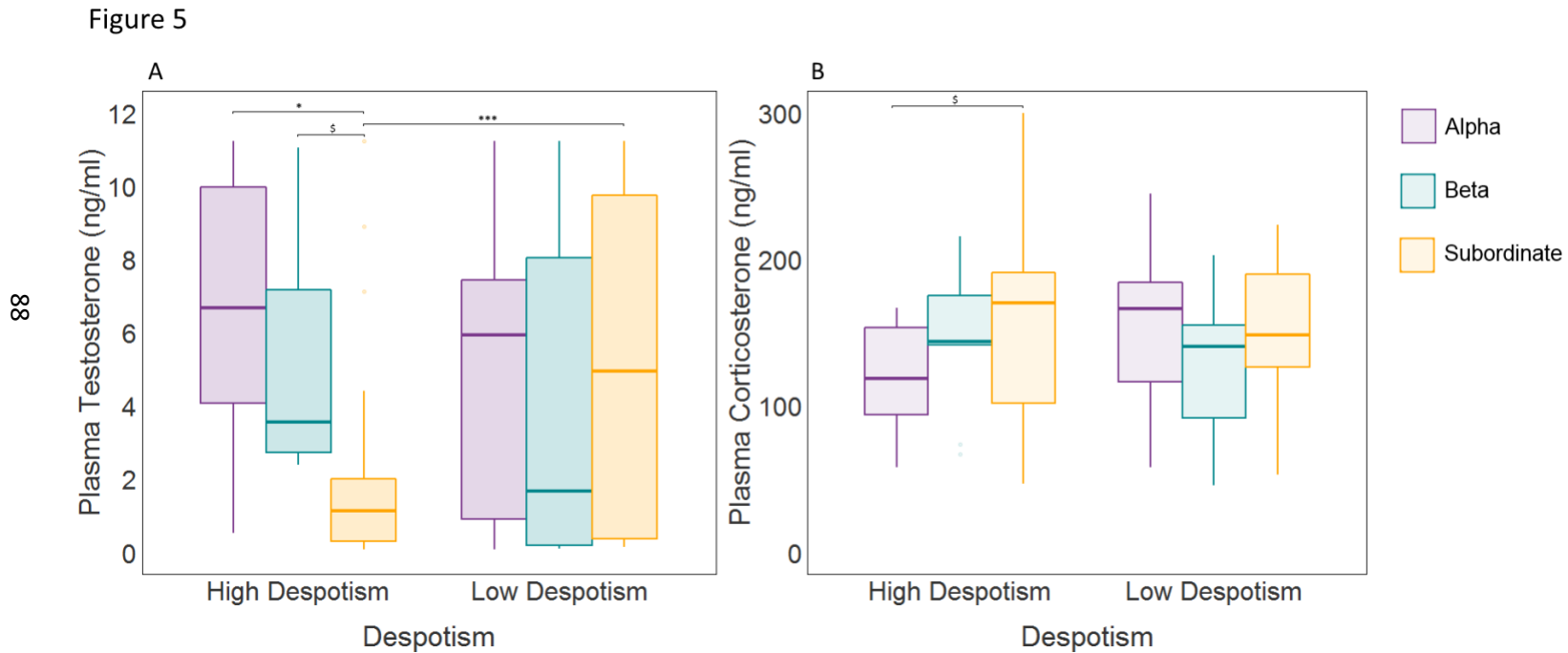
vs. betas:  $\beta = 1.935 \pm 15.657$ ,  $N = 77$ ,  $p = 0.902$ ; alphas vs. subordinates:  $\beta = 13.712 \pm 13.503$ ,  $N = 77$ ,  $p = .313$ ; betas vs. subordinates:  $\beta = 11.778 \pm 13.728$ ,  $N = 77$ ,  $p = 0.394$ ). In high despotism hierarchies, alpha males had marginally lower levels of corticosterone than subordinate animals (**Figure 3.5B**, GLMM: alphas vs. betas:  $\beta = 30.594 \pm 23.943$ ,  $N = 35$ ,  $p = 0.214$ ; alphas vs. subordinates:  $\beta = 38.271 \pm 20.957$ ,  $N = 35$ ,  $p = 0.080$ ; betas vs. subordinates:  $\beta = 7.677 \pm 20.957$ ,  $N = 35$ ,  $p = 0.717$ ). There was no significant relationship between social rank and corticosterone levels in the low despotism group (GLMM: alphas vs. betas:  $\beta = -22.219 \pm 20.518$ ,  $N = 42$ ,  $p = 0.286$ ; alphas vs. subordinates:  $\beta = -6.579 \pm 17.470$ ,  $N = 42$ ,  $p = 0.709$ ; betas vs. subordinates:  $\beta = 15.64 \pm 18.04$ ,  $N = 42$ ,  $p = 0.392$ ). When only considering despotism over the final two days the same effects were observed (**Supplemental Figure S3.2B**).

**Figure 3.4. (A) Plasma Testosterone and (B) Plasma Corticosterone levels by social rank across all hierarchies.** Boxplots show medians, interquartile ranges and 95% confidence intervals. Purple = alpha males (N=20), Green = beta males (N=19), Orange = subordinates (N=38).

Figure 4



**Figure 3.5. Plasma Testosterone and Corticosterone Levels in Hierarchies that Vary in Despotism** Plasma testosterone (A) and plasma corticosterone (B) levels by social rank in high and low despotism hierarchies. Boxplots show medians, interquartile ranges and 95% confidence intervals. Purple = alpha males, Green = beta males, Orange = subordinate males. High despotism N = 9 groups (9 alpha, 9 beta, 17 subordinate males); low despotism N = 11 groups (11 alpha, 10 beta, 21 subordinate males). Significant differences between groups are shown - \*\*\*  $p \leq 0.001$ , \*  $p < 0.05$ , \$  $p < 0.10$ .





### *Hormone Levels in Pair-Housed Versus Group-Housed Males:*

There were no significant differences in plasma testosterone levels between pair and group-housed animals (GLM: alphas -  $F_{1,27} = 0.221$ ,  $p = 0.642$ ,  $N = 30$ ; subordinates -  $F_{1,45} = 3.011$ ,  $p = 0.090$ ,  $N = 48$ ). Pair-housed subordinate males had significantly lower plasma corticosterone levels than subordinate males from both high and low despotism groups (pairs:  $85.710 \pm 13.261$  ng/ul,  $N = 9$ ; groups:  $149.769 \pm 10.131$  ng/ul,  $N = 38$ ; GLM:  $F_{1,47} = 4.923$ ,  $p = 0.032$ ,  $N = 47$ ). Alpha males had equivalent levels of corticosterone regardless of housing condition (pairs:  $124.901 \pm 9.904$  ng/ul; groups:  $136.660 \pm 8.377$  ng/ul; GLM:  $F_{1,27} = 0.180$ ,  $p = 0.675$ ).

## **DISCUSSION**

We found no relationship between dominance rank and plasma testosterone levels in pair-housed male mice. This finding is consistent with the majority of published studies in mice (Barnard et al., 1996; Ely, 1981; Hilakivi et al., 1989; Machida et al., 1981; Selmanoff et al., 1977). We also found no simple linear relationship between plasma testosterone levels and social rank across all social hierarchies. However, we did find a significant relationship between social status and plasma testosterone levels in hierarchies characterized by high alpha male despotism. Alpha males in these hierarchies won more fights per hour and won between 60-80% of all fights that occurred compared to between 20-40% by alpha males in low despotism hierarchies. In these high despotism hierarchies, alpha males had significantly higher plasma testosterone than subordinate males, whereas in low despotism hierarchies, alpha, beta, and subordinate males showed no differences in plasma testosterone levels, with subordinate males in low despotism groups showing elevated testosterone levels when compared to subordinate males in high despotism groups. Elevated levels of endogenous testosterone in highly dominant alpha males versus subordinate animals have been shown in other group-living rodents such as rats and guinea pigs (Monder et al., 1994; Sachser, 1987; Sachser and Pröve, 1986).

Previously, we have shown that highly despotic alpha males are especially effective at suppressing acts of aggression from more subordinate individuals towards other males within the social group (Curley, 2016a; Williamson et al., 2017). The current findings suggest that the presence of highly despotic alpha males may physiologically suppress subordinate males in the group, leading them to have significantly lower levels of plasma testosterone. This may be similar to African cichlid fish, where dominant males in social hierarchies have high levels of testosterone, estradiol, and 11-ketotestosterone, and are reproductively active, while subordinate fish are reproductively suppressed with nearly nonexistent levels of these HPG-regulated hormones (Maruska and Fernald, 2013). This type of reproductive suppression has been shown to exist in mammalian systems as well, in dwarf mongooses and meerkats (Creel et al., 1992; O’Riain et al., 2000). While subordinate mice are not completely reproductively suppressed, there is evidence that more subordinate individuals have a down-regulated hypothalamic-pituitary-gonadal axis resulting in lower seminal vesicle weight, decreased testes weight and decreased sperm motility (Bronson and Eleftheriou, 1964; Koyama and Kamimura, 1998; McKinney and Desjardins, 1973). The suppression of testosterone production in subordinate mice in highly despotic social hierarchies is consistent with these findings. In hierarchies characterized by lower despotism, increased levels of inter-male agonistic competition occurred throughout the group, leading to a more equitable distribution of power. Notably, subordinate males in these low despotism groups are winning significantly more aggressive encounters per hour than their counterparts in the highly despotic group. Although the total number of aggressive behaviors engaged in by subordinates is still low, it is six times higher on average than in subordinates from the high despotism group, who often completely inhibit their aggression. The higher levels of testosterone found in subordinate males in the low despotism group suggests that there is no suppression of testosterone production in these subordinate males. These individuals still exhibit meaningful levels of aggression likely because there exists greater inter-male competition and potential for all individuals to

rise up the hierarchy. This is consistent with findings from both African cichlid fish and mice where recently social ascended males have elevated plasma testosterone (Fernald, 2014; Maruska and Fernald, 2013; Williamson et al., 2017). Further, although it has been demonstrated that testosterone is necessary for hierarchy formation (Luttge, 1972; van den Berg et al., 2015) our findings suggest that elevated testosterone levels above those of other ranks are not necessary for a dominant male to maintain his alpha status once it has been attained.

Dominant pair-housed individuals had significantly higher plasma corticosterone levels than their subordinate partners. This finding is consistent with two other mouse studies (Haemisch et al., 1994; Merlot et al., 2004) as well as other studies of group-living rodents such as rats and guinea-pigs (McEwen et al., 2015; Monder et al., 1994; Sachser and Lick, 1989), but is inconsistent with the majority of previous studies in mice (**Table 3.2**). It has been assumed that higher levels of glucocorticoids should be observed in those animals experiencing the highest levels of social stress, which typically is expected to be subordinates (Sapolsky, 1992). Alternatively, dominant males have been found to have higher corticosterone than subordinates in a number of species including African wild dogs, naked mole rats, marmosets and dwarf mongooses (Abbott, 1993; Clarke and Faulkes, 1997; Creel et al., 1992; de Villiers et al., 1997), with it being argued that this elevation is related to the arousal and activation of agonistic and other behaviors. However, our pair-housed males do not engage in high levels of fighting (an average of only about 2.3 fights per hour), resulting in fewer losses being experienced by the subordinates when compared to our group-housed animals. Notably, those studies in mice that report subordinate males having higher levels of corticosterone than dominant males are those where animals have only been housed together for 1-3 days (Bronson, 1973; Louch and Higginbotham, 1967), or when males are co-housed with females (Ely & Henry 1978). In both of these contexts, there is likely to be relatively higher and consistent levels of ongoing conflict and rank uncertainty between males. Those studies that report

higher levels of basal corticosterone in dominant compared to subordinate males are in small groups of males that have been housed together for several weeks (Haemisch et al., 1994; Merlot et al., 2004) such as our study. Differences in other contextual variables may also be responsible for variability in findings. For instance, pair-housed animals have much reduced space available with no possibility for animals to avoid each other compared to group-housed animals. Dominant male mice exhibit higher levels of locomotor activity (Bartolomucci et al., 2001) and patrolling behavior (Williamson et al., 2016) than subordinate males, so it is possible that the observed elevated corticosterone in dominant versus subordinate pair-housed males is related to these males attempts to exhibit these behaviors. We propose that the higher basal corticosterone observed here in dominant males in pairs represents differences in arousal of non-agonistic behavior such as activity between dominant and subordinate males rather than differences in stress response related to social status conflict.

No straightforward linear relationship between social rank and plasma corticosterone levels was observed in social groups, although alpha males did have lower plasma corticosterone than subordinate males in highly despotic social hierarchies. Further, when comparing pair-housed and group-housed animals, subordinates in group housing had significantly higher plasma corticosterone than subordinates living in pair housing. These findings illustrate the complex association between endogenous corticosterone and social status. We suggest that differences in social context may account for the observed differences in this relationship. Living in groups appears to be particularly stressful for subordinate mice who lose far more fights and are significantly more socially suppressed than when living in pairs especially when the hierarchy is dominated by a highly despotic alpha males (Curley, 2016a; Williamson et al., 2017). Similar high levels of corticosterone are observed in males who experience repeated losses in the form of acute and chronic social defeat (Keeney et al., 2006, 2001; Pizarro et al., 2004). We also found that animals of all social statuses gained similar amounts of body weight in both pair-housing and group-housing. This is

in contrast to male rats living in groups in the visible burrow system where socially subordinate animals lose body weight (Nguyen et al., 2007; Tamashiro et al., 2007). Nevertheless, across all ranks, animals that gained less weight over the group-housing, but not pair-housing, period had significantly elevated levels of corticosterone. It is possible that other social stresses of group living independent of social status may result in both reduced body weight gain and higher endogenous corticosterone.

## **CONCLUSION**

We found no evidence for simple relationships in stable social hierarchies between social rank and either plasma testosterone or plasma corticosterone without further examining social context. In hierarchies that contained highly despotic alpha males, these alpha males had higher levels of plasma testosterone and lower levels of plasma corticosterone than subordinate males. In hierarchies with less despotic alpha males, individuals of other ranks engaged in more competitive agonistic interactions than in hierarchies with highly despotic alpha males. Subordinate males in these hierarchies also had higher levels of testosterone than subordinate males in highly despotic hierarchies. Subordinates living in hierarchies also experienced more social defeats and had significantly higher plasma corticosterone than pair-housed subordinates in stable dyadic relationships. These pair-housed subordinates likely experienced less overall social stress and indeed these males also had lower plasma corticosterone than pair-housed dominant males. These findings reinforce the importance of looking at the unique contextual characteristics of a specific social network when examining the physiological correlates of dominant or subordinate social status.

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## SUPPLEMENTAL MATERIAL

### Supplemental Table S3.1. Mouse Social Behavior Ethogram

During observations, observers code all agonistic interactions occurring between any two individuals. As multiple behaviors may occur during the same interaction, observers record the behaviors with the highest priority. For instance, if one animal fought another animal who responded by fleeing, this would be recorded as a 'Fighting' event only, as 'Fighting' takes priority to the co-occurring 'Induced-Flee'. If an animal fled when approached but was not attacked by another animal, then this would be recorded as 'Induced-Flee'. Similarly, if an animal displayed subordinate posture following a chase, this would be recorded as 'Chasing', because 'Chasing' takes priority over 'Subordinate Posture'. 'Subordinate Posture' and 'Induced-Flee' are only recorded if they occur in the absence of fighting, chasing, or mounting. These two subordinate behaviors do not co-occur so are given equal priority.

Priority	Behavior	Description
1	Fighting	The focal individual lunges at and/or bites the target individual.
2	Chasing	The focal individual follows the target individual rapidly and aggressively while the other individual attempts to flee.
3	Mounting	The focal individual mounts another individual from behind.
4=	Subordinate posture	The focal individual responds to the approach from another individual by remaining motionless and/or exposing their nape.
4=	Induced-Flee	The focal individual flees without any aggression shown by another individual.

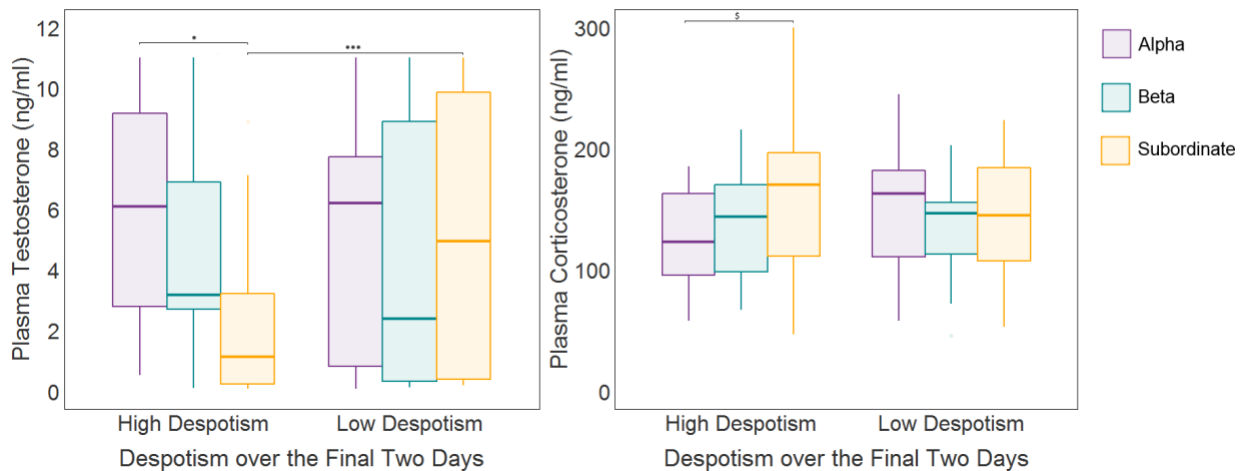
**Supplemental Figure S3.1.** Housing Vivarium.

Each individual was weighed and placed into a large custom built mouse vivarium (length 150cm, height 80cm, width 80cm; Mid-Atlantic). Vivaria consist of multiple shelves, nest boxes, and a metal backboard containing multiple holes for air circulation. Mice could explore and access each shelf and cage via ramps and tunnels. Standard chow and water were provided ad libitum at the top of the vivarium. Multiple enrichment objects such as plastic igloos and round tubes were also provided. Pine shaving bedding was used to cover the shelves and nestboxes in each vivarium.



**Supplemental Figure S3.2 A) Plasma Testosterone and B) Plasma Corticosterone levels by social rank in high and low despotism hierarchies as determined by behavior only during the final two days of group-housing.** Boxplots show medians, interquartile ranges and 95% confidence intervals. Purple = alpha males, Green = beta males, Orange = subordinates. Results of GLMMs: High despotism testosterone - alphas vs. betas:  $\beta = -0.224 \pm 0.302$ ,  $N = 39$ ,  $p = 0.462$ ; alphas vs. subordinates:  $\beta = -0.838 \pm 0.367$ ,  $N = 39$ ,  $p = 0.029$ ; betas vs. subordinates:  $\beta = -0.613 \pm 0.394$ ,  $N = 39$ ,  $p = 0.129$ . Low despotism testosterone - alphas vs. betas:  $\beta = -0.230 \pm 0.332$ ,  $N = 38$ ,  $p = 0.493$ ; alphas vs. subordinates:  $\beta = -0.153 \pm 0.239$ ,  $N = 38$ ,  $p = 0.528$ ; betas vs. subordinates:  $\beta = -0.383 \pm 0.290$ ,  $N = 38$ ,  $p = 0.196$ . High-despotism subordinate male testosterone vs. low-despotism subordinate male testosterone:  $\beta = 1.320 \pm 0.367$ ,  $N = 38$ ,  $p = 0.001$ . High despotism corticosterone - alphas vs. betas:  $\beta = 17.410 \pm 22.600$ ,  $N = 39$ ,  $p = 0.446$ ; alphas vs. subordinates:  $\beta = 34.550 \pm 19.750$ ,  $N = 39$ ,  $p = 0.089$ ; betas vs. subordinates:  $\beta = 17.140 \pm 19.750$ ,  $N = 39$ ,  $p = 0.392$ . Low despotism corticosterone - alphas vs. betas:  $\beta = -13.736 \pm 21.954$ ,  $N = 38$ ,  $p = 0.536$ ; alphas vs. subordinates:  $\beta = -7.185 \pm 18.654$ ,  $N = 38$ ,  $p = 0.699$ ; betas vs. subordinates:  $\beta = 6.451 \pm 19.322$ ,  $N = 38$ ,  $p = 0.741$ . Significant differences between groups are shown - \*\*\*  $p \leq 0.001$ , \*  $p < 0.05$ , §  $p < 0.10$ .

Supplementary Figure 2



## **CHAPTER 4 – Neuroendocrine mechanisms of stable social hierarchy dynamics in female mice**

Cait M. Williamson, Alexandra R. Decasien, Alesi Lanham, Russell D. Romeo, James P. Curley

## **ABSTRACT**

While the neurobiology of male dominance hierarchies is an extensive area of study across species, significantly less work has been done studying female social hierarchies. We have previously shown that groups of 12 CD-1 male mice form significantly linear dominance hierarchies and that one's position within the hierarchy is associated with brain gene expression and plasma hormone levels. Here, using the same behavioral paradigm we use in our study of male social groups, we examine the behavioral characteristics of female dominance hierarchies, comparing their behavior to that of males. We further investigate the relationship between estrous state and social dominance behavior, as well as analyze the associations between dominants status and plasma corticosterone, plasma estradiol, and estrogen mediated gene expression in the ventromedial hypothalamus and medial preoptic area. We show that females form significantly linear dominance hierarchies but that their characteristics differ from those of males and that estrous state does play a role in dominance behaviors. We further conclude that subordinate females exhibit higher plasma corticosterone and higher ER $\beta$ , PR, and OPRM1 mRNA levels than dominant females. In addition contributing to our knowledge of female social behavior, this work determines that there are significant sex differences in dominance hierarchy dynamics, potentially due to behavioral differences that exist across the estrous cycle, and that estrogen plays an important role in social hierarchy behaviors in females.

## INTRODUCTION

While the study of the neurobiology of male aggression and social dominance is a rich area of research across species (Lenkov, Lee, Lenkov, Swafford, & Fernald, 2015; Karen P. Maruska & Fernald, 2011; Rosvold, H. Enger, Mirsky, & Pribram, 1954; Wang et al., 2011; Williamson, Franks, & Curley, 2016a; Williamson, Romeo, & Curley, 2017), female aggression and social dominance have not been examined to the same extent. Much of the work on female aggression has focused specifically on maternal aggression, which is expressed by females when they are pregnant or early postpartum in order to defend their offspring (Erskine, Barfield, & Goldman, 1978; Haney, Debold, & Miczek, 1989). Maternal aggression is a fascinating phenomenon that occurs throughout the animal kingdom, however, it is a specific behavior driven by specific mechanisms that cannot be assumed to be the same as those that drive more general aggressive tendencies and dominance behaviors (DeVries, Winters, & Jawor, 2015; Figler, Twum, Finkelstein, & Peeke, 1995; Sinn, While, & Wapstra, 2008). If we want to have a full understanding of the neurobiology of social behavior, it is imperative to study typical social behavior in a more general context in females.

Female aggression has been shown to be expressed in a variety of contexts separate from that of maternal aggression (Ogawa et al., 1998; Ribble & Salvioni, 1990; Kimberly A. Rosvall, 2013; Stockley & Bro-Jørgensen, 2011). Females compete for resources such as food, territory, and mates and seem to do so through a host of behaviors, from overt aggression to more complex behaviors such as affiliation, alliance formation, and altruism (Stockley & Bro-Jørgensen, 2011). It is essential to understand both these complex behaviors and the physiological mechanisms underlying them. Studying these behaviors from a dominance hierarchy perspective is appropriate, because, while formation and maintenance of dominance hierarchies involves the overt expression of aggressive behaviors, it also requires a



combination of various complex social behaviors. In order to live successfully in a linear hierarchy, individuals must be able to appraise their social context and respond appropriately to individuals of higher and lower status to them (Curley, 2016; Williamson, Lee, & Curley, 2016). In many group living species, such as vervet monkeys (Whitten, 1983), caribou (Barrette & Vandal, 1986), chimpanzees (Murray, Eberly, & Pusey, 2006), and bison (Rutberg, 1986), it has been demonstrated that females form dominant-subordinate relationships. In mice, it has been documented that females establish dominant-subordinate relationships in pairs (Schuhr, 1987; van den Berg, Lamballais, & Kushner, 2015), however this has not been studied extensively in a group context. Recent work has suggested that females are capable of asserting dominant status but that they do not necessarily form significantly linear dominance hierarchies (Weidt, Gyax, Palme, Touma, & König, 2018). The complex dynamics of their group structure have not been assessed. While it is clear that females form dominant-subordinate relationships similar to those in males, they utilize different strategies than males to attain dominant status. Males primarily compete for mates and in doing so compete for resources in order to gain access to mates, while females primarily compete for resources and compete for mates or interfere with others' mating opportunities in order to secure resources for themselves and their offspring (Stockley & Bro-Jørgensen, 2011). Previously, our lab has explored the complex group dynamics and neurobiology of male social hierarchies (Williamson, Franks, et al., 2016a; Williamson, Lee, et al., 2016; Williamson, Lee, Romeo, & Curley, 2017), however, the complex dynamics and underlying neuroendocrine and neurobiological correlates of female group social behavior have not yet been investigated in a controlled manner. While we must first understand female social behavior as its own distinct phenomenon, ultimately, our understanding of group social behavior can only be complete through a comprehensive understanding of the similarities and differences in group social strategy and dominance hierarchy structure between the sexes.

One of the reasons female social behavior has not been investigated to the same extent as that of males is the potential complication of the estrous cycle, as in rodents it has been shown to have some effect on certain types of behavior, such as behavior on anxiety tests (Palanza, Gioiosa, & Parmigiani, 2001), as well as in motivation and addiction (Roberts, Bennett, & Vickers, 1989). In primates, some accounts suggest that females attempt to ascend the hierarchy and are more aggressive during menstruation, but the picture is not entirely clear (Rowell, 1972). In rodents, some species show changes in aggressive behavior across the estrous cycle but others do not (Floody, 1983). Female California mice display changes in aggression across the estrous cycle, with aggression being greatest during diestrus and lower when females were in proestrus and estrus (Ellen S. Davis & Marler, 2003). Further, even within species, there is conflicting evidence, with certain studies concluding that aggression varies across the cycle and others determining that there is no change (Floody, 1983). For example, in rats, one study showed no change across the cycle (Barr, Gibbons, & Moyer, 1976), while another showed that females in estrus are subordinate to those not in estrus (Seward, 1945). There is limited evidence that in house mice females in proestrus and metestrus display increased levels of aggression than those in estrus or diestrus (Hyde & Sawyer, 1977). While this is clearly a complicated question that likely is dependent on various contextual factors, we must begin to understand what role, if any, estrous state has in influencing female social hierarchy behavior.

Social hierarchy behavior is modulated through an intricate system of neurobiological and neuroendocrine mechanisms. One active area of research has focused on the consequences of living in a social hierarchy on hypothalamic-pituitary-adrenal (HPA) axis activation in dominant and subordinate individuals. We have previously shown that this is a complicated, context-dependent relationship in males, with subordinate males in particularly aggressive groups exhibiting higher plasma corticosterone than dominants in those groups, but not showing any significant difference from dominant males in

groups where there is a more equitable distribution of power (Williamson, Lee, et al., 2017). In female rodents, the research is limited, but it has been shown that subordinate individuals exhibit higher levels of plasma corticosterone (Schuhr, 1987). It has also been suggested that sex differences exist in terms of how females and males respond to social stress (Haller, Fuchs, Halász, & Makara, 1999). The HPA consequences for females living in a social hierarchy remain to be elucidated.

The “Social Behavior Network” is a bidirectional circuit of brain regions associated with multiple forms of social behavior across species (Goodson, 2005; Newman, 1999). This network, which includes the medial amygdala (meA), the bed nucleus of the stria terminalis (BNST), the lateral septum (LS), the medial preoptic area (mPOA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), and the periaqueductal grey (PAG) is thought to be the core of the social brain. Given this, there is no doubt that the expression of appropriate female social hierarchy behavior requires coordinated activity throughout all of these regions. However, in female social behavior, there is particular interest in the role of the VMH. The VMH has a well-established role in promoting female sexual behavior (Aou, Oomura, & Yoshimatsu, 1988; Oomura, Aou, Koyama, Fujita, & Yoshimatsu, 1988; D W Pfaff & Sakuma, 1979; Yang et al., 2013). Further, it has been shown that the VMH is activated following aggressive encounters between female California mice (E. S Davis & Marler, 2004) and specific cell populations in the VMH are essential to regulating female aggression (Hashikawa et al., 2017). The mPOA has been found to be of particular interest in the regulation of social hierarchy behavior and aggression across species (Hammond & Rowe, 1976; Hou et al., 2016; Larson, O’Malley, & Melloni, 2006; Maruska & Fernald, 2011; Pan, Xu, Young, Wang, & Zhang, 2010; Williamson, Romeo, et al., 2017), with generally more activity in the mPOA being associated with dominant status. We have previously shown that dominant individuals within the hierarchy express higher levels of corticotropin releasing factor mRNA in the mPOA than subordinates (So, Franks, Lim, & Curley, 2015), and individuals ascending a dominance hierarchy as they transition from

subdominant to dominant status show increased levels of GnRH gene expression in the mPOA (Williamson, Lee, et al., 2016).

Estrogen and the neuropeptides and genes it regulates have emerged as critical modulators of social behavior. In the brain, estrogen acts at both estrogen receptor alpha (ER $\alpha$ ) and estrogen receptor beta (ER $\beta$ ), which are expressed throughout the social behavior network in the brain, including the ventromedial hypothalamus (VMH) and medial preoptic area of the hypothalamus (mPOA) (Cushing, 2016). Activation of these receptors results in activation of second messenger systems that can modulate cell function and enact further cascades of activation (Duque-Wilckens & Trainor, 2017; Gen Murakami, 2016; Soma, Rendon, Boonstra, Albers, & Demas, 2015). Estradiol, the major estrogen steroid hormone, has been mainly associated with promoting aggressive behaviors (Albert, Petrovic, & Walsh, 1989; K. A. Rosvall et al., 2012; Rubenstein & Wikelski, 2005), but has also been found to be involved in many facets of social behavior, such as social learning and social recognition (Ervin et al., 2015). Estradiol acts to elevate a host of gene products in the hypothalamus, including progesterone receptors (PR), oxytocin receptors (OTR), opioid receptors, and GnRH (Pfaff et al., 2000), and each of these genes has been demonstrated to play a role in regulating appropriate social behavior. Progesterone acting at progesterone receptors has been shown to modulate social recognition, with progesterone treatment impairing social recognition memory (Bychowski & Auger, 2012). In females, oxytocin has been shown to inhibit aggression, and oxytocin knock out females are more aggressive towards other females than wild-type littermates (Ragnauth et al., 2005). More generally, oxytocin has been implicated in the motivation to affiliate, perhaps acting to ameliorate aggressive tendencies (Campbell, 2008). Opioid receptors, specifically mu-opioid receptor (OPRM1), have been shown to be involved in motivation to initiate social contact, with OPRM1 knockout pups showing decreased motivation to seek out their mothers (Moles, Kieffer, & D'Amato, 2004) and OPRM1 knockout males failing to exhibit typical social exploration

behaviors (Wöhr, Moles, Schwarting, & D'Amato, 2011). Further, OPRM1 allele variations are associated with differences in sociability and social dominance (Briand et al., 2015). The relationship between GnRH expression and social hierarchy behavior has been extensively studied in males, specifically in the context of hypothalamic-pituitary-gonadal (HPG) axis activation changes in response to ascent up the hierarchy, with males who ascend to alpha status showing rapid increases in GnRH gene expression in the hypothalamus (Maruska, Zhang, Neboori, & Fernald, 2013; Maruska & Fernald, 2011; Williamson, Romeo, et al., 2017).

The current study used the paradigm previously established in our lab in the study of male social hierarchy dynamics to study female social hierarchy behavior and begin to disentangle its neurobiological and neuroendocrine mechanisms. We investigated the hierarchical structure of eight female dominance hierarchies as well as plasma corticosterone and plasma estradiol concentrations for dominant and subordinate mice within these hierarchies. We further examined gene expression differences between dominant and subordinate individuals in the ventromedial hypothalamus and medial preoptic area of the hypothalamus across six genes known to modulate various aspects of social behavior and moderated by the action of estrogen: ER $\alpha$ , ER $\beta$ , PR, OTR, OPRM1, and GnRH. This work furthers our understanding of many of the questions that remain in terms of female social behavior and the neurobiological and neuroendocrine mechanisms that underlie it.

## **METHODS**

### ***Subjects and Housing***

A total of 96 female outbred CD1 mice were obtained from Charles River Laboratories at 7 weeks of age. Mice were housed in the animal facility in the Department of Psychology at Columbia University, with

constant temperature (21-24°C) and humidity (30-50%). The room was kept on a 12/12 light/dark cycle, with white light (light cycle) on at 2400 hours and red lights (dark cycle) on at 1200 hours. All mice were uniquely marked by dyeing their fur with a blue, nontoxic animal marker (Stoelting Co.), enabling individuals to be identified throughout the study. These marks remain for up to 12 weeks and only require one application. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC Protocol No. AC-AAAP5405).

### ***Group Social Behavior Observations***

Upon arrival at the Columbia University Psychology Department animal facility, mice were housed in groups of 3 for 2 weeks in standard sized cages. At 9 weeks of age, groups of 12 mice were weighed and placed into large, structurally complex, custom built vivaria (length 150cm, height 80cm, width 80cm; Mid-Atlantic; **Supplemental Figure 4.1**). The vivaria were constructed as described in (Williamson, Lee, et al., 2016). Each vivarium consists of an upper level constructed of multiple shelves connected by plastic tubes and covered in pine bedding and a lower level comprised of 5 interconnected standard sized cages filled with pine bedding and connected by a system of plastic tubes. Mice can access all levels of the vivarium at any time through this interconnecting system of ramps and tunnels. Standard chow and water were provided ad libitum on the top level of the vivarium. Social groups were created such that in each group of 12 females, each individual had previous social experience with maximum one other individual and at least 6 females per group had no experience with any of the other individuals. Mice were placed in the vivarium at the onset of the dark cycle on Day 1 of the experiment and were observed by trained observers for 2 hours directly following introduction to the group and for 2 hours each day for the next two weeks. Observations always occurred during the dark cycle at some point during the first 6 hours of red light. During these live observations, observers used all occurrence sampling to record the winner and loser in

all instances of fighting, chasing, mounting, subordinate posture, and induced-flee behaviors (see **Supplemental Table 4.1** for an ethogram of these behaviors). Winners of each encounter were considered to be those that chased, bit, mounted, or forced another individual to exhibit subordinate behavior. This method has been used multiple times previously in our lab to understand the social organization of groups of mice (Curley, 2016; Lee, Khan, & Curley, 2017; Williamson, Franks, & Curley, 2016b; Williamson, Lee, et al., 2016, 2017; Williamson, Romeo, et al., 2017). Vaginal smears were collected from every mouse each evening at the same time of day to determine if estrus state played a role in modulating social behavior. To collect the samples, trained lab members removed mice from the vivaria individually and placed them back as soon as the sample was collected. Collecting samples from each social group interrupted the group for less than 5 minutes. Smear samples were analyzed under a microscope by a single trained lab member and double checked by a second lab member to verify accuracy. At the end of group housing, the 2 most dominant and 2 most subordinate individuals were determined using the Glicko Rating System (Glickman, 1999; Williamson, Lee, et al., 2016) as well as David's Scores (De Vries, 1995; Williamson, Lee, et al., 2016). Mice were weighed, final estrus smears taken, and euthanized via decapitation 2 hours post lights off on Day 15. Trunk blood was collected into heparinized tubes, immediately placed on ice, centrifuged at 4°C in a refrigerated centrifuge, and plasma separated and frozen at -80°C until analyzed for corticosterone and estradiol levels via radioimmunoassay. Brains were collected and flash frozen in hexane and stored at -80°C until dissection. Plasma hormone and brain mRNA levels were measured for the two most dominant and two most subordinate individuals in each group, except for in two cohorts where the top three most dominant existed in intransitive relationships, so three dominant individuals and two subordinate individuals were used.

### ***Hormone Assays***

Plasma corticosterone and plasma estradiol concentrations were measured using commercially available kits (MP Biomedicals) and conducted using the manufacturer's specifications. For the corticosterone assay, the average inter-assay coefficient of variation was 9.3%, the lowest detectable was 24.78 ng/ul, and the highest detectable was 938.34 ng/ul. For the estradiol assay, the coefficient of variation was 7.2%, the lowest detectable was 8.53 pg/ul, and the highest detectable was 2455.79 pg/ul.

### ***Gene Expression***

Brains were stored at -80° C until dissection. Samples of the medial preoptic area (mPOA) and ventromedial hypothalamus (VMH) were collected using a Harris Micro-Punch with reference to the coronal plane from the Mouse Brain Atlas (Paxinos & Franklin, 2004) and the Allen Brain Atlas (Lein et al., 2007). The mPOA was collected as one 1mm diameter area along the midline from Bregma +0.14 mm to -0.7 mm. The VMH was collected as one 1mm diameter area from each hemisphere from Bregma -1.34 mm to -1.82mm. RNA was isolated from both brain regions using the AllPrep RNA Micro Kit (Qiagen) and reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR applications. Quantitative RT-PCR was performed with 1ul of cDNA using an ABI 7500 Fast Thermal Cycler and the Fast SYBR Green Master Mix reagent (Applied Biosystems). All primer probes (Sigma-Aldrich) were designed to span exon boundaries ensuring amplification of only mRNA. For each gene,  $C_T$  values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH – endogenous control). Relative expression values were obtained by the  $\Delta\Delta C_T$  method (Schmittgen & Livak, 2008) with fold difference being determined respective to subordinates individuals. The following validated quantitative PCR primers were used for mRNA analysis: GAPDH (Forward: TGTGTCCGTCGTGGATCTGA; Reverse: CCTGCTTACCACCTTCTTGA), estrogen receptor alpha (ER $\alpha$  – Forward: CGTGTGCAATGACTATGCCTCT; Reverse: TGGTGCATTGTTTGTAGCTGG), estrogen receptor beta (ER $\beta$  – Forward:



GTCAGGCACATCAGTAACAAGGG; Reverse: ATTCAGCATCTCCAGCAGCAGGTC), progesterone receptor (PR – Forward: GCGAGAGACAACACTGCTTTCAGT; Reverse: CAAACACCATCAGGCTCATCCA), gonadotropin releasing hormone (GnRH1 – Forward: AGCACTGGTCCTATGGGTTG; Reverse: GGTTCTGCCATTTGATCCAC), oxytocin receptor (OTR – Forward: TTCTTCGTGCAGATGTGGAG; Reverse: CCAAGAGCATGGCAATGATG), opioid receptor  $\mu$  1 (OPRM1 – Forward: AATGTTTCATGGCAACCACAA; Reverse: TTTGAGCAGGTTCTCCAGT).

### ***Statistical Analysis***

All statistical analyses were undertaken in R v.3.4.0 (R Core Team, 2017).

#### *Group Dominance Structure and Social Organization*

For each cohort, five measures of dominance structure and organization were calculated: Landau's modified  $h'$ , directional consistency, steepness, triangle transitivity, and despotism. The methods of calculation for these measures are detailed in (Williamson, Lee, et al., 2016), but briefly: Landau's modified  $h'$ , directional consistency, and steepness are calculated using frequency win/loss sociomatrices, which are created using the total frequency of wins and losses recorded for each individual over the observation period. Landau's modified  $h'$  evaluates the extent to which individuals in a hierarchy can be linearly ordered (De Vries, 1995) and ranges from 0-1, with a value of 1 indicating a completely linear hierarchy. Directional consistency measures the degree to which all agonistic interactions occur in the direction from the more dominant to more subordinate individual in the pair. It also ranges from 0-1, with 1 indicating that all agonistic interactions occur in the direction of dominant to subordinate. Steepness measures the unevenness of relative individual dominance within the hierarchy. It ranges from 0-1, with 1 indicating that differences in dominance ratings between adjacently ranked individuals are maximal. A score closer to 1 indicates that power is not equitably distributed across the hierarchy, but rather lies in

the hands of a few powerful individuals at the top. Triangle transitivity measures the proportion of relations between all triads that are transitive (i.e. if individual A is dominant to individual B and individual B is dominant to individual C, then individual A is dominant to individual C; a perfect hierarchy would have all transitive triads) (Shizuka & McDonald, 2012). It is calculated using a binary win/loss sociomatrix, where 1's are assigned to individuals in rows that won more often against individuals in columns and 0's are assigned to individuals in rows that lost more often to individuals in columns. Triangle transitivity ranges from 0-1, with 1 indicating that all triads are transitive (i.e. a perfectly linear hierarchy). Despotism is the proportion of all wins by the dominant male over the total number of aggressive interactions over the observation period. It is a value between 0-1, with 1 indicating that the alpha male performed 100% of all aggression within the network.

Glicko ratings were also calculated at the end of the observation period. All individuals begin with a Glicko rating of 2200, and points are added or subtracted based on the rating of each individual's opponents (i.e. if an individual with a high Glicko rating defeated an individual with a low Glicko rating, relatively few points would be added to their total; if an individual with a low Glicko rating defeated an individual with a high Glicko rating, a larger number of points would be added to their total). These Glicko ratings at the end of the period were used to determine who the most dominant and most subordinate individuals in the group were.

Landau's modified  $h'$ , directional consistency, and triangle transitivity were calculated using the R package 'compete' v.0.1 (Curley, Shen, & Huang, 2015). Steepness was calculated using the R package 'steepness' v.0.2.2 (Leiva & deVries, 2014), Glicko ratings were calculated using the 'PlayerRatings' package v.1.0 in R (Stephenson & Sonas, 2012)

#### *Comparison of Female Social Hierarchy Behavior to Male Social Hierarchy Behavior*

To measure differences in social hierarchy structure between male and female social groups, we used previously published data from Williamson, Lee, et al., 2016. In this study, we analyzed social behavior from 10 groups of 12 male CD-1 mice who were observed in exactly the same manner as the female groups from the current study. We used Wilcoxon rank sum tests to compare Landau's modified  $h'$  values, directional consistency, triangle transitivity, steepness, and despotism values for males and females.

#### *Estrous State Analysis*

To measure differences in wins and losses during each phase of the estrous cycle, we ran negative binomial mixed models, with wins or losses as the outcome variable, estrous state, hours of observation, and rank as fixed effects, and cohort, day, and an identifier of which two mice were involved in the interaction as random effects.

#### *Hormone and Gene Expression Analysis*

The two most dominant and two most subordinate individuals in each group were determined based on Glicko ratings. To assess the difference in plasma hormone and gene expression levels between dominant and subordinate individuals, we first ran linear mixed effect models with hormone/gene expression level as the outcome variable, status and estrus state as fixed effects, and cohort as a random effect (model 1). This model showed no effect of estrus state for any of the hormones or genes tested, so we then ran linear mixed effect models with hormone/gene expression level as the outcome variable, status as a fixed effect, and cohort and estrus state as random effects (model 2) as well as linear mixed effect models with hormone level as the outcome variable, status as a fixed effect, and cohort as a random effect, not including estrus state in the model (model 3). Model 3 resulted in the best AIC/BIC values for all hormones and genes tested, so we proceeded to use that model to determine the relationship between plasma hormone/gene expression levels and social status. All models were run using the R package 'lme4' (Bates

et al., 2015). Both corticosterone and estradiol levels had a normal distribution, so we ran normal LMMs for each. For both the VMH and mPOA, PR and OTR  $\Delta\Delta C_T$  values were normally distributed, so we ran normal LMMs for each. For both the VMH and mPOA, ER $\alpha$ , ER $\beta$ , GnRH, and OPRM1  $\Delta\Delta C_T$  values were found to fit a gamma distribution, so we ran GLMMs, specifying the family as “gamma”. For each model, the residuals were checked and verified to be normally distributed.

## RESULTS

### *Hierarchy Measures and Organization*

**Table 4.1** shows the modified Landau’s  $h'$ , directional consistency, steepness, triangle transitivity, and despotism values for each cohort. All cohorts except cohort A demonstrated significant a significant linear hierarchy.

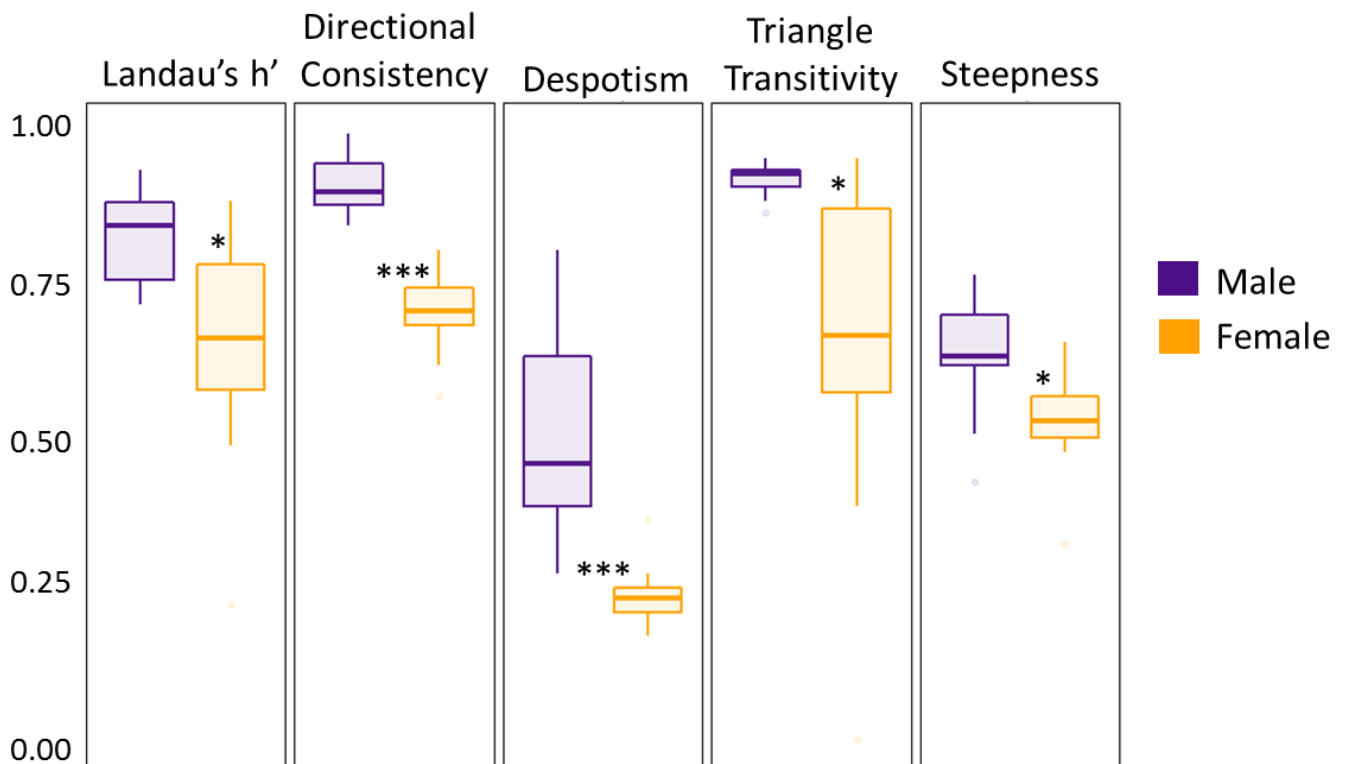
**Table 4.1: Social Hierarchy Measures for Each Cohort**

<b>Cohort</b>	<b>Modified Landau’s <math>h'</math></b>	<b>Directional Consistency</b>	<b>Steepness</b>	<b>Triangle Transitivity</b>	<b>Despotism</b>
<b>A</b>	0.22	0.56***	0.32	0	0.14
<b>B</b>	0.88***	0.79***	0.65***	0.89***	0.27
<b>C</b>	0.65***	0.70***	0.56***	0.63***	0.22
<b>D</b>	0.66***	0.70***	0.50***	0.65***	0.21
<b>E</b>	0.60***	0.70***	0.50***	0.67***	0.20
<b>F</b>	0.48**	0.72***	0.47***	0.38**	0.24
<b>G</b>	0.82***	0.80***	0.56***	0.95***	0.36
<b>H</b>	0.76***	0.61***	0.54***	0.86***	0.17

**Female dominance hierarchies are significantly different from male dominance hierarchies**

On every measure we analyzed, female social groups were significantly different from male social groups in their dominance hierarchy organization. In females, all measures had significantly lower values than those in males (**Figure 4.1**): modified Landau's  $h'$  (Wilcoxon Rank Sum test,  $W = 14.5$ ,  $p < 0.05$ ), directional consistency (Wilcoxon Rank Sum test,  $W = 0$ ,  $p < 0.001$ ), triangle transitivity (Wilcoxon Rank Sum test,  $W = 12$ ,  $p < 0.05$ ), steepness (Wilcoxon Rank Sum test,  $W = 16$ ,  $p < 0.05$ ), and despotism (Wilcoxon Rank Sum test,  $W = 2.5$ ,  $p < 0.001$ ).

