**Dynamic changes in social dominance and mPOA GnRH expression in male
mice following social opportunity**

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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.

38 INTRODUCTION

39 Social hierarchies emerge and stabilize over time as individuals engage in competitive or
40 agonistic interactions and relatively subordinate individuals learn to consistently yield to
41 individuals of a relatively higher social status (Chase, 1982). Dominance hierarchies occur
42 frequently in wild species (Muller & Wrangham, 2004; Nakano, 1995; Sapolsky, 1983, 1993) and
43 in laboratory-based studies of cichlids (Grosenick, Clement, & Fernald, 2007; Oliveira &
44 Almada, 1996), crayfish (Issa, Adamson, & Edwards, 1999), honey bees (Kucharski, Maleszka,
45 Foret, & Maleszka, 2008), and mice (Wang et al., 2011; Williamson, Lee, & Curley, 2016).
46 Individuals at the top of a social hierarchy have significantly higher reproductive success,
47 increased neurogenesis, enhanced immune function, and better overall health outcomes than
48 those at the bottom of a hierarchy (Archie, Altmann, & Alberts, 2012; Bartolomucci et al., 2001;
49 Kozorovitskiy & Gould, 2004; Sapolsky, 2005). It is therefore essential that individuals are
50 capable of recognizing their own social status relative to others in a hierarchy and of
51 dynamically shifting their behavior when a social system destabilizes (Fernald, 2014).

52

53 One approach to studying dynamic changes in social behavior within a social hierarchy is the
54 social opportunity paradigm, where sub-dominant individuals rapidly ascend a hierarchy
55 following the removal of the most dominant individual. Ascent following social opportunity has
56 been studied in African cichlid fish, with changes in both behavior and physiology occurring in
57 sub-dominants within minutes of the removal of the alpha male (Maruska, Zhang, Neboori, &
58 Fernald, 2013; Maruska & Fernald, 2013; Maruska, Levavi-Sivan, Biran, & Fernald, 2011). These
59 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 sub-dominant 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), suggesting a down-

regulation of the HPG axis. 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) , 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

102 enrichment (wooden blocks and nestlets). At 9 weeks of age, mice were weighed and put into a
103 large custom built vivarium (length 150cm, height 80cm, width 80cm; Mid-Atlantic;

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 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. 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). Following verification of the hierarchy (i.e. 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 1**.

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°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 respective to subordinates in the sham-removal condition. The following validated quantitative PCR primers were used for mRNA analysis: GAPDH (Forward: TGTGTCCGTCGTGGATCTGA; Reverse: CCTGCTTCACCACCTTCTTGA), GnRH (Forward: AGCACTGGTCCTATGGGTTG; Reverse: GGTTCCTGCCATTTGATCCAC). Samples that did not yield sufficient RNA for cDNA conversion were eliminated from the analysis.

189

190 **Hormone Assay**

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

195

196 **Statistical Analysis**

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

198 *Behavior Analysis:* The linearity of each hierarchy was calculated using Landau's Modified h' .
199 Briefly, the total number of wins by each individual against all other individuals are entered into
200 a sociomatrix. Landau's method then assesses the degree to which each individual consistently
201 dominates others in contests and whether individuals can be linearly ordered based upon their
202 wins and losses. It ranges from 0 (no linearity) to 1 (completely linear). The significance of h' is
203 determined by performing 10,000 two-step Monte Carlo randomizations of the sociomatrix and
204 comparing the observed h' against a simulated distribution of h' (De Vries, 1995; Williamson et
205 al., 2016). Temporal changes in individual dominance ratings were calculated using Glicko
206 Ratings (Glickman, 1999; So et al., 2015). Glicko ratings are a pairwise-contest model ratings
207 system where ratings points are recalculated following each successive win or loss. All
208 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).

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 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: $V=0$, $P=0.002$, **Figure 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 ($P=0.32$) or between the next two days ($P=0.62$), 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 (Wilcox paired test: $V=55$, $P=0.002$) 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.

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 $P<.051$ - mean $P=0.0184$). 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 2 & 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, $P=0.021$). 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 $X^2=8.17$, $df=1$, $P=0.004$). 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 wins compared to subdominant males from the sham-removed group (Chi-squared tests, all $P<0.05$; **Figure 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 3B**, Wilcoxon Rank Sum Test all $P < .001$). 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 subordinate 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, $W=33.5$, $P=0.033$).

Further, as shown in **Figure 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: $\beta=1.69 \pm 0.30$, $N=64$, $P < .001$). Subdominant males were significantly more aggressive when alpha males had been removed compared to sham-removed (Wilcoxon Signed Rank Test, $W=4.5$, $P < .001$) and compared to the day prior to removal (Wilcoxon Paired Signed Rank Test, $V=0$, $P < .001$). 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 ($p=.89$) or compared to subdominant males from either group the day prior to removals ($p=.66$).