**Figure 4.1: Female social hierarchies are significantly different from those of males.** Boxplots compare modified Landau's  $h'$ , directional consistency, despotism, triangle transitivity, and steepness measures from 8 female social groups and 10 male social groups. For all measures, female groups had significantly lower values than male groups.

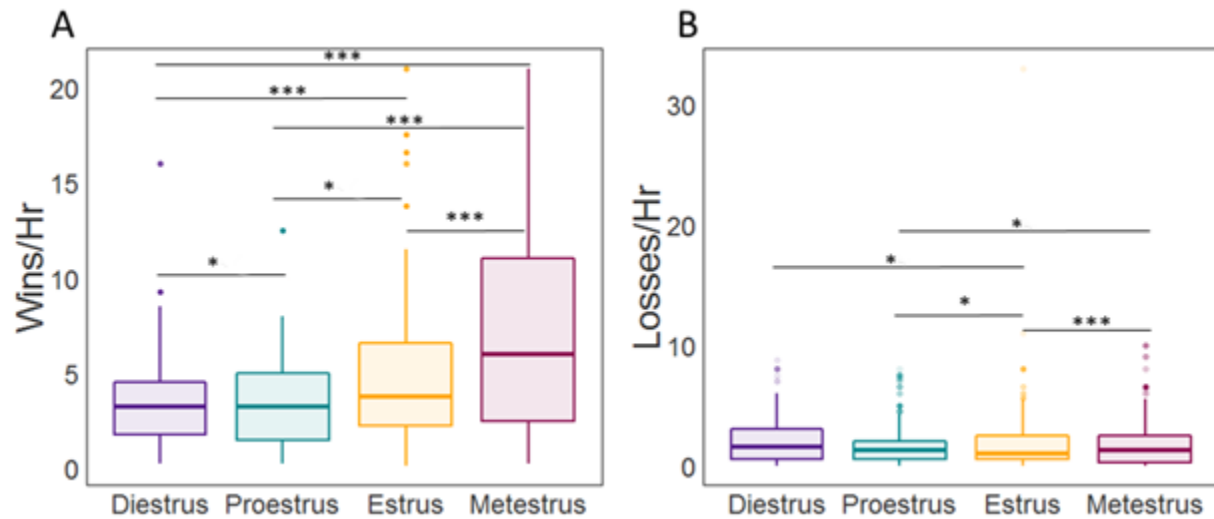


### ***Number of wins and losses differ between phases of the estrous cycle***

When in the metestrus phase of the cycle, individuals won significantly more than the other three phases (**Figure 4.2A**: Metestrus vs. Diestrus:  $\beta = 0.336 \pm 0.047$ ,  $p < 0.001$ ; Metestrus vs. Proestrus:  $\beta = 0.228 \pm 0.042$ ,  $p < 0.001$ ; Metestrus vs. Estrus:  $\beta = 0.145 \pm 0.034$ ,  $p < 0.001$ ). Individuals in estrus won significantly more than those in diestrus (**Figure 4.2A**:  $\beta = 0.191 \pm 0.046$ ,  $p < 0.001$ ) or proestrus (**Figure 4.2A**:  $\beta = 0.083 \pm 0.038$ ,  $p < 0.05$ ). Individuals in proestrus won significantly more than those in diestrus. (**Figure 4.2A**:  $\beta = 0.108 \pm 0.049$ ,  $p < 0.05$ ). These relationships were true across ranks, as we controlled for rank in the model.

Individuals in the metestrus phase of the cycle lost significantly more than those in estrus (**Figure 4.2B**:  $\beta = 0.157 \pm 0.044$ ,  $p < 0.001$ ) and those in proestrus (**Figure 4.2B**:  $\beta = 0.107 \pm 0.054$ ,  $p < 0.05$ ), but there was no significant difference in losses between metestrus and diestrus (**Figure 4.2B**:  $\beta = -0.018 \pm 0.059$ ,  $p = 0.758$ ). Individuals in estrus lost significantly less than those in diestrus (**Figure 4.2B**:  $\beta = -0.139 \pm 0.057$ ,  $p < 0.05$ ). There was no difference in number of losses between the proestrus and diestrus phases (**Figure 4.2B**:  $\beta = 0.089 \pm 0.061$ ,  $p = 0.148$ ). These relationships were true across ranks, as we controlled for rank in the model.

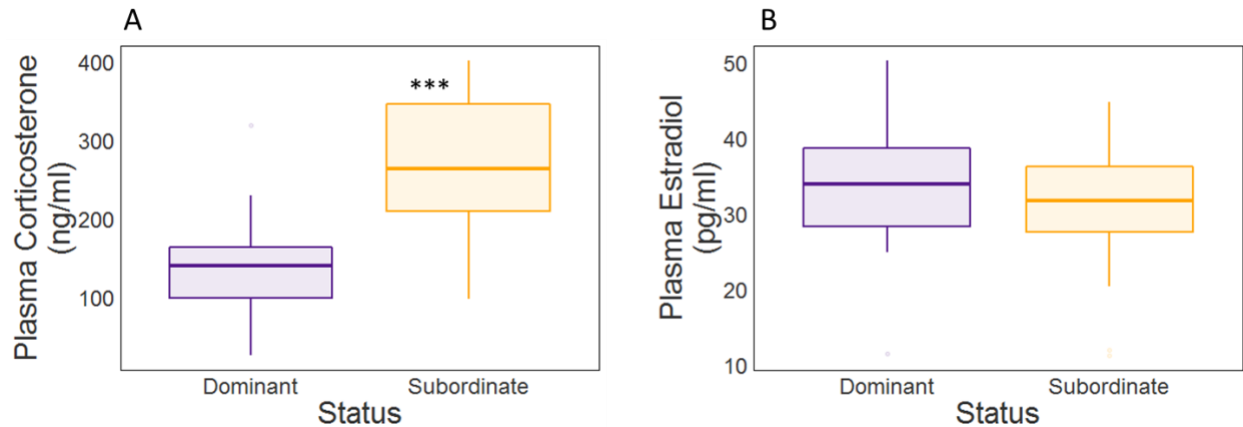
**Figure 4.2: Number of wins and losses differ between stages of the estrous cycle** (A) Wins per hour across the estrous cycle (B) Losses per hour across the estrous cycle



#### ***Plasma corticosterone and estradiol levels***

Plasma corticosterone levels were found to be significantly lower for subordinate individuals as compared to dominant individuals (**Figure 4.3A**,  $\beta = 125.96 \pm 26.97$ ,  $p < 0.001$ ). No significant difference in plasma estradiol levels was found between subordinate and dominant individuals (**Figure 4.3B**,  $\beta = -3.33 \pm 2.56$ ,  $p = 0.205$ ).

**Figure 4.3: Plasma Corticosterone and Estradiol levels for dominant and subordinate females (A)** Subordinate females have significantly higher levels of plasma corticosterone than dominant females. (B) There is no difference between dominant and subordinate individuals in plasma estradiol levels.

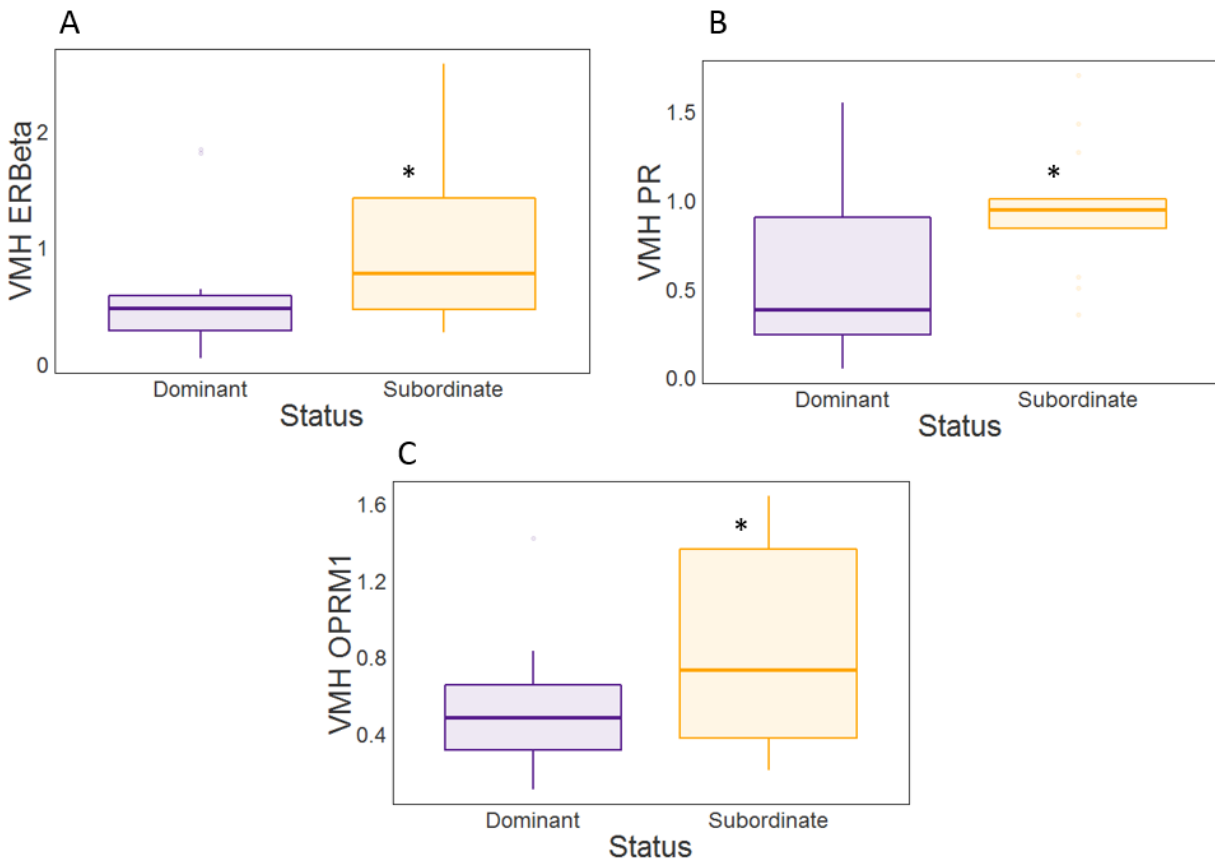


#### ***Gene expression in the VMH***

In comparison to dominant individuals, subordinate individuals displayed significantly higher levels of expression in the VMH of ER $\beta$  (**Figure 4.4A**; GLMM:  $\beta = 0.674 \pm 0.306$ ,  $p < 0.05$ ), PR (**Figure 4.4B**; LMM:  $\beta = 0.344 \pm 0.154$ ,  $p < 0.05$ ), and OPRM1 (**Figure 4.4C**; GLMM:  $\beta = 0.4175 \pm 0.2114$ ,  $p < 0.05$ ). There were no significant differences in expression levels of ER $\alpha$  (**Supplemental Figure 4.3A**; GLMM:  $\beta = 0.227 \pm 0.190$ ,  $p = 0.233$ ), GnRH (**Supplemental Figure 4.3B**; GLMM:  $\beta = 0.0004 \pm 0.151$ ,  $p = 0.998$ ), and OTR (**Supplemental Figure 4.3C**; LMM:  $\beta = 0.097 \pm 0.106$ ,  $p = 0.369$ ).



**Figure 4.4: ER $\beta$ , PR, and OPRM1 mRNA levels in the VMH are higher for subordinate females (A)** Subordinate females have higher levels of ER $\beta$  in the VMH. (B) Subordinate females have higher levels of PR in the VMH. (C) Subordinate females have higher levels of OPRM1 in the VMH.



#### **Gene expression in the mPOA**

There were no significant differences between dominant and subordinate individuals in any of the genes studied in the mPOA (**Supplemental Figure 4.4**: ER $\alpha$ : GLMM:  $\beta = 0.122 \pm 0.139$ ,  $p = 0.378$ ; ER $\beta$ : GLMM:  $\beta = -0.006 \pm 0.097$ ,  $p = 0.947$ ; PR: LMM:  $\beta = 0.054 \pm 0.143$ ,  $p = 0.710$ ; GnRH: GLMM:  $\beta = 0.214 \pm 0.175$ ,  $p = 0.222$ ; OTR: LMM:  $\beta = 0.138 \pm 0.118$ ,  $p = 0.908$ ; OPRM1: GLMM:  $\beta = 0.187 \pm 0.194$ ,  $p = 0.335$ ).

## DISCUSSION

In the present study, we show that female mice are capable of forming significantly linear dominance hierarchies, although their characteristics do differ in substantial ways from those of males. This finding fits well with prior literature demonstrating that female mammals engage in intrasexual competition for resources and are capable of forming dominant-subordinate relationships (Kaufmann, 1983; Rowell, 1974; Schuhr, 1987; Stockley & Bro-Jørgensen, 2011; van den Berg et al., 2015; Weidt et al., 2018), and it furthers the findings from previous studies not only by determining that it is possible for female mice to form significantly linear dominance hierarchies, but by thoroughly analyzing the characteristics of these hierarchies. Notably, female hierarchies differed from those of males on every metric tested, and there was one female group that did not form a linear hierarchy. Generally, female hierarchies were less linear, as measured by Landau's modified  $h'$  value and directional consistency, and the groups were significantly less despotic, with a smaller range of despotism values, than those of males. Their hierarchies were also significantly less steep, and there are more intransitive triangles within the hierarchies. In males, it is possible to linearly order the ranks, with each male occupying a unique rank, while in females, we can classify them as "dominant", "subdominant", or "subordinate", but we are not able to uniquely order them. It is difficult to deduce exactly why these differences might exist, though they are likely related to differential evolutionary selection pressures. There are fundamental differences in the reproductive strategies of males and females which can lead to different competitive goals and adaptations. Females generally exhibit higher parental investment in offspring and lower potential reproductive rates (Stockley & Bro-Jørgensen, 2011) and typically compete first over resources, as the high energetic demands of lactation and gestation (Gittleman & Thompson, 1988) mean their reproductive success is constrained by access to food (Emlen & Oring, 1977; Sterck, Watts, & Schaik, 1997; Wrangham, 1981). In contrast, males typically compete first for mates and only compete for that resources insofar as they provide further access to mates. Recent work in house mice suggests that this pattern exists in mice as well, with females

not engaging in any more agonistic behaviors when given the opportunity to compete for males (Weidt et al., 2018) These sex differences have resulted in female adaptations for intrasexual competition that are significantly different from those of males, involving more subtle behaviors such as low-level persistent aggression and alliance formation instead of overt displays of physical aggression (Stockley & Bro-Jørgensen, 2011). In our studies, females indeed exhibited significantly less aggression than males, as is demonstrated by their scores on the various measures examined. In our vivaria, individuals have unlimited access to food, water, space, and nesting material. Given that females are more likely to compete over resources, it is quite possible that if any of these resources was provided in a finite amount, there would have been increased levels of aggression, resulting in more significantly linear hierarchies.

Estrous state was significantly related to winning and losing. During the estrous and metestrus phases of the cycle, individuals were more aggressive, winning significantly more fights than those in diestrus or proestrus. Further, individuals in metestrus were more aggressive than those in estrus. While little work has examined the role the estrous cycle plays in aggression in mice, it has been previously shown that in house mice, females in proestrus and metestrus are more aggressive than those in estrus or diestrus (Hyde & Sawyer, 1977). We did not find that individuals in proestrus were more aggressive, so our findings are not in complete agreement with this study. Our finding that individuals in estrus were more aggressive than those in proestrus and diestrus is in contrast to work in rats showing that individuals in estrus are more subordinate to those not in estrus (Seward, 1945). Females in estrus also lost significantly less than those in diestrus or proestrus, with females in metestrus losing more than those in estrus. This suggests that females can be considered to be behaving in the most dominant fashion during the estrus stage and that perhaps the increase in wins during the metestrus phase is related to an increased motivation to engage in fights, resulting in both losing and winning more. These fluctuations in aggression during the estrous cycle offer crucial insight into explaining the differences between male and female hierarchies

and could explain why female hierarchies were less linear than those of males. If individuals are engaging in aggression at different levels based on their estrous state, it is likely that the hierarchy shifts a bit from day to day. This could explain why directional consistency isn't as high as that in males and could also provide an explanation for why we saw lower triangle transitivity. Females could have different dominant-subordinate relationships depending on where they and the other individuals in the group were in their cycle. Alternatively, the effect sizes were fairly small, so it is possible this variation across the cycle didn't ultimately affect behavior to a large extent and therefore might not be related to the overall hierarchical structure.

In addition to examining behavioral characteristics of female social hierarchies, we investigated their neuroendocrine underpinnings. We found that subordinate females within the hierarchy exhibit higher levels of plasma corticosterone than dominant females. This is different from what we have previously shown in males, where there was only a difference in plasma corticosterone levels between dominants and subordinates in highly despotic hierarchies (Williamson, Lee, et al., 2017). In females, none of the groups had despotism measures approaching 0.50, the number over which we considered male groups to have high despotism. Despite this, subordinate females do exhibit significantly higher levels of plasma corticosterone. There is evidence to suggest that females respond differently to different types of social stress. Females display increases in plasma corticosterone in response to social instability while males are not affected by it, and, conversely males display increases in plasma corticosterone in response to social defeat (Haller et al., 1999), while females are less susceptible to this model of social stress. In non-human primates, subordinate females exhibit high cortisol levels and are insensitive to negative feedback of the HPA axis (Shively, Laber-Laird, & Anton, 1997). Further, it is well-documented that across species females have a higher predisposition towards anxiety and depressive disorders (Breslau, Davis, Andreski, Peterson, & Schultz, 1997; Piccinelli & Simon, 1997; Shively et al., 1997; Szádóczy, Ríhmer, Papp, & Füredi, 1997),

which can be associated with chronic social stress and a more sensitive stress response. This supports the idea that females may be more susceptible to the consequences of the chronic social stress associated with their subordinate status than males, resulting in higher plasma corticosterone output.

There was no significant difference in plasma estradiol levels between dominant and subordinate individuals. This is somewhat surprising, as estrogens have been known to promote aggression in both males and females and are generally known to masculinize (Lenz, Nugent, & McCarthy, 2012; Wu et al., 2009). Given this, one would hypothesize that dominant individuals exhibit higher levels of plasma estradiol than subordinates. However, given that even dominant females displayed low levels of despotism, it is likely that they were engaging in female-typical levels of aggression and therefore not showing particularly elevated estradiol levels. Further, estradiol acts to modulate a cascade of gene expression in the brain, including that of oxytocin, which promotes affiliative behavior (Campbell, 2008). When females are under threat, estrogen-potentiated OT provides a calming effect, enabling females to engage in stress-reducing behaviors (Taylor et al., 2000). Because estrogens can have many, sometimes opposite, effects, depending on where in the brain and on what receptors they are acting, it is perhaps not surprising that estradiol levels found in plasma at one time point do not differ between dominant and subordinate individuals.

In the VMH, we found that subordinate females exhibited higher levels of ER $\beta$ , PR, and OPRM1 mRNA. Previous work studying the role of ER $\beta$  in social behavior and aggression supports our finding here. ER $\beta$  has been associated with inhibition of aggression in males (Nomura et al., 2002; Ogawa et al., 1999) and is expressed in hypothalamic neurons that synthesize oxytocin (Patisaul, Scordalakes, Young, & Rissman, 2003), suggesting that action of estrogen at ER $\beta$  receptors leads to the synthesis of oxytocin, thus

facilitating affiliative behaviors. The story is complicated however, with some work showing that acute activation of ER $\beta$  leads to increased aggression in females (Clipperton Allen, Cragg, Wood, Pfaff, & Choleris, 2010). However, this study used an ER $\beta$  agonist injected IP, which would have led to global activation of ER $\beta$ . Given that this was not an approach targeted at a specific brain region, it is likely that ER $\beta$  in the hypothalamus could have an effect in reducing aggression and increasing affiliation, while action at ER $\beta$  receptors elsewhere plays a role in increasing aggression. More targeted administration of an ER $\beta$  agonist to specific brain regions could elucidate the complexities of ER $\beta$  activation in social behavior in females.

While the role of progesterone and its actions on progesterone receptors in female sexual behavior has been extensively studied (Blaustein, 2008; J. P. Lydon et al., 1995; John P. Lydon, DeMayo, Conneely, & O'Malley, 1996; Moguilewsky & Raynaud, 1979), its role in female aggression and dominance is not well understood. We show here that subordinate individuals have increased levels of PR mRNA in the VMH, suggesting that action at these receptors is associated with subordinate behaviors. In hamsters, some work shows that progesterone administration reduces aggression (Fraile, McEwen, & Pfaff, 1987), while other work suggests the opposite effect, with ovariectomized females who are given progesterone responding with increased aggression (Payne & Swanson, 1972). In female bank voles, progesterone administration also increases intrasexual aggression, in both ovariectomized and intact females (Kapusta, 1998). While work clearly remains to be done to elucidate the role of progesterone in modulating aggression, dominance in our system is not only tied to aggression. In order to be successful in a social hierarchy and attain dominant status, an individual not only must win fights, but must be able to appropriately respond to the social context at hand. Progesterone receptors have been related to a variety of social behaviors other than aggression, the most pertinent of which is social recognition. Progesterone treatment has been shown to lead to impaired social recognition in male rats, and it is clear that this

impairment is due to action at PR receptors, as administration of a PR antagonist blocked this impairment of social recognition (Bychowski & Auger, 2012). Our subordinate mice therefore might exhibit some impairments in social recognition, either as a cause or consequence of their subordinate status. An individual cannot ascend to dominant status in a social hierarchy with poor social recognition skills, so given this relationship between progesterone receptors and social recognition, it is consistent that subordinate individuals would show increased PR mRNA in the VMH.

We also found that subordinate mice exhibit significantly higher levels of OPRM1 mRNA in the VMH. OPRM1 has been implicated in a host of social behaviors, but findings are not consistent as to its exact role in regulating appropriate social behavior. It appears to be involved in motivation to initiate social contact, as OPRM1 knockout pups show impaired social attachment behaviors (Moles et al., 2004), and OPRM1 knockout males fail to exhibit typical social exploration behaviors (Wöhr et al., 2011). MECP2 duplication mice, which show increased OPRM1 expression, exhibit impairments in social interaction and social approach behavior (Samaco et al., 2012). Further, a single nucleotide polymorphism of the OPRM1 gene, has been associated with both increases in social dominance behaviors and resilience to social defeat. These social behavior phenotypes are thought to be a result of increased endogenous opioid release in response to social interactions (Briand et al., 2015). Our finding here adds to the growing literature surrounding the role of OPRM1 in regulating typical social behavior and may help to further elucidate its actions. Subordinate mice in our paradigm typically exhibit decreased social exploration and increased affiliation behaviors. They tend to group together in the lower levels of the vivarium while the dominant individuals patrol and seek out agonistic interactions in order to assert their alpha status. That they have increased levels of OPRM1 mRNA supports the idea that OPRM1 mediates social attachment and motivation to initiate affiliative social contact.

We did not find any mRNA differences between dominant and subordinate females in ER $\alpha$ , OTR, or GnRH in the VMH. ER $\alpha$  has been implicated in social behavior, but more typically through action in the medial amygdala than action in the VMH (Murakami, Hunter, Fontaine, Ribeiro, & Pfaff, 2011). Social recognition is impaired if ER $\alpha$  expression in the amygdala is knocked down, but there is no effect of knocking down ER $\alpha$  in the VMH on social recognition or adult aggression (Spiteri et al., 2010). This helps to explain why we see no differences in ER $\alpha$  expression in the VMH between dominant and subordinate mice. GnRH, while it has been shown to be related to male social hierarchy behavior, has more typically been associated with reproductive behavior in females (Moss & McCann, 1973; Pfaff, 1973), and has yet to be implicated in female social dominance or aggression. As with ER $\alpha$ , OTR actions modulating social dominance behaviors are typically more site-specific in the central amygdala or mPOA (Duque-Wilckens & Trainor, 2017), and have not been implicated in the VMH.

We did not find any mRNA differences for any of the genes studied (ER $\alpha$ , ER $\beta$ , PR, OTR, GnRH, and OPRM1). The mPOA is known to be crucial in male dominance behavior (So et al., 2015; Williamson, Romeo, et al., 2017) and aggression (Nelson & Trainor, 2007) and is relevant to many sociosexual behaviors in females (Turkenburg, Swaab, Endert, Louwerse, & van de Poll, 1988; Whitney, 1986). However, in terms of estrogen-mediated gene expression, there does not seem to be any role of the mPOA in regulating female intrasexual behavior. This is supported by findings that there is a sex specific mechanism of social hierarchies in mice that is mediated by testosterone (van den Berg et al., 2015). Given that GnRH in the mPOA is a fundamental part of production of testosterone via the HPG axis, it is consistent that the mPOA would play a more integral role in male than female social behavior. While we



show here that females do exhibit social network behaviors similar to those of males, it is clear that we cannot assume that the mechanisms driving these behaviors are the same between the sexes.

## **CONCLUSION**

In the present study, we establish that females are capable of forming significant linear hierarchies. These hierarchies possess significantly different characteristics from those of males and do not appear to be affected by changes in behavior across the estrous cycle. We show that subordinate females display significantly higher levels of corticosterone than dominant females, suggesting that subordinate females are perhaps more susceptible to the social stress of living at the bottom of a hierarchy than males. We also show that subordinate females have higher levels of ER $\beta$ , PR, and OPRM1 mRNA than dominant females, suggesting that these genes, which are all modulated by the actions of estrogen, are involved in differentiating subordinate and dominant individuals. This work furthers our understanding of group female social behavior, explores sex differences between male and female social hierarchy formation and maintenance, and provides evidence that the actions of estrogen may play a role in modulating female social hierarchy behavior. From this work, it also is clear that the VMH plays an important role in regulating non-sexual female social behavior, and future studies should investigate how the VMH is involved in differentiating between dominant and subordinate individuals. Given the clear role of estrogen, it continues to be essential to examine sex differences in female social behavior and further understand how sex hormones play a role in non-sexual social behavior.

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## SUPPLEMENTAL MATERIAL

### Supplemental Table S4.1: Mouse Social Behavior Ethogram:

During observations, observers code all agonistic interactions occurring between any two individuals. As multiple behaviors may occur during the same interaction, observers record the behaviors with the highest priority. For instance, if one animal fought another animal who responded by fleeing, this would be recorded as a 'Fighting' event only, as 'Fighting' takes priority to the co-occurring 'Induced-Flee'. If an animal fled when approached but was not attacked by another animal, then this would be recorded as 'Induced-Flee'. Similarly, if an animal displayed subordinate posture following a chase, this would be recorded as 'Chasing', because chasing takes priority over 'Subordinate Posture'. Subordinate posture and Induced-Flee are only recorded if they occur in the absence of fighting, chasing, or mounting. These two subordinate behaviors do not co-occur so are given equal priority.

Priority	Behavior	Description
1	Fighting	The focal individual lunges at and/or bites the target individual.
2	Chasing	The focal individual follows the target individual rapidly and aggressively while the other individual attempts to flee.
3	Mounting	The focal individual mounts another individual from behind.
4=	Subordinate posture	The focal individual responds to the approach from another individual by remaining motionless and/or exposing their nape.
4=	Induced-Flee	The focal individual flees without any aggression shown by another individual.

Supplemental Figure S4.1: Housing Vivarium

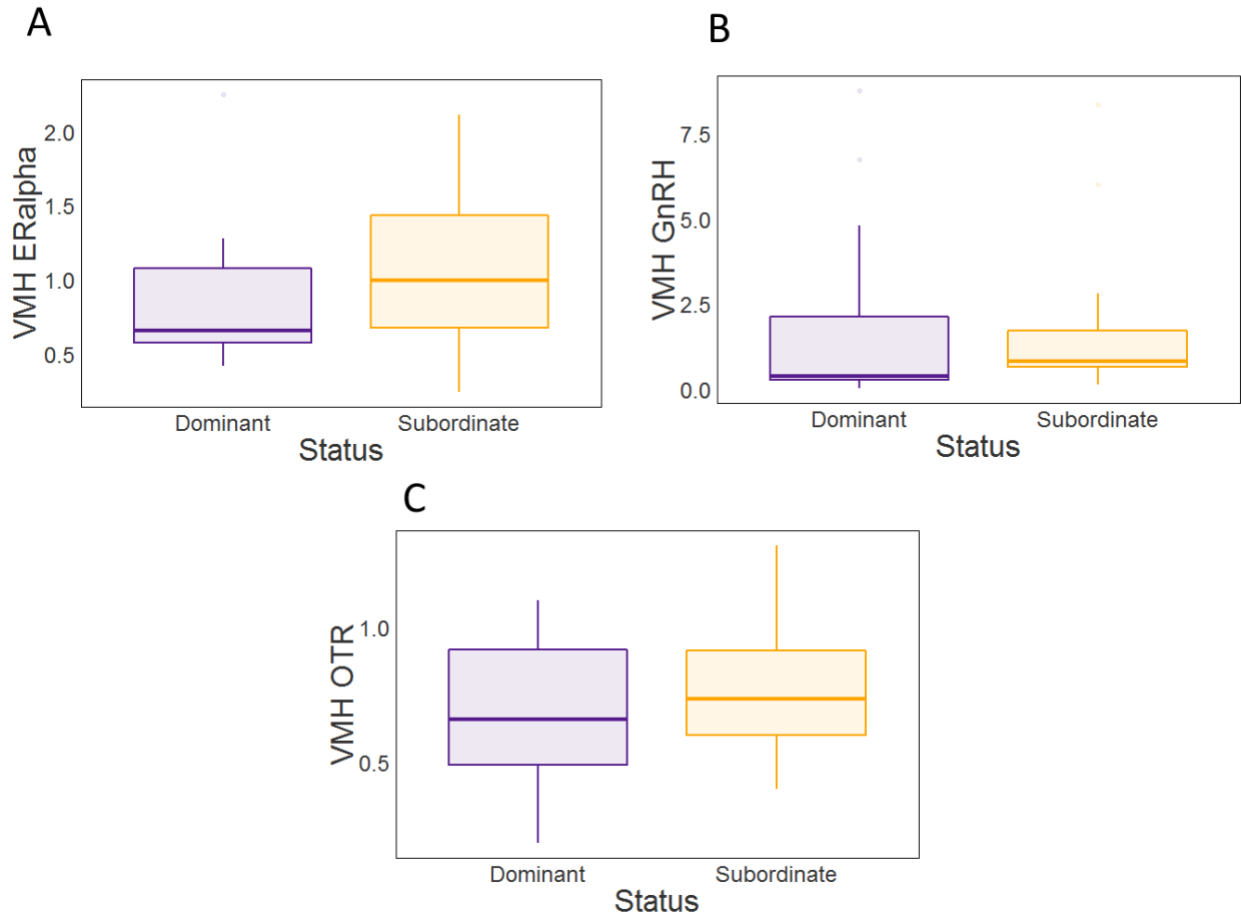




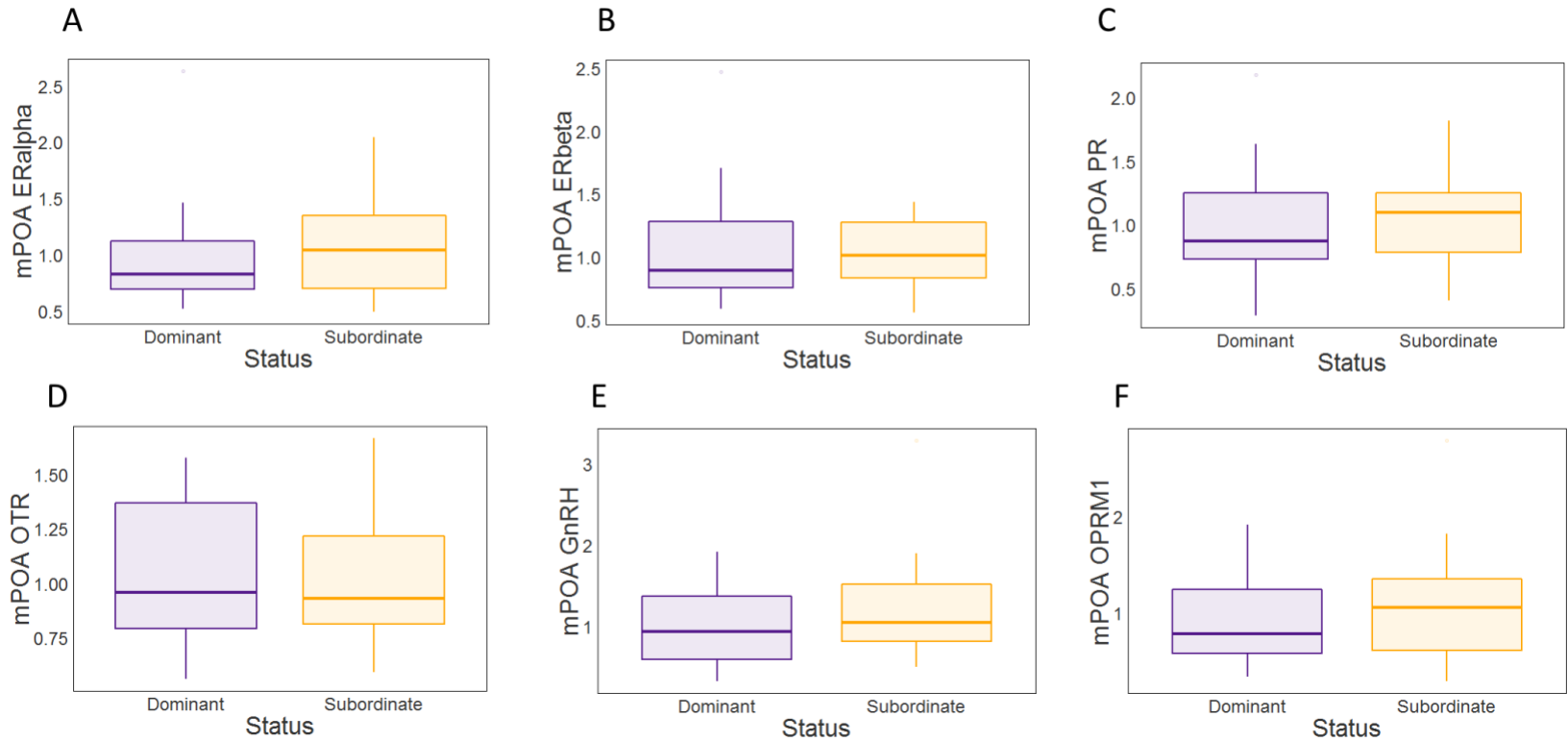
Supplemental Figure S4.2 Glicko scores over the group housing period for all cohorts



Supplemental Figure S4.3 ER $\alpha$ , GnRH, and OTR mRNA levels in the VMH do not differ between dominant and subordinate females



Supplemental Figure S4.4 ER $\alpha$ , ER $\beta$ , PR, OTR, GnRH, and OPRM1 mRNA levels in the mPOA do not differ between dominant and subordinate females



## CHAPTER 5 – Mouse social network dynamics and community structure are associated with plasticity-related gene expression

Cait M. Williamson, Becca Franks, James P. Curley

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## **ABSTRACT**

Laboratory studies of social behavior have typically focused on dyadic interactions occurring within a limited spatiotemporal context. However, this strategy prevents analyses of the dynamics of group social behavior and constrains identification of the biological pathways mediating individual differences in behavior. In the current study, we aimed to identify the spatiotemporal dynamics and hierarchical organization of a large social network of male mice. We also sought to determine if standard assays of social and exploratory behavior are predictive of social behavior in this social network and whether individual network position was associated with the mRNA expression of two plasticity-related genes, DNA methyltransferase 1 and 3a. Mice were observed to form a hierarchically organized social network and self-organized into two separate social network communities. Members of both communities exhibited distinct patterns of socio-spatial organization within the vivaria that was not limited to only agonistic interactions. We further established that exploratory and social behaviors in standard behavioral assays conducted prior to placing the mice into the large group was predictive of initial network position and behavior but were not associated with final social network position. Finally, we determined that social network position is associated with variation in mRNA levels of two neural plasticity genes, DNMT1 and DNMT3a, in the hippocampus but not the mPOA. This work demonstrates the importance of understanding the role of social context and complex social dynamics in determining the relationship between individual differences in social behavior and brain gene expression.

## INTRODUCTION

Laboratory studies of mouse social behavior typically involve observations of dyadic interactions of non-familiar social partners in a novel environment (Brodkin, 2007; Kas et al., 2014). While these tests provide some basic information on the behavior of a laboratory mouse, there is increasing concern that these tests do not provide sufficient insight into more complex social behaviors such as social competence that may be relevant for translational research (Hofmann et al., 2014; Peters, Pothuizen, & Spruijt, 2015). A critical issue to be resolved is what complex, ethologically-relevant social behaviors are laboratory mice able to exhibit? It is known from field studies that the ancestors of laboratory mice (*Mus musculus*) live in large social groups with a high degree of spatial organization (Berry, 1970; Crowcroft, 1973). Additionally, previous studies have shown that both wild mice and laboratory mice in semi-natural environments form territories each with dominant mice that patrol and defend resources such as food or females (Gray, Jensen, & Hurst, 2000; Mackintosh, 1970; Mondragón, Mayagoitia, López-Luján, & Diaz, 1987; Perony, Tessone, König, & Schweitzer, 2012). More recently, studies using automated tracking technologies to look at the behavior of laboratory mice living in large groups have revealed similar patterns of spatial and temporal organization, suggesting it is feasible to study such social behavior in the laboratory (Freund et al., 2015; Freund et al., 2013; Ohayon, Avni, Taylor, Perona, & Roian Egnor, 2013; Perony et al., 2012; Thanos, Restif, O'Rourke, Lam, & Metaxas, 2015; Weissbrod et al., 2013).

One of the most well-understood types of social organization is the dominance hierarchy, which has been studied in many different species, including insects (Röseler, Röseler, Strambi, & Augier, 1984), fish (Maruska & Fernald, 2011), primates (Enger, Mirsky, & Pribram, 1954; Machado & Bachevalier, 2006; Noonan et al., 2014), and humans (Kumaran, Melo, & Duzel, 2012). These hierarchies may be determined through competitive dominance where animals out-compete each other for access to resources or

agonistic dominance where animals are judged to be dominant based upon wins and losses against each other during agonistic contests (De Waal, 1989). They may also be represented by formal dominance whereby individuals express behaviors that communicate dominance or subordinate behavior without engaging in fighting (De Waal, 1989). In the wild, social rank in a dominance hierarchy is primarily determined by an individual's ability to monopolize resources (e.g. food, space, mates) and higher rank is strongly associated with improved reproductive success and fitness (Franz, McLean, Tung, Altmann, & Alberts, 2015; Mooney, Peragine, Hathaway, & Holmes, 2014). In the laboratory mouse, the majority of social dominance studies have focused on social rank acquisition in dyads or a small number of competing individuals (Curley, 2011). It has also been shown that male mice may form elementary linear dominance hierarchy when animals are repeatedly tested against each other in pairs (van den Berg, Lamballais, & Kushner, 2015; Wang et al., 2011). We have previously shown that groups of twelve male mice living together in an ethologically relevant visible burrow system form stable linear dominance hierarchies based upon their expressions of agonistic and formal dominance (So, Franks, Lim, & Curley, 2015; Williamson, Lee, & Curley, 2016).

The aim of the present study was to determine whether thirty male mice living in large social groups of thirty individuals would form a dominance hierarchy. Previously we have identified that male mice in groups of twelve reliably form dominance hierarchies (Curley., In press; So et al., 2015; Williamson et al., 2016), but it is not yet known if individuals would be able to hierarchically organize themselves in larger groups which would potentially require greater social learning and competence by all individuals. Additionally, using statistical modeling and social network analysis, we aimed to identify more complex spatiotemporal patterns of social interactions between individuals, particularly whether individuals would preferentially associate into sub-communities within the larger network. A further aim was to determine

whether individual differences in standard tests of social and exploratory behavior were predictive of the social behavior of individuals when living in large stable social groups. Previous studies in a number of species have reported positive and negative associations between personality types such as boldness, exploration and sociability, and dominance rank (Boogert, Reader, & Laland, 2006; Carere, Drent, Privitera, Koolhaas, & Groothuis, 2005; David, Auclair, & Cézilly, 2011; Fox, Ladage, Roth II, & Pravosudov, 2009; Verbeek, Goede, Drent, & Wiepkema, 1999), and that animals spatially organize themselves according to similarities and dissimilarities in these personalities (Aplin et al., 2013; Carter, Lee, Marshall, Ticó, & Cowlshaw, 2015; Croft et al., 2009; Massen & Koski, 2014; Pike, Samanta, Lindström, & Royle, 2008). Thirdly, we examine whether individual differences in social network position are related to individual differences in gene expression of two markers of brain plasticity, DNA (cytosine-5)-methyltransferase 1 (DNMT1) and DNA (cytosine-5)-methyltransferase 3 alpha (DNMT3a), in the hippocampus and medial preoptic area of the hypothalamus (mPOA). While DNMT1 is primarily known to mediate the maintenance of DNA methylation patterns established in early development, this enzyme may also play a role in DNA methylation in post-mitotic neuronal cells and therefore mediate brain plasticity (Champagne, 2010; Jensen Peña, Monk, & Champagne, 2012). DNMT3a mediates de novo methylation patterning and is required for synaptic plasticity, learning and memory (Feng et al., 2010). Indeed, changes in the expression of DNMTs have been associated with behavioral plasticity including learning and memory processes (Feng et al., 2010; Miller & Sweatt, 2007; Yu, Baek, & Kaang, 2011). Establishing and maintaining position within a social network requires individuals to learn about their relationships with multiple other individuals and to be able to express socially contextual appropriate behavior to all other individuals within their social network (Fernald, 2014). Acquiring such social information and responding to changes in social context has been shown to be associated with a suite of neuroplastic changes in animals of different social status across species (Cardoso, Teles, & Oliveira, 2015; Fernald, 2015; Taborsky & Oliveira, 2012). Further, manipulations of DNMT-dependent DNA methylation



has also been shown to lead to changes in social status. In African cichlid fish, upregulating DNA methylation through L-methionine administration leads individuals to become socially dominant, while inhibition of DNMT activity through zebularine administration prohibits individuals from becoming dominant (Lenkov, Lee, Lenkov, Swafford, & Fernald, 2015). Silencing DNMT3a through RNA interference in honeybees leads to increased development of queen versus worker bees (Kucharski, Maleszka, Foret, & Maleszka, 2008). Given the role of DNMTs in modulating neural plasticity, learning, memory, and social status and the importance of these mechanisms in regulating both the formation of social hierarchies and the maintenance of socially competent behavior, we hypothesized that changes in the expression of DNMT1 and DNMT3a in two brain regions associated with social behavior and learning and memory would be associated with an individual's ability to maintain a central social network position.

## **METHODS**

### ***Subjects and housing***

Male outbred CD1 mice (N=60) aged 7 weeks were purchased from Charles River and housed in standard sized cages (27cm x 17cm x 12cm) with pine shaving bedding in groups of three for 10 days prior to the start of behavioral testing and throughout the behavioral testing period. Each male placed in the vivarium (1-2 individuals selected randomly from each cage) was given a unique ID (1-30) and distinctively marked with a blue, nontoxic, non-hazardous marker (Stoelting Co.). These marks remain for up to 12 weeks enabling each animal to be clearly identified throughout the study. These 30 males were used as subject animals in the study (Table 1). The remaining 30 animals were only used in this study as stimulus animals in social tests. Standard behavioral testing took place over a 15 day period, 3 days after which subject mice were all weighed and randomly placed into one of four custom built mouse vivaria (length 150cm,

height 80cm, width 80cm; Mid-Atlantic) (**Supp. Figure S5.1**), which were inter-connected by tubes such that mice could move from one vivarium to another. Each vivarium consisted of three sides of Plexiglas with sliding front doors and a metal backboard containing multiple holes for air circulation. Standard food chow and water was provided ad libitum at the top shelf via cage lids that protruded through the vivarium roof. Animals could access each shelf via a system of ramps and tunnels that connected each shelf and side. These same types of tunnels connected each vivarium to the one next to it. Multiple enrichment objects such as plastic igloos and wooden blocks were also provided. The floors of each vivarium were covered with pine shaving bedding. The floors of each vivarium were covered with pine shaving bedding. Bedding was not changed during the vivarium observation period to avoid disturbing mice and interfering with the group structure. Sufficient clean bedding was provided at the beginning of observations in all burrows and shelves that animals could nestbuild with and use throughout observations. The animals were kept in a room at constant temperature (21–24°C) and humidity (30-50%) on a 12/12 light/dark cycle, with white lights (light cycle) coming on at 2400 hours and red lights (dark cycle) coming on at 1200 hours. Mice were housed in the Department of Psychology at Columbia University. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol No: AC-AAAG0054). At the end of the experiment, all animals were euthanized via decapitation with each individual's brain and blood being stored for future analyses.

**Table 5.1. Timeline of Experimental Procedures**

Day	Event
1 -10	60 male CD-1 mice arrive and housed in standard sized cages in groups of 3 while habituating to facility
11-28	Mice remain housed in same groups of 3 and undergo standard behavioral testing
11	Open-field testing on the 30 subject mice, chosen randomly from the group of 60
14	Novel-object testing on the same 30 subject mice
19	All 60 mice habituated to the social test arena for 10 minutes
20	Social interaction testing; each subject mouse is paired with a novel stimulus mouse
25	Social approach-avoidance testing; each subject mouse is paired with a novel stimulus mouse (different partner to the previous social test)
29	30 subject mice placed in the vivarium and social group observations and census counts begin
29-48	Two hours of behavioral observations occur each day and census counts occur each day at 3 separate time points: 2 hours prior to dark cycle onset, 1 hour post dark cycle onset, and 3 hours post dark cycle onset
48	At the conclusion of the 2 hours of behavioral observations, mice are euthanized via cervical dislocation and brains are collected for gene expression analysis

***Social group observations***

Live behavioral observations were conducted in red light conditions for 2 hours per day for 19 consecutive days by three trained observers, all observing at the same time in order to assure that all behaviors were

accurately observed. Observations took place each day between 12pm and 4pm, during the first 4 hours of the dark cycle. Behavioral observations were conducted as previously described (Williamson et al., 2016) (Table 5.2), with additional recording of the location of each behavioral event (see Supp. Figure S5.1). Observers were trained to recognize the unique ink patterns, and they are consistent with an exceptionally high degree of inter-rater reliability. 11 observers were used in total, each with a minimum of 50 hours of coding experience prior to this study (mean 80 hours).

**Table 5.2. Ethogram of Behaviors Coded during Vivaria Observations**

Behavior	Description
Fighting	Individual lunges at or bites another individual
Chasing	Individual follows the target individual rapidly and aggressively whilst other individual attempts to flee.
Mounting	Individual mounts another individual from behind
Subordinate posture	Individual reacts to the movements of another individual by remaining motionless
Induced-Flee	Individual flees without any aggression shown by another individual

The total number of aggressive acts directed from one individual towards another were inputted into a frequency win/loss sociomatrix with winners in rows and losers in columns. As individuals cannot engage in agonistic interactions with themselves no data exists in the diagonal of each matrix. These data are referred to as directed or asymmetric data in social network analysis as individuals may direct behaviors more frequently to individuals than they receive from those individuals. From this, a binarized win/loss sociomatrix was calculated [see (So et al., 2015; Williamson et al., 2016) for more information]. Briefly, if

individual  $i$  wins more contests against individual  $j$  than individual  $j$  wins against individual  $i$  then a 1 is allocated to the matrix cell  $[i,j]$  indicating that  $i$  dominates  $j$  and a 0 is allocated to the matrix cell  $[j,i]$  indicating that  $j$  is dominated by  $i$ . Following the rule proposed by Appleby, if there is a tie in the number of wins then both  $[i,j]$  and  $[j,i]$  are allocated a 0 (Appleby, 1983). Social network analysis was conducted using the binarized win/loss matrices. All statistical analyses were undertaken in R version 3.1.2 (R Core Team, 2015).

### ***Hierarchical network organization***

Network metrics were calculated and analyzed using the 'igraph v0.7.1', 'sna v2.3-2' and 'compete v0.1' packages in R (Butts, 2014; Csardi & Nepusz, 2006; Curley, 2016). The following network-level metrics were evaluated to assess hierarchical organization of the network: *i) Density* – the proportion of all possible network ties that exist; *ii) Average Path Length* – the mean number of steps between any two individuals in the network. Unreachable nodes are given the maximum path length; *iii) Out-degree Centralization* – the degree to which the distribution of out-degrees across all individuals is skewed such that relatively few individuals have the highest out-degrees relative to the maximum possible. Individuals with high out-degrees dominate many other individuals. *iv) Out-closeness Centralization* – the degree to which the distribution of out-closeness scores across all individuals is skewed such that relatively few individuals have the highest out-closeness relative to the maximum possible. Individuals with high out-closeness centrality are highly connected to many individuals in short steps (Freeman, 1978). *v) Triangle transitivity* – this measure represents the proportion (Pt) of relations between all combinations of three individuals (A, B, C) in the network that are transitive (i.e. individual A dominates individual B, individual B dominates individual C, therefore individual A dominates individual C) (McDonald & Shizuka, 2012). This is scaled (t.tri) between 0 (the number of transitive three-way relations are no higher than random

expectation) and 1 (all possible three-way relations are transitive as would occur in a completely linear dominance hierarchy). We tested for the significance of  $t_{tri}$  using a Monte-Carlo randomization of 1,000 generated random graphs using the method outlined by Shizuka & McDonald (2012) P-values are obtained by calculating the proportion of times that the randomly generated  $t_{tri}$  values are greater than the observed value. *vi) Degree assortativity* – Out-degree and in-degree assortativity measure the extent to which individuals associate with other individuals that are of a similar out- and in- degree respectively. Assortativity ranges between -1 (individuals of equivalent degrees never associate with each other) and 1 (individuals of equivalent degrees always associate with each other). We tested whether networks had significantly high assortativity by randomizing the degree distribution of each network 10,000 times. P-values are obtained by calculating the proportion of times that the randomly generated assortativity values are greater than the observed value (Newman, 2002, 2003; Noldus & Mieghem, 2015). *vii) Maximum out-degree and minimum in-degree* – We also tested whether networks had a hierarchical structure by testing whether the maximum out-degree and minimum in-degree of each network significantly differed from that expected by chance. We computed the maximum out-degree and minimum in-degree for 5,000 random networks drawn from a Bernoulli graph distribution possessing the same number of individuals (nodes) and graph density as each network. Mean and standard deviations of P-values were obtained by comparing the proportion of times that the observed maximum out-degree and minimum in-degree were greater and lower respectively than those values generated from the distribution of randomized networks for 20 replicates of each set of 5,000 randomizations (Butts, 2011). Networks were visualized using Gephi v0.8.2. Additionally, using the win-loss frequency sociomatrix, the following metrics of hierarchical organization were calculated and tested for their significance *i) De Vries' modified h' value, ii) steepness, iii) directional consistency* (Williamson et al., 2016) using the 'compete v0.1' R package (Curley, 2016).

### ***Network communities***

All analyses were undertaken using the 'igraph v0.7.1' R package (Csardi & Nepusz, 2006). To examine the community structure of the network, we first generated a symmetricized association matrix of all agonistic interactions by summing the frequency win/loss sociomatrix and its transpose. This represents the total number of interactions occurring between each pair of animals. The community membership of individuals is then determined using the Girvan-Newman method (Girvan & Newman, 2002; Lusseau, Whitehead, & Gero, 2008; Newman & Girvan, 2004). Briefly, this method calculates the edge betweenness of all edges in the network and removes the edge with the highest value. Betweenness is recalculated for all remaining edges and the process continues until all edges are removed. The order in which edges are removed results in a hierarchically ordered dendrogram. The modularity (Q) of each sub-division of each subgraph is calculated. Q is an index of how interconnected edges within each sub-division are compared to a random graph with  $Q=0$  representing that community ties are random. The sub-divisions that give the maximum value of Q for any graph represent the communities of the network. Following (Lusseau et al., 2008), to assess confidence in community membership assignment we bootstrapped our original data with replacement 1000 times. Each replicate had the same total number of observations as the original data. For each bootstrap replicate we reassigned community membership according to the Girvan-Newman community method. A community comembership matrix was then produced containing the total number of times that each pair of animals was assessed to be members of the same community out of the 1000 replicates. The community detection algorithm was then carried out on this comembership matrix to determine community membership. Differences in the frequency of aggressive behaviors between members of communities were assessed using Wilcoxon Signed Rank tests in R. We further tested community structure by applying non-metric multidimensional scaling (nMDS) to a distance matrix

generated from a summary table of the total number of aggressive interactions in each vivarium by each individual.

### ***Non-agonistic behavioral observations***

Census counts of the location of observable mice in the vivaria were undertaken daily at three time points (at 1000hrs, 1300hrs, 1600hrs). A trained observer recorded the identity of all visible mice in each vivarium at each time point. From these data we determined which individuals were in close association (within the same vivarium) at each census period. We then calculated a half-weight association index for each of the 435 dyads ranging between 0 and 1 (0 indicating that the animals were never associated and 1 that they were always associated) (Whitehead, 2008). Specifically, for two individuals *A* and *B*, their half-weight association index is calculated by  $HWI = x / (x + y_{AB} + 0.5 * (y_A + y_B))$  where *x* = number of census periods where *A* and *B* are associated, *y<sub>A</sub>* = number of census periods with only *A* identified, *y<sub>B</sub>* = number of census periods with only *B* identified, *y<sub>AB</sub>* = number of census periods with *A* and *B* both identified but not associated. We also tested for a correlation between the association index matrix and the social network community comembership matrix using a Mantel Test using the vegan R package (Oksanen et al., 2015).

### ***Individual network position***

All analyses were undertaken using the 'igraph v0.7.1' R package (Csardi & Nepusz, 2006). The following individual network measures were calculated: *i) Out- and in-degree* – The number of ties directed to (out) and from (in) to each individual; *ii) Out- and in-closeness* – A measure of how many individuals an individual directs connections to (out) or receives connections from (in) across relatively short paths; *iii) Kleinberg's Hub Score Centrality* – a measure of how influential an individual is to the network based upon the number of its outgoing ties (Kleinberg, 1999; So et al., 2015). Additionally, the rank order of individuals



was assessed using the improved algorithm for the Inconsistencies and Strength of Inconsistencies (I&SI) ranking method (Schmid & de Vries, 2013; Williamson et al., 2016). Inter-correlations between network measures and ranks were analyzed using Spearman rank tests in R adjusting p-values for multiple comparisons using Holm's method (Benton, Ruta, Dunhill, & Sakamoto, 2013).

### ***Network position and pre-vivarium behavior***

Prior to housing in the vivarium, all 30 males underwent testing on two social and two non-social standard behavioral tests. The purpose of performing these tests was to determine whether measures of sociability and exploratory activity prior to being placed in a large social group corresponded in any way with dominance, network position, or community membership. All testing was conducted under red (dark phase) lighting conditions 1-6 hours after lights off. The following tests were carried out: *i) Open-Field:* The open-field test is a behavioral assessment of exploratory activity in an unfamiliar environment (Prut & Belzung, 2003). Open-field testing was conducted as previously described (Champagne, Curley, Swaney, & Keverne, 2009). *ii) Novel Object:* The novel object test is typically described as a test of exploratory behavior (Crawley, 2007). Novel Object testing was conducted 2 days after the open-field test, in the same 59.5cm x 59.5cm square plastic box that subjects had previously been tested in. A novel object (small ceramic flower pot – height 3.8cm, diameter 4.45cm) was placed in the center of the open field. The subject mouse was removed from its home-cage and placed in the bottom-right corner of the box. The movement of the mouse through the arena as well as its interaction with the novel object was recorded with a video camera for 10 minutes. The mouse was then removed and returned to its home-cage. Fecal boli emitted during the test session were counted. The arena was cleaned with 70% ethanol between trials. Analysis of the video was completed using Observer (Noldus, V11.5). The frequency and durations of the following behaviors were coded: subject moving but not in proximity to the novel object, subject idle and not in proximity to the novel object, moving and in proximity to the novel object, idle and in

proximity to the novel object, and sniffing the novel object. Proximity was defined as being within 7cm of the novel object. *iii) Social Interaction:* Social behavior was assessed using a social interaction test (File & Seth, 2003). This test was conducted 5 days after the novel object test. Day 1 consisted of a habituation phase. Mice were habituated to a square plastic box (31.75cm x 27.3cm) with pine bedding on the floor alone for 15 minutes. On Day 2, each mouse was placed in the corner of the box with another unfamiliar CD1 male mouse of the same age and approximate weight. The interactions between the mice were video recorded for 10 minutes. The mice were then removed and returned to their home-cages. Analysis of the video was conducted using Observer (Noldus, V11.5) with the time and duration of all behavioral events being coded (see **Supp. Table S5.1** for ethogram). *iv) Social Approach-Avoidance:* Social behavior was assessed using the social approach-avoidance test (Crawley, 2007). This test was conducted 5 days after the social interaction test. Animals were first placed into a (31.75cm x 27.3cm) square plastic box for 10 minutes in order to habituate to the environment. The floor of the box was covered in pine bedding and contained two upside-down cups (height = 5.1cm, diameter = 2.54cm) placed in opposite corners. At the end of the 10 minute habituation phase, animals were removed from the box and returned to a holding cage. A novel object (plastic brick – height = 5.1cm, width = 2.54cm, length = 2.54cm) was then placed under one cup and a neutral unfamiliar stimulus mouse (a male CD1 of the same age and approximate weight) was placed under the other cup. The mouse was then placed into the box for 10 minutes for the test phase and subsequently returned to his home cage. All testing was video recorded and conducted under red (dark phase) lighting conditions. Analysis of the video was conducted using Observer (Noldus, V11.5) with the time and duration of all behavioral events being coded (see **Supp. Table S5.2** for ethogram).

Following the guidelines for factor analysis in animal behavior research laid out by Budaev (Budaev, 2010), the Kaiser-Meyer-Olkin and Bartlett's tests were used to determine sufficient sampling adequacy and

parallel analysis was used to determine the appropriate number of factors for all analyses. Briefly, initial exploratory factor analyses were run for each behavioral test (open-field, novel-object, social interaction, social approach-avoidance) using the main behavioral variables coded in each test. Behavioral variables loading greater than 0.5 and less than -0.5 were considered to load onto each factor identified by parallel analysis (**Supp. Table S5.3**). One behavioral variable from each factor from each test was then included in an overall factor analysis. As time spent sniffing all three body parts of the novel mouse loaded onto one factor in the social interaction test, we used total sniffing duration as a composite behavioral variable. The data included in the exploratory analysis for the social approach-avoidance test did not pass sampling adequacy so the most theoretically significant behavioral variable 'Duration Sniffing Novel Animal' was included in the overall factor analysis along with frequency of rearing. Variables with loading scores greater than 0.40 or less than -0.40 were considered as significant loadings in the overall factor analysis. We purposefully used selected variables from preliminary factor analyses to ensure the observation to variable ratio was kept to a minimum and was suitable for 30 subjects (Budaev, 2010). Factor scores were calculated using Thurstone's method with the validity of score estimates being tested with the calculation of the maximum proportion of determinacy  $\rho^2$  (Grice, 2001). This is equivalent to the squared multiple correlation between each factor and original variables and should significantly exceed 0.5 for factor scores to be considered valid (Budaev, 2010; Grice, 2001). We then tested whether factor scores of behavior prior to being placed into the vivarium was associated with final network position or network position after day 4 using linear regression and Spearman Rank correlations. We also determined whether there was significant assortativity of individuals within the social network based upon factor scores. Finally, we assessed whether members of each community differed in their pre-vivarium behavior using Mann-Whitney tests. All factor analysis was carried out in R using the psych R package (Revelle, 2015).

### ***Network position and gene expression***

After the final behavioral observation, mice were immediately euthanized by cervical dislocation and brains removed and placed into hexane cooled by dry ice. Brains were stored at  $-80^{\circ}\text{C}$  until dissection. Samples of the whole hippocampus (ventral and dorsal) and medial preoptic area (mPOA) were collected using a Harris Micro-Punch with reference to coronal cross-sections from the Mouse Brain Atlas (Paxinos & Franklin, 2004). The hippocampus was collected bilaterally from Bregma  $-0.82\text{mm}$  to  $-1.46\text{mm}$  and the mPOA was taken as one  $1\text{mm}$  diameter area along the midline from Bregma  $+0.14\text{mm}$  to  $-0.7\text{mm}$ . RNA was isolated from the hippocampus of each individual using the AllPrep RNA Micro Kit (Qiagen) and reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR applications (Invitrogen). Quantitative RT-PCR was performed with  $1\ \mu\text{L}$  of cDNA using an ABI 7500 Fast Thermal Cycler and the Fast SYBR Green Master Mix reagent (Applied Biosystems). All primer probes (Sigma-Aldrich) were designed to span exon boundaries ensuring amplification of only mRNA. For each gene,  $C_T$  values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH - endogenous control). Relative expression values were obtained by the  $\Delta\Delta C_T$  method with fold-difference being determined relative to the average expression value for each gene in each brain region across all animals. The following validated quantitative PCR primers were used for mRNA analysis: GAPDH (Forward: TGTGTCCGTCGTGGATCTGA; Reverse: CCTGCTTCACCACCTTCTTGA), DNMT1 (Forward: GCCATGTGAACAGGAAGATGAC; Reverse: GTCCAAGTGAGTTTCCGGTCTT), DNMT3a (Forward: TCTTGAGTCTAACCCCGTGATG; Reverse: CCTCACTTTGCTGAACTTGCT). Samples that did not yield sufficient RNA for cDNA conversion were eliminated from the analysis. Relative gene expression of each gene was compared to each measure of network position using Spearman rank correlations. To test for separate relationships between relative gene expression and dominance in each community, separate correlations were run if a linear model determined a significant interaction between community

membership and network position. Outliers were determined using an iterated Grubbs Test (Grubbs, 1969), and results are reported for analyses including and excluding these outliers.

## **RESULTS**

We conducted observations for 38 hours over 19 days which led to collection of data on 1230 agonistic interactions.

The valued and binary sociomatrices for all aggression directed between pairs of animals living in the large vivaria are shown in **Figure 5.1**.



### ***Male mice establish a hierarchically organized agonistic social network***

The network of agonistic interactions has a low density (0.34), high average path length (2.12), high out-closeness centralization (0.54) and relatively high out-degree centralization (0.39) indicating that relationships are selective and that the power and influence within the network is unequally distributed. Congruently, randomization tests indicated the maximum out-degree was significantly higher than expected ( $p = 0.000 \pm 0.000$ ; mean  $\pm$  SD from Monte-Carlo simulations) and the minimum in-degree was significantly smaller than expected ( $p=0.003 \pm 0.001$ ) for random networks of the same size and density. Moreover, the out-degree assortativity ( $r_{out} = 0.28, p < 0.001$ ) and in-degree assortativity ( $r_{in} = 0.26, p < 0.001$ ) are both significantly positive indicating that individuals are more likely to connect to other individuals with a similar out-degree and in-degree meaning that the network has a core-periphery structure (Noldus & Miegheem, 2015). Triangle transitivity was also significantly higher than expected by chance ( $P_t=0.94, t_{tri}=0.76, p < 0.001$ ) indicating a highly hierarchically organized network with minimal cyclic relationships. Hierarchical organization was confirmed by the significantly higher than chance values of Landau's modified  $h'$  (0.42,  $p < 0.001$ ), directional consistency (0.79,  $p < 0.001$ ) and steepness (0.31,  $p < 0.001$ )

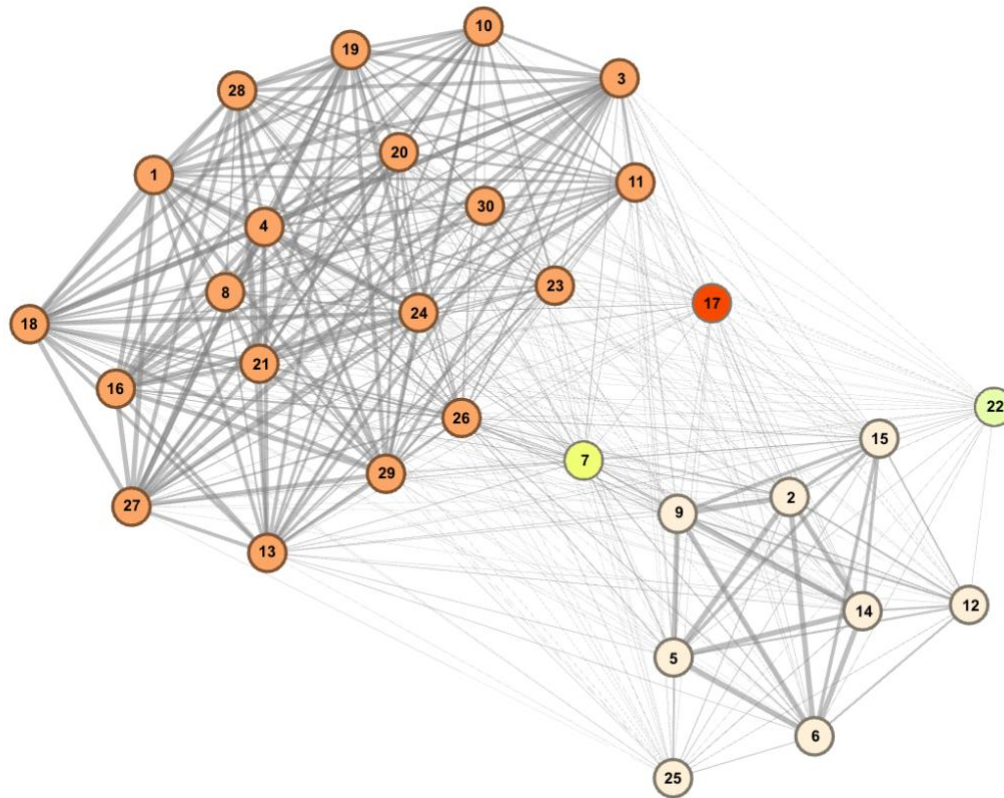
### ***Male mice establish distinct social network communities***

Community detection identified two major sub-communities within the overall agonistic network ( $Q_{max} = 0.24$ ) (**Figure 5.2**). One consisted of 19 individuals (community A) and the other 8 individuals (community B). Additionally three individuals could not be placed within either community. Members of each community showed distinct preferences in the location of their agonistic interactions (**Figure 5.3**). Individuals from community A were more aggressive (Wilcoxon Signed-Rank Test:  $V=180, p < 0.001$ ) and received more aggression ( $V=163, p < 0.001$ ) in vivaria 1&2 compared to vivaria 3&4 (**Supp. Figure S5.2**).

Conversely, individuals from community B were more aggressive ( $V=5$ ,  $p=0.078$ ) and received more aggression ( $V=0$ ,  $p=0.008$ ) in vivaria 3&4 compared to vivaria 1&2. We confirmed this community structure by performing non-metric multidimensional scaling of total agonistic interactions of each individual by location (**Figure 5.4**). Notably, the most dominant individuals of each community are at the furthest extremes of each dimension with subordinate individuals from both communities more clustered close together. Further, the nMDS analysis indicated that two of the three extra individuals belonged to community B and one to community A (**Figure 5.4**)



**Figure 5.2. Mice within Overall Social Hierarchy Establish Separate Hierarchically Organized Communities.** Community detection determined 19 individuals to belong to community A (orange), eight individuals to belong to community B (cream) and three individuals to not conclusively belong to either community. Tie strength is equivalent to the proportion of times that each subject pair were identified to belong to the same community from bootstrapped replications of original data (See **METHODS**). Numbers refer to I&SI ranks



**Figure 5.3. Location and Frequency of Agonistic Interactions by Subject.** Schematics showing the frequency of aggressive contests that occurred in each vivarium. The largest squares refer to the top section of each of the four vivarium with each row representing the three shelves. Underneath each large square, five small squares represent the five nest-boxes in the bottom section of the vivarium. Tubes connecting vivaria 1-2, 2-3 and 3-4 are shown. Each number refers to the overall I&SI rank. IDs are ordered by community (A & A/other = rows 1-4; B & B/other = rows 5-6). Individuals in community A and B win and lose more frequently in vivaria 1&2 and vivaria 3&4 respectively.

A) Total frequency of wins. Colors range from white (0 fights won in location) through yellow and red to black.

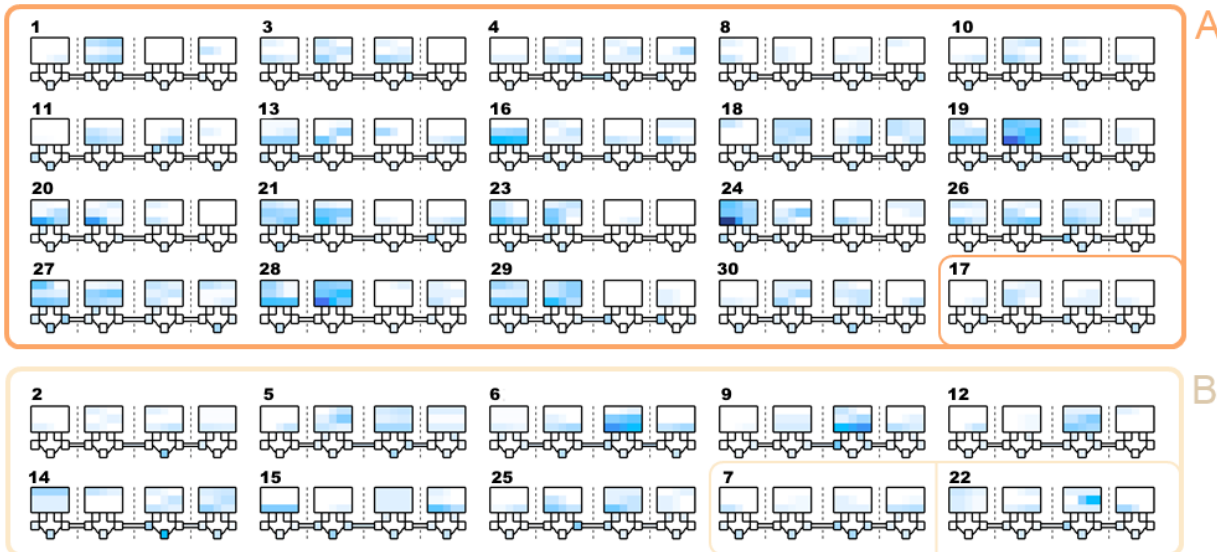
Figure 5.3A

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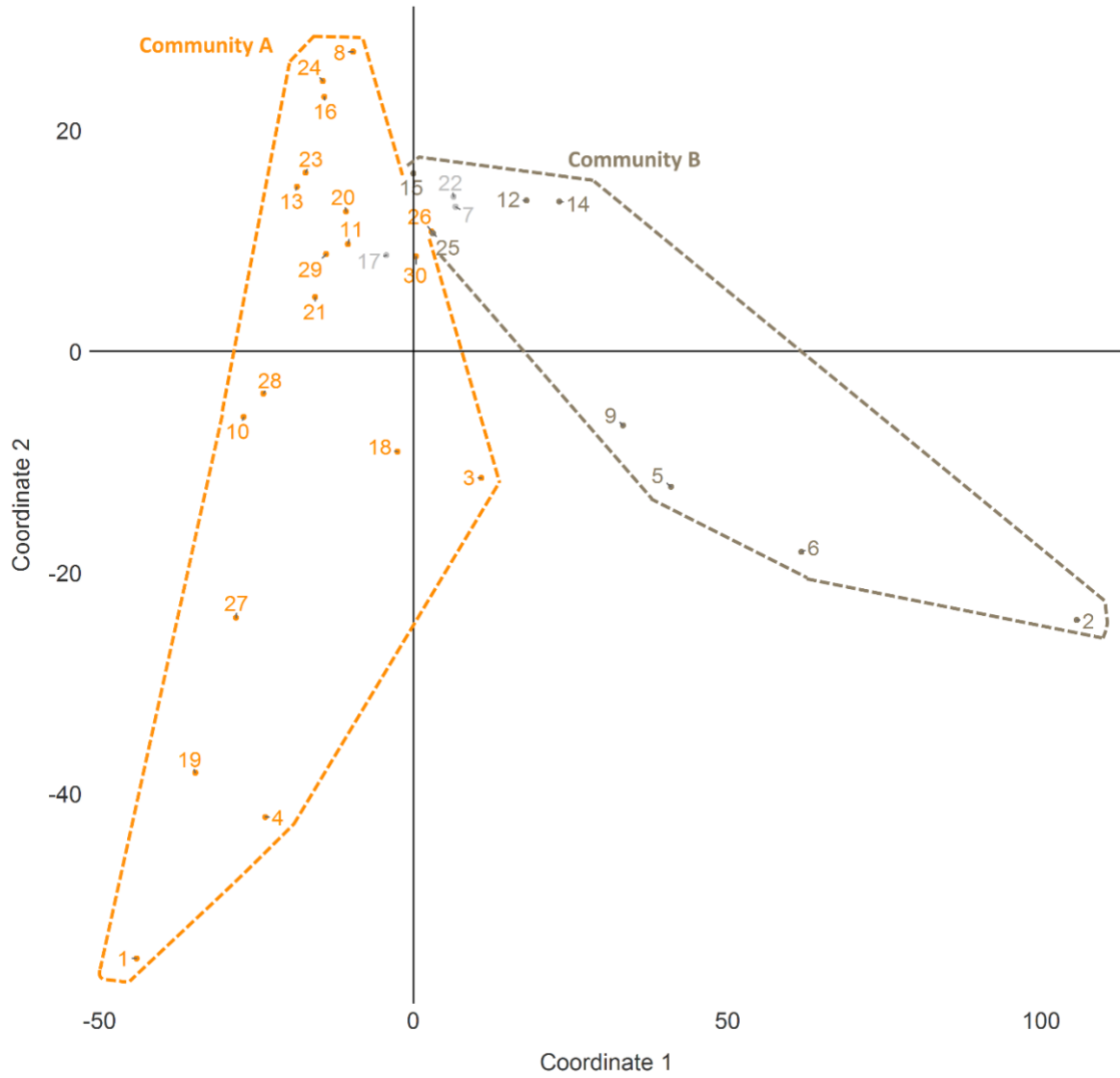
B) Total frequency of losses. Colors range from white (0 fights lost in location) through light and dark blue.

FIGURE 5.3B



**Figure 5.4. Non-metric Multidimensional Scaling (nMDS) Plot of Individual Space Usage**

Scaling plot of the first two coordinates generated from nMDS analysis of the number of agonistic interactions undertaken by each subject in each vivarium. Numbers refer to I&SI ranks.



### ***Network community structure predicts non-aggressive social interactions***

The half-weight association of each relationship was calculated from the census count data of non-agonistic social interactions that was obtained at three time-points each day. This measure gives an index of overall likelihood of social interaction of each pair of individuals. The average association index for dyads within communities (i.e. Community A – Community A dyads or Community B – Community B dyads) are significantly greater than for those between communities (i.e. Community A – Community B dyads; Wilcoxon Rank Sum Test: AA vs AB –  $W=20268$ ,  $p<.001$ ; BB vs AB –  $W = 2705$ ,  $p=.023$ , **Supp. Figure S5.3**). Further, the difference between the medians of association indices occurring within and between communities are significantly larger than expected by chance as determined by 10,000 Monte Carlo randomizations ( $p<0.001$ ). We also found that the half-weight association index matrix is significantly correlated with the community comembership matrix (Mantel Test -  $r=0.38$ ,  $p=0.001$ ). Therefore, community membership predicts social association between even non-agonistically interacting individuals.

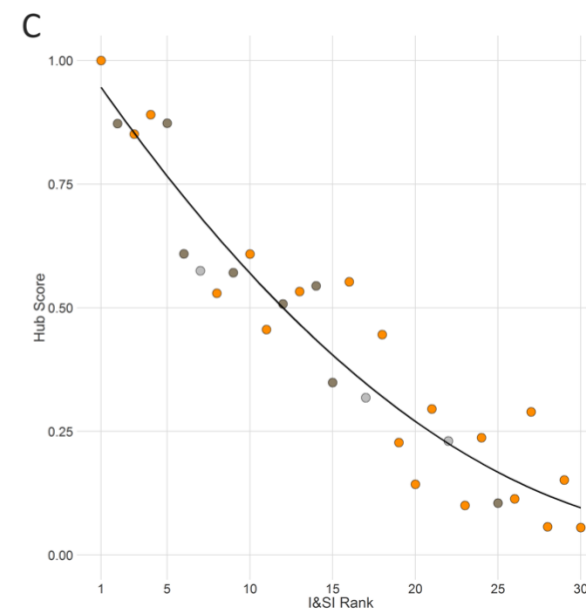
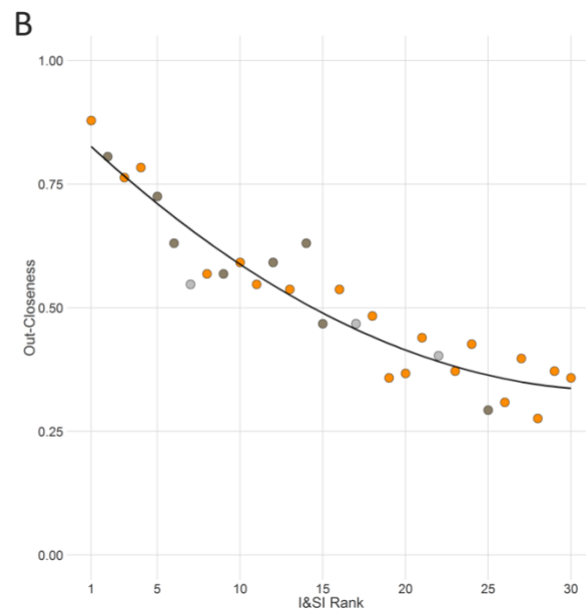
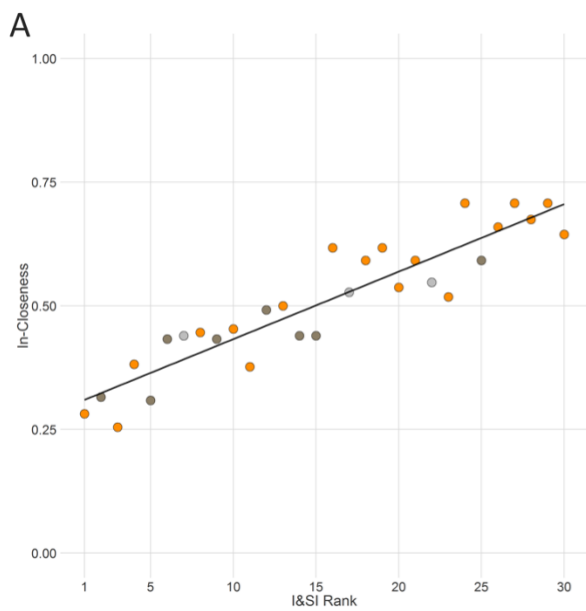
### ***Male mice have stable individual differences in network position and power***

The out-degree, in-degree, out-closeness, in-closeness and hub score of each individual in the agonistic network were found to be highly significantly inter-correlated with each other as well as with the I&SI ranking of individuals (absolute rhos 0.78-0.99, all Holm's  $p < 0.001$ ). Dominant animals have higher out-degrees, out-closeness and hub scores and lower in-degrees and in-closeness than subordinate animals (**Figure 5.5**). Notably, body weight prior to entering the vivarium, after removal or the change in body weight between these time points did not predict dominance rank or network position (all Holm's adjusted  $p = 1$ ).

Each individual's Shannon's evenness of the spatial distribution of giving or receiving aggression was not associated with network position or dominance rank (all  $p > 0.34$ ). However, higher ranked individuals were significantly more likely to exhibit significant unevenness in their spatial distribution of giving aggression (Logistic Regression:  $\beta = -0.11 \pm 0.06$ ,  $z = -1.96$ ,  $p = 0.049$ ). Lower ranked individuals were significantly more likely to exhibit significant unevenness in the spatial distribution of receiving aggression (Logistic Regression:  $\beta = 0.12 \pm 0.06$ ,  $z = 2.04$ ,  $p = 0.042$ ). Across all individuals, there was no significant difference in evenness between giving and receiving aggression between days 1-6, but during days 7-12 (Wilcoxon Rank Sum test,  $W = 114.5$ ,  $p < 0.001$ ) and days 13-19 ( $W = 158.5$ ,  $p < 0.001$ ) giving aggression was significantly less equitable than receiving aggression (**Supp. Figure S5.4**). This suggests that more dominant individuals become more localized in their space usage over time.

**Figure 5.5. Individual Network Positions are associated with Dominance Rank.** Individuals with a higher I&SI dominance rank have decreased in-closeness **(A)** and increased out-closeness **(B)** and hub scores **(C)** in the agonistic network. The best-fitting relationship is linear for A and quadratic for B & C. Each point represents one individual with color representing the network community of that individual (orange – community A, dark gray – community B, light gray – other).

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### ***Behavior prior to group formation predicts early but not final social network position***

Factor analysis of the behavior exhibited by each mouse on the four standard tests conducted prior to vivarium housing resulted in two main factors which we named 'activity' and 'exploration' (see **METHODS**). Activity and exploration accounted for 25% and 21% of total variance in behavior respectively. Network position (out-degree, in-degree, in-closeness, out-closeness or hub score) was not related to each individual's activity or exploration factor scores (all  $R^2 < 0.035$ ). Further, individual activity ( $r = -0.04$ ,  $p = .67$ ) or exploration ( $r = 0.00$ ,  $p = .19$ ) scores did not predict assortativity in the overall network, though at the community level, we did find that eventual members of community A were significantly less active in pre-vivarium behavioral tests than eventual members of community B (Mann-Whitney Test,  $W = 25$ ,  $p = .005$ , Community A median =  $-0.35$  (IQRs:  $-0.67 - 0.36$ ), Community B median =  $0.85$  (IQRs:  $0.28 - 1.40$ )).

As behavior pre-vivarium may be more reflective of initial behavior in the vivarium we examined if individual network position at the end of Day 4 was associated with pre-vivarium behaviors. Early out-degree ( $\rho = -0.41$ ,  $p = 0.025$ ) and out-closeness ( $\rho = -0.44$ ,  $p = 0.016$ ) were significantly negatively associated with exploration scores whereas in-degree ( $\rho = 0.39$ ,  $p = 0.034$ ) and in-closeness ( $\rho = 0.38$ ,  $p = 0.039$ ) were significantly positively associated with exploration scores. No relationship between early network scores and activity scores were found. Notably, time spent sniffing the novel animal in both the social interaction and social approach tests was negatively associated with early out-degree (SI:  $\rho = -0.48$ ,  $p = 0.007$ ; SA:  $\rho = -0.54$ ,  $p = 0.002$ ) and out-closeness (SI:  $\rho = -0.47$ ,  $p = 0.008$ ; SA:  $\rho = -0.55$ ,  $p = 0.001$ ), but time spent sniffing the novel object or time in the inner area of the open-field was not (**Suppl Figure S5.5**).

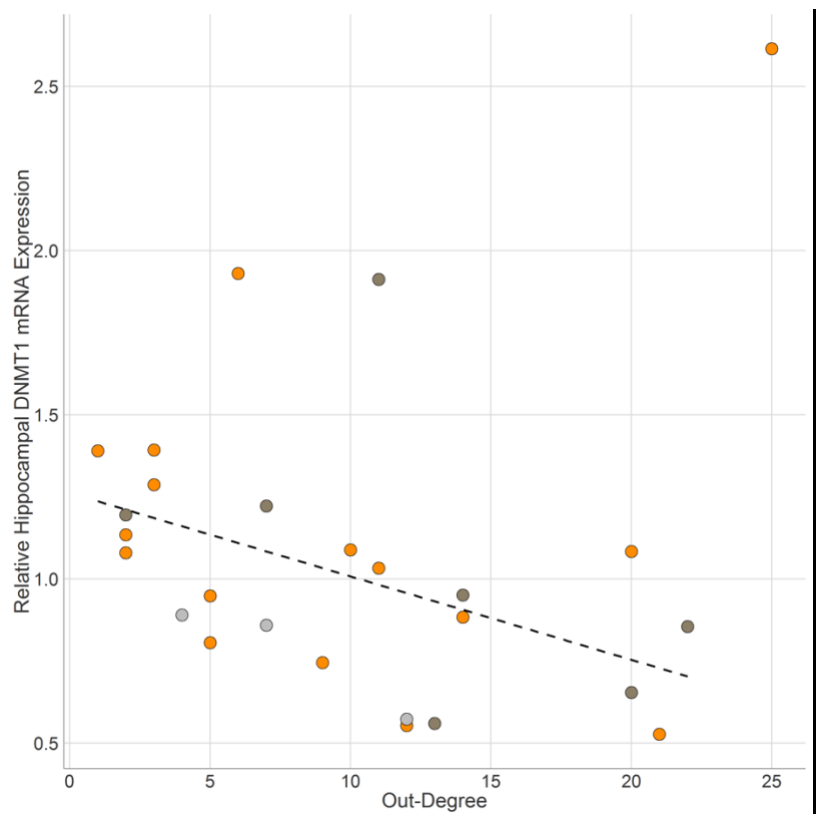


### ***Hippocampal expression of plasticity related genes are associated with network position***

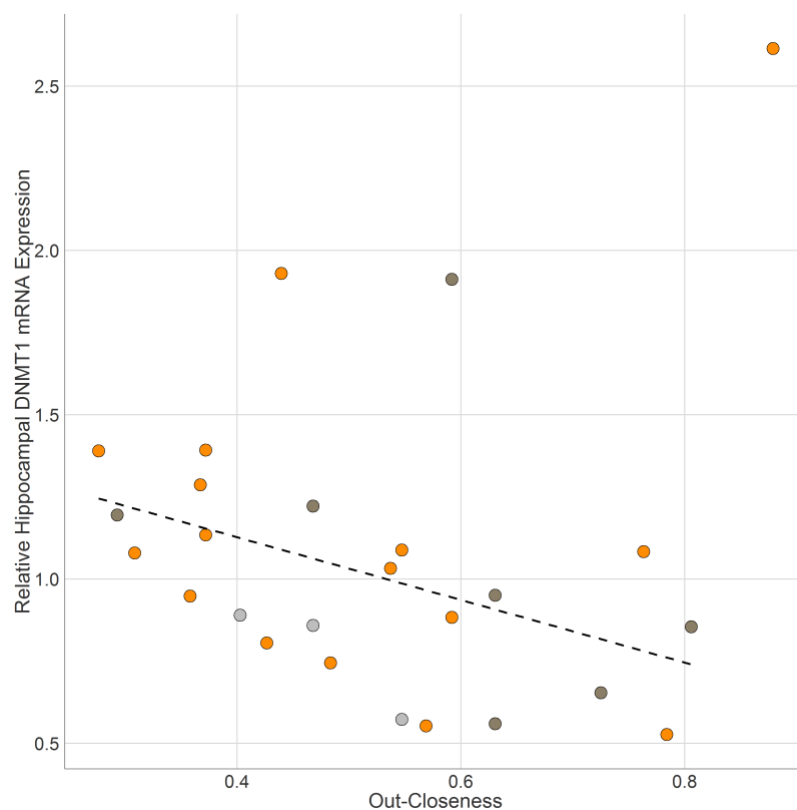
DNMT1 gene expression in the hippocampus was significantly negatively associated with out-degree ( $\rho = -.40$ ,  $p=.042$ ) and hub score ( $\rho = -.40$ ,  $p=.042$ ) and marginally negatively associated with out-closeness ( $\rho=-.35$ ,  $p=.080$ ) (**Figure 5.6**). One individual with the highest DNMT1 gene expression value was determined to be an outlier using an iterated Grubbs Test. This individual had the highest out-closeness score. He was an alpha male that rarely lost any fights until the last 3-4 days of observations when he began to lose a series of fights to one other dominant individual. Removing this outlying data point leads to much higher significant negative associations between DNMT1 gene expression and out-degree ( $\rho=-.58$ ,  $p=.003$ ), out-closeness ( $\rho=-.52$ ,  $p=.008$ ) and hub score ( $\rho = -0.58$ ,  $p=0.003$ ). In-degree and In-closeness were not related to hippocampal DNMT1 expression, though there was a trend for a positive association with the outlier removed (in-degree:  $\rho = 0.36$ ,  $p=0.078$ ; in-closeness:  $\rho = 0.37$ ,  $p=0.072$ ). Across all individuals in the network, hippocampal DNMT3a expression was not associated with any network measure. However, when examining each community (as defined in **Figure 5.4**) separately, out-degree ( $\rho = -0.71$   $p=0.057$ ), out-closeness ( $\rho = -0.73$ ,  $p=.040$ ) and hub score ( $\rho = -0.74$ ,  $p=0.046$ ) were negatively associated with DNMT3a expression amongst community B individuals (**Suppl Figure S5.6**). No relationship was observed among community A individuals. There was no significant relationship between DNMT1 or DNMT3a and network measures in the medial preoptic area.

**Figure 5.6. Brain Gene Expression and Social Dominance.** Hippocampal DNMT1 expression is negatively associated with **A) Out-Degree** and **B) Out-Closeness**. Black hashed lines represent best-fit with outlier removed. Each point represents one individual with color representing the network community of that individual (orange – community A, dark gray – community B, light gray – other).

**Figure 5.6A**



**Figure 5.6B**



## DISCUSSION

### *Mice establish a hierarchically organized dominance network*

We found that a group of 30 communally living male outbred CD1 mice formed a remarkably hierarchically organized social dominance network. The agonistic social network had a very low overall density, high average path length and high out-closeness centralization. These features demonstrate that the power within the network is disproportionately distributed with most network power being monopolized by relatively few individuals. The triangle transitivity was also significantly higher than chance evidencing a highly linear hierarchical structure (Shizuka & McDonald, 2015). Degree assortativity and out-degree assortativity were also significantly positive indicating that individuals were more likely to be connected to other individuals of similar out- and in-degrees, indicating that there exists a core-periphery structure to the social network (Noldus & Mieghem, 2015). These findings were consistent with the highly significant Landau's modified  $h'$ , steepness and directional consistency values that indicated that the social hierarchy was both highly linear and steep. These results extend our previous findings that male CD1 mice living in groups of 12 form hierarchically organized dominance networks (So et al., 2015; Williamson et al., 2016). The observed degree of linearity are also similar to those observed in other non-primate mammalian societies with equivalent group sizes (Chase, 1980; Chase & Seitz, 2011; Fournier & Festa-Bianchet, 1995; Sigurjonsdottir et al., 2012).

We also found that mice further organized themselves into network communities using the Newman-Girvan modularity matrix clustering algorithm. This approach has been well developed and validated for identifying community structure in species as diverse as whales, dolphins, birds and primates (Aplin et al., 2013; Girvan & Newman, 2002; Griffin & Nunn, 2011; Lusseau & Newman, 2004; Lusseau et al., 2008). Based upon the frequency of agonistic interactions, we found strong evidence for two main communities

comprised of 19 and 8 individuals respectively. It was not possible to identify with certainty using this method the community membership of the other three remaining individuals. Individuals in the larger community A were more likely to engage in aggressive interactions with each other and focused these interactions within vivaria 1 and 2. Individuals in the smaller community B were also more likely to be aggressive towards one another with these interactions more commonly occurring in vivaria 3 and 4.