313

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

321

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

323 One hour following the subdominant male's rise to dominant status both subdominant males
324 and subordinate males showed elevated GnRH mRNA levels in the mPOA, as compared to sub-
325 dominant and subordinate males in the sham-removed group (GLM: $F_{2,38}=3.018$, $P<.05$, **Figure**
326 **4A**).

327

328 Social status was significantly associated with plasma testosterone levels with subordinate male
329 mice having lower testosterone than subdominant mice (GLM, $F_{2,58}=2.46$, $P<.05$., **Figure 4B**).

330 There was no significant interaction between alpha removal and social status in the GLM.

331 However, it is notable that in the alpha male removed group, we did find a significant effect of

status on plasma testosterone levels (GLM: $F_{1,30}=4.589$, $P<.05$), which was not observed in the sham-removal group (GLM: $F_{1,28}=1.221$, $P=.279$).

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 2 and 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 2 and 3**).

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).

353

354 The social ascent by subdominant male mice observed in the current study is consistent to what
355 has been observed to occur during a social opportunity in African cichlid fish (Maruska et al.,
356 2013). In this species, individual fish respond behaviorally and physiologically within seconds to
357 minutes to the change in social context. Likewise, we observed that rising subdominant male
358 mice respond to the removal of alpha males rapidly with the first fight occurring in less than
359 three minutes. This is remarkable given that we removed the alpha male at the change of light
360 cycle (white light to dark light) – at time when the subdominant male was not always active or
361 even awake. Regardless, removal of the alpha male always led to individuals attempting to take
362 advantage of the social opportunity which in turn aroused the subdominant male even if he
363 was not originally aware of the opportunity. Although it has long been established that
364 individuals across all species are able to re-establish social hierarchies over time following the
365 death, removal or other disturbance of dominant individuals (Chase & Seitz, 2011; Franz,
366 McLean, Tung, Altmann, & Alberts, 2015; Rosvold, Mirsky, & Pribram, 1954), we and others
367 have argued that this ability to respond rapidly and dynamically to changes in social context is a
368 fundamental feature of group-living social cognition and social competence (Desjardins et al.,
369 2012; Fernald, 2014; Oliveira, 2009; Williamson et al., 2016). Individuals that are unable to
370 respond flexibly to social challenges such as these are likely to be at a great social, reproductive
371 and health fitness disadvantage (Hofmann et al., 2014; Taborsky & Oliveira, 2012). Our study
372 adds to the work in cichlid fish, suggesting that rapid social ascent following social opportunity
373 may be a universal feature of linear social hierarchies.

374

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 following the removal of the alpha male compared to individuals in the sham-removal condition. The increase in hypothalamic GnRH mRNA of subdominant males during such a social opportunity is 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.

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.

419

420 Although there may be multiple mechanisms through which changes in GnRH mRNA levels may
421 be modulated, we did not observe any relationship between total wins or losses or the number
422 of total contests that occurred in each hour following removal or sham-removal and mPOA
423 GnRH mRNA levels. This suggests that the relationship between behavior and gene expression
424 is not a simple linear association. Nevertheless, it is evident that all individuals in each social
425 hierarchy, including those undergoing transition from subdominant to alpha status as well as
426 subordinate males, are exhibiting a behavioral and neuroendocrine response to the increased
427 social instability induced by removal of the alpha male.

428

429 We observed elevated circulating plasma testosterone levels in subdominant individuals
430 compared to subordinate males. In animals undergoing social opportunity, although both
431 individuals exhibited increased levels of GnRH mRNA, subdominant males had significantly
432 higher testosterone than the subordinates. This dissociation between increased GnRH and
433 testosterone levels may be related to an inability of subordinate individuals to respond to GnRH
434 as occurs in group-living subordinate sugar gliders (Bradley & Stoddart, 1997). In this species,
435 exogenous administration of GnRH to dominant and subordinate individuals leads to an
436 increase in plasma testosterone in dominant but not subordinate individuals. Similarly, male
437 wild dark-eyed juncos alter their behavioral and physiological responses to GnRH
438 administration dependent upon the particular social context (McGlothlin, Jawor, Ketterson,
439 Adkins-Regan, & Whitlock, 2007). Thus, it is possible that subordinate individuals 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, & Maestriperi, 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 & Vandenberg, 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 sub-dominant 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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FIGURE LEGENDS

Figure 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 a 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. (D) At this one-hour time point, the most subdominant and subordinate animal in each hierarchy is removed and brains and trunk-blood collected. (E) 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.

Figure 2. Behavioral changes following social opportunity. (A) Glicko Dominance Rating as recalculated after each observed agonistic interaction. (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.

Figure 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. (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. N=16 males per group.

Figure 4. HPG measures correlated to social status and impacted by social opportunity. (A)

Log-transformed fold difference in mPOA GnRH mRNA levels and (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.

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