These results were further confirmed and extended by applying a nonmetric multidimensional scaling to the frequency of agonistic interactions in each vivaria by each individual. This strongly agreed with the finding that there were indeed two main communities of mice that could be identified based upon space usage. This analysis was also able to identify the community membership of the remaining three individuals. These community memberships were also confirmed by non-agonistic data. More dominant individuals were at the extremes of the nMDS plot and more subordinate individuals were closer to the boundary of the two clusters. Dominant individuals were also more likely to show significant unevenness in their utilization of those locations where they attack other individuals. This unevenness also increased over time. This is highly suggestive that more dominant individuals were attempting to form territories, a finding consistent with previous reports that male wild mice living in large semi-natural environments will form territories which they will seek to defend from intruders (Crowcroft, 1973; Hurst et al., 2001; Mackintosh, 1970; Perony et al., 2012).

Using our daily census counts of mice, we found that the average half-weight association index for those relationships within each network community was significantly higher than for between community relationships. Community membership and association index matrices were also significantly correlated

with one another demonstrating that these network communities are not only related to the aggressive interactions between mice but to their overall social lives.

We demonstrate in this study that by providing sufficient space that it is possible to collect social behavior data on a large group of laboratory mice that can then be used to determine and assess changes in the social network patterning at the individual, relationship and group structural level in the laboratory. Using such data we are able to show that mice navigate social environments that vary over time and are spatially complex. Understanding how mice manage and maintain their multiple social relationships across time and social contexts enables us to gain insight into the neurobiological processes underlying social learning and competence that are integral aspects of healthy social functioning for all species (Cardoso et al., 2015; Fernald, 2015; Hofmann et al., 2014; Taborsky & Oliveira, 2012).

### ***Behavior prior to group formation does not predict individual network position***

In the directed agonistic network, individuals with high out-degree, out-closeness and hub-score were indicative of more powerful and socially dominant individuals. Individuals with higher in-degree and in-closeness scores were more subordinate. These network metrics were highly inter-correlated with each other likely due to the highly organized network structure. We confirmed the accuracy of these metrics for assessing social power by demonstrating that they correlated extremely highly with the dominance ranking produced using the I&SI ranking algorithm (Schmid & de Vries, 2013).

Assessing the behavior of mice on standard laboratory tests of social and non-social behavior prior to group housing, we found two factors which we named “activity” and “exploration” that significantly

accounted for a large proportion of the variance in behavior. The two factors were not related to one another congruent with other studies (Berton, Ramos, Chaouloff, & Mormède, 1997). Other research in laboratory mice supports our finding that motor activity levels of individual mice are consistent across time and in different contexts indicative of a robust personality trait (Paulus, Dulawa, Ralph, & Geyer, 1999; Tang, Orchard, & Sanford, 2002). The behavioral variable that most strongly correlated with “exploration” factor scores was time spent sniffing in the social interaction test though all other variables also correlated with “exploration” factor scores more than  $r=0.4$  (**Supp. Table S5.4**). We therefore did not clearly observe a distinction between social and asocial exploration as others have noted (Berton et al., 1997; Maier, Vandenhoff, & Crowne, 1988; Makino, Kato, & Maes, 1991), although the highest correlation observed between exploration behavior variables was between time spent sniffing the novel animal in the two social tests. Others have reported similar associations between these two tests in mice (Brodkin, 2007; Crawley, 2007).

Previous studies have suggested both positive (Boogert et al., 2006; David et al., 2011) and negative associations (Fox et al., 2009; Verbeek et al., 1999) between activity levels/exploration and dominance rank. It has been argued that ecological, social and life-history contextual factors may mediate the relationship between these variables (Dingemanse & Goede, 2004). In this study, we could not find any relationship between any behavioral measure made prior to group-formation and eventual social network position. Our data are consistent with one other study that found no pre-group formation differences in activity, anxiety-like or exploratory behavior between male mice that would later become dominant and subordinate in groups of five (Hilakivi-Clarke & Lister, 1992). We did however find that investigation of novel social stimuli prior to group formation was negatively associated with initial out-degree and out-closeness in the social network. This provides evidence that these standard tests of social behavior do reliably a social phenotype that is related to initial social approach behavior and might suggest that social

behavior styles prior to group formation can modulate early social interactions in groups. However, these tests are not reliable for predicting long-term social behavior of animals in social networks being not related to ultimate social network position or dominance rank suggesting that as the group context changes these initial behavior styles become less important than concurrent experiential factors for governing social interaction (Chase & Seitz, 2011; Hsu, Earley, & Wolf, 2006).

We also found that animals of similar activity or exploration factor scores did not preferentially assort or disassort with one another in the social dominance network. Homophily, the preferential association of phenotypically similar individuals, has been observed in human and animal social groups (McPherson, Smith-Lovin, & Cook, 2001). For instance, Aplin et al (2014) found in a natural population of great tits that they assort their social interactions based on their exploratory personality type. Chimpanzees and baboons also assort based on personality measures such as sociability and boldness (Carter et al., 2015; Massen & Koski, 2014). In our social system, however, it seems most likely that the social dominance structure of the population is most critical to determining the associations of individuals as we did find that animals showed significant in-degree and out-degree assortativity. Curiously, we found that the pre-group-formation activity levels of individuals were significantly lower in individuals in community A compared to community B. It is not immediately clear why individuals of lower activity levels would exist in larger communities and more activity in smaller communities, though it has been argued that the number and strength of ties in a social network may relate to personality type (Croft et al., 2009; Pike et al., 2008). It is possible that the less active mice remained in the main large community and more active mice split from it, but this hypothesis requires further investigation.

Our findings have significant implications for social behavior research carried out in laboratory mice. Over the last decade, the majority of work on social behavior of mice has utilized short and simple behavioral battery tests, often using only one outcome behavioral parameter (Peters et al., 2015). This behavioral assay approach fails to incorporate the complexity of any behavior but especially social behavior. The social approach-avoidance test which exists in several different guises (Moy et al., 2004; Nadler et al., 2004; Yang, Silverman, & Crawley, 2001) and the social interaction test (File & Seth, 2003) are the most commonly used social behavior assays in laboratory mice. Both use the total time spent sniffing the novel animal as an index of the sociality of the subject animal. We would argue, as others have (Hofmann et al., 2014; Peters et al., 2015), that it is not clear whether exhibiting high or low social investigation in these tests is a reliable indicator of something as complex as social behavior. It is possible that the investigation of novel individuals in a novel environmental context is actually more related to behavioral inhibition or exploratory behavior than social behavior. Indeed, our findings that time spent investigating both social and non-social stimuli are grouped together in the same 'exploration' factor would seem to support the hypothesis that these tests are not specific to social behavior. It is also not clear from our results that these social behavior assays have strong predictive value for the social behavior of individuals in a group context. Therefore, we suggest that there is a much larger and more complex aspect of the social lives of mice that is not captured by these tests and ought to be considered when investigating the effects of genetic or pharmacological treatments on social behavior.



### ***Social network position is associated with differential brain gene expression***

Hippocampal DNMT1 mRNA expression levels are significantly negatively related to network measures of power and dominance (i.e. out-degree, out-closeness, hub score) across all individuals. The hippocampus is critical for the integration of social information and regulation of learning about social status (Curley, Jensen, Mashoodh, & Champagne, 2011; van der Kooij & Sandi, 2012). While DNMT1 has traditionally been viewed as important for the maintenance of DNA methylation, it is expressed at high levels in the adult hippocampus (Brown, Weaver, Meaney, & Szyf, 2008), and recent studies have found that expression of DNMT1 dynamically shifts in relation to differential environmental experiences that may be related to aggression (Gudsruk & Champagne, 2012; Kundakovic et al., 2013; Zhang et al., 2010). Additionally, studies have shown that variation in social experience can be associated with changes in DNA methylation patterns that are dependent upon the activity of DNA methyltransferases (Alvarado, Fernald, Storey, & Szyf, 2014; Alvarado, Lenkov, Williams, & Fernald, 2015; Borghol et al., 2012; Elliott, Ezra-Nevo, Regev, Neufeld-Cohen, & Chen, 2010; Provençal et al., 2013). Specifically, chronic social defeat stress leads to long-term demethylation of the *Crf* promoter in mice and consequently leads to an increase in social avoidance behaviors (Elliott et al., 2010). In cichlid fish, social crowding during development results in decreased methylation of the *GnRH1* gene (Alvarado et al., 2015) and pharmacological induction of increased methylation leads to development of socially dominant individuals while pharmacological inhibition of DNMT activity leads to development of socially subordinate individuals (Lenkov et al., 2015). Taken together with our findings it is plausible that changes in social network position and social status may be regulated via DNA methyltransferase-dependent epigenetic mechanisms in the hippocampus.

Higher levels of DNMT1 in more subordinate less powerful mice may suggest that these mice are experiencing a social suppression of gene expression in the hippocampus. Subsequent differences in gene

expression between more and less dominant individuals in a brain region specific manner may enable individuals of different social statuses to learn how to express socially contextually appropriate behaviors (Cardoso et al., 2015). Interestingly, the individual with the highest DNMT1 mRNA expression was an extremely dominant individual who lost a number of fights to one other dominant male immediately prior to the end of observations requiring them to learn to express subordinate behavior in a socially specific manner.

While there was no overall relationship between DNMT3a expression and dominance and social network measures, it is relevant to note that we found that more subordinate individuals in the smaller community B exhibited greater levels of relative DNMT3a mRNA expression than dominant individuals. DNMT3a is well known to functionally modulate the effect of environmental experiences on brain gene expression and specifically regulate learning about socioemotional behavior including social defeat (Hammels et al., 2015; Yu et al., 2011). In honeybees, inhibition of DNMT3a in larvae leads to their development as a queen bee (Evans & Wheeler, 1999; Kucharski et al., 2008), further demonstrating that DNMT3a can play a plastic role in regulating social roles. Although we do not see this association in the larger community, this may be due to temporal differences in when each community is undergoing changes in social roles and thus brain plasticity, or related to differential social dynamics (e.g. the higher rate of repeated social interactions) that occur in small versus large communities.

Finally, although changes in the DNA methylation of specific genes (e.g. *GnRH1*) in the mPOA is integral for the ability to transition from dominant to subordinate status in cichlid fish undergoing social ascent (Maruska & Fernald, 2011), we found no relationship between social network position or rank and DNMT1

or DNMT3a mRNA expression in the mouse mPOA, suggesting that plasticity in the mPOA may not be important to the maintenance of social status in mouse stable hierarchies.

## **CONCLUSION**

We have demonstrated that a large group of 30 male mice form a hierarchically organized agonistic social network. This network is further sub-organized into two main network communities that are spatially dissociated. We also demonstrate that behavior of males prior to group-formation in commonly used laboratory behavior tests (the open-field test, novel object test, social interaction test, and approach-avoidance paradigm), is not predictive of later social network position though is somewhat associated with initial behavior in the network prior to the group stabilizing its hierarchical organization. We further show that dominance rank and network position are associated with differential hippocampal DNMT1 and DNMT3a expression suggesting that increased hippocampal neural plasticity may be associated with the development of contextually specific subordinate behavior. Future studies will need to mechanistically address the functional significance of changes in hippocampal DNMT expression in regulating social competence within a social hierarchy. Studying the neurobiology of complex social behavior of mice requires the development of improved paradigms of behavioral assessments that go beyond mice interacting in dyads in novel contexts for brief periods of time. Here, we have shown that using ethologically relevant housing of male mice over three weeks is sufficient to reveal complex spatiotemporal patterns of agonistic behavior between male mice with context-specific consequences for brain gene expression.

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**SUPPLEMENTAL MATERIAL**

**Supplemental Table S5.1. Ethogram of Behaviors Coded in the Social Interaction Test**

<b>Behavior</b>	<b>Description</b>
Idle/nothing	Individual is not moving or interacting with other individual
Sniff head	Individual approaches and makes an olfactory investigation of the head of other individual
Sniff body	Individual approaches and makes an olfactory investigation of the body of other individual
Sniff anogenital	Individual approaches and makes an olfactory investigation of the anogenital region of other individual
Sniff and follow	Individual approaches and makes an olfactory investigation of other individual while following around the space
Rearing	Individual rears on hind legs
Digging	Individual digs into pine bedding on floor
Self-grooming	Individual grooms self with mouth and/or paws
Jumping	Individual jumps
Contact side by side	Individual has contact with other individual while neither sniffing nor biting
Pursuing	The focal individual follows the target individual rapidly and aggressively whilst the target individual attempts to flee
Allogrooming	Individual grooms with their paws and mouth the fur and/or face of another individual
Biting	Individual bites other individual
Lunging	Individual moves towards other individual as if to attack
Tail Rattle	Individual displays a fast tail vibration of the tail, often observed in a distance ambivalence situation
Defensive freeze	Individual freezes as other individual moves to attack
Display of subordinate posture	Individual reacts to the movements of the partner by remaining motionless
Fleeing	Individual moves rapidly away from the partner

**Supplemental Table S5.2. Ethogram of Behaviors Coded in the Social Approach-Avoidance Test**

<b>Behavior</b>	<b>Description</b>
Near Novel Object	Individual is close to cup containing the novel object but not sniffing or engaging with it
Sniff Novel Object	Individual is sniffing the cup containing the novel object
Near Social Stimulus	Individual is close to the cup containing the social stimulus but not sniffing or engaging with it
Sniff Social Stimulus	Individual is sniffing the cup containing the social stimulus
Rearing	Individual rears on hind legs
Idle/Nothing	Individual is not moving or interacting with other individual or the novel object
Moving	Animal is moving through the space but not engaging with social stimulus or novel object
Digging	Individual digs into pine bedding on floor
Self-grooming	Individual grooms self with mouth and/or paws
Jumping	Individual jumps

**Supplemental Table S5.3. Summary of Factor Loadings for Each Standard Behavioral Test**

a) Open-Field, KMO = 0.53, Bartlett's Test  $p < .001$

<b>Behavior</b>	<b>Factor 1</b>	<b>Factor 2</b>
Latency to Center of Area (s)	0.03	<b>0.97</b>
Rearing Frequency	<b>-0.59</b>	-0.48
Duration Immobile (s)	<b>0.99</b>	0.11
Duration in Inner Area (s)	-0.01	<b>-0.62</b>
Number of boli	-0.20	0.03
Proportion Variance	0.27	0.31

b) Novel-object, KMO = 0.55, Bartlett's Test  $p < .001$

<b>Behavior</b>	<b>Factor 1</b>	<b>Factor 2</b>
Latency to Move Near (s)	<b>0.99</b>	-0.14
Latency to Sniff Near (s)	<b>0.64</b>	-0.08
Duration Move Near (s)	-0.10	<b>0.71</b>
Duration Sniff Near (s)	0.02	<b>0.73</b>
Time immobile (s)	0.36	<b>-0.54</b>
Number of boli	-0.10	0.43
Proportion Variance	0.26	0.26

c) Social Interaction, KMO = 0.61, Bartlett's Test  $p < .001$

<b>Behavior</b>	<b>Factor 1</b>	<b>Factor 2</b>
Duration Sniff Anogenital (s)	0.20	<b>0.83</b>
Duration Sniff Body (s)	0.09	<b>0.71</b>
Duration Sniff Follow (s)	-0.08	0.19
Duration Sniff Head (s)	0.27	<b>0.50</b>
Time immobile (s)	<b>0.88</b>	0.07
Frequency of Rearing	<b>-0.92</b>	-0.11
Proportion Variance	0.29	0.25



**Supplemental Table S5.4. Correlation of Behavioral Variables and Factor Scores**

OF: open-field, NO: novel object, SI: social interaction, SA: social approach/avoidance

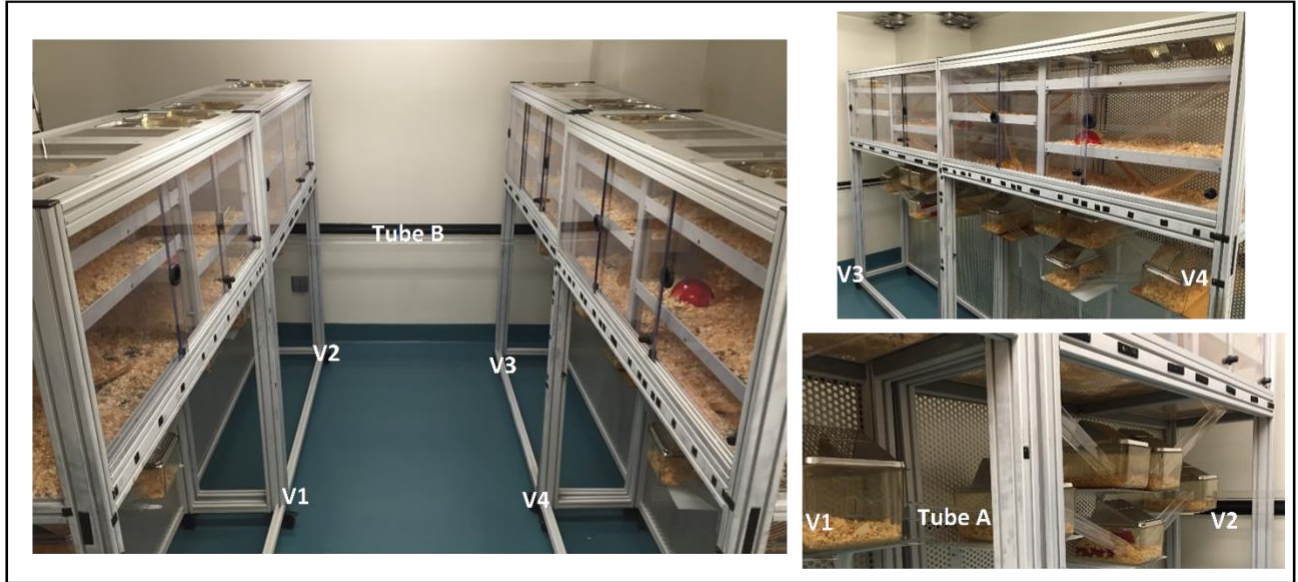
a) Exploration - \*\*\* p<.001, \*\*p<.01, \*p<.05, †p<.10

<b>Behavior</b>	OF: Duration in Inner Area (s)	NO: Duration Sniffing Novel Object (s)	SI: Duration Sniffing Novel Animal (s)	SA: Duration Sniffing Novel Animal (s)
Exploration Factor Score	<b>0.55**</b>	<b>0.48**</b>	<b>0.94***</b>	<b>0.43*</b>
OF: Duration in Inner Area (s)		0.19	0.33†	0.22
NO: Duration Sniffing Novel Object (s)			<b>0.37*</b>	0.11
SI: Duration Sniffing Novel Animal (s)				<b>0.44*</b>

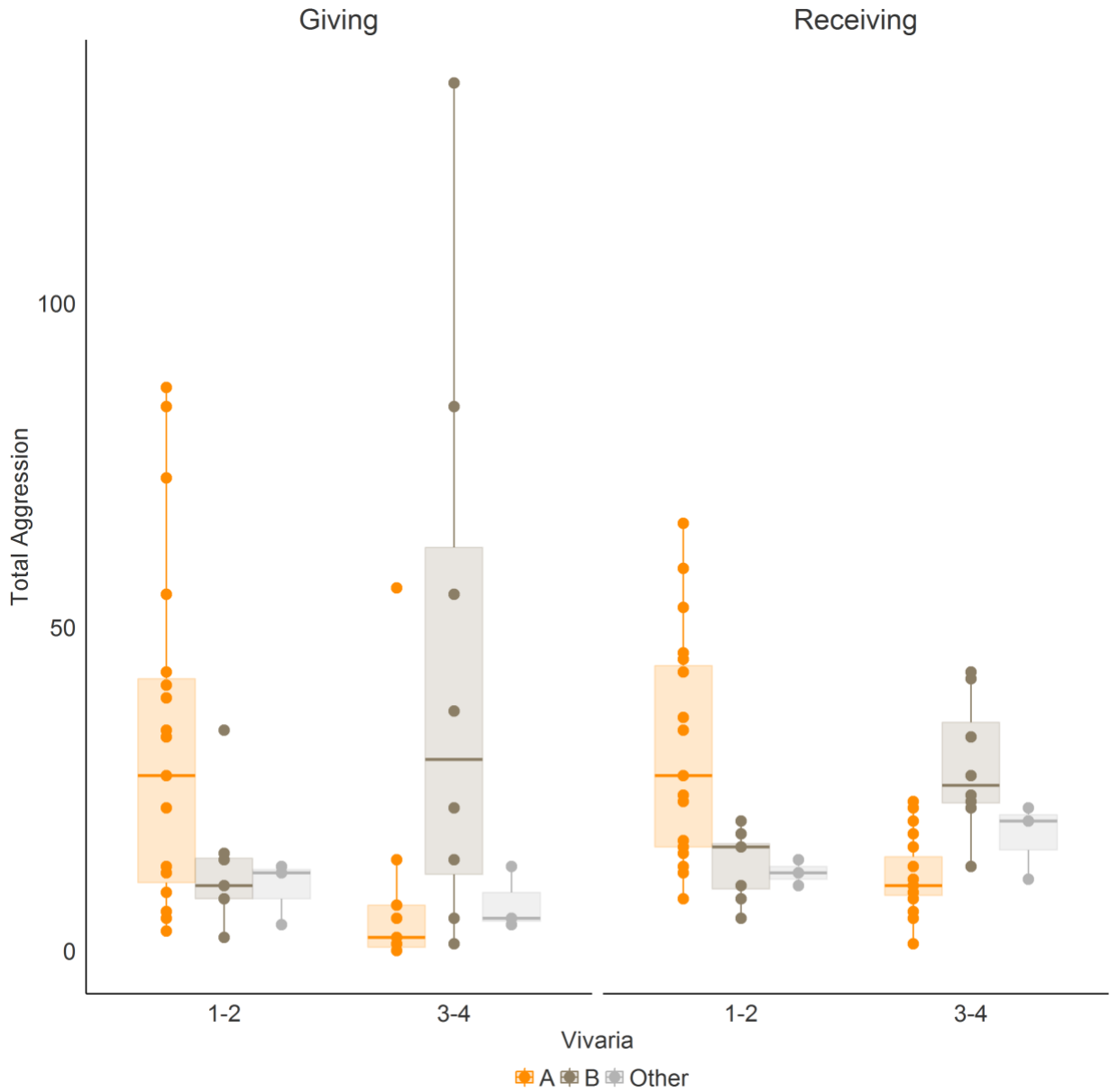
b) Activity - \*\*\* p<.001, \*\*p<.01, \*p<.05

<b>Behavior</b>	OF: Rearing Frequency	SI: Rearing Frequency	SA: Rearing Frequency
Activity Factor Score	<b>0.85***</b>	<b>0.73***</b>	<b>0.73***</b>
OF: Rearing Frequency		<b>0.50**</b>	<b>0.45*</b>
SI: Rearing Frequency			<b>0.41*</b>

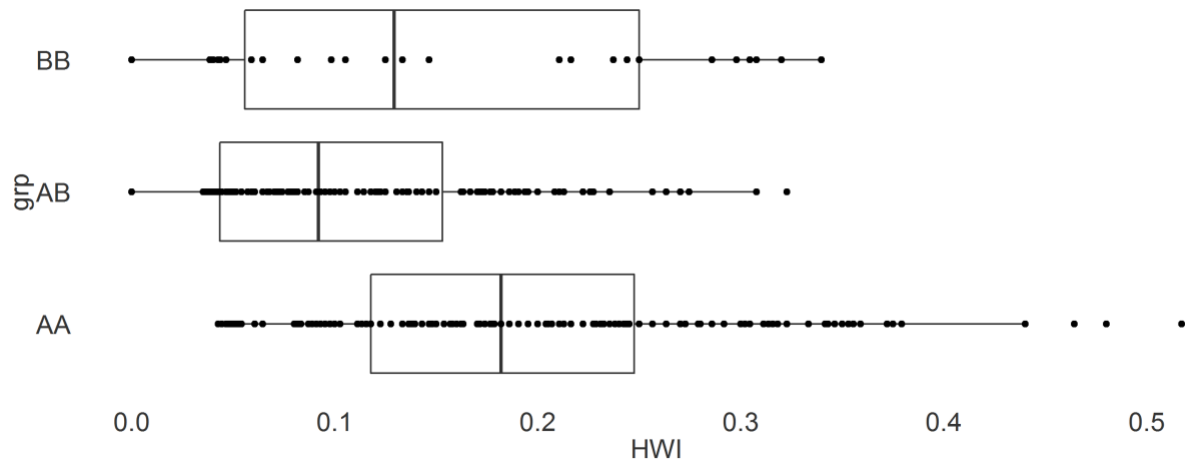
**Supplemental Figure S5.1. Housing Vivaria** - View of all 4 inter-connected vivaria (V1, V2, V3, V4) connected via a long tube (Tube B) across the room (Left). View of connection between the nest-boxes of adjacent vivaria V1 & V2 connected via a short tube (Tube A) (Bottom Right). An identical tube (Tube C) connects V3 & V4. View of vivaria V3 and V4 showing food and water at top of upper section and nest-boxes underneath.



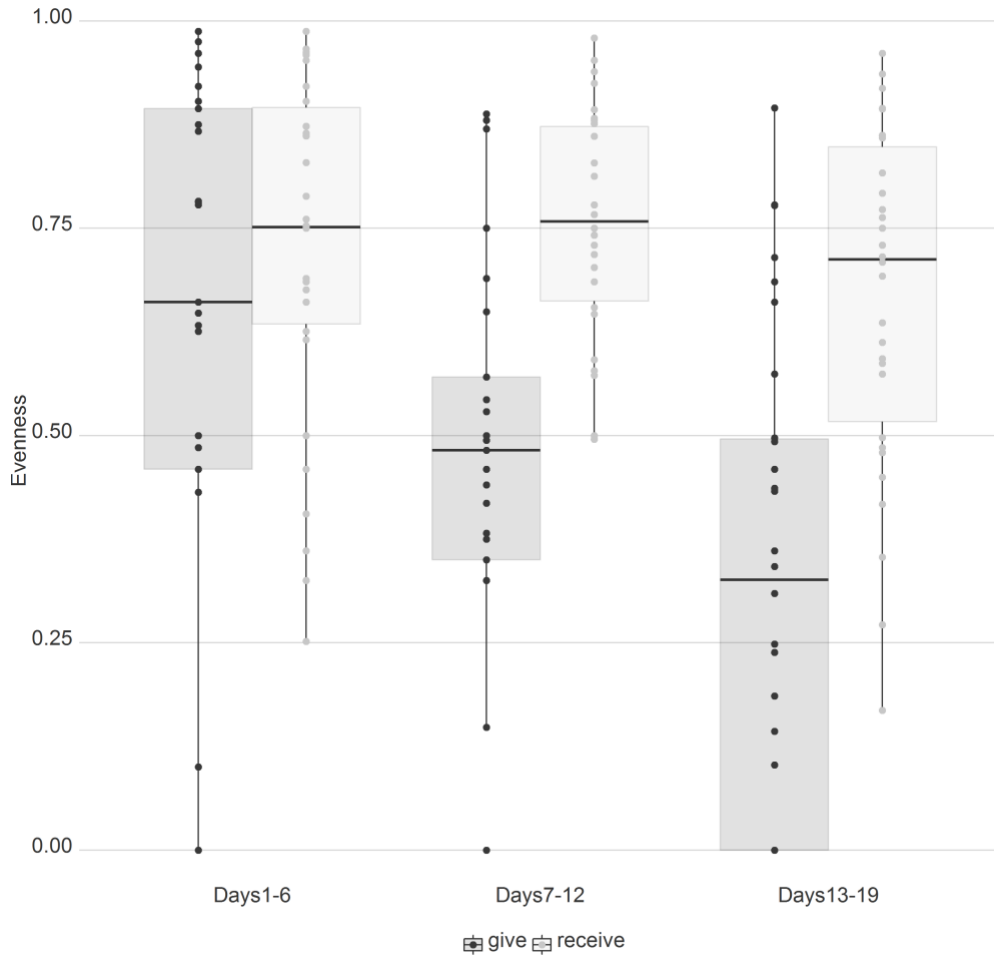
**Supplemental Figure S5.2. Location of Agonistic Interactions By Community** Boxplots of the total number of aggressive interactions that occurred in vivaria 1-2 or 3-4 separated by community (A, B or Other). Each point refers to a unique individual.



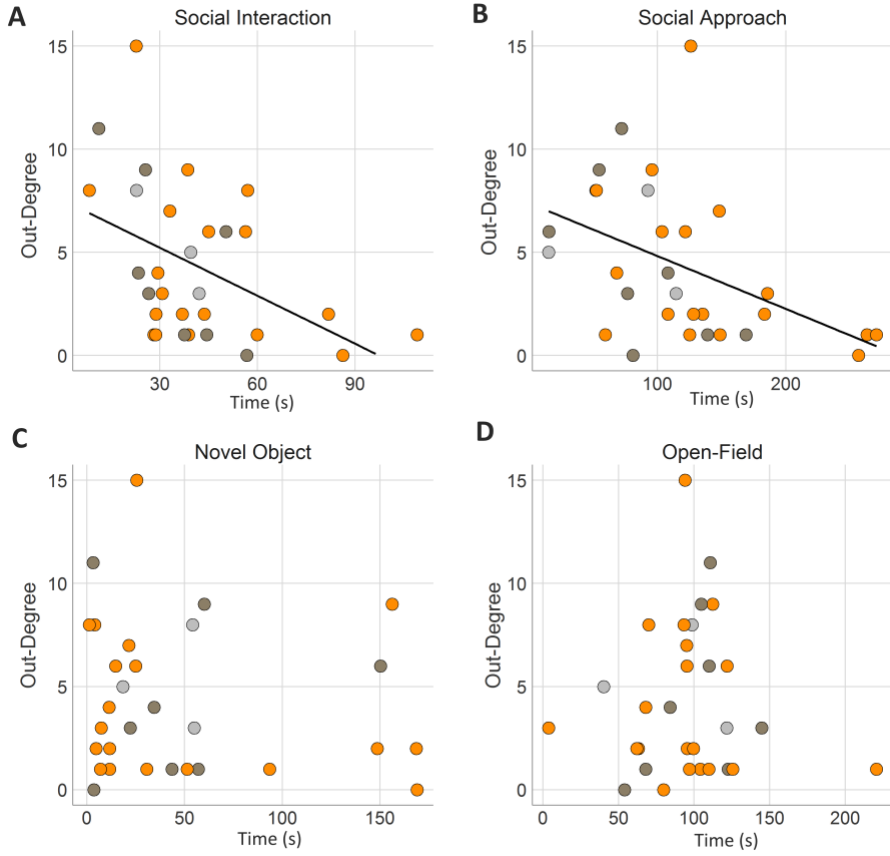
**Supplemental Figure S5.3. Half-Weight Association Indices Within and Between Communities** Boxplots of the half-weight-association indices (HWI) occurring between individuals in community A (AA) or community B (BB) or between individuals in each community (AB).



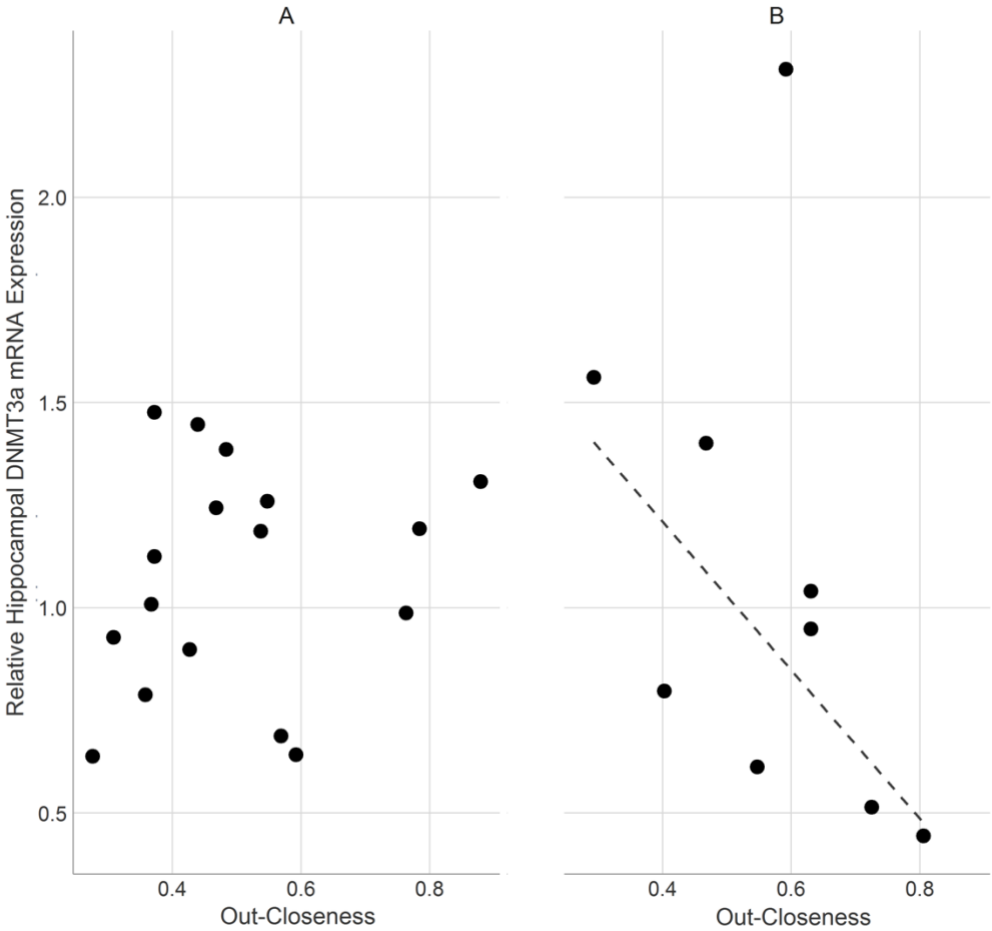
**Supplemental Figure S5.4. Changes in Space Usage Evenness of Giving and Receiving Aggression by Days**  
Boxplots of Shannon's Evenness of giving and receiving aggression of individuals over days.



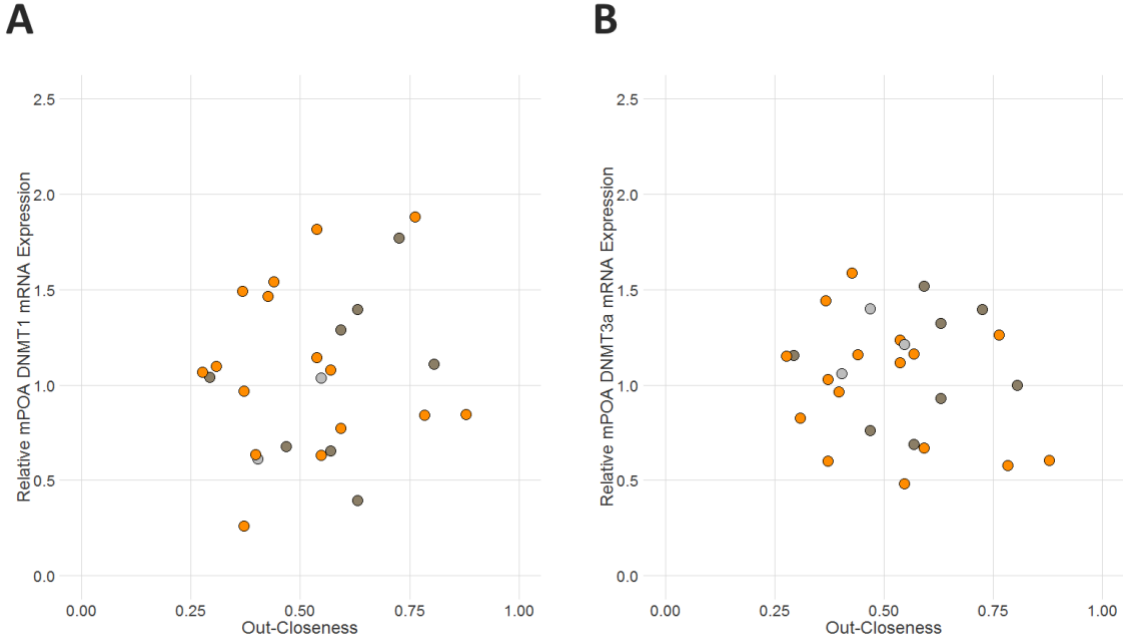
**Supplemental Figure S5.5. Investigation of novel social stimuli are negatively associated with Initial Individual Network Position.** Individuals who investigate a novel social animal for longer on (A) the social interaction test and (B) the social approach test have a smaller out-degree after four days of group formation. No relationship between out-degree and behavior on (C) the novel object or (D) the open-field tests were found. Behavior on all tests was not related to final network position. Each point represents one individual with color representing the network community of that individual (orange – community A, dark gray – community B, light gray – other).



**Supplemental Figure S5.6.** Hippocampal DNMT3a expression is negatively associated with Out-Closeness in individuals in Community B but not Community A. Trendline represents the line of best fit with the outlier removed.



**Supplemental Figure S5.7.** mPOA (A) DNMT1 and (B) DNMT3a expression are not associated with Out-Closeness. Each point represents one individual with color representing the network community of that individual (orange – community A, dark gray – community B, light gray – other).





## CHAPTER 6 – The behavioral, neuroendocrine, and brain plasticity response to social opportunity

### *Study #1: Dynamic changes in social dominance and mPOA GnRH expression in male mice following social opportunity*

Cait M. Williamson, Russell D. Romeo, James P. Curley

Please note, study published as:

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## **ABSTRACT**

Social competence - the ability of animals to dynamically adjust their social behavior dependent on the current social context – is fundamental to the successful establishment and maintenance of social relationships in group-living species. The social opportunity paradigm, where animals rapidly ascend a social hierarchy following the removal of more dominant individuals, is a well-established approach for studying the neural and neuroendocrine mechanisms underlying socially competent behavior. In the current study, we demonstrate that this paradigm can be successfully adapted for studying socially competent behavior in laboratory mice. Replicating our previous reports, we show that male laboratory mice housed in a semi-natural environment form stable linear social hierarchies. Novel to the current study, we find that subdominant male mice immediately respond to the removal of the alpha male from a hierarchy by initiating a dramatic increase in aggressive behavior towards more subordinate individuals. Consequently, subdominants assume the role of the alpha male. Analysis of brain gene expression in individuals one hour following social ascent indicates elevated gonadotropin-releasing hormone (GnRH) mRNA levels in the medial preoptic area (mPOA) of the hypothalamus compared to individuals that do not experience a social opportunity. Moreover, hormonal analyses indicate that subdominant individuals have increased circulating plasma testosterone levels compared to subordinate individuals. Our findings demonstrate that male mice are able to dynamically and rapidly adjust both behavior and neuroendocrine function in response to changes in social context. Further, we establish the social opportunity paradigm as an ethologically relevant approach for studying social competence and behavioral plasticity in mammals.

## INTRODUCTION

Social hierarchies emerge and stabilize over time as individuals engage in competitive or agonistic interactions and relatively subordinate individuals learn to consistently yield to individuals of a relatively higher social status (Chase, 1982). Dominance hierarchies occur frequently in wild species (Muller & Wrangham, 2004; Nakano, 1995; Sapolsky, 1983, 1993) and in laboratory-based studies of cichlids (Grosenick, Clement, & Fernald, 2007; Oliveira & Almada, 1996), crayfish (Issa, Adamson, & Edwards, 1999), honey bees (Kucharski, Maleszka, Foret, & Maleszka, 2008), and mice (Wang et al., 2011; Williamson, Lee, & Curley, 2016). Although findings vary across species, individuals at the top of a social hierarchy may have significantly higher reproductive success, increased neurogenesis, enhanced immune function and better overall health outcomes than those at the bottom of a hierarchy (Archie, Altmann, & Alberts, 2012; Bartolomucci et al., 2001; Kozorovitskiy & Gould, 2004; Maruska & Fernald, 2013; Sapolsky, 1993). It is therefore essential that individuals are capable of recognizing their own social status relative to others in a hierarchy and of dynamically shifting their behavior when a social system destabilizes (Fernald, 2014).

One approach to studying dynamic changes in social behavior within a social hierarchy is the social opportunity paradigm, where subdominant individuals rapidly ascend a hierarchy following the removal of the most dominant individual. Ascent following social opportunity has been studied in African cichlid fish, with changes in both behavior and physiology occurring in subdominants within minutes of the removal of the alpha male (Maruska, Zhang, Neboori, & Fernald, 2013; Maruska & Fernald, 2013; Maruska, Levavi-Sivan, Biran, & Fernald, 2011). These physiological changes include alterations within the hypothalamic-pituitary-gonadal (HPG) axis such as increased levels of circulating 11-ketotestosterone (Maruska & Fernald, 2010) and increased brain gonadotropin-releasing hormone 1 (GnRH1) mRNA levels (Maruska & Fernald, 2013) within socially ascending sub-dominant males.

Previously, we have shown that outbred CD-1 male mice housed in groups of 12 will consistently and rapidly form linear dominance hierarchies in the laboratory (So, Franks, Lim, & Curley, 2015; Williamson et al., 2016). Housing male mice in large, complex environments for a period of three weeks, we have established that each mouse has a unique rank and behaves appropriately to individuals of relatively higher and lower social status (Williamson et al., 2016). Similar to cichlid fish (Desjardins, Hofmann, & Fernald, 2012), we have also shown that subdominant and subordinate mice are aware of social context, inhibiting their aggressive behavior in the social hierarchy when the alpha male is actively aggressive to other individuals and increasing their aggression when the alpha male is inactive (Curley, 2016b).

The aim of the current study was to first determine whether, following removal of the alpha male mouse from a social hierarchy, subdominant male mice (beta males) would recognize and take advantage of this social opportunity by increasing their aggression to all other individuals in the hierarchy and ascending to alpha male status. The second aim was to determine if such rapid behavioral changes are associated with physiological changes in the HPG axis similar to those observed in cichlid fish. Although subordinate male mice are not fully reproductively suppressed, they do have decreased testes weight (Bronson & Eleftheriou, 1964; Mckinney & Desjardins, 1973) and sperm motility (Koyama & Kamimura, 1998) compared to dominant males, suggesting a down-regulation of the HPG axis. In one study investigating groups of three males, subdominant male mice appear to be similar in HPG activation to subordinate males (Mckinney & Desjardins, 1973). We hypothesized that compared to subdominant males in stable hierarchies, where no social opportunity occurred, we would observe increased hypothalamic GnRH mRNA levels in subdominant males undergoing social ascent as well as a corresponding increase in plasma testosterone.

## **METHODS**

### **Subjects and Housing**

Throughout the study, subjects were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21–24°C) and humidity (30-50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red lights (dark cycle) on at 1200 hours. For each experiment, all mice were individually and uniquely marked by dyeing their fur with a blue, nontoxic, non-hazardous animal marker (Stoelting Co.). These marks remain for up to 12 weeks and only require one application, thus enabling each animal to be visually identified throughout the study. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol Nos: AC-AAAG0054, AC-AAAP5405).

### **Experiment #1: Behavioral dynamics of a social hierarchy following social opportunity**

Twelve male outbred CD1 mice aged 6 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 3 weeks in standard-sized cages containing environmental enrichment (wooden blocks and nestlets). At 9 weeks of age, all twelve mice were weighed and put into a large custom built vivarium (length 150cm, height 80cm, width 80cm; Mid-Atlantic; **Supplemental Figure S6.1**). The vivarium was constructed as described in So et. al. (2015) and Williamson et. al. (2016). Briefly, each vivarium consists of an upper level consisting of multiple shelves covered in pine bedding and a lower level consisting of a series of nestboxes filled with pine bedding connected by tubes. Mice can access all levels of the vivarium via a system of ramps and tunnels. Standard chow and water were provided ad libitum at the top of the vivarium, encouraging movement and exploration of all the shelves. The one cohort of twelve animals were put into the vivarium just before onset of the dark cycle on Day 1 of the experiment and were observed for 40 days for up to 5 hours per day with an average of 3 hours of observation per day. All

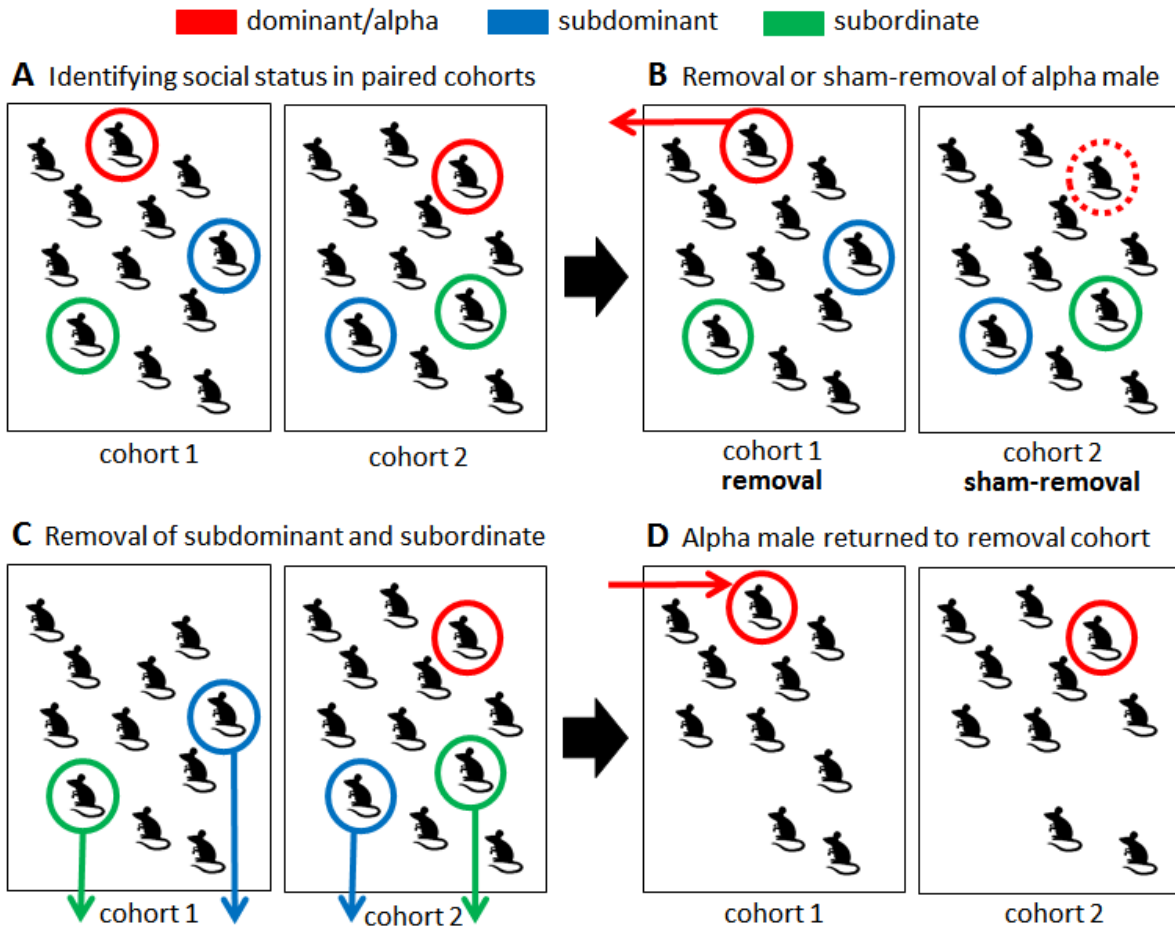
observations were conducted during the first seven hours of the dark cycle. During these observations, trained observers recorded all instances of fighting, chasing, mounting, subordinate posture and induced-flee behaviors recording the identity of the individuals that were dominant and subordinate in the interaction (contests) using all occurrence sampling. **Supplemental Table S6.1** contains an ethogram of these behaviors. On Day 5, the first alpha male was removed from the system. Upon removal, the alpha male was never returned to the social group. The most dominant male within the social hierarchy continued to be removed every 3-4 days until there were only 2 mice remaining in the system. These removals occurred on Days 8, 12, 15, 19, 22, 26, 29, 33, 36, and 40, and observations were conducted following each alpha removal and on all days in between removals.

**Experiment #2: Behavioral and neuroendocrine changes following exposure to social opportunity vs. social stability**

To determine how rapidly individual males socially ascend and the association between ascent and changes in gene expression and circulating hormone levels, we designed a social opportunity manipulation comparing individuals from socially stable groups to those from a group undergoing a social transition. A total of 96 male outbred CD1 mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 2 weeks in standard sized cages. At 9 weeks of age, groups of 12 mice were placed into custom built vivaria (see **Supplemental Figure S6.1**). Social groups (N=12 mice) were paired such that each group was introduced into the vivarium on the same day as one other group, creating 4 sets of paired cohorts. Live behavioral observations occurred as described in Experiment #1 for 2 hours a day for each group on Days 1-5 of the experiment. At the end of this initial observation period, a linear dominance hierarchy was verified to have emerged through analysis of the collected behavioral data through calculation of Landau's modified  $h'$  (De Vries, 1995). The identity of the alpha male and all

other ranks was determined through calculation of Glicko Ratings. In the Glicko Rating system (Glickman, 1999; Williamson et al., 2016), animals gain or lose points based on the number of wins and losses relative to the difference in ratings between themselves and their opponent (see Williamson et al., 2016 for a more detailed description of the calculations). All social groups formed a linear hierarchy with identifiable individual ranks by Day 5. On Day 6, immediately following the onset of the dark/red light cycle, the alpha male from one of the paired cohorts was removed from the vivarium (social opportunity condition) and placed in a standard cage with food and water. In the other paired cohort, the alpha male was sham-removed. The sham-removal consisted of an experimenter opening the Perspex windows to the vivarium, placing their hand into the vivarium and reaching towards the alpha mouse but not removing him from the vivarium. Thus, in this condition the alpha male was not removed from the social group. This condition controls for behavioral changes that may be occurring in response to a disturbance of the housing system that does not impact the presence of the alpha male. Live behavioral observations occurred for the one-hour period directly following alpha removal or sham-removal. Ascending subdominant males were confirmed as the individual who won most contests post-removal without consistently losing to other males. One hour after the subdominant male in the social opportunity group had won three fights, two mice were removed from each group. From the social opportunity group, the subdominant individual who had risen to dominant status and the most subordinate individual were removed. From the sham-removal group, the subdominant individual who had remained subdominant and the most subordinate individual were taken. This experimental design is detailed in **Figure 6.1**.

**Figure 6.1. Schematic of the social opportunity experimental design.** (A) Two cohorts of twelve mice are put into separate vivaria and a stable social hierarchy emerges, with clearly defined dominant, subdominant and subordinate individuals. (B) The alpha male is removed from one stable hierarchy and sham-removed from the paired hierarchy. (C) Following removal/sham-removal, behavioral observations are conducted on both cohorts until one hour after a subdominant rises in the alpha-removed group. At this one-hour time point, the most subdominant and subordinate animal in each hierarchy is removed and brains and trunk-blood collected. (D) One-hour following this removal of the subdominant and subordinate, the alpha male is returned to its social group. This procedure is repeated three more times five days apart for each pair of cohorts.



Following removal from the social group, mice were immediately euthanized via cervical dislocation, and brains were flash frozen in hexane. Trunk blood was collected into heparinized tubes and plasma was separated and then stored at  $-80^{\circ}\text{C}$ . Following brain and blood collection (subdominant and subordinate), the alpha male who had been removed within the social opportunity condition was returned to his social group. This procedure was repeated at five day intervals for a total of four “removals”. However,



manipulations were counter-balanced between paired cohorts (i.e. one vivarium had alpha removal for removals 1 and 3 and sham-removals for removals 2 and 4 and the opposite was true of the paired vivarium). Each removal/sham-removal decreased the size of the social group by 2, resulting in N=10 (first removal), N=8 (second removal) and N=6 (third removal). This experimental design yielded N=16 mice per group from four groups: subdominant/alpha-removed, subordinate/alpha-removed, subdominant/alpha sham-removed, subordinate/alpha sham-removed.

### **Gene Expression**

Brains were stored at -80°C until dissection. Samples of the medial preoptic area (mPOA) were collected using a Harris Micro-Punch with reference to coronal plane from the Mouse Brain Atlas (Paxinos & Franklin, 2004). The mPOA was collected as one 1mm diameter area along the midline from Bregma +0.14mm to -0.7mm. RNA was isolated from the mPOA of each individual using the AllPrep RNA Micro Kit (Qiagen) and reverse transcribed to cDNA using the SuperScript III First-Strand Synthesis System for RT-PCR applications (Invitrogen). Quantitative RT-PCR was performed with 1µL of cDNA using an ABI 7500 Fast Thermal Cycler and the Fast SYBR Green Master Mix reagent (Applied Biosystems). All primer probes (Sigma-Aldrich) were designed to span exon boundaries ensuring amplification of only mRNA. For each gene,  $C_T$  values were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH - endogenous control). Relative expression values were obtained by the  $\Delta\Delta C_T$  method (Schmittgen & Livak, 2008) with fold-difference being determined relative to subordinates in the sham-removal condition. The following validated quantitative PCR primers were used for mRNA analysis: GAPDH (Forward: TGTGTCCGTCGTGGATCTGA; Reverse: CCTGCTTACCACCTTCTTGA), Gnrh1 (Forward: AGCACTGGTCCTATGGGTTG; Reverse: GGTTCTGCCATTTGATCCAC). Samples that did not yield sufficient RNA for cDNA conversion were eliminated from the analysis.

## **Hormone Assay**

Plasma testosterone concentrations were measured using a commercially available kit (MP Biomedicals, LLC; Orangeburg, NY) and conducted using the manufacturer's specifications. Samples were run in duplicate and values were averaged. The intra-assay coefficient of variation was 16.2% and lower limit of detectability for the assay was 0.09 ng/ml.

## **Statistical Analysis**

All statistical analyses were undertaken in R version 3.2.3 (R Core Team, 2016).

*Behavior Analysis:* The linearity of each hierarchy was calculated using Landau's Modified  $h'$ . Briefly, the total number of wins by each individual against all other individuals are entered into a sociomatrix. Landau's method then assesses the degree to which each individual consistently dominates others in contests and whether individuals can be linearly ordered based upon their wins and losses. It ranges from 0 (no linearity) to 1 (completely linear). The significance of  $h'$  is determined by performing 10,000 two-step Monte Carlo randomizations of the sociomatrix and comparing the observed  $h'$  against a simulated distribution of  $h'$  (De Vries, 1995; Williamson et al., 2016). Temporal changes in individual dominance ratings were calculated using Glicko Ratings (Glickman, 1999; So et al., 2015). Glicko ratings are a pairwise-contest model ratings system where ratings points are recalculated following each successive win or loss. All individuals start with a rating of 2200. Ratings are gained after wins and lost after losses with the magnitude of points gained or lost dependent upon the difference in ratings scores between the two individuals in each contest (Glickman, 1999; Williamson et al., 2016). Landau's modified  $h'$  was calculated using the R package *compete* v0.1 (Curley, 2016a). Glicko ratings were calculated using the *PlayerRatings* package v1.0 in R (Stephenson & Sonas, 2012).

Differences in proportions of individuals engaging in behavior were assessed using a Binomial test or Chi-Squared test as appropriate. Differences between two groups in the frequency or latency of behaviors were assessed using paired or unpaired Wilcoxon Signed Rank Tests as appropriate. Individual David's Scores were calculated based on wins and losses in the hour after alpha removal or sham-removal to compare individual dominance scores during these periods (De Vries, 1995). To compare changes in total wins between the day prior to alpha removal/sham-removal and the day of alpha-removal/sham-removal, we used a zero inflated negative binomial generalized mixed effect model with counts of wins as the outcome variable, alpha removal status (removal or sham-removal) and day (day prior to removal or day of removal) as fixed effects and cohort and removal number as random effects using the R package glmmADMB (Skaug, Fournier, Bolker, Magnusson, & Nielsen, 2015) and lmerTest (Kuznetsova, Brockhoff, & Christensen, 2015).

*GnRH Expression and Testosterone Analysis:* To examine the effect of alpha removal and social status (subdominant or subordinate) on GnRH mRNA levels and circulating plasma testosterone, we used general linear models using the R package lme4 (Bates et al., 2015). GnRH and testosterone data were log-transformed to ensure assumptions of normal distribution were satisfied. To determine the effect of number of wins, number of losses, and total fights occurring within each social group in the hour post-alpha removal or sham-removal on GnRH mRNA levels and circulating plasma testosterone we performed Spearman rank correlations between number of wins, losses, total fights and GnRH or testosterone levels. These correlations were run separately for each condition and male social status giving four groups (subdominant/alpha-removed; subdominant/sham-removal; subordinate/alpha-removed; subordinate/sham-removal).

*Effect size calculations:* For all Wilcoxon Rank Sum tests and Wilcoxon Signed Rank tests, effect sizes were calculated with the formula  $r = \frac{z}{\sqrt{N}}$ . An  $r$  value below 0.3 indicates a low effect, between 0.3 and 0.5 indicates a moderate effect, between 0.5 and 0.7 indicates a large effect. Cohen's  $d$  was calculated for all Chi-Squared tests. A Cohen's  $d$  value between 0.2 and 0.5 indicates a small effect size, 0.5 to 0.8 indicates a medium effect size, and values above 0.8 indicate a large effect size.

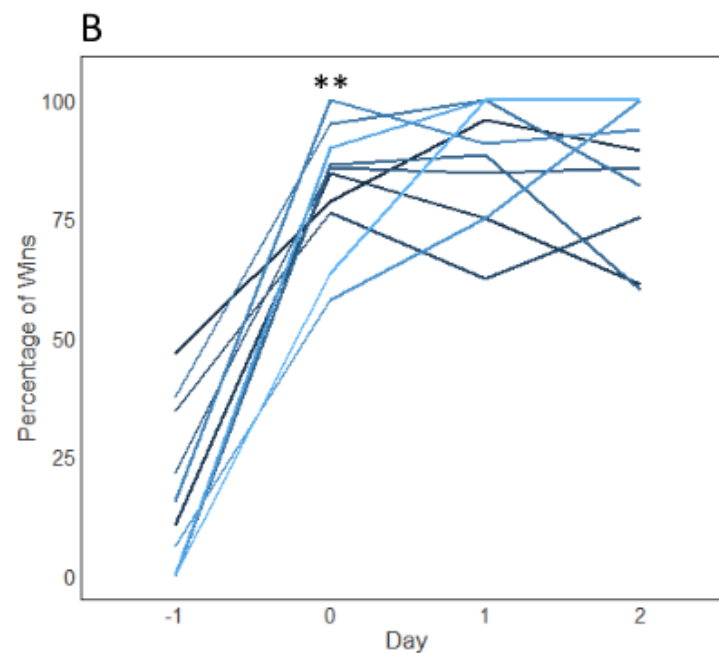
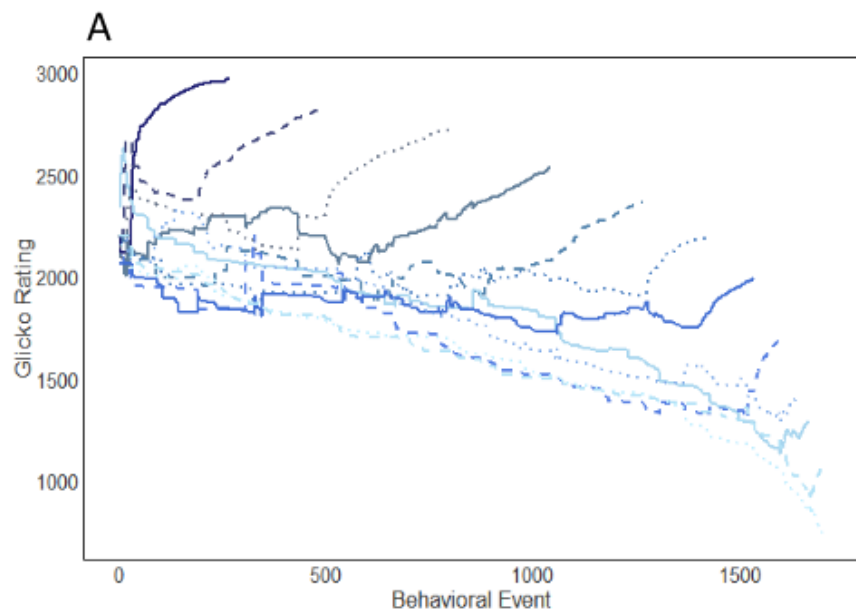
## RESULTS

### **Experiment #1: Subdominant males socially ascend and assume alpha male status following social opportunity**

Following each of the 10 removals of the most dominant alpha male from the social group, the next most socially dominant male ascended to become the new alpha male within one day (Binomial test,  $N=10$ ,  $p = 0.002$ ). Glicko dominance ratings indicated that the socially ascending male rapidly increases their dominance rating relative to all other males following each alpha male removal (**Figure 6.2A**). All rising subdominant males showed a significant increase in their daily relative share of aggressive behaviors in the hierarchy on the day of alpha removal compared to the day prior to alpha removal (Wilcoxon paired test:  $V = 0$ ,  $p = 0.002$ ,  $r = 0.63$ , **Figure 6.2B**). There was no difference in relative share of aggression by each male between the day of ascending to alpha rank and the day after (Wilcoxon paired test:  $V = 17$ ,  $p = 0.32$ ,  $r = 0.24$ ) or between the next two days (Wilcoxon paired test:  $V = 22$ ,  $p = 0.62$ ,  $r = 0.09$ ), indicating that socially ascended males maintain their new alpha male status over several days. Additionally, the day before the alpha male was removed, subdominant males lost a median of 12.9% of all fights in the hierarchy (nearly all to alpha males), whereas after alpha removal this value significantly dropped to a median of 0% fights lost (Wilcoxon paired test:  $V = 55$ ,  $p = 0.002$ ,  $r = 0.62$ ) and stayed at this value for the next three days. Thus,

the most dominant subdominant male socially ascends to become the new and stable unequivocal alpha male following removal of the previous alpha male.

**Figure 6.2.** Behavioral changes following social opportunity. (A) Glicko Dominance Rating as recalculated after each observed agonistic interaction. Separate lines represent different individuals. (B) The percentage of all contests occurring in a social hierarchy that are won by the subdominant male on the day of social ascent (0), the following two days (1,2) and the day before social ascent when a more dominant alpha male was present (-1). Separate lines represent different individuals. Asterisks denote a significant difference in percentage of wins ( $p < 0.01$ ) from the day prior to removal of the dominant to the day of dominant removal.



## **Experiment #2: Social ascent dynamics and neuroendocrine impact**

Prior to the first alpha or sham-removal, all eight social groups of twelve males had formed a stable social hierarchy with a clear alpha male (All  $h'$  values  $> 0.43$  – mean  $h' = 0.54$ ; all  $p < 0.05$  - mean  $p = 0.018$ ). All alpha males maintained their social rank for the duration of the experiment.

### **Subdominant males rapidly socially ascend following social opportunity**

After each of the 16 removals of alpha males, one subdominant male clearly rapidly ascended within one hour. Rising subdominants had on average 10 times as many wins as the individual with the second most wins in this time period and 15/16 rising males never lost any fight (the remaining rising male only lost one fight, **Supplemental Figures S6.2 & S6.3**).

The identity of the rising subdominant could be predicted from analyzing the behavior in the five days prior to each alpha removal. A significant proportion of males that rose (13/16) were those with the second highest Glicko ranking (i.e. second to alpha male) prior to removal (Binomial Test,  $p=0.02$ ). In 3/16 instances, individual that ascended was another subdominant male with a slightly lower Glicko rating than the highest subdominant. Notably, two of these instances were during the fourth removal (i.e. following several manipulations of the social group).

We compared the frequency of aggressive behavior exhibited by rising subdominant males compared to subdominant males of the equivalent rank when the alpha male was sham-removed. A significantly higher proportion of subdominant males showed aggression within one-hour following the alpha male being removed versus sham-removed (alpha removed = 16/16 males, alpha sham-removed = 8/16 males; Chi-

Squared Test  $X^2 = 8.17$ ,  $df = 1$ ,  $p = 0.004$ ,  $d = 1.17$ ). When the alpha male was sham-removed, no beta male won more than ten contests. Conversely, one beta male in the alpha removed group won 48 contests in one hour. A significantly higher proportion of subdominant males from the alpha male removed group achieved each number of wins compared to subdominant males from the sham-removed group (Chi-squared tests, all  $p < 0.05$ ; **Figure S6.3A**).

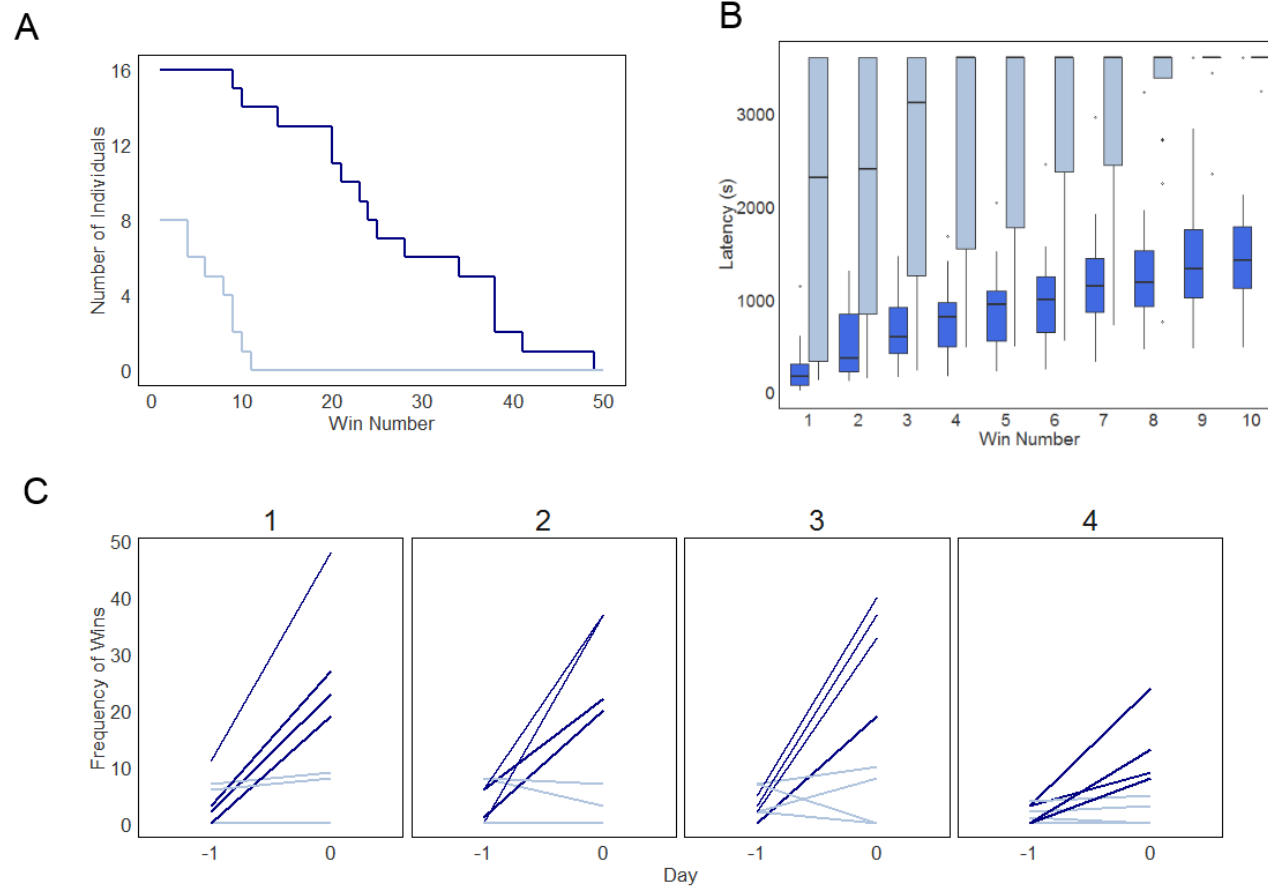
The social ascent of rising subdominants was rapid. The latency to each successive win was significantly shorter when the alpha male was removed compared to when the alpha male was sham-removed (**Figure S6.3B**, Wilcoxon Rank Sum Test all  $p < 0.001$ , all  $r$  between 0.53 and 0.62). Most strikingly, the average latency to winning a fight was under 3 minutes after the alpha male was removed (median[IQR] = 165s [78s,300s]) but was over 38 minutes for subdominant males following sham-removal (2306s [338s,3600s]). Even when considering only those males that were aggressive during the observation (alpha removed  $N=16$ , alpha sham-removed  $N=8$ ), subdominant males were significantly faster to record their first win when the alpha male was removed (Wilcoxon Rank Sum Test,  $W = 33.5$ ,  $p = 0.03$ ,  $r = 0.38$ ).

Further, as shown in **Figure S6.3C**, in the one-hour period directly following alpha male removal, subdominant males displayed significantly increased aggression; compared to behavior during the same one-hour on the previous day (alpha male present) and compared to behavior of subdominants following sham-removal. Using a negative binomial mixed effect model with frequency of aggressive behavior as the outcome variable and cohort and removal number as random effects, there was a significant interaction between alpha removed/sham-removed and day (NB-GLMM:  $\beta=1.69\pm0.30$ ,  $N=64$ ,  $P<0.001$ ). Subdominant males were significantly more aggressive when alpha males had been removed compared to sham-removed (Wilcoxon Signed Rank Test,  $W = 4.5$ ,  $p < 0.001$ ,  $r = 0.62$ ) and compared to the day prior



to removal (Wilcoxon Paired Signed Rank Test,  $V = 0$ ,  $P < 0.001$ ,  $r = 0.62$ ). There was no significant difference in the frequency of aggression of subdominant males when the alpha male was sham-removed; compared to behavior the day prior to sham-removal ( $p = 0.89$ ) or compared to subdominant males from either group the day prior to removals ( $p = 0.66$ ).

**Figure 6.3. Behavioral changes in subdominant males following removal of the alpha male.** (A) Total number of subdominant beta males winning each number of social contests after alpha removal (dark blue) or sham removal (light blue). (B) Latency of subdominant males to win successive contests within one hour after the alpha male was removed (dark blue) or sham-removed (light blue). (C) Frequency of all wins won by subdominant males during one hour time-matched observations on the day prior (-1) or day of (0) the alpha male being removed (dark blue) or sham-removed (light blue) at each removal, separated by removal number. Removal 1 occurred when there were 12 mice in the group, removal 2 occurred when there were 10 mice in the group, removal 3 when there were 8 mice in the group, and removal 4 when there were 6 mice in the group. N=16 males per condition.



We also assessed how subordinate mice changed their behavior in response to the alpha-removal or sham-removal. In 5/16 alpha-removals, the most subordinate animal had one win post-removal and in 1/16 removals the subordinate animal had two wins. Proportionally this is a significantly higher number of subordinate animals showing any aggression during removals than was observed during sham-removals (0/16, Chi-Squared Test:  $X^2 = 5.13$ ,  $df = 1$ ,  $p = 0.024$ ,  $d = 0.87$ ). It is also a significantly smaller proportion of animals showing any aggression than the proportion of subdominant animals that exhibited aggression (Chi-Squared Test:  $X^2 = 11.78$ ,  $df = 1$ ,  $p < 0.001$ ,  $d = 1.53$ ).

#### **GnRH mRNA gene expression and plasma testosterone levels following social opportunity**

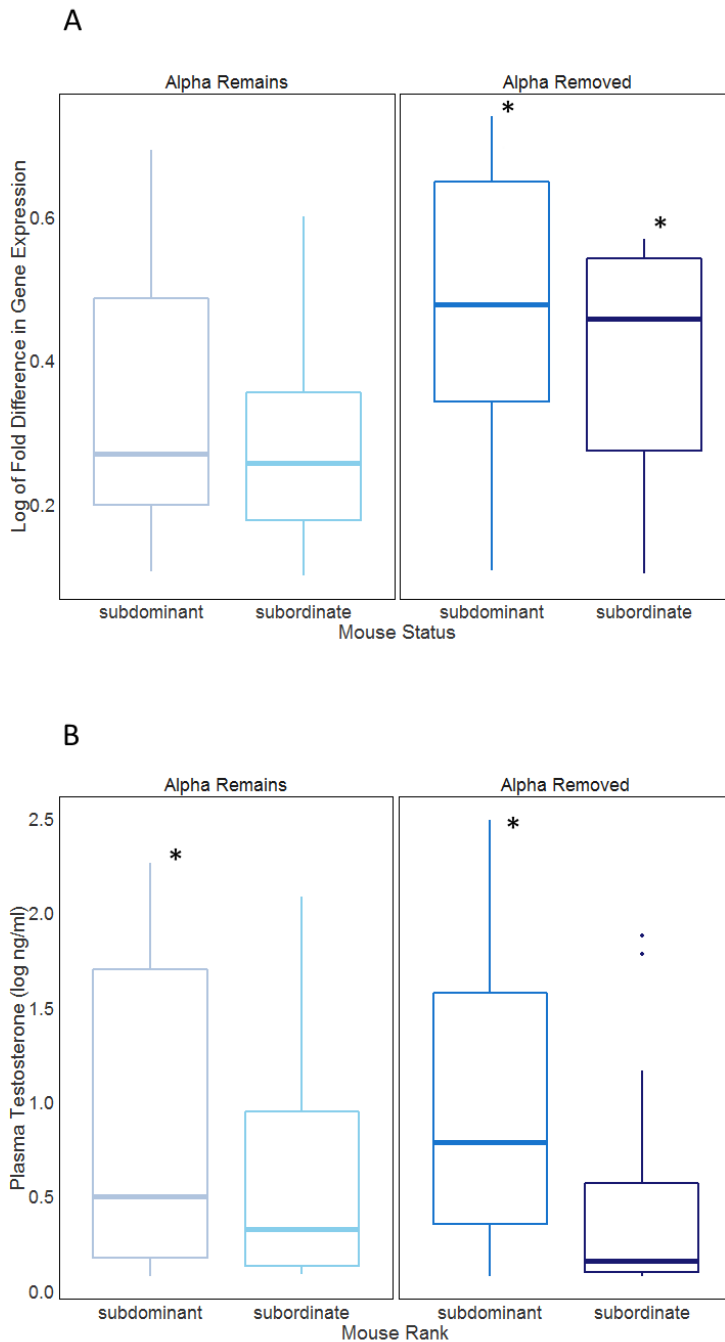
One hour following the subdominant male's rise to dominant status both subdominant males and subordinate males showed elevated GnRH mRNA levels in the mPOA, as compared to sub-dominant and subordinate males in the sham-removed group (GLM:  $F_{2,38} = 3.02$ ,  $p = 0.04$ , **Figure 6.4A**).

Social status was significantly associated with plasma testosterone levels with subordinate male mice having lower testosterone than subdominant mice (GLM,  $F_{2,58} = 2.46$ ,  $p = 0.03$ , **Figure 6.4B**). There was no significant interaction between alpha removal and social status in the GLM. However, it is notable that in the alpha male removed group, we did find a significant effect of status on plasma testosterone levels (GLM:  $F_{1,30} = 4.59$ ,  $p = 0.04$ ), which was not observed in the sham-removal group (GLM:  $F_{1,28} = 1.221$ ,  $p = 0.28$ ).

There was no relationship between the frequency of wins or losses by each individual and their mPOA GnRH mRNA levels or circulating plasma testosterone levels in any of the four groups (**Supplemental**

**Tables S6.2 and S6.3).** There was also no relationship between the frequency of all contests that occurred between all animals in the group and GnRH mRNA or circulating plasma testosterone levels in any of the four groups (**Supplemental Tables S6.2 and S6.3**).

**Figure 6.4. HPG measures correlated to social status and impacted by social opportunity.** (A) Log-transformed fold difference in mPOA GnRH mRNA levels. Asterisks denote a significant difference between GnRH levels in individuals in the group undergoing social ascent and the stable group ( $p < 0.05$ ). (B) plasma testosterone levels in subdominant and subordinate males following alpha removal (dark blue) or sham-removal (light blue). Boxplots show median, IQR and 95% confidence interval of data. Asterisks denote a significant difference between plasma testosterone levels in subdominant individuals and subordinate individuals, regardless of alpha removal ( $p < 0.05$ ).



## DISCUSSION

In the current study, we show that removing an alpha male mouse from a social hierarchy leads to a rapid increase in aggression and a subsequent ascent to alpha status by the most subdominant male. This is a robust effect that occurred following every single removal of alpha males regardless of whether the group consisted of as many as 12 individuals or as few as 3 individuals in the social group. Subordinate males expressed aggression during social opportunity but these males were quickly defeated by the socially ascending subdominant males. Though subordinate individuals clearly respond to the dynamic change in social context, these individuals are unable to take advantage of the opportunity. These findings support and extend previous findings of an attentional hierarchy - where we observed that the aggressive behavior of subdominant and subordinate males is suppressed when alpha male mice are actively aggressive within a social hierarchy (Curley, 2016).

The social ascent by subdominant male mice observed in the current study is consistent with what has been observed to occur during a social opportunity in African cichlid fish (Maruska et al., 2013). In this species, individual fish respond behaviorally and physiologically within seconds to minutes to the change in social context. Likewise, we observed that rising subdominant male mice respond to the removal of alpha males rapidly with the first fight occurring in less than three minutes. This is remarkable given that we removed the alpha male at the change of light cycle (white light to dark light) – a time when the subdominant male was not always active or even awake. Regardless, removal of the alpha male always led to individuals attempting to take advantage of the social opportunity which in turn aroused the subdominant male even if he was not originally aware of the opportunity. Although it has long been established that individuals across all species are able to re-establish social hierarchies over time following the death, removal or other disturbance of dominant individuals (Chase & Seitz, 2011; Franz, McLean,

Tung, Altmann, & Alberts, 2015; Rosvold, Mirsky, & Pribram, 1954), we and others have argued that this ability to respond rapidly and dynamically to changes in social context is a fundamental feature of group-living social cognition and social competence (Desjardins et al., 2012; Fernald, 2014; Oliveira, 2009; Williamson et al., 2016). Individuals that are unable to respond flexibly to social challenges such as these are likely to be at a great social, reproductive and health fitness disadvantage (Hofmann et al., 2014; Taborsky & Oliveira, 2012). Our data are consistent with experimental findings in cichlid fish (Burmeister, Jarvis, & Fernald, 2005; Carpenter, Maruska, Becker, & Fernald, 2014; Maruska et al., 2013; Maruska & Fernald, 2010, 2011; Maruska et al., 2011) and rhesus monkeys (Rosvold et al., 1954), as well as naturalistic observations of hierarchy maintenance in primates (Chase & Seitz, 2011; Franz et al., 2015), suggesting that rapid social ascent following social opportunity may be a universal feature of linear social hierarchies.

Increased aggression exhibited by the subdominant and recognition of the absence of the alpha male by the group as a whole leads to physiological as well as behavioral shifts. We find that both the ascending subdominant and the most subordinate male in the group express higher levels of GnRH mRNA in the mPOA of the hypothalamus one hour following the removal of the alpha male compared to individuals in the sham-removal condition. Similar rapid increases in mRNA expression are observed in male mice exposed to soiled bedding from an unfamiliar male (Gore, Wersinger, & Rissman, 2000) and in doves following a one hour courtship period (Mantei, Ramakrishnan, Sharp, & Buntin, 2008). Increases in hypothalamic GnRH mRNA of subdominant males during such a social opportunity are observed in African cichlid fish where up-regulation of GnRH and the HPG axis occurs during social ascent (Maruska & Fernald, 2013; Maruska et al., 2011). Importantly, in cichlid fish subordinate males are truly reproductively suppressed, and the transition from being reproductively inactive to becoming reproductively active upon social ascent requires large changes in reproductive physiology that are regulated by the HPG axis

(Maruska & Fernald, 2011). In mice, there is some evidence that more subordinate individuals do have a down-regulated HPG axis, as subordinates have been found to have lower seminal vesicle weight and decreased testes weight (Bronson & Eleftheriou, 1964; McKinney & Desjardins, 1973) as well as decreased sperm motility (Koyama & Kamimura, 1998). Therefore, although subordinate male mice are not necessarily entirely reproductively suppressed, it is not entirely unexpected that ascent to dominant status would involve changes along the HPG axis. The mechanism for this GnRH plasticity is still poorly understood, however, it is possible that changes in social experience could trigger dynamic changes in GnRH expression through direct neural input from different sensory modalities rather than via steroid hormone effects (Stevenson, Hahn, MacDougall-Shackleton, & Ball, 2012).

Increased hypothalamic GnRH mRNA levels in subordinate individuals was not expected, as these animals are not consistently engaging in increased aggression and do not socially ascend during the social opportunity. One potential explanation is that subordinates sense that the social context has altered and observing changes in social interactions between other individuals leads to the increased GnRH. Several species including cichlid fish and corvids are able to infer social ranks through observation (Bond, Kamil, & Balda, 2003; Grosenick et al., 2007) and are able to adjust their own behavior by closely monitoring the behavior of other more dominant individuals and recognizing when these animals are absent (Desjardins et al., 2012; Freniere & Charlesworth, 1983). Indeed, we have previously described how subdominant and subordinate male mice attend to alpha males and change their own behavior when alpha males are less active (Curley, 2016b). Further, watching fights leads to increased androgen levels in observers across species including fish and humans (Bernhardt, Dabbs Jr, Fielden, & Lutter, 1998; Oliveira, Lopes, Carneiro, & Canário, 2001), suggesting that simply observing the changes in social interactions may be sufficient to change GnRH gene expression in all group members. Alternatively, increased engagement in aggressive interactions, even if the vast majority of those interactions resulted in losses, may underlie the increased



hypothalamic GnRH expression in subordinate individuals. Though losing fights has not been previously associated with an increase in GnRH, losers and winners in social contests between male mice exhibit similar decreases in c-fos activation of RFamide-related peptide (RFRP) cells (Jennings et al., 2016). Given that RFRP (gonadotropin-inhibitory hormone in birds), is a negative regulator of the reproductive axis (Kriegsfeld, Ubuka, Bentley, & Tsutsui, 2015), this decrease in RFRP activation due to any type of aggressive encounter (win or loss) could lead to increases in GnRH both following winning and subsequent social ascent in subdominants and following losing experienced as others socially ascend. Although there may be multiple mechanisms through which changes in GnRH mRNA levels may be modulated, we did not observe any relationship between total wins or losses or the number of total contests that occurred in each hour following removal or sham-removal and mPOA GnRH mRNA levels. This suggests that the relationship between behavior and gene expression is not a simple linear association. Nevertheless, it is evident that all individuals in each social hierarchy, including those undergoing transition from subdominant to alpha status as well as subordinate males, are exhibiting a behavioral and neuroendocrine response to the increased social instability induced by removal of the alpha male.

We observed elevated circulating plasma testosterone levels in subdominant individuals compared to subordinate males. In animals undergoing social opportunity, although both individuals exhibited increased levels of GnRH mRNA, subdominant males had significantly higher testosterone than the subordinates. This dissociation between increased GnRH and testosterone levels may be related to an inability of subordinate individuals to respond to GnRH as occurs in group-living subordinate sugar gliders (Bradley & Stoddart, 1997). In this species, exogenous administration of GnRH to dominant and subordinate individuals leads to an increase in plasma testosterone in dominant but not subordinate individuals. Similarly, male wild dark-eyed juncos alter their behavioral and physiological responses to GnRH administration dependent upon the particular social context (McGlothlin, Jawor, Ketterson, Adkins-

Regan, & Whitlock, 2007). A related phenomenon also occurs in naked mole rats – nonbreeding females do not show an LH surge of the same magnitude as breeding females in response to exogenous GnRH administration (Faulkes, Abbott, Jarvis, & Sherriff, 1990). Thus, it is possible that subordinate individuals in our social hierarchies are able to increase GnRH in the mPOA but are unable to successfully respond to that GnRH increase with increased testosterone levels and ultimately higher HPG activation.

In the current study, socially subdominant males in the sham-removal group had equivalent levels of testosterone to subdominant males in the alpha-removal group. There is an extensive literature on the relationship between circulating testosterone and aggression and social dominance with higher circulating testosterone levels being commonly observed in the dominants of many species (Gesquiere et al., 2011; Higham, Heistermann, & Maestripieri, 2012; Mendonça-Furtado et al., 2014; Sapolsky, 2005). In mice, more dominant males have been reported to have higher circulating testosterone levels than subordinates but these findings are inconsistent (Bronson, 1973; Ely & Henry, 1978; Haemisch, Voss, & Gärtner, 1994; Hiadlovská et al., 2015; Oyegbile & Marler, 2005; Selmanoff, Goldman, & Ginsburg, 1977; Zielinski & Vandenbergh, 1993). One possible explanation for this inconsistency is variation in social context. Indeed, it has been proposed that testosterone will be more highly correlated with dominance status and agonistic behavior during times of social instability (Wingfield, Hegner, Dufty, & Ball, 1990) when it is essential for individuals to attempt to rise in social status (Liening, Mehta, & Josephs, 2012). Evidence in support of this “challenge hypothesis” has been seen in fish (Almeida, Gonçalves-de-Freitas, Lopes, & Oliveira, 2014), lizards (Greenberg & Crews, 1990), and chimpanzees (Cavigelli & Pereira, 2000). It is likely that given the repeated removals of beta subdominant males from our social system every five days that these subdominant males may have been consistently exerting their dominance to maintain their newly established social position and as such exhibited higher circulating plasma testosterone compared to more subordinate individuals. While these subdominant individuals do exhibit higher levels

of testosterone, they do not have the elevated GnRH mRNA levels that the socially ascending subdominants do immediately after alpha removal. This finding could be due to consistently increased testosterone levels over the previous 5-day period leading to an overall down-regulation of GnRH in the hypothalamus (Lee, Lee, & Chow, 2008) in these males that is overridden in the subdominants in the alpha-removal group .

## **CONCLUSION**

We have demonstrated that following the removal of the alpha male from a stable social hierarchy, the subdominant male responds within minutes to this social opportunity by increasing their aggression against all other individuals. If the alpha is permanently removed, this leads to the subdominant assuming the alpha male role. Other males also respond behaviorally to the social opportunity but are not as capable at ascending the social hierarchy. Associated with these behavioral changes are rapid increases in mPOA GnRH gene expression which may lead to further changes in the HPG axis regulation of behavior. Further, recently socially risen subdominant males possess higher circulating plasma testosterone which is likely associated with their increased aggression following social ascent. Such dramatic and rapid behavioral and physiological modifications in response to dynamic alterations in social contexts are consistent with individuals engaging in socially competent behaviors similar to those that occur in other animals that similarly live in dynamically transitioning social hierarchies.

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**SUPPLEMENTAL MATERIAL**

**Supplemental Table S6.1. Mouse Social Behavior Ethogram:**

During observations, observers code all agonistic interactions occurring between any two individuals. As multiple behaviors may occur during the same interaction, observers record the behaviors with the highest priority. For instance, if one animal fought another animal who responded by fleeing, this would be recorded as a 'Fighting' event only, as 'Fighting' takes priority to the co-occurring 'Induced-Flee'. If an animal fled when approached but was not attacked by another animal, then this would be recorded as 'Induced-Flee'. Similarly, if an animal displayed subordinate posture following a chase, this would be recorded as 'Chasing', because chasing takes priority over 'Subordinate Posture'. Subordinate posture and Induced-Flee are only recorded if they occur in the absence of fighting, chasing, or mounting. These two subordinate behaviors do not co-occur so are given equal priority.

Priority	Behavior	Description
1	Fighting	The focal individual lunges at and/or bites the target individual.
2	Chasing	The focal individual follows the target individual rapidly and aggressively while the other individual attempts to flee.
3	Mounting	The focal individual mounts another individual from behind.
4=	Subordinate posture	The focal individual responds to the approach from another individual by remaining motionless and/or exposing their nape.
4=	Induced-Flee	The focal individual flees without any aggression shown by another individual.

**Supplemental Table S6.2.** Spearman rho correlations between wins, losses and total contests that occurred within one-hour of alpha male or sham-removal and mPOA GnRH mRNA expression in subdominant and subordinate males.

Condition	Male Social Status	N	Wins	Losses	Total Contests
Alpha removed	Subdominant	11	-0.04	-0.41	-0.07
	Subordinate	11	-0.32	-0.31	-0.41
Alpha remained	Subdominant	12	-0.50	0.08	-0.47
	Subordinate	10	NA	-0.03	0.21

*All rho values are  $p > 0.05$ , NA indicates correlations that were not computable due to all individuals having zero wins.*

**Supplemental Table S6.3.** Spearman rho correlations between wins, losses and total contests that occurred within one-hour of alpha male or sham-removal and circulating plasma testosterone in subdominant and subordinate males.

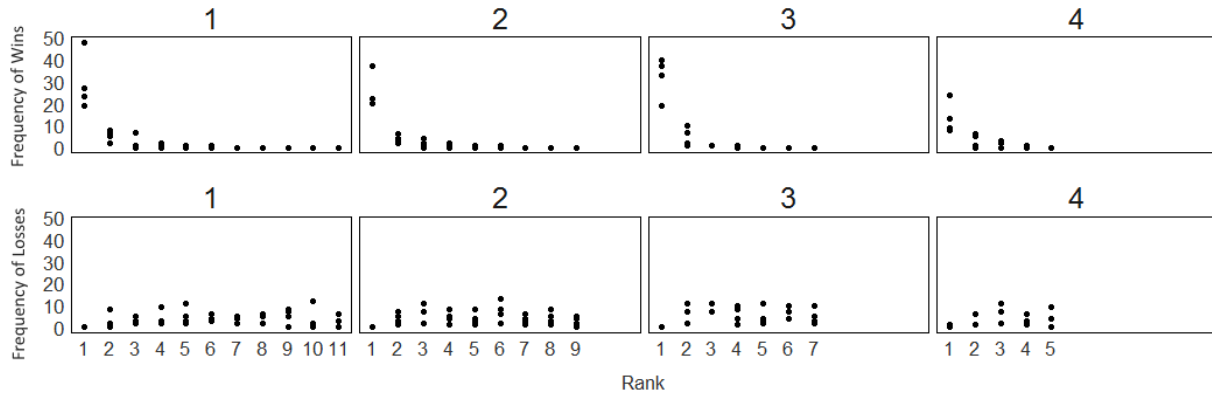
Condition	Male Social Status	N	Wins	Losses	Total Contests
Alpha removed	Sub-Dominant	16	-0.17	0.00	-0.12
	Subordinate	15	0.07	-0.29	0.45
Alpha remained	Sub-Dominant	15	0.05	0.21	-0.03
	Subordinate	15	NA	-0.08	-0.25

*All rho values are  $p > 0.05$ , NA indicates correlations that were not computable due to all individuals having zero wins.*

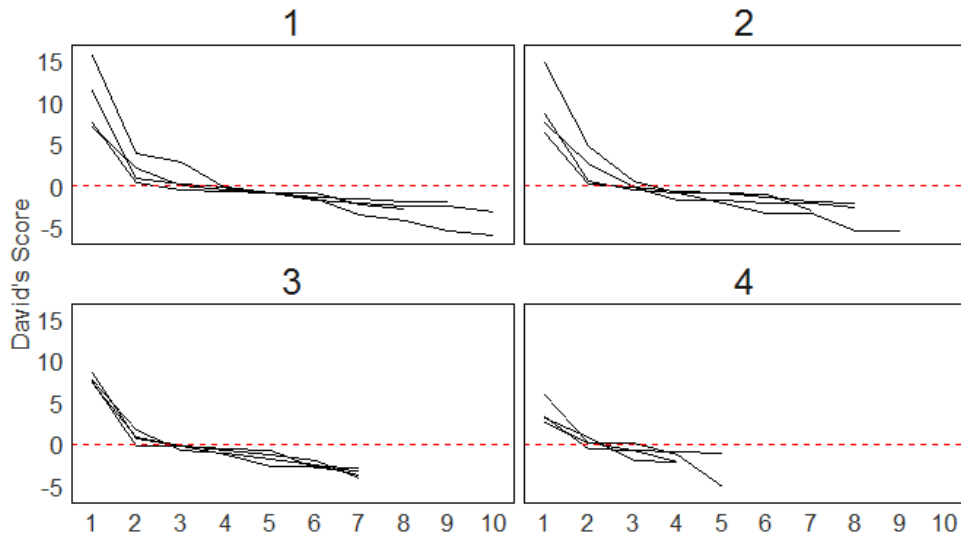
**Supplemental Figure S6.1.** Housing Vivarium.



**Supplemental Figure S6.2.** Frequency of wins and losses within one hour of the alpha male being removed. Animals are ranked on the x-axis in order of total wins. Panels 1-4 refer to the whether the alpha male removal was the first, second, third or fourth removal for that group.



**Supplemental Figure S6.3.** David's Scores of animals during the one hour period following alpha male removal. David's Scores are a metric used to determine the relative competitive ability of individuals by assessing their total wins and losses weighted against the ratings of their opponents. Panels 1-4 refer to the whether the alpha male removal was the first, second, third or fourth removal for that group. Note if an animal had 0 wins and 0 losses it is not possible to calculate a David's Score. In all alpha male removals (N=16), the rising subdominant had higher David's Scores than all other individuals (Binomial Test,  $p < 0.001$ ).



***Study #2: Short-term changes in circulating plasma testosterone levels are not associated with social ascent***

Cait M. Williamson, Russell D. Romeo, James P. Curley

## **ABSTRACT**

Social ascent, where beta individuals attain dominant status after removal of the alpha male, is a complex behavioral phenomenon associated with a host of physiological changes. In Study #1, we showed that as individuals undergo ascent up a hierarchy, they display increased GnRH mRNA in the medial preoptic area of the hypothalamus. Further, subordinate individuals from these groups where the alpha has been removed also display this increased GnRH. Plasma testosterone levels, however, were not different between subdominant males undergoing social ascent and those in a stable social hierarchy. Here, we use the same paradigm as this previous study to determine if there are changes in testosterone 30 minutes following social rather than one hour following social ascent. We find a similar pattern in circulating testosterone levels to the one found in Study #1.

## INTRODUCTION

The relationship between social dominance and circulating plasma testosterone is a complex one that is dependent on many factors (Williamson, Lee, Romeo, & Curley, 2017). In Study #1 of this chapter, we demonstrated that as subdominant males ascend to alpha status, they display increased levels of GnRH mRNA in the medial preoptic area of the hypothalamus. Subordinate individuals from the same social groups display a corresponding increase – merely being in the presence of social instability leads to this change in GnRH levels (Williamson, Romeo, & Curley, 2017). This did not translate to simple changes in testosterone levels between groups. Both subdominant males from the group where the alpha male was removed and where the alpha male remains in the group display increased circulating plasma testosterone levels as compared to subordinate individuals from both groups. This leaves two questions: first, why are subordinate individuals displaying increased GnRH but not the subsequent testosterone pulse? And second, why are the testosterone levels of the individuals ascending to dominant status, who are exhibiting significantly higher levels of aggression, not any different from the subdominant individuals in the group where the alpha was not removed?

To determine if the time at which we measured the circulating testosterone levels could explain these questions, we performed the same experiment as in Study #1 but took blood samples thirty minutes following alpha removal rather than one hour following alpha removal, as we did in Study #1. We chose 30 minutes as the time point after finding that the majority of studies across species show that individuals start to display increased plasma testosterone in response to performing aggressive behavior within 10-30 minutes (Landys, Goymann, Raess, & Slagsvold, 2007; Marler, Oyegbile, Plavicki, & Trainor, 2005; Maruska, Zhang, Neboori, & Fernald, 2013; Wingfield & Wada, 1989) All other variables remained consistent between Study #1 and the present study.



## **METHODS**

### **Subjects and Housing**

Throughout the study, subjects were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21–24°C) and humidity (30-50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red lights (dark cycle) on at 1200 hours. All mice were individually and uniquely marked by dyeing their fur with a blue, nontoxic, non-hazardous animal marker (Stoelting Co.). These marks remain for up to 12 weeks and only require one application, thus enabling each animal to be visually identified throughout the study. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol Nos: AC-AAAG0054, AC-AAAP5405).

### **Thirty Minute Social Opportunity Manipulation**

We used the same social opportunity paradigm described in the methods of Study #1. There were only two differences from the procedure described above: 1) A total of 48 male outbred CD1 mice were used, creating 2 sets of paired cohorts, rather than 4 and 2) Upon dominant removal, live behavioral observations occurred for the 30-minute period directly following alpha removal or sham-removal, rather than 1 hour. Thirty minutes after the subdominant male had won three fights, the subdominant mouse who had risen to dominant status or the subdominant mouse who had remained subdominant, in the case of the sham-removal group, and the most subordinate mouse were removed.

Following removal from the vivarium, mice were euthanized via cervical dislocation. Trunk blood was collected into heparinized tubes and plasma was separated and then stored at -80°C. Following blood collection, the alpha male who had been removed from the social opportunity group was returned to his

group. This procedure was repeated at five day intervals for a total of four “removals”. As above, manipulations were counter-balanced between paired cohorts (i.e. one vivarium had alpha removal for removals 1 and 3 and sham-removals for removals 2 and 4 and the opposite was true of the paired vivarium). Each removal/sham-removal decreased the size of the social group by 2, resulting in N=10 (first removal), N=8 (second removal) and N=6 (third removal). This experimental design yielded N=8 mice per group from four groups: subdominant/alpha-removed, subordinate/alpha-removed, subdominant/alpha sham-removed, subordinate/alpha sham-removed.

### **Hormone Assay**

Plasma testosterone concentrations were measured using a commercially available kit (MP Biomedicals, LLC; Orangeburg, NY) and conducted using the manufacturer’s specifications. Samples were run in duplicate and values were averaged. The intra-assay coefficient of variation was 15% and lower limit of detectability for the assay was 0.1 ng/ml.

### **Statistical Analysis**

All statistical analyses were undertaken in R version 3.4.0 (R Core Team, 2016).

*Behavior Analysis:* The same analyses as in Study #1 were conducted to determine the linearity of the hierarchies as well as who the subdominant and most subordinate individuals were before each removal. As in Study #1, to compare changes in total wins between the day prior to alpha removal/sham-removal and the day of alpha-removal/sham-removal, we used a zero inflated negative binomial generalized mixed effect model with counts of wins as the outcome variable, alpha removal status (removal or sham-removal) and day (day prior to removal or day of removal) as fixed effects and cohort and removal number

as random effects using the R package glmmADMB (Skaug, Fournier, Bolker, Magnusson, & Nielsen, 2015) and lmerTest (Kuznetsova, Brockhoff, & Christensen, 2015). We used separate Wilcoxon rank sum tests for subdominants and subordinates to compare wins by individuals in the alpha removed group to wins by individuals in the alpha remained group on the day of removal or sham-removal and Wilcoxon signed rank tests to compare wins by individuals in each group on the day of alpha removal to the day before alpha removal.

*Testosterone Analysis:* As in Study #1, we used general linear models using the R package lme4 (Bates et al., 2015) to analyze the effect alpha removal and social status on circulating plasma testosterone levels. Testosterone data was log-transformed to ensure assumptions of normal distribution were satisfied. We ran spearman correlations between number of wins and circulating plasma testosterone levels for all four groups to determine if there was a relationship between winning and testosterone output.

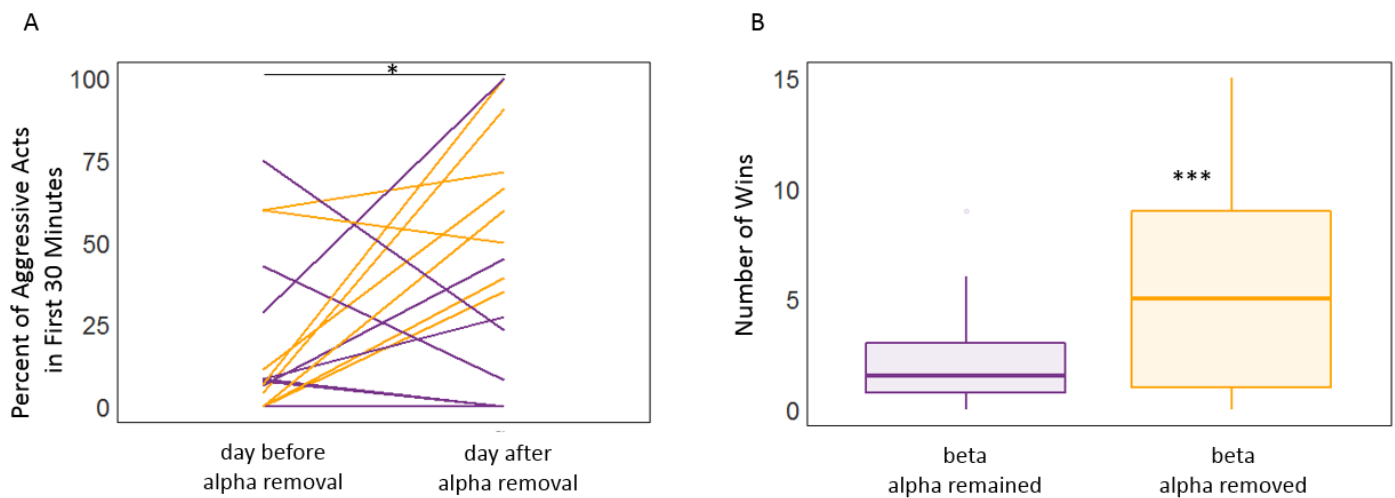
## RESULTS

### *Social Opportunity Behavior*

We successfully replicated our behavioral results from Study #1. In the 30 minute period directly following alpha male removal, subdominant males displayed significantly higher levels of aggression as compared to the same 30 minute period on the previous day when the alpha male was present and compared to the behavior of subdominant individuals following sham-removal. We used a negative binomial mixed effect model with frequency of aggressive behavior as the outcome variable and cohort and removal number as random effects to determine that there was a significant interaction between alpha removed/sham removed and day (**Figure 6.5A:** NM-GLMM:  $\beta=1.676 \pm 0.572$ ,  $N=32$ ,  $p < 0.005$ ). Subdominant males won

significantly more when alpha males had been removed compared to the sham-removal group (**Figure 6.5B**: Wilcoxon rank sum test,  $W = 60$ ,  $p < 0.005$ ) and compared to the day prior to removal (Wilcoxon signed rank test,  $V = 1$ ,  $p < 0.05$ ). Subdominant males in the alpha remained group did not win significantly more on the day of alpha removal as compared to the day before alpha removal (Wilcoxon signed rank test,  $V = 15$ ,  $p = 0.932$ ). Subordinate behavior did not change with alpha removal or sham removal. There was no significant difference between subordinate wins in the alpha removed group as compared to the sham-removal group on the day of removal/sham-removal (Wilcoxon rank sum test,  $W = 40$ ,  $p = 0.170$ ). Further, in the alpha removed group, there was no significant difference between subordinate wins the day before alpha removal as compared to the day of alpha removal (Wilcoxon signed rank test,  $V = 2$ ,  $p = 0.773$ ). For the sham-removal group, there was no significant difference between subordinate wins the day before sham-removal as compared to the day of sham-removal (Wilcoxon signed rank test,  $V = 1$ ,  $p = 1$ ).

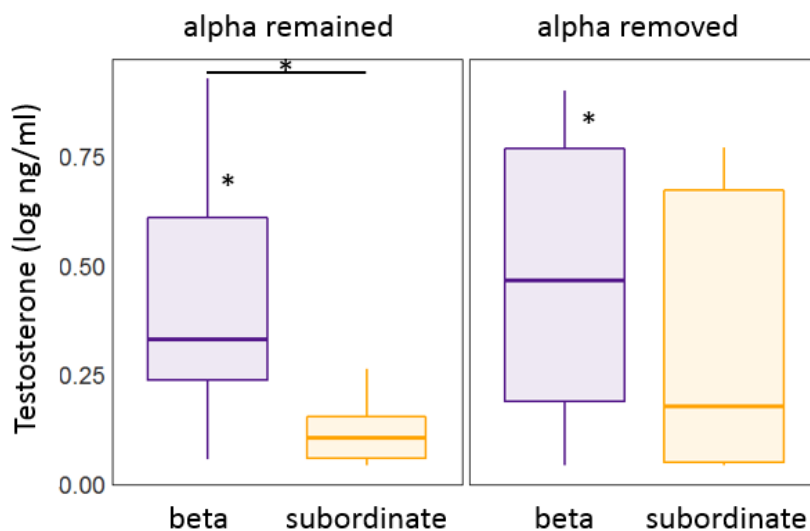
**FIGURE 6.5 Replication of behavioral changes following social opportunity:** Following alpha removal, beta males (purple) display significantly higher levels of aggression as compared to their own behavior the day before (A) and beta males in the sham-removal group (yellow) (B).



### ***Testosterone Response to Social Opportunity***

There is a main effect of social status, such that subordinates have significantly lower T than dominants (**Figure 2:**  $\beta = -0.221 \pm 0.103$ ,  $p < 0.05$ ). There is no effect of alpha removed, such that individuals in the alpha removed group did not have significantly different testosterone levels from individuals in the alpha remained group (**Figure 2:**  $\beta = 0.308 \pm 0.144$ ,  $p = 0.833$ ) and no interaction between status and alpha removal existed (**Figure 2:**  $\beta = 0.180 \pm 0.208$ ,  $p = 0.395$ ). Interestingly, in contrast to our findings from Study #1, when looking separately at the alpha remained and alpha removed groups, in the alpha remained group, there was a significant difference in plasma testosterone levels between the beta and subordinate individuals, with subordinate mice displaying significantly lower levels (**Figure 2:**  $\beta = -0.314 \pm 0.121$ ,  $p < 0.05$ ). In the alpha removed group, there was no significant difference between beta and subordinate plasma testosterone levels (**Figure 2:**  $\beta = -0.134 \pm 0.166$ ,  $p = 0.432$ ).

**FIGURE 6.6 Plasma testosterone changes in response to social opportunity** Plasma testosterone levels in subdominant (purple) and subordinate (yellow) males following sham removal (left) or alpha removal (right). Beta individuals, regardless of removal condition, display significantly higher levels of plasma testosterone than subordinate individuals.



There was no relationship between the frequency of wins and circulating plasma testosterone levels in any of the four groups (Spearman correlations; beta, alpha removed:  $\rho = 0.265$ ,  $p = 0.526$ ; beta, alpha remained:  $\rho = -0.400$ ,  $p = 0.326$ ; subordinate, alpha removed:  $\rho = -0.317$ ,  $p = 0.445$ ; subordinate, alpha remained: could not run correlation because all win values were 0).

## DISCUSSION

In this study, we replicated the behavioral results from Study #1, showing that upon alpha removal, subdominant individuals increase aggression and ascend to dominant status. In contrast to Study #1, we found here that there was actually no effect of alpha removal on subordinate behavior, as here subordinate males did not display increased aggression during the social opportunity period. This could be due to the fact that there was less time in which they could express that aggression (30 minutes vs. the 1 hour in Study #1). In Study #1, while subordinate individuals did show increased aggression in the alpha removed condition as compared to the sham-removal condition, the amount of aggression was still quite small – 5/16 subordinates in the alpha removed group had one win while 1/16 had two wins. If you were to divide the number of wins per mouse in half to normalize to wins in a 30 minute period, this amount of aggression is consistent with what we see in the present study, where 2/8 subordinates in the alpha removed group had one win while 0 had two wins.

We further replicated the finding that in terms of the plasma testosterone response to social opportunity, there is no interaction between alpha removal condition (removal or sham-removal) and social status; the only effect is that beta males have higher levels of T than subordinate males, regardless of alpha removal condition. However, looking separately at the alpha removed and alpha remained groups, in the alpha removed group, you do not see a significant difference between dominant and subordinate T levels, which

is different from our finding in Study #1. This is interesting, as, even though there is no significant interaction, it appears that some subordinate individuals might be responding to social opportunity with a testosterone pulse, and this is detectable on a 30 minute timeline. As in Study #1, there was no correlation between wins and circulating plasma testosterone in any of the groups.

Generally speaking, the present study does not answer some of the central questions that arose from our findings in Study #1. While subordinate individuals show a GnRH increase in response to social opportunity, they do not show a subsequent increase in circulating plasma testosterone. Further, beta males in groups where there was no alpha removal have circulating plasma T that is not significantly different from that of beta males in groups where the alpha was removed. We posited that these two findings could have been due to the timeline at which we looked at the testosterone in the blood. However, our findings here replicate our findings from Study #1 and so do not conclude that the timeline is the reason for this result. The increased testosterone we see in subdominant individuals from both groups could be due to something other than the acute stimulus of alpha removal directly before we collected the blood samples. As mentioned in the discussion of Study #1, testosterone is highly correlated with dominance status during times of instability (Liening, Mehta, & Josephs, 2012; Wingfield, Hegner, Dufty, & Ball, 1990). This phenomenon, known as the “challenge hypothesis” seems to be at play in this paradigm. Subdominant individuals in both the alpha removed and alpha remained groups can be considered to be existing in somewhat unstable groups, as the group makeup is shifting every five days. This is likely causing testosterone levels to be already elevated in subdominant individuals, thus making it difficult to detect if there is increased testosterone in response to the acute stimulus of the removal of the alpha male. Further some work shows that after only a single act of aggression, circulating plasma testosterone levels begin to rise around the 30 minute time point (Marler et al., 2005). Given that subdominant individuals in the alpha remained group are generally winning at least one fight during this

30 minute period, it is perhaps not surprising that they exhibit elevated testosterone levels similar to those in subdominant individuals ascending to alpha status. Further work is required to determine what is modulating the discrepancy between the elevated GnRH we see in subordinate individuals in the alpha removal group and their circulating plasma testosterone levels.

## **CONCLUSION**

We successfully replicated our behavioral findings from Study #1, and our findings for circulating plasma testosterone levels also follow a similar pattern to those in Study #1. Given these findings, it is clear that either at a 30 minute time point or a one hour time point, beta males consistently rise to alpha status. This rise, however, is not necessarily accompanied by a change in testosterone levels in either beta or subordinate individuals.



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<https://doi.org/10.1007/BF00193463>

***Study #3: Social status is associated with a behavioral, but not plasma testosterone, response to exogenous GnRH administration in mice***

Cait M. Williamson, Giovanna Regis, Russell D. Romeo, James P. Curley

## **ABSTRACT**

The role HPG axis activity plays in social dominance is complex. We have previously shown that the relationship between testosterone levels and social status is dependent on social context as well as that subordinate individuals are capable of responding to changes in social context with elevated GnRH mRNA, but not testosterone, levels. In the present study, we use a paradigm known as a GnRH challenge, where we administer exogenous GnRH to both dominant and subordinate individuals, to determine if subordinate individuals have a dampened response to GnRH. We show that GnRH administration leads to increased levels of aggression in dominant, but not subordinate, individuals but that both dominant and subordinate individuals respond to GnRH with increased testosterone levels. Our findings provide further evidence that social context is an important modulator of behavior and that the relationship between dominance and testosterone is a complex one.

## INTRODUCTION

Although it is commonly reported that elevated plasma testosterone is associated with dominant behaviors and ultimately dominant social status across species, social context is an important modulator of neuroendocrine output. Previous studies from our lab have demonstrated that the relationship between plasma testosterone levels and social dominance is dependent on social context, namely, the despotism of the alpha male, a measure of the aggression demonstrated by the dominant individual compared to the aggression across the group as a whole. Only in groups where the alpha male was extremely despotic, performing over 50% of all aggression within the group, did we see that dominant individuals had significantly higher plasma testosterone than subdominant and subordinate individuals (Williamson, Lee, Romeo, & Curley, 2017). Further, in Study #1 of this chapter, we showed that upon removal of the alpha male from a stable, linear social hierarchy, both subdominant and subordinate individuals in groups where the alpha male has been removed leading to a social opportunity display an increase in GnRH. However only the subdominant individuals exhibit increased testosterone levels (Williamson, Romeo, & Curley, 2017), providing further evidence that social context can disrupt normal neuroendocrine output. In Study #2, we explored whether this finding was due to the time at which we looked at circulating plasma T by taking blood only 30 minutes instead of 60 minutes post social ascent. The finding in Study #2 mirrored that from the previous study – subordinate individuals undergoing social ascent do not show an increase in testosterone to correspond with their increased GnRH mRNA levels. This suggests there may be another factor preventing the lack of increased testosterone in subordinate individuals in response to the GnRH pulse.

Here, we explore another potential explanation for this finding. As mentioned in the discussion of Study #1, subordinate individuals may not respond to GnRH to the same extent as dominant individuals. Research in sugar gliders (a small marsupial) provides good evidence for this phenomenon. Administration of exogenous GnRH to sugar gliders leads to increased testosterone in dominants but not subordinates

(Bradley & Stoddart, 1997a). Similarly, male wild dark-eyed juncos have a differential response to GnRH dependent on the social context (McGlothlin, Jawor, Ketterson, Adkins-Regan, & Whitlock, 2007), with males who exhibited higher post-GnRH administration testosterone showing increased territorial behavior. A related phenomenon occurs in naked mole rat hierarchies, where females are the most dominant individuals -- nonbreeding females do not show an LH surge of the same magnitude as breeding females in response to exogenous GnRH administration (Faulkes, Abbott, Jarvis, & Sherriff, 1990). The present study aims to determine whether this phenomenon also exists in mice by examining plasma testosterone levels following exogenous administration of GnRH to both dominant and subordinate individuals. We further examine the relationship between social context and neuroendocrine output by analyzing the behavioral response to exogenous GnRH in both dominant and subordinate individuals.

## **METHODS**

### ***Subjects and Housing***

A total of 76 male outbred CD1 mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 2 weeks in standard sized cages before behavioral testing. All subjects were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21-24°C) and humidity (30-50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400h and red light (dark cycle) on at 1200h. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol No. AC-AAAM1450).

### ***Establishment of Dominant-Subordinate Pairs***

At 9 weeks of age, mice were paired with a novel partner and placed in standard cages. One mouse in each dyad had its tail colored with nontoxic permanent marker, allowing for visual identification

throughout the study. Mice remained paired with the novel partner for one week without disturbance in order to allow for the establishment of dominant-subordinate relationships. Pairs were video recorded using a GoPro Hero 3 for 2 hours on Day 1 directly after pairing, 2 hours on Day 6, and 1 hour directly after GnRH administration on Day 7. All behavioral video recording occurred at the same time of day during the dark cycle.

### ***GnRH Administration***

Gonadotropin Releasing Hormone (GnRH) (Sigma, St. Louis, MO ) was dissolved in 0.9% sterile saline to final concentrations of 0.6mg/kg (high dose), 0.1mg/kg (medium dose), and 0.03mg/kg (low dose). Doses were chosen based on previous literature and a pilot dose response study conducted in our lab. GnRH or saline was administered subcutaneously at a volume of .01ml/g on Day 7 of pair housing, directly prior to the final hour of video recording. Each mouse in a pair received the same dose, and doses (or saline) were randomly assigned to each pair.

### ***Video Coding and Behavioral Analysis***

Videos were coded using the Observer XT Software (Noldus, V11.5). Behaviors coded included aggressive behaviors (such as fighting, subordinate, chasing, fleeing), sniffing behaviors (sniffing the head, body or anogenital area) and grooming behavior (See **Table 1** for ethogram of coded behaviors). The individual with a higher total number of aggressive behaviors in the pair was determined to be the dominant individual. If dominance status could not be determined, the dyad was dropped from further analysis.

**Table 6.1 Ethogram of social behaviors**

<b>Behavior</b>	<b>Description</b>
Fighting	The focal individual lunges at and/or bites the target individual.
Chasing	The focal individual follows the target individual rapidly and aggressively while the other individual attempts to flee.
Subordinate posture	The focal individual responds to the approach from another individual by remaining motionless and/or exposing their nape.
Flee	The focal individual flees without any aggression shown by another individual.
Sniffing	The focal individual is sniffing another individual's head, body or anogenital area
Grooming	The focal individual is grooming another individual.

### ***Hormone Assay***

One hour post GnRH or saline administration, mice were immediately euthanized via cervical dislocation, trunk blood was collected into heparinized tubes, and plasma was separated and then stored at -80°C until radioimmunoassay. Plasma testosterone concentrations were measured via radioimmunoassay using a commercially available kit (MP Biomedicals, LLC; Orangeburg, NY) and conducted using the manufacturer's specifications. Samples were run in duplicate and values were averaged. The intra-assay coefficient of variation was 13.4%, the lower limit of detectability for the assay was 0.11 ng/ml and the highest 11.22 ng/ml.

### ***Statistical Analysis***

All statistical analyses were conducted in R version 3.4.0 (R Core Team, 2017) in RStudio 1.0.143 (RStudio Team, 2016).



To compare the change in total wins by the dominants on the day before GnRH administration to the day of GnRH administration, we used a zero-inflated negative binomial generalized mixed effect model with number of wins per hour as the outcome variable, dose (low, medium high) and day (day prior to administration, Day 6, or day of administration, Day 7) as fixed effects and pair ID as a random effect using the R packages glmmADMB (Skaug, Fournier, Bolker, Magnusson, & Nielsen, 2015) and lmerTest (Kuznetsova, Brockhoff, & Bojesen Chistensen, 2015).

To test the relationship between plasma testosterone levels, GnRH dose, and social rank, we ran a linear mixed model with dose, dominance status, and fights (including both wins and losses) in the hour post GnRH administration as fixed effects and pair ID as a random effect.

To analyze the dose response effect, we ran a Friedman multiple comparison test and then compared each dose using Wilcoxon signed-rank tests.

## **RESULTS**

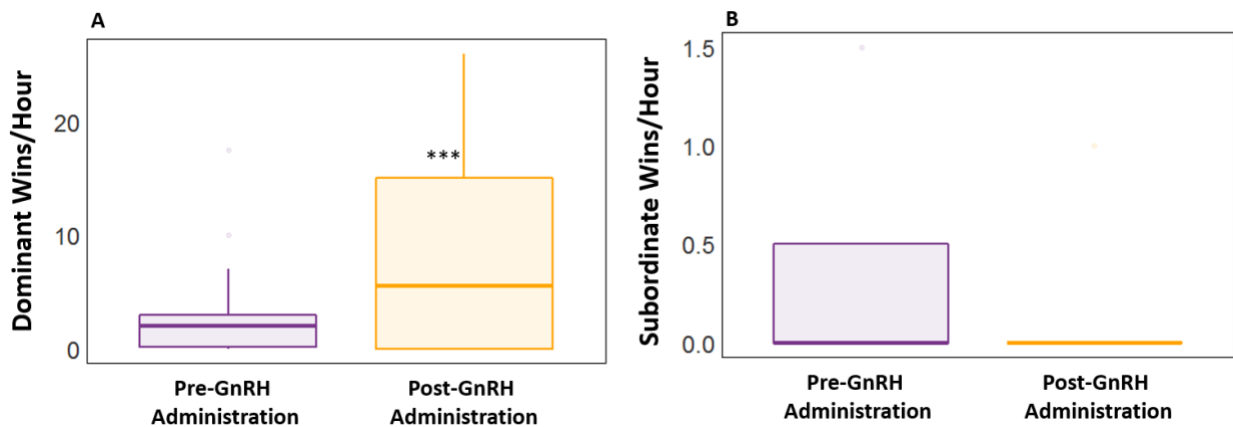
### ***Establishment of Dominant-Subordinate relationships***

In 18 of the 19 dyads, a clear dominant-subordinate relationship formed. One pair did not form a dominant-subordinate relationship because they did not fight. This pair was dropped from the analyses. Dominants were determined as the mouse which won more fights against their partner over the final two days of pair housing.

### ***GnRH administration leads to dominant, but not subordinate, individuals winning more***

Comparing dominant behavior the day prior to GnRH administration to data collected directly after GnRH administration, we found that individuals who were administered any dose of GnRH won significantly more directly after GnRH administration when compared to the day before GnRH administration (**Figure 6.7A**,  $\beta = 1.351 \pm 0.373$ ,  $p = 0.0003$ ), regardless of dose. There was no dose response effect of wins (low vs. medium:  $\beta = 0.470 \pm 0.548$ ,  $p = 0.392$ ; low vs. high:  $\beta = -0.191 \pm 0.572$ ,  $p = 0.738$ ; medium vs. high:  $\beta = -0.661 \pm 0.536$ ,  $p = 0.218$ ). There was no effect of GnRH administration on subordinate individuals' wins, as the majority won 0 fights the day before administration and won 0 fights the day after administration (**Figure 6.7B**).

**Figure 6.7: Effect of GnRH administration on aggression in dominant and subordinate individuals** (A) Dominant individuals win significantly more after GnRH administration. (B) GnRH administration does not lead to increased aggression in subordinate individuals. *Note: y-axes have different scales due to the disparity between dominant and subordinate wins/hr.*



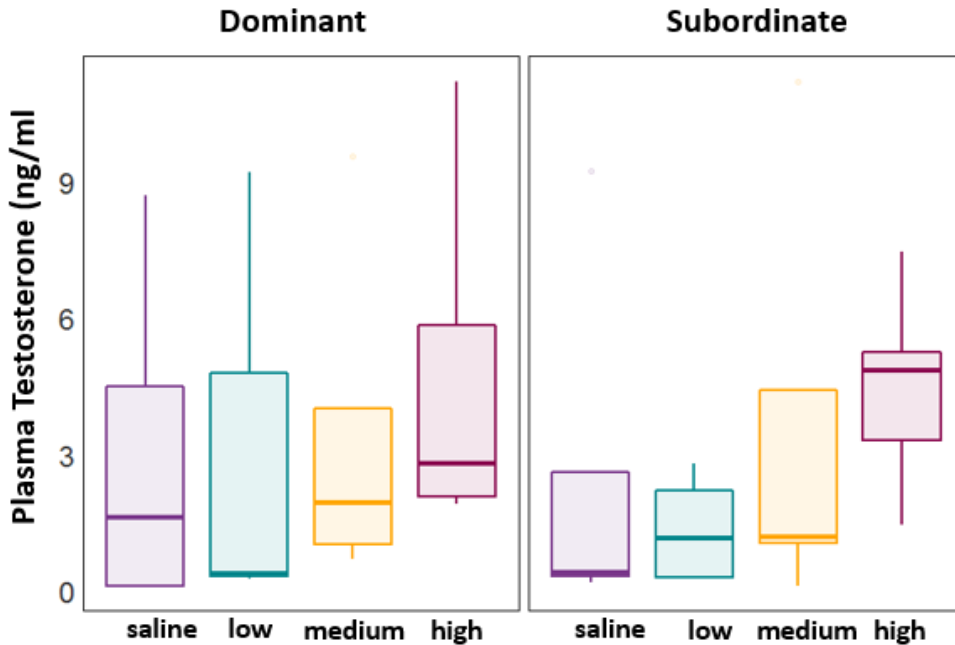
### ***Testosterone response to GnRH or saline administration***

Subordinate individuals did not show significantly different levels of plasma testosterone as compared to dominant individuals (**Figure 6.8**  $\beta = 0.4000 \pm 6.569$ ,  $p = 0.952$ ). Further, there was no interaction between

dose of GnRH and rank, such that there was no differential response to GnRH administration based on social rank (**Figure 6.8**: high dose vs. saline:  $\beta = -1.400 \pm 9.854$ ,  $p = 0.889$ ; high dose vs. low dose:  $\beta = -2.585 \pm 10.332$ ,  $p = 0.806$ ; high dose vs. medium dose:  $\beta = -2.000 \pm 9.290$ ,  $p = 0.833$ ; medium dose vs. saline:  $\beta = -0.600 \pm 9.854$ ,  $p = 0.952$ , low dose vs. saline:  $\beta = -1.185 \pm 10.842$ ,  $p = 0.915$ ; medium dose vs. low dose:  $\beta = -0.585 \pm 10.332$ ,  $p = 0.956$ ).

The groups that received different drug treatments (GnRH – low, medium, and high – and saline) showed a significant difference in testosterone levels (Friedman multiple comparison test  $\chi^2 = 6.8889$ ,  $p < 0.05$ ). When each dose of GnRH was compared separately to each other dose, the only statistically different testosterone level was between high dose v. low dose (**Figure 6.8**,  $W = 79$ ,  $p < 0.05$ ). There was no statistically significant difference in the high dose v. the medium dose (**Figure 6.8**,  $W = 69.5$ ,  $p = 0.151$ ) or in the medium v. low dose (**Figure 6.8**,  $W = 62$ ,  $p = 0.182$ ). When the separate doses were compared to the saline group there was no statistical difference between their testosterone levels (high v. saline:  $W = 58$ ,  $p = 0.1198$ ; medium v. saline:  $W = 54$ ,  $p = 0.2294$ ; low v. saline:  $W = 40$ ,  $p = 0.7361$ ). Across dominant and subordinate individuals, when all three doses were collapsed into a single drug group, there was no significant difference in plasma testosterone levels between the drug group and the control (saline) group ( $W = 143$ ,  $p = 0.188$ ). The same was true just for subordinate individuals ( $W = 20$ ,  $p = 0.442$ ) and just for dominant individuals ( $W = 18$ ,  $p = 0.396$ ).

**Figure 6.8: Effect of GnRH administration on testosterone response in dominant and subordinate individuals** There was no significant difference in testosterone levels between dominant and subordinate individuals, regardless of dose. There was a dose response effect such that groups receiving different drug treatments showed a significant difference in testosterone levels.



## DISCUSSION

In this study, we found that exogenous GnRH administration leads to an increase in aggression in dominant but not subordinate mice. This behavioral change is not, however, due to a differential testosterone response, as dominant and subordinate individuals both responded to GnRH with equivalent increases in plasma testosterone. This suggests that social context, in this case the rank of the individual, is an important modulator of behavior, as despite showing increased testosterone, subordinate individuals were unable to win more fights. While this does not explain our findings in Study #1 in this chapter (Williamson, Romeo, et al., 2017), that subordinate individuals increase GnRH but not testosterone levels in response to social opportunity, it is consistent with the behavioral findings in that study, where increased GnRH levels were not sufficient to lead to winning in subordinate individuals.

Notably, we found that exogenous GnRH administration leads to corresponding testosterone increases in both dominant and subordinate mice. This refutes our initial hypothesis that subordinate mice have a dampened response to GnRH, based on work in sugar gliders, dark eyed juncos, and mole rats (Bradley & Stoddart, 1997b; Faulkes et al., 1990; McGlothlin et al., 2007). It also cannot explain why in our previous work, we found that subordinates increase GnRH in response to social opportunity but do not show a subsequent testosterone surge (Williamson, Romeo, et al., 2017). It is possible that this is because in pairs, subordinates are not reproductively suppressed to the same extent that they are in groups. Some of our previous work shows that in highly despotic hierarchies, there is a significant suppression of testosterone production in subordinates in highly despotic groups, but not in pairs (Williamson, Lee, et al., 2017). This suppression of testosterone could be due to reduced levels of GnRH receptors in the brain. If we want to further understand this phenomenon, it would be important to conduct a similar experiment to this but in a group setting in the vivarium.

We found that there was a dose response effect, proving that by administering exogenous GnRH we were able to influence testosterone production beyond that of a normal surge elicited by pulsatile GnRH release. This demonstrates that while the pulsatile nature of GnRH release can make these phenomena difficult to study in a controlled manner, by administering large enough doses, we are able to control testosterone output.

## **CONCLUSION**

While we were not able to determine that subordinate mice show a dampened testosterone response to GnRH, we did find that GnRH administration leads to higher amounts of aggression in dominant but not subordinate individuals. This provides further evidence for the idea that social context, in addition to physiology, can modulate behavior, as even though subordinate individuals were given GnRH and responded with a testosterone surge, that testosterone surge alone was not sufficient to make them dominant. As we have demonstrated through previous work, social dominance is a complex phenomenon, and it is clear that there is not just one factor (i.e. testosterone levels) influencing whether an individual expresses dominant or subordinate behavior.

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## CHAPTER 7 – Immediate early gene activation throughout the brain is associated with dynamic changes in social context

Cait M. Williamson, Inbal S. Klein, Won Lee & James P. Curley

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## **ABSTRACT**

Social competence is dependent on successful processing of social context information. The social opportunity paradigm is a methodology in which dynamic shifts in social context are induced through removal of the alpha male in a dominance hierarchy, leading to rapid ascent in the hierarchy of the beta male and of other subordinate males in the social group. In the current study, we use the social opportunity paradigm to determine what brain regions respond to this dynamic change in social context, allowing an individual to recognize the absence of the alpha male and subsequently perform status-appropriate social behaviors. Replicating our previous work, we show that following removal of the alpha male, beta males rapidly ascend the social hierarchy and attain dominant status by increasing aggression towards more subordinate individuals. Analysis of patterns of Fos immunoreactivity throughout the brain indicates that in individuals undergoing social ascent, there is increased activity in regions of the social behavior network, as well as the infralimbic and prelimbic regions of the prefrontal cortex and areas of the hippocampus. Our findings demonstrate that male mice are able to respond to changes in social context and provide insight into the how the brain processes these complex behavioral changes.

**KEYWORDS:** social context, social hierarchy, prefrontal cortex, social behavior network

## INTRODUCTION

Organization into dominance hierarchies is a fundamental feature of group social behavior across species, including non-human primates (Muller & Wrangham, 2004; Sapolsky, 1993), cichlid fish (Grosenick, Clement, & Fernald, 2007; Huffman, Hinz, Wojcik, Aubin-Horth, & Hofmann, 2015; Oliveira & Almada, 1996), naked mole rats (Holmes, Goldman, & Forger, 2008), honey bees (Kucharski, Maleszka, Foret, & Maleszka, 2008), mice (Wang et al., 2011; Williamson, Lee, & Curley, 2016a), and humans (Zink et al., 2008). Individuals form these dominance structures through a complicated appraisal of their social context in order to ascertain their position relative to that of the other individuals within their social network (Curley, 2016b; Fernald, 2014; Grosenick et al., 2007; Oliveira, 2009). There has been characterization of the complex behavioral features of the formation and maintenance of dominance hierarchies (Chase & Seitz, 2011; Chase, Tovey, Spangler-Martin, & Manfredonia, 2002; Curley, 2016b; Williamson, Lee, et al., 2016a; Williamson, Lee, Romeo, & Curley, 2017), as well as identification of the neural correlates associated with social status in stable social hierarchies (So, Franks, Lim, & Curley, 2015; Wang et al., 2011; Williamson, Franks, & Curley, 2016; Zerubavel, Bearman, Weber, & Ochsner, 2015; Zink et al., 2008).

Although hierarchies are commonly stable, there often occurs times when individuals change in social rank. One particularly salient example of this is when a power vacuum emerges at the top of a hierarchy following the removal or deposition of the alpha individual. When such social opportunities occur, subdominant animals typically rapidly ascend to the alpha position. Such behavior has been observed experimentally in hierarchies of both cichlid fish (Maruska & Fernald, 2010) and CD1 outbred mice (Williamson, Romeo, & Curley, 2017) associated with changes along the hypothalamic-pituitary-gonadal (HPG) axis. Ascent to dominant status in cichlid fish is also associated with increased immediate early gene

expression in several regions specific to fish social behavior (Burmeister, Jarvis, & Fernald, 2005; Maruska, Zhang, Neboori, & Fernald, 2013). However, there has been no comprehensive, whole brain analysis of the neural response to changes in social context in mammals. This ability to process this dynamic social context information and behave in a socially competent manner when the structure of a social hierarchy shifts is critical for successful social living.

The “Social Behavior Network” (SBN) is a bidirectional circuit of brain regions associated with multiple forms of social behavior (i.e. aggression, sexual behavior, communication, social recognition, affiliation and bonding, parental behavior, and social stress responses) across species (Goodson, 2005; Newman, 1999). This network was first described to include the medial amygdala (meA), the bed nucleus of the stria terminalis (BNST), the lateral septum (LS), the medial preoptic area (mPOA), the anterior hypothalamus (AH), the ventromedial hypothalamus (VMH), and the periaqueductal grey (PAG) (Newman, 1999). These brain regions are thought to be the core of the social brain, with much supporting evidence for their role in regulating relatively simple social behavior (see Goodson, 2005 for a comprehensive review). However, for complex social behaviors, such as the formation, maintenance, and dynamic adjustment of social hierarchies, which are reliant on an individual’s ability to perceive changes in their social environment, it is important to understand how activity within the SBN is modulated and complemented by brain regions associated with executive functioning (i.e. prefrontal cortex (Wang et al., 2011; Zink et al., 2008)) and memory (i.e. hippocampus (Noonan et al., 2014; Williamson, Franks, et al., 2016)).

In previous studies, we have demonstrated differential gene expression throughout the brains of outbred CD1 mice of different social rank living in linear hierarchies, specifically in the medial amygdala, central amygdala, medial preoptic area (So et al., 2015) and in the whole hippocampus (Williamson, Franks, et al., 2016). We have shown that within minutes of the removal of the dominant male from a social group,

the subdominant male exhibits increased aggression as well as rapid changes in GnRH gene expression in the medial preoptic region of the hypothalamus (Williamson, Romeo, et al., 2017). In the current study, we aimed to generate a map of immediate early gene activity throughout the SBN and areas related to the monitoring of social context and social memory to assess how the brain of subdominant animals responds to a changing social context when a social opportunity to ascertain alpha status arises. Specifically, we assessed the pattern of Fos immunoreactivity in subdominant mice in response to the removal of the alpha male (a dynamic social change) and compared this neural response to that of subdominant mice living in a stable social system.

## **METHODS**

### ***Subjects and Housing***

A total of 48 male outbred CD1 mice aged 7 weeks were obtained from Charles River Laboratories and housed in groups of 3 for 2 weeks in standard sized cages prior to the behavioral experiment. Throughout the study, mice were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21–24°C) and humidity (30-50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red light (dark cycle) on at 1200 hours. All mice were individually and uniquely marked by dyeing their fur with a blue, nontoxic, non-hazardous animal marker (Stoelting Co.). These marks remain for up to 12 weeks and only require one application, enabling each animal to be visually identified throughout the study. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol No: AC-AAAP5405).

### ***Behavioral Manipulation***

To determine Fos activation associated with social ascent, we performed a social opportunity manipulation comparing subdominant individuals in socially stable groups to those ascending in a

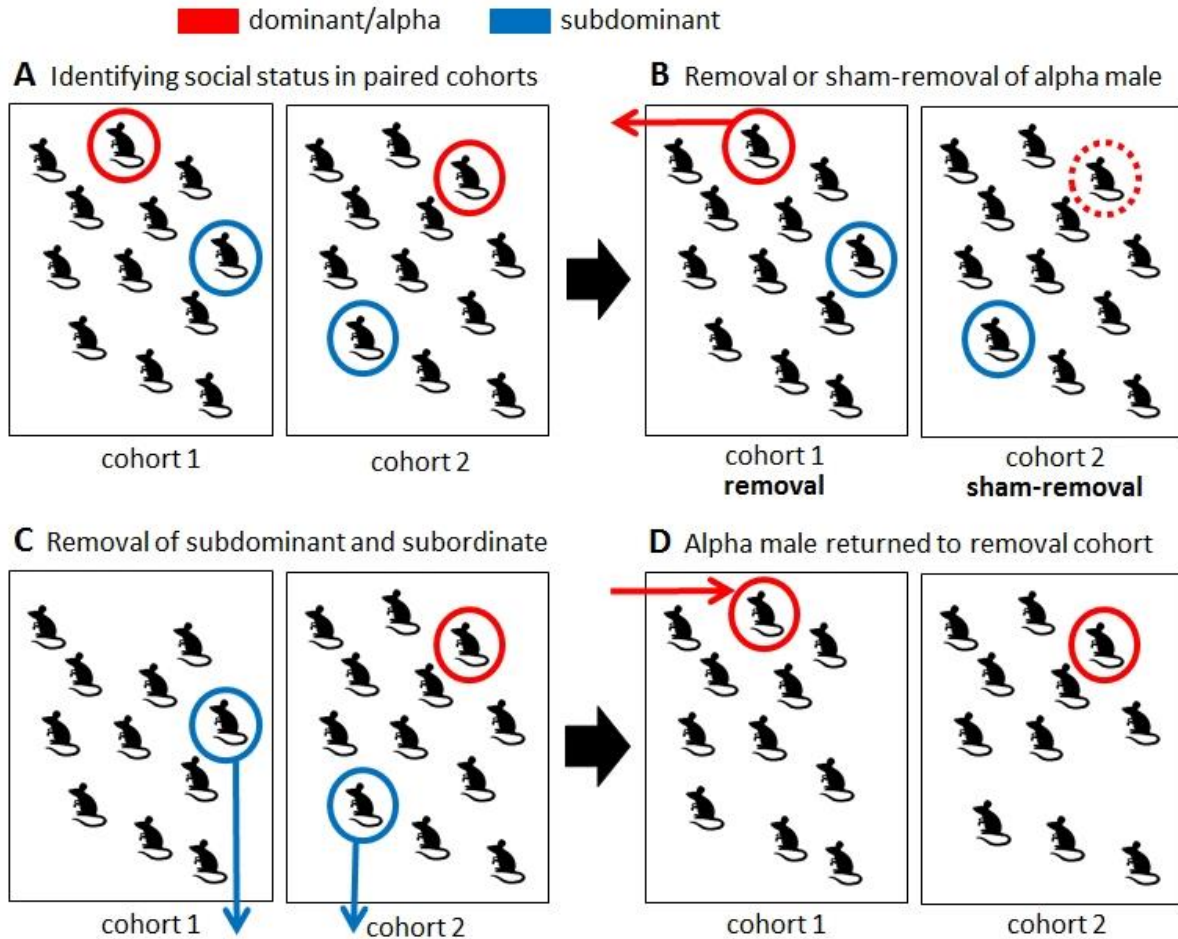
hierarchy from subdominant to dominant status. This procedure is similar to that previously described (Williamson, Romeo, & Curley, 2017). At 9 weeks of age, 4 groups of 12 mice were placed into custom built vivaria (length 150cm, height 80cm, width 80cm; Mid-Atlantic; **Supplemental Figure 1**). The vivarium was constructed as previously described (Williamson, Lee, & Curley, 2016). Each vivarium consists of an upper level consisting of multiple shelves covered in pine bedding and a lower level consisting of a series of nest-boxes filled with pine bedding connected by tubes. Mice can access all levels of the vivarium via a system of ramps and tunnels. Standard chow and water were provided ad libitum at the top of the vivarium, encouraging movement and exploration of all the shelves. Social groups were introduced into the vivarium directly before onset of the dark cycle on Day 1. There were 4 social groups, each with 12 mice per group. Each group was paired with one other group for experimental control and counterbalancing purposes. For example, when group 1 was experimentally manipulated, group 2, its paired group, served as the control condition. Live behavioral observations were conducted each day during the dark cycle. These observations consisted of trained observers recording all instances of fighting, chasing, mounting, subordinate posture, and induced-flee behaviors. Each trained observer was responsible for observing one cohort at a time, so observers were entirely focused on one group during their observation. The identity of the dominant and subordinate individuals in each interaction were recorded using all occurrence sampling. Data was collected directly into electronic tablets and uploaded live to a google spreadsheet. **Supplemental Table 1** contains an ethogram of the behaviors recorded. Live observations were conducted for 2 hours during the first four hours of the dark cycle each day on Days 1-4 of group housing. At the end of Day 4, it was verified that a dominance hierarchy had emerged in each group, and the identity of the alpha and beta male in each group was determined. The presence and linearity of the hierarchies was confirmed through calculating Landau's modified  $h'$  values, and the identity of the alpha and beta male was confirmed using Glicko scores and examination of the sociomatrix of wins and losses. These analyses are described below in the statistical analysis section. On Day 5, at the

onset of the dark cycle, the alpha male from one of the paired cohorts was removed from the vivarium and placed in a standard cage with food and water. In the other paired cohort, the alpha male was sham-removed, which entailed an experimenter opening the Perspex windows to the vivarium, placing their hand in the vivarium, and reaching towards the alpha mouse but not removing it from the vivarium. This condition, which does not involve removing any mouse from the social group, controls for behavioral changes that may be occurring in response to a non-social disturbance to the environment. Live behavioral observations occurred for the period directly following the removal or sham-removal. Ascending males were confirmed as the individual who won most aggressive contests post-alpha removal without consistently losing to other individuals. Ninety minutes after this ascending individual had won three fights, the ascending male was removed from the alpha removal group and the non-ascending subdominant male was removed from the sham-removal group.

Following removal from the social group, mice were anesthetized with ketamine/xylazine and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were stored at 4°C in 4% paraformaldehyde for the first six hours following perfusion and then switched to a 30% sucrose solution. Following the perfusions, the alpha male who had been removed in the social ascent condition was returned to his social group. Alpha males always retained their alpha status on return to the social group.

This procedure was repeated at four day intervals for a total of six "removals". Manipulations were counter-balanced between paired cohorts (i.e. one vivarium had alpha removal for removals 1, 3, and 5, and sham removals for removals 2, 4, and 6, and the opposite was true for the paired vivarium). Each removal/sham-removal decreased the size of the social group by 1, resulting in N = 12 (removal one), N = 11 (removal two), N = 10 (removal three), N = 9 (removal 4), N = 8 (removal 5), N = 7 (removal 6). This design yielded 12 mice per group from two groups: beta male/alpha removed (ascending males) and beta

male/alpha remained (sham-removal subdominants). See **Figure 7.1** for a schematic of the behavioral manipulation.



**Figure 7.1. Schematic of the social opportunity experimental design.** (A) Two cohorts of twelve mice are put into separate vivaria and a stable social hierarchy emerges, with clearly defined dominant, and subdominant individuals. (B) The alpha male is removed from one stable hierarchy and sham-removed from the paired hierarchy. (C) Following removal/sham-removal, behavioral observations are conducted on both cohorts until ninety minutes after a subdominant rises in the alpha-removed group. At this ninety-minute time point, the sub-dominant (rising to alpha) animal in each hierarchy is removed and brain extracted for analyses. (D) One-hour following this removal of the subdominant, the alpha male is returned to its social group. This procedure is repeated five more times four days apart for each pair of cohorts.

### ***Immunohistochemistry***

Brains were stored in 30% sucrose in 0.1M PB at 4°C until slicing. Perfused whole brains were sliced coronally into 40 µm sections and stored in 0.1 M PB azide until processing according to the avidin– biotin procedure, using the Vectastain ABC Elite peroxidase rabbit IgG kit (Vector Laboratories, Burlingame, CA). Free-floating sections were transferred into wells and washed three times in 0.1 M PB for five minutes each rinse. The sections were then washed once in hydrogen peroxide for five minutes and then washed three times in PBT for five minutes each rinse. The sections were then placed in a solution of 2% Normal Goat Serum (NGS, Vector Laboratories) in 0.1% Triton-X in 0.1M PB (PBT) for an hour, and then incubated in primary Fos rabbit polyclonal IgG (Santa Cruz, USA, SC-52) at a concentration of 1:5000 overnight at 4°C with 2% NGS block. The next day, the sections were washed 3 times in PBT for 5 minutes each rinse and then incubated in biotinylated anti-rabbit IgG (Vecstastain ABC Kit, Vector Laboratories) at a concentration of 1:200 in PBS for 1 hour. Once the hour was complete, sections were once again washed 3 times in PBT for 5 minutes each rinse. Sections were then incubated for 1 hour in an avidin– biotin–peroxidase complex in 0.1 M PBT (A and B solutions of the Vectastain ABC Kit, Vector Laboratories) at a concentration of 40ul A: 40ul B: 10ml PBT and then washed 3 times in 0.1M PBS for 5 minutes each rinse. Fos immunoreactivity was visualized by incubating the sections in 0.02% 3,3'-diaminobenzidine (DAB) solution for 2-4 minutes. Sections were then washed once for 1 minute in 0.1M PBS and then washed 3 times in 0.1M PBS for 5 minutes each rinse. All sections were then stored in 0.1M PB at 4°C for up to 24 hours until mounting. Sections were mounted onto FisherBrand Plus slides and then coverslipped with DePeX mounting medium (Sigma-Aldrich, St. Louis, MO).

### ***Photos and Image Analysis***

Images were taken of brain sections under a 10x objective microscope at a magnification x100 and a digital camera. Localization of specific brain regions was determined using the Allen Mouse Brain Atlas (Lein et



al., 2007). For each brain region, 2-3 brain sections per mouse were imaged. Images were then cropped to include only the exact portion of each brain region by overlaying images from *The Mouse Brain in Stereotaxic Coordinates* (Paxinos & Franklin, 2004) over the photos in an image editing program. Particles were then analyzed with the batch function using a macro in ImageJ (Schneider, Rasband, & Eliceiri, 2012). Twenty-five separate brain regions were processed: bed nucleus of the stria terminalis (BNST), lateral septum (LS), anterior hypothalamus (AH), medial preoptic area (mPOA), ventromedial hypothalamus (VMH), medial amygdala (meA), dorsolateral periaqueductal grey (dIPAG), ventrolateral periaqueductal grey (vIPAG), dorsal and ventral premammillary nuclei (PMd and PMv), cingulate cortex, infralimbic and prelimbic regions of the prefrontal cortex (IL, PrL), piriform cortex, retrosplenial cortex (RC), area CA1 of the hippocampus (CA1), area CA3 of the hippocampus (CA3), the dentate gyrus (DG), anterior cortical amygdala (ACA), central amygdala (CeA), basolateral amygdala (BLA), arcuate nucleus (Arc), lateral hypothalamus (LH), primary auditory cortex, primary visual cortex. All subjects for each brain region were analyzed concurrently, with each brain region being analyzed separately.

### ***Statistical Analysis***

All statistical analyses were undertaken in R version 3.4.0 (R Core Team, 2016) in RStudio version 1.0.143 (RStudio Team, 2015).

*Behavioral analysis:* For each cohort, the linearity of the social hierarchy was calculated using Landau's Modified  $h'$ . Briefly, the total number of wins by each individual against all other individuals are entered into a sociomatrix. Landau's method then assesses the degree to which each individual consistently dominates others in contests and whether individuals can be linearly ordered based upon their wins and losses. The  $h'$  value ranges from 0 (no linearity) to 1 (completely linear). The significance of  $h'$  is determined by performing 10,000 two-step Monte Carlo randomizations of the sociomatrix and comparing the observed  $h'$  against a simulated distribution of  $h'$  (De Vries, 1995; Williamson, Lee, &

Curley, 2016b). Temporal changes in individual dominance ratings were calculated using Glicko Ratings (Glickman, 1999; So et al., 2015). Glicko ratings are a pairwise-contest model ratings system where ratings points are recalculated following each successive win or loss. All individuals start with a rating of 2200. Ratings are gained after wins and lost after losses with the magnitude of points gained or lost dependent upon the difference in ratings scores between the two individuals in each contest (Glickman, 1999; Williamson, Lee, et al., 2016b). Landau's modified  $h'$  was calculated using the R package *compete* v0.1 (Curley, 2016a). Glicko ratings were calculated using the *PlayerRatings* package v1.0 in R (Stephenson & Sonas, 2012).

*Social ascent analysis:* To compare wins and losses between betas in the alpha removed group to those in the alpha remained group, we used Wilcoxon rank sum tests. To compare wins and losses between betas in the alpha removed and sham-removed group on the day of removal or sham-removal to their behavior the day before, we used Wilcoxon signed rank tests.

*Fos Analysis:* To determine the effect of alpha removal on the number of immunoreactive cells in each brain region, we used the R package *lme4* (Bates et al., 2015) to run negative binomial mixed models, with social status (alpha removed or alpha remained) as a fixed effect and cohort, removal number, side of the brain, number of wins, and number of losses as random effects. This model was run separately for each of the 25 brain regions. We chose  $p = 0.01$  as our alpha level in order to decrease the chance of type 1 error without inflating type 2 error.

*Hierarchical clustering analysis:* To determine brain region activation patterns in both the alpha removed and alpha remained groups, we created correlation matrices for each group and visualized them using the

R package lattice (Sarkar, 2017). We then used the package pvclust (Suzuki & Simodaira, 2015) to determine hierarchical clusters and generate p-values for each cluster using multiscale bootstrap resampling.

## RESULTS

### *All cohorts form significantly linear hierarchies*

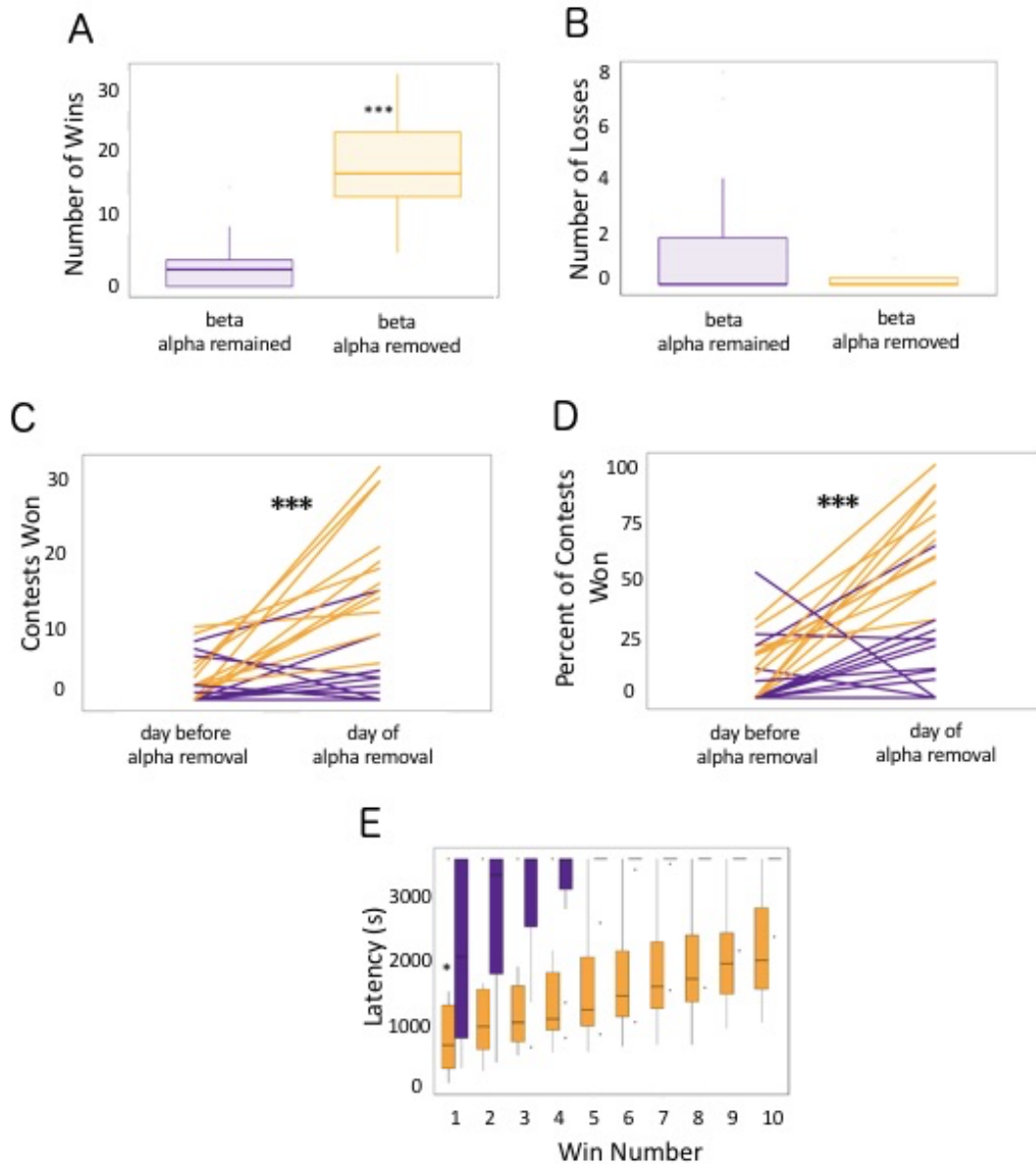
All social groups formed significantly linear dominance hierarchies with a clear alpha and beta male after the first four days of group housing prior to the first alpha or sham-removal (all  $h'$  values  $> 0.45$ , mean  $h' = 0.59$ , all  $p < 0.05$ , mean  $p = 0.016$ ). All alpha males maintained their alpha status for the duration of their presence in the established social hierarchy.

### *Subdominant males socially ascend following removal of the dominant male*

After each of the 12 alpha male removals, a subdominant male ascended within 1 hour. Rising subdominants had significantly more wins than the subdominant males in the sham-removal group (Wilcoxon rank sum test  $W = 138$ ,  $p = 0.00018$  – **Figure 7.2A**). Further, the majority (9/12) of rising subdominant individuals never lost a fight during this period, however there was no significant difference in number of losses between rising subdominants in the alpha removed group and those in the sham removal group (Wilcoxon rank sum test  $W = 56.5$ ,  $p = 0.3006$  – **Figure 7.2B**). When compared to their behavior the day before alpha removal, rising subdominants had significantly more wins (Wilcoxon signed rank test  $V = 1$ ,  $p = 0.003$ ) and significantly fewer losses (Wilcoxon signed rank test  $V = 48$ ,  $p = 0.037$ ). There was no significant difference in wins (Wilcoxon signed rank test  $V = 21$ ,  $p = 0.54$ ) or losses (Wilcoxon signed rank test  $V = 16$ ,  $p = 0.832$ ) in non-rising subdominants on the day of sham removal when compared to

the day before alpha removal (**Figure 7.2C**). In the alpha removed group, latency to first win occurred on average at 14.9 minutes, with some individuals winning their first fight within 15 seconds. This was significantly different from in the sham removal group, where latency to first win occurred on average at 34.9 minutes (Wilcoxon rank sum test:  $W = 36.5$ ,  $p = 0.042$ ; **Figure 7.2D**).

In 11/12 of the removals, the rising subdominant was predicted based on data from the previous three days prior to the alpha removal. In these 11 cases, the rising subdominant was the male with the second highest Glicko ranking (i.e. the beta male) prior to removal. In the one instance where this was not the case, the individual that ascended had a slightly lower Glicko ranking than the previous beta male. However, it is worth noting that this was in the sixth removal after many manipulations of the social group, and the alpha male in this group was extremely despotic performing over 80% of all aggressive acts within the group. Consequently, fewer social contests occurred between lower-ranked individuals making it difficult to unequivocally identify the ranks of all other lower-ranked males at this time-point.



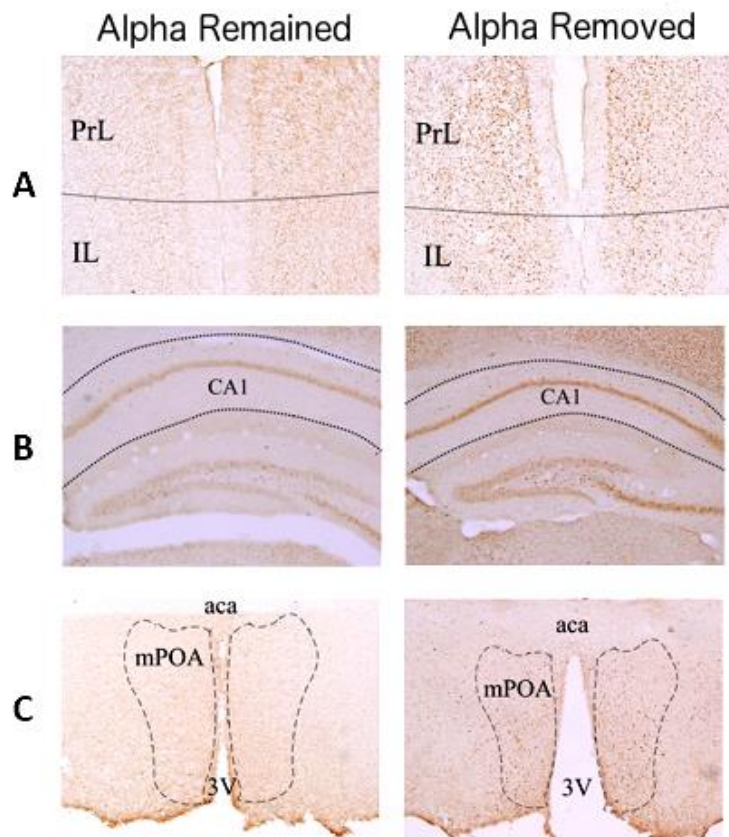
**Figure 7.2. Behavioral changes in subdominant male following removal or non-removal of the alpha male.** (A) Beta males in groups where the alpha male was removed win significantly more contests (fights, chases, mounts, instances of subordinate behavior, and instances of induced flee) than beta males in groups where the alpha male remained in the group. (B) Beta males in groups where the alpha male was removed do not lose significantly fewer contests than beta males in groups where the alpha male was removed from the group. (C) The number of contests won by the beta male on the day before removal or sham removal compared to the day after removal or sham removal. Yellow lines represent individuals in the removal group, purple lines represent individuals in the sham removal group. (D) The percentage of all contests won by the beta male on the day before removal or sham removal compared to the day after removal or sham removal. Yellow lines represent individuals in the removal group, purple lines represent individuals in the sham removal group. (E) Latency of subdominant males to win successive contests within one hour after the alpha male was removed (yellow) or sham-removed (purple)

***Social ascent is associated with differential Fos immunoreactivity throughout the brain***

**Table 7.1** describes Fos immunoreactivity pattern for beta males in the alpha removed and alpha remained conditions for each brain region studied. For 15/25 brain regions, there was a significant difference in Fos immunoreactivity, with individuals from the alpha removed group displaying significantly higher numbers of immunoreactive cells (**Table 7.1**). Consistent with our predictions, 5 of these regions (BNST, LS, AH, mPOA, dIPAG) are areas within the Social Behavior Network. The remaining 9 regions included prefrontal cortex (cingulate, infralimbic, prelimbic) as well as the retrosplenial cortex, hippocampal regions (CA1 and dentate gyrus), and a hypothalamic region (arcuate nucleus). Both the auditory cortex and visual cortex displayed increased immunoreactivity in the alpha removed condition. See **Figure 7.3** for sample images of Fos staining.

Table 7.1. Table of Fos cell counts and results of mixed models for all brain regions examined

Region	Network	Bregma (mm)	Alpha Removed Mean $\pm$ SEM	Alpha Remained Mean $\pm$ SEM	$\beta \pm$ SEM	p-value
<b>BNST</b>	<i>SBN</i>	<i>0.38 to 0.14</i>	<i>44.6 <math>\pm</math> 11.3</i>	<i>21.8 <math>\pm</math> 4.9</i>	<i>1.1 <math>\pm</math> 0.4</i>	<i>0.008</i>
<b>LS</b>	<i>SBN</i>	<i>0.38 to 0.14</i>	<i>96.0 <math>\pm</math> 27.8</i>	<i>19.7 <math>\pm</math> 4.2</i>	<i>1.9 <math>\pm</math> 0.7</i>	<i>0.004</i>
<b>AH</b>	<i>SBN/social def.</i>	<i>-0.46 to -0.58</i>	<i>69.0 <math>\pm</math> 12.2</i>	<i>39.3 <math>\pm</math> 7.4</i>	<i>0.5 <math>\pm</math> 0.2</i>	<i>0.004</i>
<b>mPOA</b>	<i>SBN/social def.</i>	<i>0.14 to -0.10</i>	<i>91.5 <math>\pm</math> 12.3</i>	<i>66.5 <math>\pm</math> 14.4</i>	<i>1.1 <math>\pm</math> 0.1</i>	<i>2.00E-16</i>
<b>VMH</b>	<i>SBN/social def.</i>	<i>-1.46 to -1.70</i>	<i>19.5 <math>\pm</math> 4.8</i>	<i>8.2 <math>\pm</math> 2.5</i>	<i>0.3 <math>\pm</math> 0.4</i>	<i>0.492</i>
<b>meA</b>	<i>SBN/social def.</i>	<i>-1.06 to -1.22</i>	<i>67.9 <math>\pm</math> 16.9</i>	<i>27.5 <math>\pm</math> 5.3</i>	<i>0.2 <math>\pm</math> 0.4</i>	<i>0.654</i>
<b>dIPAG</b>	<i>SBN/social def.</i>	<i>-2.92 to -3.16</i>	<i>20.6 <math>\pm</math> 3.5</i>	<i>12.5 <math>\pm</math> 2.2</i>	<i>1.6 <math>\pm</math> 0.3</i>	<i>6.23E-06</i>
<b>vIPAG</b>	<i>SBN/social def.</i>	<i>-2.92 to -3.16</i>	<i>12.7 <math>\pm</math> 3.2</i>	<i>6.3 <math>\pm</math> 1.4</i>	<i>1.0 <math>\pm</math> 0.4</i>	<i>0.023</i>
<b>PMd</b>	<i>social def.</i>	<i>-2.70 to -2.92</i>	<i>24.2 <math>\pm</math> 3.6</i>	<i>19.2 <math>\pm</math> 3.1</i>	<i>0.3 <math>\pm</math> 0.2</i>	<i>0.1</i>
<b>PMv</b>	<i>social def.</i>	<i>-2.70 to -2.92</i>	<i>37.5 <math>\pm</math> 5.0</i>	<i>35.9 <math>\pm</math> 5.9</i>	<i>0.3 <math>\pm</math> 0.2</i>	<i>0.159</i>
<b>Cingulate</b>	<i>Cortical</i>	<i>2.34 to 2.10</i>	<i>48.8 <math>\pm</math> 14.8</i>	<i>10.2 <math>\pm</math> 1.8</i>	<i>1.6 <math>\pm</math> 0.3</i>	<i>2.67E-06</i>
<b>IL</b>	<i>Cortical</i>	<i>1.94 to 1.54</i>	<i>90.4 <math>\pm</math> 14.0</i>	<i>12.9 <math>\pm</math> 2.3</i>	<i>1.5 <math>\pm</math> 0.3</i>	<i>5.49E-07</i>
<b>PrL</b>	<i>Cortical</i>	<i>1.94 to 1.54</i>	<i>142.2 <math>\pm</math> 24.9</i>	<i>17.9 <math>\pm</math> 3.4</i>	<i>2.0 <math>\pm</math> 0.3</i>	<i>1.33E-11</i>
<b>Piriform</b>	<i>Cortical</i>	<i>1.94 to 0.02</i>	<i>198.5 <math>\pm</math> 30.8</i>	<i>81.3 <math>\pm</math> 18.1</i>	<i>0.6 <math>\pm</math> 0.4</i>	<i>0.111</i>
<b>RC</b>	<i>Cortical</i>	<i>-0.94 to -1.94</i>	<i>34.7 <math>\pm</math> 11.7</i>	<i>5.2 <math>\pm</math> 1.6</i>	<i>1.4 <math>\pm</math> 0.5</i>	<i>0.004</i>
<b>CA1</b>	<i>limbic</i>	<i>-1.94 to -2.18</i>	<i>98.0 <math>\pm</math> 22.1</i>	<i>17.6 <math>\pm</math> 5.1</i>	<i>1.9 <math>\pm</math> 0.4</i>	<i>3.32E-06</i>
<b>CA3</b>	<i>Limbic</i>	<i>-1.06 to 1.22</i>	<i>125.9 <math>\pm</math> 28.3</i>	<i>61.6 <math>\pm</math> 14.7</i>	<i>0.6 <math>\pm</math> 0.3</i>	<i>0.085</i>
<b>DG</b>	<i>Limbic</i>	<i>-1.94 to -2.18</i>	<i>76.3 <math>\pm</math> 12.6</i>	<i>25.1 <math>\pm</math> 4.2</i>	<i>1.0 <math>\pm</math> 0.3</i>	<i>0.003</i>
<b>ACA</b>	<i>Limbic</i>	<i>-0.46 to -0.82</i>	<i>44.5 <math>\pm</math> 11.8</i>	<i>24.9 <math>\pm</math> 7.4</i>	<i>0.6 <math>\pm</math> 0.4</i>	<i>0.169</i>
<b>ceA</b>	<i>Limbic</i>	<i>-1.06 to -1.34</i>	<i>16.5 <math>\pm</math> 4.5</i>	<i>14.5 <math>\pm</math> 3.4</i>	<i>0.1 <math>\pm</math> 0.7</i>	<i>0.730</i>
<b>BLA</b>	<i>Limbic</i>	<i>-1.06 to -1.34</i>	<i>91.3 <math>\pm</math> 29.7</i>	<i>63.4 <math>\pm</math> 17.8</i>	<i>1.1 <math>\pm</math> 0.6</i>	<i>0.068</i>
<b>Arc</b>	<i>Limbic</i>	<i>-1.46 to -1.70</i>	<i>37.1 <math>\pm</math> 10.8</i>	<i>10.8 <math>\pm</math> 2.1</i>	<i>2.0 <math>\pm</math> 0.3</i>	<i>3.78E-09</i>
<b>LH</b>	<i>Limbic</i>	<i>-0.46 to -0.58</i>	<i>31.6 <math>\pm</math> 6.2</i>	<i>19.5 <math>\pm</math> 2.4</i>	<i>0.5 <math>\pm</math> 0.3</i>	<i>0.062</i>
<b>Auditory</b>	<i>Sensory</i>	<i>-2.18 to -2.80</i>	<i>120.2 <math>\pm</math> 44.3</i>	<i>18.7 <math>\pm</math> 4.1</i>	<i>2.1 <math>\pm</math> 0.6</i>	<i>4.60E-04</i>
<b>Visual</b>	<i>Sensory</i>	<i>-2.54 to -2.92</i>	<i>143.4 <math>\pm</math> 34.6</i>	<i>17.4 <math>\pm</math> 4.0</i>	<i>1.7 <math>\pm</math> 0.4</i>	<i>3.05E-05</i>



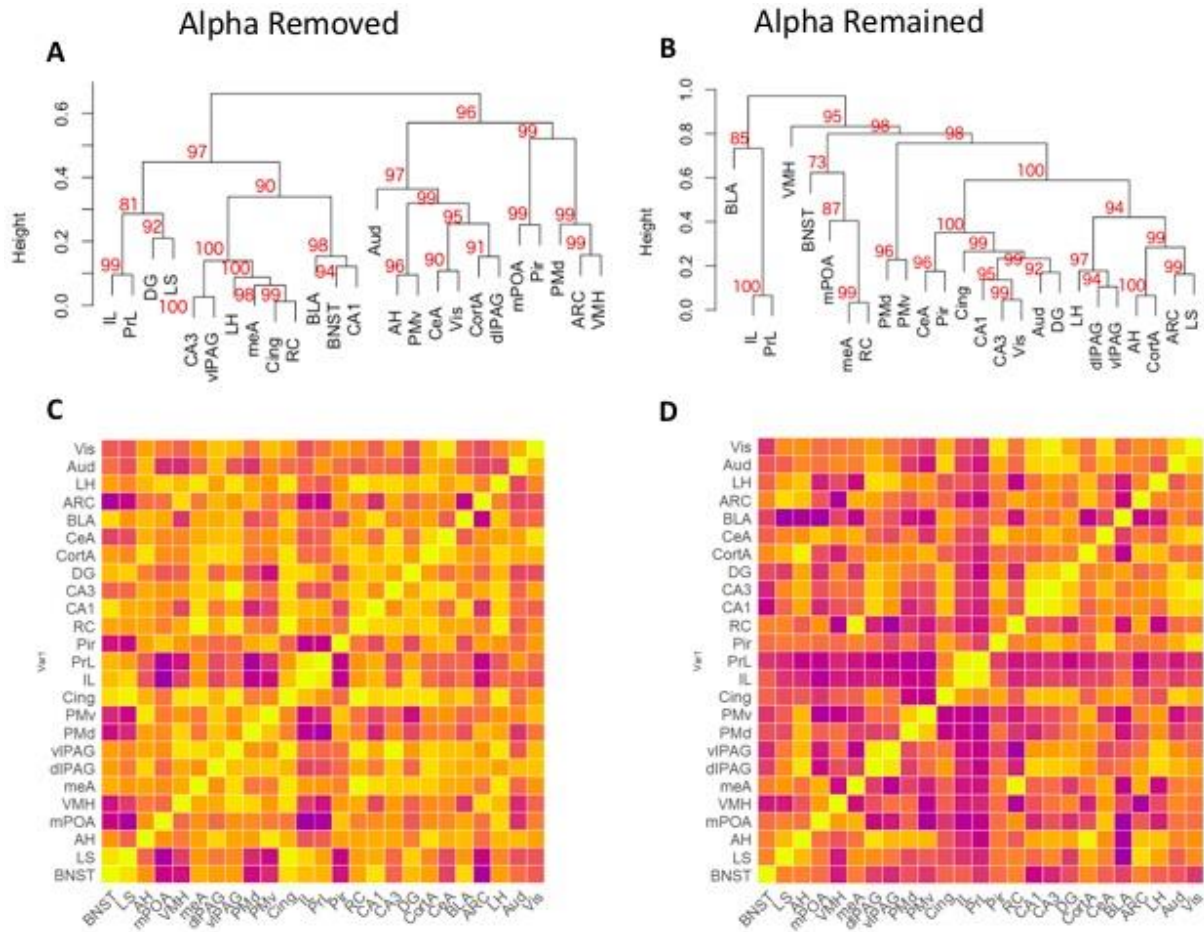
**Figure 7.3. Sample images of Fos staining in individuals from the alpha remained (left) and alpha removed (right) groups.** (A) Prelimbic and infralimbic regions of the medial prefrontal cortex. The dotted line indicates the separation between the IL and PrL regions. (B) CA1 region of the hippocampus. Dotted lines indicate the edges of CA1 (C) Medial preoptic area of the hypothalamus. Lines indicate the edges of the mPOA.

***Hierarchical clustering analysis suggests differential patterns of activation in individuals undergoing social ascent***

To examine whether social ascent lead to differential co-activation patterns throughout the brain, we performed a hierarchical clustering analysis. Significantly different clusters between individuals undergoing social ascent and those in stable social groups were identified. In the alpha removed group,



two distinct clusters formed, one including IL, PrL, DG, LS, CA3, vIPAG, LH, meA, Cing, RC, BLA, BNST, and CA1 and one including Aud, AH, PMv, CeA, Vis, CortA, dIPAG, mPOA, Pir, PMd, ARC, and VMH (**Figure 7.4A**). In the alpha remained group one cluster contained all regions but the BLA, IL, and PrL, with the IL and PrL splitting off into their own cluster (**Figure 7.4B**). Notably, in the alpha removed group we saw greater positive correlation between brain regions (**Figure 7.4C**) and in the alpha remained group, we saw greater negative correlation between brain regions (**Figure 7.4D**), suggesting that there was generally more coordinated activation of pathways in the individuals undergoing social ascent.



**Figure 7.4. Beta individuals undergoing social ascent have distinct neural activation patterns as compared to beta individuals from stable groups. (A and B) Cluster dendrogram displaying results of**

hierarchical clustering analysis for beta males where the alpha was removed (A) and for beta males where the alpha remained (B). Red numbers indicate the approximately unbiased p-value generated through multiscale bootstrap resampling. Values higher than 95 indicate statistical significance. Red boxes denote significant clusters that are strongly supported by data. (C and D) Pearson correlation coefficients were used to create a heatmap of neural co-activation across examined brain regions. (C) Heatmap for beta males where the alpha was removed (D) Heatmap for beta males where the alpha remained

## **DISCUSSION**

In the present study, we successfully replicated the behavioral findings from our previous work (Williamson, Romeo, et al., 2017), illustrating that following removal of the alpha male, beta males recognized the emergence of a power vacuum and use this opportunity to ascend to alpha status. Ascending males won significantly more and lost significantly less in comparison to their own behavior the day before, as well as compared to non-ascending beta males in hierarchies whose alpha had not been removed. Moreover, this change occurs rapidly with beta males beginning their ascent on average within 15 minutes. These findings provide further evidence that individuals in social groups recognize and behaviorally respond to dynamic changes in social context. This ability appears to be a fundamental feature of living within a social group, and has been seen to occur in a similarly controlled manner in African cichlid fish (Maruska, Zhang, et. al., 2013; Maruska & Fernald, 2010) where beta males begin to change color and increase aggression in response to alpha removal within minutes, and in primates, where beta males quickly and forcefully ascended to alpha status following alpha males receiving amygdaloid lesions (Rosvold, Mirsky, & Pribram, 1954). This ability for individuals to recognize and rapidly respond to changes in social status is an essential feature of social competence and is associated with greater social, reproductive, and health outcomes (Hofmann et al., 2014; Taborsky & Oliveira, 2012).

In the current study, we also demonstrate that response to a change in social context is associated increases in neural activity. Notable increases in immediate early immunoreactivity were observed throughout the SBN as well as in the prefrontal cortex and hippocampus. It appears that coordinated activation of these regions is required to facilitate the assessment of a change in the social context combined with the increase in aggressive behavior to facilitate social ascent. In the SBN, we saw significant differences in cell counts between ascending beta males and stable beta males in the BNST, lateral septum, mPOA, anterior hypothalamus, and the dIPAG. Each of these regions has well-established roles in the modulation of social behavior (Goodson, 2005). Significantly, we did not see any difference in neural activity in the VMH or medial amygdala. The VMH has been demonstrated to be of particular interest in female social behavior (Goodson, 2005), for example when females are assessing the social dominance of potential mates (Desjardins, Klausner, & Fernald, 2010) or in female aggression (Hashikawa et al., 2017). Further, the VMH has been shown to be involved in response to territorial challenge and stress (Goodson & Evans, 2004) but does not appear to be involved in the processing of more general changes in social context. The lack of difference in the medial amygdala is more remarkable, as it has been heavily implicated in social dominance (Bolhuis, Fitzgerald, et. al., 1984; Rosvold, Enger et al., 1954; So et al., 2015; Timmer, Cordero, et. al., 2011). However, these previous findings appear to be specific to stable social groups and to understanding the physiology of individuals of dominant vs. subordinate status and cannot be assumed to extend to individuals responding to a changing social context and subsequently undergoing a change in social status.

The largest fos immunoreactivity differences observed between ascending beta males and non-ascending beta males were in the prelimbic and infralimbic regions of the prefrontal cortex. In non-ascending beta males we find very little activation in these regions, whereas in ascending males we find very large levels of activation. These regions of the medial prefrontal cortex have been established as essential to the processing of rodent social behavior, including aggressive behavior, affiliative behaviors, and dominance

behavior (Ko, 2017; Wang, Kessels, & Hu, 2014). In mice, prelimbic neurons have also been implicated in processing social preference as well as social-spatial information (Murugan et al., 2017). Further, the mPFC has been implicated in humans in the processing of social status information (Silk et al., 2017; Wang et al., 2014; Zerubavel et al., 2015) and the social network position of others (Parkinson, Kleinbaum, & Wheatley, 2017) as well as processing information in relation to self – i.e. how one fits into the broader social context (Pfeifer et al., 2009). Other studies have shown activation of the mPFC when processing unstable social hierarchies (Zink et al., 2008). In pairs of rhesus monkeys, the dominant individual's PFC becomes locked in an “up-state” while the subordinate individual's becomes locked in a “down-state”. This state rapidly switches when relative hierarchical status is switched (Fujii, Hihara, Nagasaka, & Iriki, 2009). Our findings provide further evidence for the crucial role of the medial prefrontal cortex in processing information about social context. Moreover, we purposefully chose to examine brains 90 minutes after each male had behaviorally demonstrated that they had begun to socially ascend. The aim of this methodological approach was to ensure we could identify regions involved in processing the change in social context and that might promote further downstream brain activation. Taking into account the previous literature and our current findings, we suggest that the IL and PL are key regions in the tracking of changes in the social environment and in facilitating further social-decision making, i.e. social ascent.

Our analyses also identified neural activity within the hippocampus, specifically the CA1 and dentate gyrus, as being associated with dynamic change in social status. Neurons in the ventral hippocampus are necessary for social memory storage and are specifically activated in response to familiar mice (Okuyama, Kitamura, Roy, Itohara, & Tonegawa, 2016). Before a beta individual begins his ascent, we observe patrolling and olfactory exploration of the vivarium by these males. During this exploration, the beta male

is coming into contact with the urine of the familiar alpha male, a highly salient signal of social status (Lee, Khan, & Curley, 2017). This exploration could potentially lead to activation of the CA1 in response to social memory of interaction with the alpha. Notably, there is a clear, excitatory pathway from CA1 to the prelimbic region of the prefrontal cortex (Thierry, Gioanni, Dégénétais, & Glowinski, 2000), suggesting that the processing of social memory information in CA1 could be integrated with social context information being processed in PrL through this excitatory pathway. However, additional studies are required to elucidate the exact activation patterns connecting these brain regions. Further, both the dentate gyrus and the retrosplenial cortex have been implicated in regulating spatial memory (Ibi et al., 2008; Jessberger et al., 2009; Nilsson, Perfilieva, Johansson, Orwar, & Eriksson, 1999; Ophir, Wolff, & Phelps, 2008; Czajkowski et al., 2014). In a changing complex social environment, determining the physical presence or absence of more dominant individuals is critical and the observed activation of these brain regions in ascending males may be related to utilization of spatial memory. Moreover, oxytocin receptor signaling in the dentate gyrus has also been shown to be necessary for discrimination of social stimuli (Raam, McAvoy, Besnard, Veenema, & Sahay, 2017), providing further evidence for the importance of this brain region in processing social contextual cues.

We observed significantly elevated activation in the primary visual cortex and primary auditory cortex of ascending males. While studies of mouse social behavior often do not focus on the primary visual cortex, it is essential to social processing in humans – social visual signals provide information about emotional expression, direction of gaze, body posture, and movement, all important social cues (Adolphs, 2003). Studies in non-human primates have demonstrated that neuronal responses in the visual cortex appear to encode highly specific social stimuli such as those described above (i.e. faces, gaze, etc.) (Perrett, Rolls, & Caan, 1982). While there is limited work to suggest that processing of visual stimuli is essential to mouse

social behavior, studies of mouse models of autism suggest that excitatory/inhibitory balance and plasticity in the visual cortex during critical periods in development is important for the development of social behavior (Gogolla et al., 2009). Further, lack of proper gamma oscillations generated in the primary visual cortex are similarly implicated in autism, and have been shown to be important for information processing and learning, suggesting that these oscillations are important for appropriate social behavior (Gogolla et al., 2009; Singer, 1993). It is likely that the activation of the visual cortex during social opportunity is related to visual monitoring of the social environment. The impact of changing social context on the primary auditory cortex is consistent with the established role of auditory cues in communication in mice. Ultrasonic vocalizations (USVs) have been demonstrated to facilitate social interactions in mice (Liu, Miller, Merzenich, & Schreiner, 2003). Dominant males have been shown to elicit significantly more of these vocalizations in mating situations (Lumley, Sipos, Charles, Charles, & Meyerhoff, 1999; Nyby, Dizinno, & Whitney, 1976), though not necessarily during aggressive encounters (Nyby & Whitney, 1978; Portfors, 2007). These USVs may function as territorial signals between males mice (Gourbal, Barthelemy, Petit, & Gabrion, 2004; Hammerschmidt, Radyushkin, Ehrenreich, & Fischer, 2012). These findings lead us to hypothesize that alpha males most likely emit USVs on a regular basis in the vivarium and that subdominants process the auditory inputs from the environment to determine if the alpha male is present or absent.

Our analyses of immediate early gene activation indicated different co-activation patterns in the beta males undergoing social ascent from those in a stable social group. Most notably, those in the alpha removed group had overall increased, positively correlated activation throughout the regions studied, whereas those in the alpha remained group showed a more negative correlation throughout these regions. Specifically, we show through hierarchical clustering analysis that individuals in the alpha

removed group have specific regional clusters of co-activation. The first cluster contains several brain regions (IL, PL, cingulate and retrosplenial cortices, CA1, CA3 and dentate gyrus) that are recognized as being critically important for the retrieval of social, emotional and spatial memories as well as the monitoring of contextual information and prediction of future events (Bicks, Koike, Akbarian, & Morishita, 2015; Eichenbaum, 2017; Tovote, Fadok, & Luthi, 2015). It is conceivable that this cluster's co-activation occurs following the removal of the alpha male from the social group when beta males recognize the changes in social and spatial contexts occurring within their environment. We propose that the coordinated patterns of activation identified in the second cluster may be related to the output of behavior. This cluster includes hypothalamic and midbrain regions of the SBN including the AH and mPOA. The increased frequency of aggressive behavior as well as increased activity such as patrolling behavior may be associated with the increased activation throughout these areas. Clearly, successful social ascent requires the integration of activation in those regions in cluster 1 that are associated with social cognition and those nodes of the SBN in cluster 2 that promote behavioral output. Although we do not know where this integration occurs, it is notable that two nodes of the SBN, the BNST and LS, both showed increased levels of Fos immunoreactivity in socially ascending males and clustered with cluster 1. These more anterior brain regions along with the MeA (which also clustered with cluster 1 but did not show a significant difference between groups) are known to be key relays between hypothalamic and midbrain regions of the SBN and other brain regions that comprise a social-decision making network including the hippocampus and frontal cortex (O'Connell & Hoffman, 2011). Taken together with our data, we hypothesize that the BNST and LS integrate intrinsic and environmental cues in response to the removal of the alpha male from the social group to produce the contextually appropriate behavioral responses of increased aggression in beta males. Future research will mechanistically address the biological significance of each of these regions in facilitating social ascent of beta males during social opportunities.

## **CONCLUSION**

In the present study, we established that following removal of the alpha male from a stable dominance hierarchy, the beta male recognizes the absence of the alpha and responds by rapidly increasing aggressive behaviors. We have demonstrated that this salient social stimulus of removing the alpha male and subsequent change in behavior by the beta male leads to increased Fos immunoreactivity throughout the brain, specifically in regions of the SBN, as well as the medial prefrontal cortex, retrosplenial cortex and area CA1 of the hippocampus. These findings suggest that the complex social competence required to assess one's social context and respond appropriately is modulated by a synchronous and integrated increase in activity throughout the brain.

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## **DISCLOSURE OF INTEREST**

The authors report no conflicts of interest.

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## SUPPLEMENTAL MATERIAL

### Supplemental Table S7.1. Mouse Social Behavior Ethogram:

During observations, observers code all agonistic interactions occurring between any two individuals. As multiple behaviors may occur during the same interaction, observers record the behaviors with the highest priority. For instance, if one animal fought another animal who responded by fleeing, this would be recorded as a 'Fighting' event only, as 'Fighting' takes priority to the co-occurring 'Induced-Flee'. If an animal fled when approached but was not attacked by another animal, then this would be recorded as 'Induced-Flee'. Similarly, if an animal displayed subordinate posture following a chase, this would be recorded as 'Chasing', because chasing takes priority over 'Subordinate Posture'. Subordinate posture and Induced-Flee are only recorded if they occur in the absence of fighting, chasing, or mounting. These two subordinate behaviors do not co-occur so are given equal priority.

Priority	Behavior	Description
1	Fighting	The focal individual lunges at and/or bites the target individual.
2	Chasing	The focal individual follows the target individual rapidly and aggressively while the other individual attempts to flee.
3	Mounting	The focal individual mounts another individual from behind.
4=	Subordinate posture	The focal individual responds to the approach from another individual by remaining motionless and/or exposing their nape.
4=	Induced-Flee	The focal individual flees without any aggression shown by another individual.

**Supplemental Figure S7.1.** Housing Vivarium.





## **CHAPTER 8 – Hypofunction of the infralimbic and prelimbic prefrontal cortex disrupts social opportunity behavior**

Cait M. Williamson & James P. Curley

## **ABSTRACT**

Successful processing of social context information is essential to success in a dominance hierarchy. In the social opportunity paradigm, the alpha male is removed from a social group, leading to a salient change in social context. We have previously shown in chapters 7 and 8 that upon removal of the alpha male, the beta male ascends to dominance status, and this ascent is accompanied by increased immediate early gene expression throughout the brain, most notably in the infralimbic (IL) and prelimbic (PrL) regions of the medial prefrontal cortex. In the current study, we selectively and reversibly induce hypofunction of the IL/PrL using the DREADD system to determine if activity in these regions is required for proper response to a change in social context and subsequent rise to dominant status. We show that hypofunction of the mPFC causes mice to lose significantly more fights during a social opportunity, and they are unable to successfully rise to dominance status. These findings suggest the crucial role of the mPFC in regulating social competence and appropriate response to changing social contexts.

## INTRODUCTION

Social context is an important modulator of both behavior and physiology in group-living animals across species (Bugnyar & Heinrich, 2006; Desjardins, Hofmann, & Fernald, 2012; Emery & Clayton, 2001; Galeotti, Saino, Sacchi, & Møller, 1997; Schuett & Dall, 2009; Visalberghi & Fragaszy, 1995; Williamson, Lee, Romeo, & Curley, 2017). The ability of individuals to respond appropriately to changing social contexts is fundamental to their survival. One way in which researchers have studied how animals respond to a change in social context is through the social opportunity paradigm (Maruska, Zhang, Neboori, & Fernald, 2013; Williamson, Klein, Lee, & Curley, 2018; Williamson, Romeo, & Curley, 2017), where the alpha male in a dominance hierarchy is removed from the group, leading to a power vacuum at the top of the hierarchy. This paradigm allows us to examine the behavioral and physiological consequences associated with the recognition of a change in the social environment and the subsequent behavioral response to that change (Williamson et al., 2018; Williamson, Romeo, et al., 2017). This ascent to dominant status has been found to be associated with changes along the HPG axis (Maruska & Fernald, 2010; Williamson, Romeo, et al., 2017), as well as with immediate early gene response throughout the Social Behavior Network in the brain (Maruska et al., 2013; Williamson et al., 2018). Further, we have found that the infralimbic (IL) and prelimbic (PrL) regions of the medial prefrontal cortex (mPFC) also exhibit a large immediate early gene in response to social opportunity, and we suggest that the IL and PrL are tracking the changes in the social environment (i.e. the removal of the alpha male), leading to further social decision making and behavioral output associated with social ascent (Williamson et al., 2018).

The medial prefrontal cortex has been established as a brain region essential to the processing of rodent social behavior (Ko, 2017; Wang, Kessels, & Hu, 2014) and has further been implicated in the processing of social-spatial information (Murugan et al., 2017). In humans, activation of the mPFC has been shown

to occur during the processing of information about how oneself fits into the broader social context (Pfeifer et al., 2009) as well as while processing unstable social hierarchies (Zink et al., 2008). Taken together, this evidence suggests a role of the medial prefrontal cortex in processing social information, however, it has yet to be shown that the medial prefrontal cortex is necessary for the processing of social context information and thus for the ability of individuals to respond properly to changing social context. One way to prove that the infralimbic/prelimbic regions of the medial prefrontal cortex is necessary for proper processing of a change in social context and subsequent behavioral response is to deactivate these regions during a period of social opportunity.

The Designer Receptor Exclusively Activated by a Designer Drug (DREADD) system is a technology that has been developed to test causal relationships between brain region activity and behavior in freely moving animals, without requiring them to be connected to wires or requiring bulky cannulas to be inserted into the brain (Roth, 2016). To use this system to study the effect of hypofunction of a specific brain region on behavior, a modified muscarinic receptor, human muscarinic acetylcholine receptor 4 "DREADD" (hM4D), the sequence for which has been inserted into the multiple cloning site of the pAAV5-CaMKIIa-mCherry vector, is injected specifically into the brain region of interest. This expression is selectively contained to the brain region of interest through viral-mediated gene transfer. In this virus, both the hM4D and mCherry expression are under the control of the CaMKIIa promoter, meaning that the receptor will be expressed in excitatory cells containing this promoter sequence. The mCherry is included for visualization of expression at the end of the experiment. This specialized hM4D receptor is exclusively activated by a pharmacologically inert chemical compound, clozapine-N-oxide (CNO). Upon CNO activation, hM4D hyperpolarizes neurons through a G-protein mediated activation of potassium channels (Armbruster, Li, Pausch, Herlitze, & Roth, 2007).

In the present study, we aimed to examine the effect of hypoactivity of the IL/PrL region of the prefrontal cortex on social opportunity behavior in mice. We have previously shown that removal of the alpha male leads to a rapid increase in aggression and ascent to dominant status by the beta male (Williamson et al., 2018; Williamson, Romeo, et al., 2017). We used the DREADD system to decrease neuronal activity in the IL/PrL in beta males during a social opportunity, where the alpha male was removed from the hierarchy as well as during a stable social period, where the alpha male remained in the group in order to examine the behavioral consequences of deactivating the IL/PrL. Based on our previous work implicating this region of the prefrontal cortex in social opportunity behavior, we expect that IL/PrL hypofunction will impair an individual's ability to ascend a social hierarchy.

## **Methods**

### ***Subjects and Drugs***

Eighteen male CD1 mice were obtained from Charles River Laboratories at 6 weeks of age. Mice were housed in the animal facility in the Department of Psychology at Columbia University, with constant temperature (21–24°C) and humidity (30-50%) and a 12/12 light/dark cycle with white light (light cycle) on at 2400 hours and red light (dark cycle) on at 1200 hours. All behavioral observations occurred during the red light cycle. Clozapine-N-oxide (CNO) was dissolved in sterile 0.9% saline to a final concentration of .2 mg/mL. All procedures were conducted with approval from the Columbia University Institutional Animal Care and Use Committee (IACUC – Protocol No: AC-AAAR6456).

### ***Virus and Surgical Procedures***

The pAAV5-CaMKIIa-hM4D(Gi)- mCherry adeno-associated virus was obtained from Addgene (Catalogue # 50477-AAV5, Cambridge, MA). In this virus, hM4D and mCherry are under the control of the CaMKIIa promoter, so both hM4d and mCherry are only expressed in excitatory cells expressing this promoter. At 7 weeks of age, after one week of adjustment to the animal facility, 16 mice were pressure injected with

0.44  $\mu$ L of the virus using a glass pipette (15-20  $\mu$ M thick). Injections were made bilaterally into the infralimbic/prelimbic region of the medial prefrontal cortex (coordinates: A/P: 2.0, M/L:  $\pm$  0.4, D/V: -2.3). Virus was slowly injected over a three-minute period, and the pipette remained in place for 4 minutes after injection was complete to minimize leaking. The pipette was then brought back up slowly over a two-minute period to avoid displacing the virus. Mice were given 4 weeks to recover and for the receptors to be expressed. After 4 weeks had passed, behavioral testing began.

### ***Social Interaction in Pairs***

We tested whether hypoactivity of the IL/PrL regions of the PFC affected behavior in pairs. Three individuals were injected intraperitoneally (i.p.) with 2 mg/kg CNO 30 minutes prior to behavioral testing and were paired with novel social partners who had been injected i.p. with saline 30 minutes prior to behavioral testing. Live observations were conducted on each pair, with observers recording all instances of aggressive and subordinate behavior (See **Supplemental Table S7.1** for ethogram of behaviors coded). One hour after pairing, mice were anesthetized with ketamine/xylazine and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were stored at 4°C in 4% paraformaldehyde for the first six hours following perfusion and then switched to a 30% sucrose in PBS solution.

### ***Social Opportunity Behavioral Paradigm***

We used a modified version of the social opportunity paradigm previously used by our lab (Williamson, Lee, Romeo, & Curley, 2017) to investigate the behavioral consequences of selective and temporary hypofunction of the infralimbic/prelimbic region of the prefrontal cortex. After a 4-week recovery period, the 16 mice who had been bilaterally injected with the virus were placed into two vivaria (8 mice per group). The vivaria (length 150cm, height 80cm, width, 80cm; Mid-Atlantic; **Supplemental Figure S7.1**) were constructed as previously described (Williamson, Lee, & Curley, 2016), with an upper level consisting

of multiple shelves connected by tubes and a lower level consisting of five inter-connected nest-boxes. Pine bedding covered the shelves and filled the nest-boxes. Standard chow and water were provided ad libitum. Mice were uniquely marked with non-toxic animal marker (Stoelting Co.) on their fur so they could be identified throughout the study. These marks remain for up to 12 weeks and did not have to be reapplied. Live behavioral observations occurred each day for 3 hours during the dark (red light) cycle. During these observations, trained observers recorded all instances of aggressive behavior (i.e. fighting, chasing, mounting, instances of subordinate behavior and instances of induced flee; see **Supplemental Table S7.1** for an ethogram of recorded behaviors). After 4 days of observations, both social groups were confirmed to have developed significantly linear dominance hierarchies, as verified through Modified Landau's  $h'$  values, directional consistency, and Glicko scores, all measures we have used previously to measure dominance (Williamson, Franks, & Curley, 2016; Williamson, Lee, et al., 2016, 2017; Williamson, Romeo, et al., 2017). Once the groups had been verified to have formed dominance hierarchies, behavioral manipulations began. There were 4 experimental conditions: Condition 1: beta males injected with CNO whose alpha male was removed, Condition 2: beta males injected with saline whose alpha male was removed, Condition 3: beta males injected with CNO whose alpha male remained, and Condition 4: beta males injected with saline whose alpha male remained. At the end of the study, each experimental condition had been tested on 7-8 individuals, with some individuals being tested in all four conditions and some not, as the chosen experimental animals were dependent on who the beta males were the day before the manipulation was to occur. See **Table 8.1** for a timeline of the behavioral paradigm. On the manipulation days (Days 5, 8, 11, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, and 50), 30 minutes before onset of the dark cycle, the beta male from one cohort was injected i.p. with CNO (2mg/kg) and while the beta male from the other cohort was injected i.p. with sterile saline. On alpha removed days, at the onset of the dark cycle, the alpha male was removed from both groups, and observations began as usual. After a 2-hour observation period, the alpha male was placed back into the group, and one further hour of

observations was conducted. On alpha remained days, a sham-removal was conducted, where an experimenter placed her hand into the vivarium and disturbed the group but did not remove the alpha male, and observations proceeded as usual. After each condition was completed once in each group, the alpha male was permanently removed from the group in order to allow a new beta male to ascend the hierarchy and serve as our subject mouse. This was repeated again after each condition had been repeated once for each new alpha male for each group, resulting in permanent alpha male removals occurring three times, on days 12, 28, and 39. One note about the first permanent alpha removal, in one social group the alpha male was extremely aggressive and had to be removed earlier than planned, resulting in each group only having been tested in 3 conditions prior to that permanent removal. After the extremely aggressive mouse had been removed, we allowed five days for the group to reset before continuing with the experiment.

At the end of the experiment, mice were anesthetized with ketamine/xylazine and perfused intracardially with phosphate-buffered saline (PBS) followed by 4% paraformaldehyde. Brains were stored at 4°C in 4% paraformaldehyde for the first six hours following perfusion and then switched to a 30% sucrose solution in PBS. Brains were sliced and mounted for later visualization of transgene expression.



**Table 8.1: Experimental Timeline**

DAY	GROUP 1	GROUP 2	NOTES
1	normal observations	normal observations	
2	normal observations	normal observations	
3	normal observations	normal observations	
4	normal observations	normal observations	
5	<b>alpha remove, CNO</b>	<b>alpha remove, saline</b>	
6	normal observations	normal observations	
7	normal observations	normal observations	
8	<b>alpha remove, saline</b>	<b>alpha remove, CNO</b>	
9	normal observations	normal observations	
10	normal observations	normal observations	
11	<b>alpha remain, CNO</b>	<b>alpha remain, saline</b>	
12	normal observations	normal observations	<b>alphas removed permanently</b>
13	normal observations	normal observations	
14	normal observations	normal observations	
15	normal observations	normal observations	
16	normal observations	normal observations	
17	<b>alpha remove, saline</b>	<b>alpha remove, CNO</b>	
18	normal observations	normal observations	
19	normal observations	normal observations	
20	<b>alpha remove, CNO</b>	<b>alpha remove, saline</b>	
21	normal observations	normal observations	
22	normal observations	normal observations	
23	<b>alpha remain, CNO</b>	<b>alpha remain, saline</b>	
24	normal observations	normal observations	
25	normal observations	normal observations	
26	<b>alpha remain, saline</b>	<b>alpha remain, CNO</b>	
27	normal observations	normal observations	<b>alphas removed permanently</b>
28	normal observations	normal observations	
29	<b>alpha remain, saline</b>	<b>alpha remain, CNO</b>	
30	normal observations	normal observations	
31	normal observations	normal observations	
32	<b>alpha remain, CNO</b>	<b>alpha remain, saline</b>	
33	normal observations	normal observations	
34	normal observations	normal observations	
35	<b>alpha remove, saline</b>	<b>alpha remove, CNO</b>	
36	normal observations	normal observations	
37	normal observations	normal observations	
38	<b>alpha remove, CNO</b>	<b>alpha remove, saline</b>	
39	normal observations	normal observations	<b>alphas removed permanently</b>
40	normal observations	normal observations	
41	<b>alpha remain, CNO</b>	<b>alpha remain, saline</b>	
42	normal observations	normal observations	
43	normal observations	normal observations	
44	<b>alpha remain, saline</b>	<b>alpha remove, CNO</b>	
45	normal observations	normal observations	
46	normal observations	normal observations	
47	<b>alpha remove, CNO</b>	<b>alpha remove, saline</b>	
48	normal observations	normal observations	
49	normal observations	normal observations	
50	<b>alpha remove, saline</b>	<b>alpha remove, CNO</b>	

## ***Histology***

Brains were sliced into 40  $\mu\text{m}$  thick coronal sections on a cryostat and stored in PB azide until mounting. Slices were mounted on FisherBrand Plus slides using Krystalon mounting media. mCherry fluorescence was visualized with a fluorescent microscope to assess correct expression within the IL/PRL regions of the mPFC.

## ***Statistical Analysis***

*Social interaction in pairs:* To determine the effect of CNO treatment on number of wins in pairs, we ran a Wilcoxon rank sum test comparing wins in saline injected mice to wins in CNO injected mice.

*Difference in wins and losses the day of alpha removal/sham-removal:* To assess the difference in number of wins and number of losses between experimental groups, we ran negative binomial mixed effect models with wins or losses as the outcome variable, group as a fixed effect, and cohort and number of mice remaining in the group as random effects.

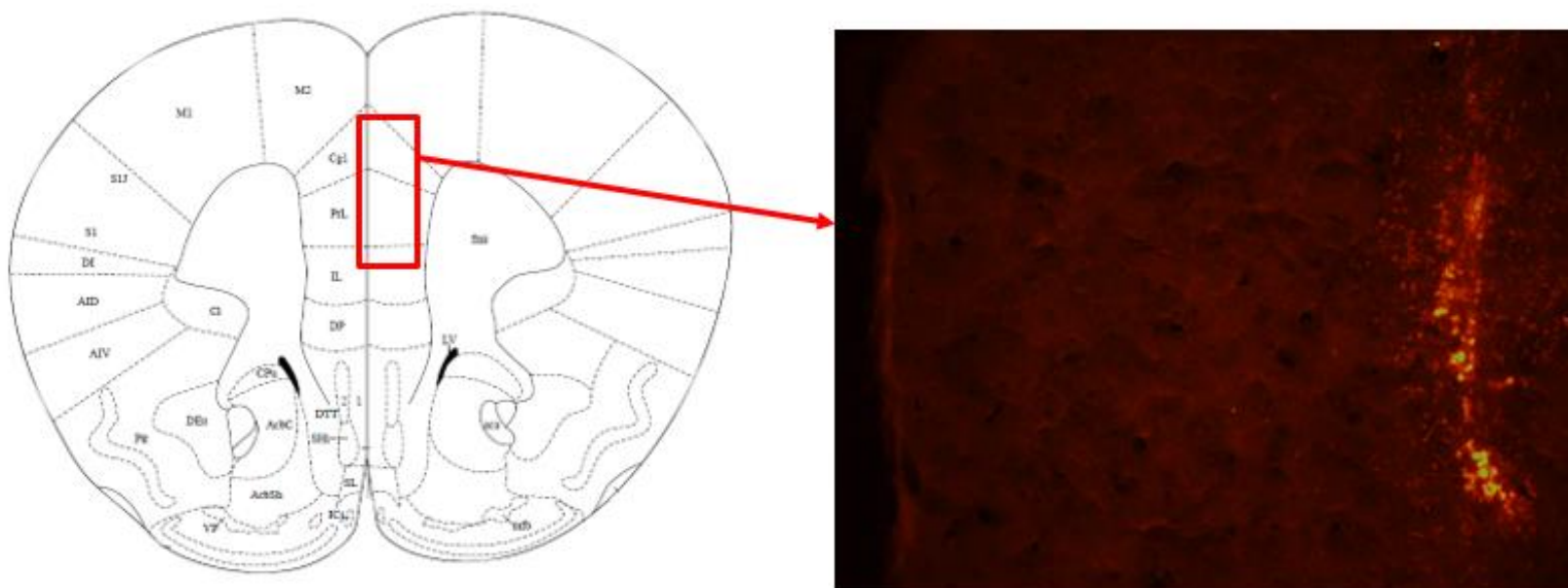
*Change in behavior from the day before alpha removal or sham-removal to the day of:* To assess the difference in proportion of wins and losses within groups between the day before removal/sham-removal and the day of removal/sham-removal, we ran Wilcoxon signed rank tests for each group for wins and losses.

## RESULTS

### *Histology*

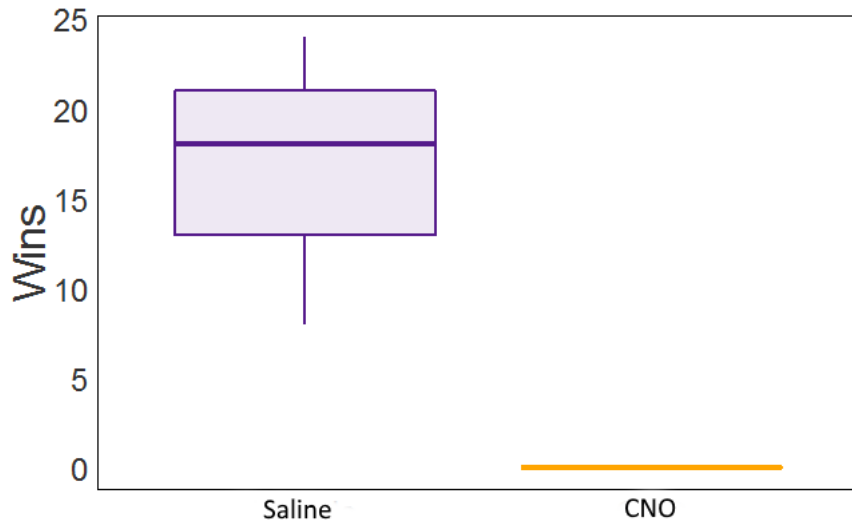
We stereotactically injected an adeno-associated virus enabling the co-expression of hM4D with mCherry (AAV5-hM4D). For all mice, we analyzed the pattern of transgene expression after behavioral testing by visualizing mCherry expression (**Figure 8.1**). The virus spread was contained to the prefrontal cortex, although some expression extended above the IL/PrL into the cingulate cortex. In one mouse, expression extended below the IL region down to the dorsal peduncular area. This mouse was never tested in the CNO condition, so was kept in the behavioral analyses. If anything, virus expression was too contained, not extending medially/laterally the width of the IL/PrL.

Figure 8.1 – Representative image of transgene expression in the medial prefrontal cortex. Expression was contained to the mPFC.



### ***Effect of CNO treatment on behavior in pairs***

In each pair, none of the individuals who were treated with CNO had a single win (**Figure 8.2**). Due to the low sample size (N = 3/group), this effect was not statistically significant (Wilcoxon rank sum test: W = 9, p = 0.064).



**Figure 8.2: Number of wins in saline-injected and CNO injected mice in pairs.** Mice given CNO won zero fights over the 30-minute social interaction period.

### ***Effect of alpha removal/sham-removal and CNO/saline treatment on number of wins***

The saline, alpha removed group won significantly more than the saline, alpha remained group (**Figure 8.3A**:  $\beta = 2.118 \pm 0.932$ ,  $p < 0.05$ ). The CNO, alpha removed group had significantly more wins than the saline, alpha remained group (**Figure 8.3A**:  $\beta = 2.19 \pm 0.932$ ,  $p < 0.05$ ). There was no significant difference in number of wins between the CNO, alpha removed group and the CNO, alpha remained group (**Figure 8.3A**:  $\beta = -0.511 \pm 0.856$ ,  $p = 0.550$ ).

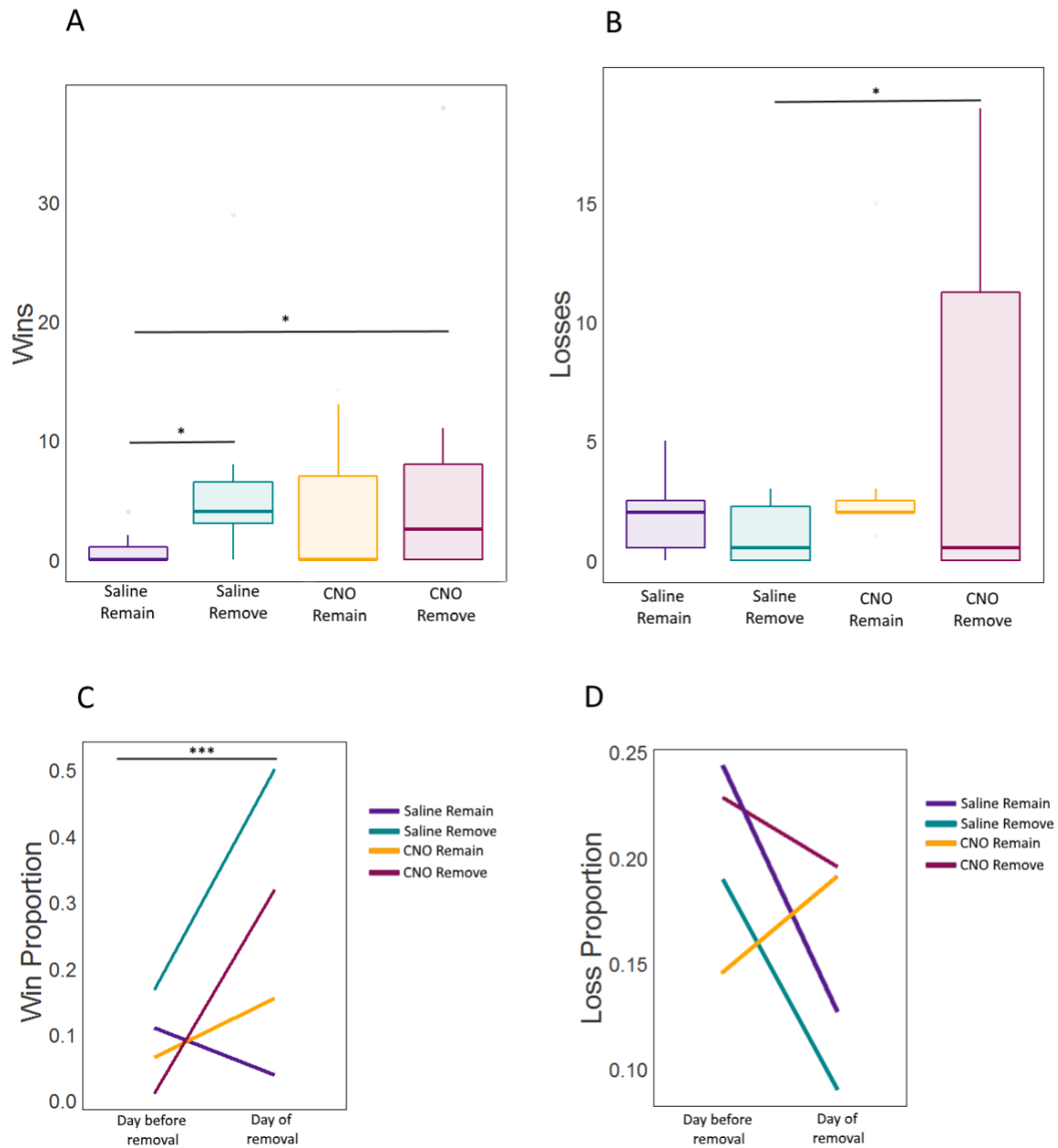
### ***Effect of alpha removal/sham-removal and CNO treatment on number of losses***

There was no significant difference in number of losses between the saline, alpha remained group and the saline, alpha removed group (**Figure 8.3B**:  $\beta = -0.501 \pm 0.741$ ,  $p = 0.499$ ). The CNO, alpha removed group lost significantly more than the saline, alpha removed group (**Figure 8.3B**:  $\beta = 1.564 \pm 0.687$ ,  $p < 0.05$ ). Further, there was a trend towards the CNO, alpha removed group losing more than the saline, alpha remained group (**Figure 8.3B**:  $\beta = 1.063 \pm 0.679$ ,  $p = 0.118$ ). In the CNO, alpha remained group, there was a slight trend towards losing more than both the saline, alpha remained group (**Figure 8.3B**:  $\beta = 0.731 \pm 0.707$ ,  $p = 0.301$ ) as well as the saline, alpha removed group (**Figure 8.3B**:  $\beta = 1.232 \pm 0.714$ ,  $p = 0.084$ ).

### ***Comparing the day before removal to the day after removal***

In the saline condition, there was no significant difference in proportion of wins or losses in the alpha remained group (wins: **Figure 8.3C**: Wilcoxon signed rank test:  $V = 5$ ,  $p = 0.423$ ; losses: **Figure 8.3D**:  $V = 15$ ,  $p = 0.402$ ). There was a significant difference between proportion of wins the day before and proportion of wins the day after in the alpha removed group (**Figure 8.3C**: Wilcoxon signed rank test:  $V = 0$ ,  $p < 0.05$ ), but there was no significant difference in proportion of losses (**Figure 8.3D**: Wilcoxon signed rank test:  $V = 22$ ,  $p = 0.205$ ).

In the CNO condition, there was no significant difference in proportion of wins or losses in the alpha remained group (wins: **Figure 8.3C**: Wilcoxon signed rank test:  $V = 3$ ,  $p = 0.584$ ; losses: **Figure 8.3D**: Wilcoxon signed rank test:  $V = 15$ ,  $p = 0.938$ ) or in the alpha removed group: (wins: **Figure 8.3C**:  $V = 0$ ,  $p = 0.059$ ; losses: **Figure 8.3D**:  $V = 15$ ,  $p = 0.402$ ).



**Figure 8.3. Behavioral response to induced mPFC hypofunction and alpha removal or sham-removal.** (A) Wins on the day of alpha removal or sham-removal: Beta males in the alpha removed group given saline win significantly more than those given saline in the alpha remained group. Beta males in the alpha removed group given CNO also win significantly more than beta males in the alpha remained group given saline. (B) Losses on the day of alpha removal or sham-removal. Beta males given CNO in the alpha-removed group lose significantly more fights than beta males given saline in the alpha removed group. (C) Change in proportion of wins from day before alpha removal or sham-removal to the day of removal or sham-removal. Beta males in the alpha removed group giving saline win proportionally more the day of alpha removal as compared to the day before alpha removal. (D) Change in proportion of losses from day before alpha removal or sham-removal to day of removal or sham-removal. There were no significant differences in number of losses the day before as compared to the day of alpha removal or sham-removal in any of the groups.

## DISCUSSION

We successfully replicated findings from previous studies showing that upon alpha removal, the subdominant male ascends to dominant status through increased aggression (Williamson et al., 2018; Williamson, Romeo, et al., 2017). The saline, alpha removed group won significantly more than the saline, alpha remained group, and won proportionally more fights the day of alpha removal as compared to the day before alpha removal. However, this increase in aggression was not as large as we have shown previously. In previous studies, ascending beta males won between 20-50 fights, where on average here, ascending beta males only won 5-10 fights. This could be due to the smaller group size (a maximum of 7 here, versus a maximum of 11 in those studies), as in those previous studies we showed that as the group reduced in size, the amount of aggression decreased (Williamson, Romeo, et al., 2017). Another factor affecting this behavior could be the age of the mice when they went into the vivarium, as typically they are placed together around 9 weeks of age. Here, we had to perform the surgeries and wait 4 weeks post-surgery to begin behavioral testing, so individuals were closer to 12 weeks of age when placed together. Finally, removing the animals to administer the CNO/saline could have affected their behavior. We have previously removed animals from the vivarium during their housing period, to collect urine (Lee, Khan, & Curley, 2017) or conduct vaginal smears (see **chapter 4**). However, these disturbances to the system occurred at the end of behavioral observations for the day, rather than 30 minutes before. Our removal and injection 30 minutes prior to removal of the alpha male could have disrupted their typical behavior. Individuals in the saline, alpha remained group behaved as expected, winning the same number of fights the day before sham-removal as the day of sham-removal and winning significantly fewer fights than mice in the saline, alpha removed group.

In the CNO, alpha removed group, beta males who had been given CNO to induce hypofunction of the IL/PrL region won around the same number of fights as ascending beta males in the saline, alpha removed



group. This demonstrates that despite the induced hypofunction of the mPFC, they were still able to win some fights. However, there was no significant difference in the proportion of fights won the day of alpha removal as compared to the day before alpha removal, suggesting that this mPFC hypofunction dampened their ability to fully socially ascend. Further, they lost significantly more fights than the mice in the saline, alpha removed condition. Given that the alpha male, the only male they had consistently lost to in the days leading up to the alpha male removal, was gone, this means that these individuals were losing to mice of lower rank than them to whom they had not previously been losing fights. This suggests an impaired ability to process their own rank in relation to that of others, an ability that is crucial to success in a dominance hierarchy (Chase, 1982; Curley, 2016; Williamson, Lee, et al., 2016). Typically, we see extremely high levels of directional consistency, with the vast majority of interactions occurring in the direction of the more dominant individual to the less dominant individual (Williamson, Lee, et al., 2016). As we have discussed previously, this demonstrates that all mice within the social group display a high degree of social competence, consistently behaving in a contextually appropriate manner, acting subordinate to individuals of higher rank and behaving in a dominant manner to those of lower rank. That the mice with IL/PrL hypofunction were not displaying the same degree of social competence as is typically demonstrated in our social hierarchies suggests that this region is crucial to proper processing of one's rank in relation to the ranks of those around them. Indeed, the medial prefrontal cortex has been previously implicated in the processing of social status information in mice as well as humans (Silk et al., 2017; Wang et al., 2014; Zerubavel, Bearman, Weber, & Ochsner, 2015). Further, it has been demonstrated to be active when processing the social network position of others in a group in humans (Parkinson, Kleinbaum, & Wheatley, 2017), as well as when processing one's own position in a group (Pfeifer et al., 2009). Other work in rodents demonstrates that increasing the synaptic efficacy of mPFC neurons causes mice to rise in social rank, where decreasing synaptic efficacy has the opposite effect, causing mice to fall in social rank (Wang et al., 2011). This was found using the tube test as a measure of

dominance, so it is difficult to determine if the same mechanism (i.e. loss of social competence) is at play in these findings, but they lend further evidence for the critical role of the mPFC in regulating social status.

The story is more complicated than merely that excitation of the mPFC leads to a rise in social rank and inhibition leads to a fall in social rank, as beta males in the alpha remained group who were injected with CNO did not lose significantly more fights than those given saline. There was a trend towards these individuals losing more than both the alpha remained and alpha removed groups who were given saline, but this was not statistically significant. We have shown previously that upon removal of the alpha male, even subordinate individuals in the group engage in greater amounts of aggression. This overall increased aggression can explain why the CNO, alpha removed individuals lost significantly more fights than the saline, alpha removed group, but that the CNO, alpha remained groups did not. It is not enough to just inhibit the mPFC -- there must be an opportunity for others to engage in aggression, thereby forcing the beta male into situations requiring high levels of social competence. When forced into these situations, our data here shows that if their mPFC is impaired, they will not always engage in the socially appropriate behavior. This study does have some limitations. It appears that our virus did not spread as far as could have been desired, and did not cover the entire PL/IrL region. It would therefore be important to conduct further studies where we inject more of the virus or work to improve the spread through the placement of the pipette during surgery. Additionally, changes to the experimental design could help us more clearly understand what the PL/IrL region is doing during these periods of social opportunity. For example, our protocol here involved leaving the mice in the vivarium for 7 weeks, where typically we house groups together for 3 weeks. This was in an effort to reduce the number of mice we used by conducting a within-subjects experiment. Given that we ended up not being able to completely conduct a within-subjects design, due to changes in the social hierarchy that were out of our control, it might work better to use our typical design and conduct a between-subjects experiment.

Here, we also show that in pairs, when one mouse in each pair is given saline and the other CNO, those given CNO lose 100% of fights and do not win a single fight. While this data is purely anecdotal, given the small sample size, it is notable that none of the mice with an impaired mPFC were able to win a single fight. It suggests that in smaller groups, even when there are not difficult social decisions to be made, mPFC hypofunction leads to a decreased ability to assert dominance. Given this data, in the future, we could benefit by first understanding these effects at a pair or small group level with a simpler experiment and then expanding to a study of the role of the mPFC in social opportunity phenomenon in large groups.

Given recent research demonstrating that CNO is not pharmacologically inert, as has been previously believed, and undergoes in vivo conversion of CNO to clozapine (Gomez et al., 2017; Manvich et al., 2018), which is a potent antipsychotic that can rapidly cross the blood-brain barrier, it is necessary to discuss how the presence of clozapine in the brains of the CNO-administered mice may have affect behavior in this paradigm. While doses of clozapine close to the amount of clozapine that would have been converted from CNO based on the 7.4% conversion ratio determined by Manvich and colleagues can exert behavioral effects such as reduced locomotion and increased anxiety (Ilg, Enkel, Bartsch, & Böhner, 2018), in rats, it has been shown not to affect social interaction behavior (Ilg et al., 2018). Despite this finding, In our experiment, one could argue that the significant increase in number of losses experienced by CNO-treated mice could be attributed to reduced locomotion caused by clozapine converted from CNO. Unfortunately, the only way to be certain if this result is due to the hypofunction of the mPFC or due to inhibited locomotion as a result of clozapine binding in the brain, is to carry out a control where mice not expressing the DREADD are treated with CNO. Based on these recent findings, it will be necessary to include this additional control group in all future DREADD studies.

## **CONCLUSION**

In this chapter, we show that hypofunction of the mPFC leads to decreased social competence, as is demonstrated through beta males losing fights to mice they had previously dominated and through an inability to effectively socially ascend to dominant status. This suggests a crucial role of the mPFC in regulating socially appropriate behaviors. Further work is necessary to clarify and extend these findings, but our result is consistent with other work implicating the mPFC in processing of one's own social status in relation to others' as well as the position of others' within a social network.

## **ACKNOWLEDGMENTS**

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## CHAPTER 9 – General Conclusion

Social context is a critical modulator of both behavior and physiology. Although an extensive literature exists on the neurobiology of social behavior, the vast majority of this work studies individuals in pairs, where there is no social context besides a single social partner. Research has examined group social dynamics in semi-natural environments in prairie voles (Ophir, Wolff, & Phelps, 2008), as well as in the field in desert rodents (Randall, Rogovin, Parker, & Eimes, 2005) and California mice (Ribble & Salvioni, 1990). However, due to the difficulties of studying large groups in the laboratory, there have been limited studies investigating the neurobiology of group social behavior in mammals in a controlled manner. Given that all social species live in groups, it is essential for us to investigate the role of social context – which includes both direct social experiences and the complex characteristics that comprise a group – in mediating the relationships between neurobiology, neuroendocrinology, and behavior. Throughout this thesis, I have demonstrated the broad role social context plays in regulating neurobiology and neuroendocrine response in individuals of varying social status.

To examine how social context contributes to differences in neural activity and neuroendocrine output, I first extensively analyzed the social dynamics of both male and female mouse dominance hierarchies. Armed with this foundation, I explored the neurobiological and neuroendocrine correlates of social status in stable social groups and found that social context is a critical modulator of these relationships. In an effort to further understand the behavioral and physiological changes associated with social context, I developed a novel behavioral paradigm to examine the behavioral and neural response to a social opportunity, finding that individuals are capable of responding rapidly to this change in social context, and this behavioral response is associated with immediate early gene activation throughout the brain. This work provided the basis for the final chapter of this dissertation, where I manipulated the brain to begin

to understand the crucial role of the medial prefrontal cortex in modulating the appropriate response to changing social contexts. In this conclusion, I highlight some of the key insights gained through this comprehensive study of the role of social context in modulating behavior and physiology.

**Male mice reliably form significantly linear dominance hierarchies, but each group possesses unique characteristics.** In **chapter 2**, I investigated the social dynamics of 10 male social groups. I found that they all form significantly linear hierarchies, but they all possess unique characteristics that distinguish them from one another. Notably, I showed that there exists a range of how unequally distributed power is within each social hierarchy, with some alpha males being highly despotic (i.e. winning over 50% of all the aggressive interactions within the group), and others being less despotic, winning less than 50% of all the aggressive interactions. Each individual within these hierarchies possesses a distinct social rank, and the directional consistency of all behaviors is highly significant, with the vast majority of all agonistic behaviors occurring in the direction of the more dominant to the more subordinate individuals. **This high degree of directional consistency shows that individuals behave consistently and appropriately according to their social rank, suggesting a high degree of social competence in each animal within the hierarchy.** It also appears that individuals can rapidly update this information, as in cases where the alpha male suddenly loses their dominance status, this high degree of directional consistency remains. While some studies have examined male mouse social hierarchies (Lewejohann et al., 2009; Poole & Morgan, 1975; Poshivalov, 1980), none have investigated male mouse social hierarchy dynamics to this extent. My findings, while interesting in their own right, are essential to the remainder of the work in this dissertation, as they lay the groundwork for us to understand both how the unique characteristics of each social group contribute to differences in neuroendocrine output and how the brain processes social context, enabling individuals to respond appropriately to changes in their social group.

**Unique characteristics of social groups are associated with differential neuroendocrine output.** In **chapter 3**, I investigated the relationships between social status and testosterone and corticosterone. As is discussed at great length throughout the chapter, there is disagreement over the nature of these relationships. Some studies suggest dominant individuals have higher levels of T, some suggest there is no difference in T between dominant and subordinate mice (see **Table 3.1**). In the corticosterone literature, there are discrepancies as well, with some studies showing higher levels of corticosterone in subordinates, some showing higher levels in dominants, and some showing no relationship between corticosterone levels and social status (see **Table 3.2**). In pairs, I found that there is no significant difference in testosterone levels between dominant and subordinate individuals and that dominant individuals actually have significantly higher levels of corticosterone. When looking across 20 separate, stable social hierarchies, I found no relationships between either testosterone or corticosterone and social status. **However, taking into account the social context in which individuals were living, significant relationships emerged.** Specifically, in groups where there was a highly despotic dominant male, dominant individuals did display significantly higher levels of testosterone than sub-dominant individuals and subordinate individuals. Corticosterone levels were also related to despotism – with subordinate individuals in highly despotic groups displaying significantly higher levels of corticosterone than dominant individuals. In these highly despotic groups, subordinate individuals were also behaviorally suppressed, engaging in significantly lower levels of aggression than those in less despotic groups. These findings can help explain the inconsistencies in the testosterone and corticosterone and social status literature, as in each study, there were considerably different social contexts (See **Table 3.1 and Table 3.2**). Some studies used large (>8 mice) groups (i.e. Ely, 1981; Hilakivi et al., 1989; Selmanoff, Goldman, & Ginsburg, 1977), with others housing mice in smaller groups (i.e. Machida, Yonezawa, & Noumura, 1981), and some included females (i.e. Ely, 1981; Selmanoff et al., 1977). My findings here indicate that the social context

of groups housed in exactly the same manner for the same period of time differs and these differences affect neuroendocrine output, so it is not surprising that studies utilizing vastly different methods came to contradictory conclusions. My findings are in line with the challenge hypothesis, which suggests that differences in testosterone between individuals of different social ranks fluctuate based on the group dynamics. Specifically, this hypothesis suggests that during times when dominant individuals must engage in higher levels of competition in order to maintain their dominance status, they will display higher levels of testosterone than subordinate individuals in the group (Wingfield, Hegner, Dufty, & Ball, 1990), but during times of relative stability, where they do not need to constantly assert dominance, this difference in testosterone levels will not exist. Evidence supporting this hypothesis has been found in birds (Wingfield et al., 1990), cichlid fish (Oliveira, Almada, & Canario, 1996), and non-human primates (Sapolsky, 1982). My findings extend this hypothesis, by suggesting that in mice, even in stable social groups, the despotism of the alpha male is a critical modulator of both the behavior and neuroendocrine output of individuals within the group. The work in this chapter determines that relationships between hormones, behavior, and social status are incredibly complicated and require a complex analysis of the behavior and social context in order to understand the intricate mechanisms regulating them.

**Female mice form linear social hierarchies, but their structures are significantly different from those of males.** In **chapter 4** I examined female social hierarchy dynamics in an effort to understand how female social groups differ from those of males and to begin to explore the associations between female social status and neurobiology and neuroendocrinology. While studies have shown that females do form dominant-subordinate relationships (Kaufmann, 1983; Rowell, 1972; Schuhr, 1987), and one recent study determined that females are capable of asserting dominance status but do not form dominance hierarchies (Weidt, Gyax, Palme, Touma, & König, 2018), no work has extensively examined female group dynamics or compared them to those of males. Females have distinct evolutionary mechanisms from

those of males that drive intra-female conflict, as they compete primarily for resources to increase chances of survival for themselves and their offspring, where males compete primarily for mates (Stockley & Bro-Jørgensen, 2011). Given these evolutionary differences, it is likely that female dynamics are distinct from those of males. Indeed, I found that while female social groups can be considered to be significantly linear in their structure, they are distinct in their characteristics, displaying significantly lower despotism than those of males, as well as significantly lower directional consistency and triangle transitivity. In addition to these behavioral differences, in contrast to the findings in chapter 3 in males, subordinate females also have significantly higher levels of plasma corticosterone in relation to that of despotic females, regardless of the level of despotism of the most dominant female. This is particularly interesting, as the human depression and anxiety literature shows that females are more susceptible than males to these disorders (Breslau, Davis, Andreski, Peterson, & Schultz, 1997; Piccinelli & Simon, 1997; Shively, Laber-Laird, & Anton, 1997; Szádóczy, Ríhmer, Papp, & Füredi, 1997). My finding here suggests that this differential susceptibility between males and females may be due to increased HPA sensitivity to social stress in females.

I further determined that subordinate females express higher levels of estrogen-mediated gene expression, namely ER $\beta$ , PR, and OPRM1 in the ventromedial hypothalamus. This finding is consistent with work showing the importance of these genes in regulating aggression (Fraile, McEwen, & Pfaff, 1987; Nomura et al., 2002; Ogawa, Taylor, Lubahn, Korach, & Pfaff, 1996), affiliative behaviors (Patisaul, Scordalakes, Young, & Rissman, 2003; Samaco et al., 2012; Wöhr, Moles, Schwarting, & D'Amato, 2011), and social recognition (Bychowski & Auger, 2012). These findings support the importance of estrogen and estrogen-mediated gene expression in regulating social behavior in females, but much work remains to be done to study female social dominance behavior and its neuroendocrine and neurobiological correlates more extensively.

**Large social groups form distinct network communities, and these network communities display unique relationships between social status and plasticity-related gene expression.** In **chapter 5**, I study the social dynamics of a large group of 30 mice from a social network perspective. The findings from this chapter further our understanding of the behavioral dynamics of large social groups, in addition to demonstrating how social context can change both behavior and gene expression in the brain. I find that even a large group of 30 individuals forms a significantly linear dominance hierarchy. I also show that in groups of this size, individuals split into distinct network communities. These communities are important to understand, as community membership was found to be indicative of brain gene expression differences. Specifically, in Community B there was a significant relationship between DNMT3a gene expression and social status, with DNMT3a mRNA levels being negatively correlated with social status. DNMT3a is a DNA methyltransferase that modulates *de novo* DNA methylation and as such is an essential mechanism by which environmental experiences can affect gene expression. Increased levels of DNMT3a indicate increased levels of DNA methylation, which are associated with inhibition of gene expression. Specifically, DNMT3a has been implicated in regulating learning about socioemotional behavior, including social defeat (Hammels et al., 2015; Yu, Baek, & Kaang, 2011). Further, work in honeybees shows that inhibiting DNMT3a in larvae leads to their development as queen bees (Evans & Wheeler, 1999; Kucharski, Maleszka, Foret, & Maleszka, 2008), providing additional evidence for the role of DNMT3a in regulating social status. This relationship between social status and DNMT3a expression did not exist in Community A or in the network as a whole. **This provides further evidence that the social context, in this case the network community of which individuals were a part, is an important variable to consider when studying the relationships between social behavior and neurobiology and neuroendocrine output.** Here, Community B was the smaller of the two communities, so there were different social dynamics at play

which possibly played a role in regulating this relationship between DNMT3a gene expression and social status.

Notably, I also find that commonly used tests of behavior (the open field test, novel object test, social interaction test, and approach-avoidance test) are largely not indicative of behavior in a group setting. I found that higher levels of exploration in the open field predict higher levels of aggression in the first few days, but this was not related to ultimate dominance status. **Given this, it is clear that social context is a modulator of behavior, as neither individual behavior nor behavior in pairs could predict an individual's behavior and ultimate network position in a large social group.** This should be a consideration for any researchers studying social behavior, especially those working to develop therapeutics for disorders of social behavior, as these studies often use these social behavior tests to determine the efficacy of their drugs, and certain therapies may be effective in one contextual setting but not another.

**Social opportunities lead to robust behavioral changes, as well as changes in HPG axis activity and increased immediate early gene expression throughout the brain.** In chapter 6 I explored how manipulating a group's social structure can lead to rapid behavioral and physiological changes. I first investigated the consequences of removing the alpha male from a social group on both beta and subordinate male behavior and HPG axis activity, showing that removal of the alpha led to a rapid and robust increase in aggression by the beta male. This increased aggression was associated with increased GnRH mRNA expression in the mPOA. This rapid response to the removal of the alpha male provides further evidence that individuals living in social hierarchies are highly socially competent, capable of recognizing the change in social context, in this case the opportunity to ascend the hierarchy, and rapidly responding. Notably, subordinate males also responded to this change in social context, displaying

increased GnRH mRNA expression and significantly more of the subordinate males in the alpha-removed condition engaging in aggressive behavior than those in the sham-removal condition. This demonstrates that the change in social context was extremely salient, affecting all mice within the group, not just the individual ascending to dominant status. Further, this shows that even the most subordinate individuals in the group display social competence, responding appropriately to this change in social context. These findings – that HPG axis activity increased with social ascent -- are consistent with work in African cichlid fish, where subordinate individuals who transition to dominant status also display HPG axis activation (Maruska & Fernald, 2011; Maruska, Levavi-Sivan, Biran, & Fernald, 2011). **Both from my work and the work in cichlid fish demonstrate that individuals are capable of rapidly responding to a change in social context, both behaviorally and through gene expression changes in the brain.**

Interestingly, we found that while subordinate males displayed increased GnRH gene expression, they did not display a corresponding testosterone pulse. This led us to further explore how subordinate individuals respond to GnRH, questioning whether they have reduced sensitivity to GnRH, as has been shown in sugar gliders (Bradley & Stoddart, 1997) and naked mole rats (Holmes, Goldman, & Forger, 2008). We did not find this to be the case – subordinate males responded to GnRH with a testosterone pulse to the same extent as dominant males in pairs. However, there were differences in terms of the behavioral response, with dominant males responding to exogenous GnRH administration with significantly increased aggression, while subordinate males did not respond behaviorally to GnRH. **This finding is additional evidence in support of the central role of social context in regulating behavior and physiology. Here, the social status of the individual was a significant modulator of behavior, suppressing subordinate male aggression, even while they displayed the same physiological response to that of dominant males.**



In **chapter 7**, I used a modified version of the social opportunity paradigm to study how the brain responds to social opportunity. Here, I replicated the behavioral finding that subdominant males respond to the removal of the alpha male with a rapid and robust increase in aggression. This recognition of the change in social context and subsequent rise to dominant status is associated with increased immediate early gene expression throughout the brain, notably in regions of the social behavior network, as well as in the infralimbic/prelimbic region of the medial prefrontal cortex and hippocampus. While these regions have all been previously implicated in regulating social behavior (Burmeister, Jarvis, & Fernald, 2005; Goodson, 2005; Maruska, Zhang, Neboori, & Fernald, 2013; Noonan et al., 2014; Wang et al., 2011), **my analyses found that activation throughout the regions studied was positively correlated suggesting that a coordinated response throughout the brain is required to recognize and respond appropriately to a change in social context.**

**The medial prefrontal cortex is crucial to socially competent behaviors and proper response to changing social contexts.** My immediate early gene study implicated the infralimbic and prelimbic regions of the prefrontal cortex in appropriate recognition of and response to a social opportunity. Building off this conclusion, in **chapter 8** I aimed to determine if the IL/PrL is necessary for proper response to this salient change in social context. I used the DREADD system to selectively inhibit the IL/PrL during times of social opportunity and found that, while mice still possessed the ability to win some fights, their social competence appeared to be compromised, as they lost significantly more fights than mice in the control condition. **That they were losing fights to individuals they had previously consistently been dominant to demonstrates that they were no longer behaving in a manner appropriate to the social context. This suggests an important role of the IL/PrL regions of the medial prefrontal cortex in regulating socially competent and context-appropriate behavior.** While there are some limitations to this work, future studies will help determine the exact role of the mPFC and the cell types involved in regulating appropriate

social context as well as extend our understanding of the specific behaviors impacted by this type of hypofunction.

**Taken together, my findings throughout this dissertation provide strong evidence for the broad role of social context in regulating both the brain and behavior.** I investigated social groups from two perspectives: stable social groups and groups undergoing a social opportunity. Using these two approaches, I can conclude that social context has an extensive role in regulating gene expression, neuroendocrine output, neural activation, and behavior. I show that these relationships are incredibly complex, and change based on social context (both the direct experience of the individual and the characteristics of the group as a whole). The implications of this work are broad, as my findings suggest that it is essential for researchers engaged in the study of social behavior, from neurobiologists to social psychologists, to consider the role of complex social dynamics when designing and carrying out their work. A discovery that is true for one social context is not guaranteed to hold up in another, and as we strive to understand both the brain and behavior in a manner that is relevant and translational, this is a phenomenon we must not ignore.

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