Social context-dependent relationships between mouse dominance rank and plasma hormone levels

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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 1** and **Table 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,

89 where each mouse has a unique rank and behaves in a socially appropriate manner to
90 individuals of relatively higher and lower social status(Williamson et al., 2016).Additionally, we
91 have shown that each social hierarchy possesses unique social dynamic characteristics. In
92 particular, we have demonstrated that alpha males vary in their ability to inhibit the aggression
93 of other males in their group, an ability referred to as despotism. In hierarchies with highly
94 despotic alpha males, other males are much less likely to express aggressive behaviors towards
95 each other, whereas in hierarchies with less despotic alpha males, power is more equally
96 distributed among sub-dominant mice (Curley, 2016a; Williamson et al., 2017, 2016). In the
97 present study, we sought to determine the role of despotic social context in modulating the
98 relationship between testosterone, corticosterone and social rank. We examined the
99 relationship between endogenous plasma testosterone and corticosterone with social status
100 within social hierarchies that were characterized either by high or low alpha male despotism.
101 Additionally, we compared endogenous levels of testosterone and corticosterone in males of
102 dominant and subordinate social status living in groupsocial hierarchies, where individuals
103 flexibly express both aggressive and subordinate behaviors, to those males living in stable
104 dyadic social relationships where individuals almost only ever express either aggressive or
105 subordinate behavior once their relative social status has been determined.

Table 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>
CD-1	4-6	N/A	N/A	No	Dominant > Subordinate	Marchida, Yonezawa, & Nourmura (1981)
CBA/J	17 (5 M, 12 F)	Eight 23 x 11 x 11cm inter-connected cages	28	Yes	Dominant > Subordinate	Ely (1981)
DBA/1/Bg	2	16 x 26.5 x 11.5cm	5	No	Dominant = Subordinate	Selmanoff, Goldman, & Ginsberg (1977)
CFLP	6	30 x 30 x 30cm	5	No	Dominant = Subordinate	Barnard, Behnke, & Sewell (1996)
DBA/1/Bg and DBA/2/Bg	7-8 (2 M, 5-6 F)	16 x 26.5 x 11.5cm	120-180	Yes	Dominant = Subordinate	Selmanoff, Goldman, & Ginsberg (1977)
DBA/1/Bg	8	16 x 26.5 x 11.5cm	150	No	Dominant = Subordinate	Selmanoff, Goldman, & Ginsberg (1977)
Swiss	10	N/A	21	No	Dominant = Subordinate	(Hilakivi et al., 1989)

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

99 **METHODS**

100 ***Literature Search***

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

116

117 ***Husbandry***

118 Throughout the study, subjects were housed in the animal facility in the Department of
119 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 dying 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 cm³/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 cm³/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 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 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 cm³/animal; Mid-Atlantic; **Supplemental Figure 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 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 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 ± 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.

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

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

207 *Pair Behavioral Analysis:*The dominant and subordinate mouse within each pair was
208 determined based on wins and losses. Dominant mice were those that consistently exhibited
209 wins without losing in the last week of pair housing (during observations conducted on days 19
210 and 22). Subordinate mice were those that consistently exhibited losses without winning in the
211 last week of pair housing. Individuals in all pairs except one could be identified as dominant or
212 subordinate.

213 *Group Behavioral Analysis:* The total number of wins and losses experienced by each individual
214 over the course of the housing period were aggregated into frequency win/loss sociomatrixes
215 for each cohort. From these sociomatrixes, we calculated the Landau's modified h' (De Vries,
216 1995) to confirm the presence of a linear social hierarchy (See Williamson et al., 2016 for a more
217 detailed description). The significance of h' is determined by performing 10,000 two-step
218 randomizations of the win/loss frequency sociomatrix and comparing the observed h' value
219 against a simulated distribution of h' . Significant h' values indicate a linear social hierarchy. We
220 also calculated the triangle transitivity ($ttri$) of each group as a further characterization of the
221 hierarchical organization of each cohort. In brief, this measure determines the proportion of
222 relationships within all triads (group of three individuals) of the hierarchy that are transitive (i.e.
223 if A is dominant over B who is dominant over C then A also is dominant over C), versus
224 intransitive. We derived a binarized 1/0 win/loss sociomatrix from the frequency sociomatrix
225 and used this binarized matrix to calculate $ttri$ (see (Williamson et al., 2016). Both h' and

226 *ttri*were calculated using the R package ‘compete’ (Curley, 2016b). Ranks of each individual in
227 each cohort were calculated using Glicko ratings. Briefly, all individuals in each group start with
228 the same initial rating and gain or lose points following each agonistic interaction based on the
229 rating difference between themselves and the individual they defeat or lose to (Glickman, 1999;
230 Williamson et al., 2016). Individuals with the highest Glicko ratings are considered alpha males,
231 those with the second highest ratings are beta males. To compare alphas, betas, and
232 subordinate individuals across groups, we normalized Glicko scores by dividing each score by
233 the square root of the sum of each score squared. Glicko ratings were calculated using the R
234 package ‘PlayerRatings’ (Stephenson and Sonas, 2012). Stability of each social group was
235 verified through observation of stabilization of Glicko ratings by the end of the second week.
236 Further, the Stability Index, a metric for the overall stability of a hierarchy during a time period,
237 was calculated using the R package ‘EloRating’ (Neumann et al., 2011; Neumann and Kulik, 2014).
238 This index analyzes rank reversals, the closer to 1 the index is, the fewer rank reversals have
239 occurred throughout the time period being analyzed (Neumann and Kulik, 2014). Alpha males,
240 beta males, and the two most subordinate males (ranks 11 and 12) were used in the analyses.

241 The despotism of each alpha male was calculated by determining the proportion of all wins
242 over the entire observation period attributed to the alpha male. Alpha male despotism was also
243 calculated only over the final two days by calculating the proportion of all wins over the final
244 two days attributed to the alpha male. Social hierarchies with alpha males having despotism
245 scores >0.5 were considered to be highly despotic whereas alpha males with despotism scores
246 <0.5 were considered to have low despotism (See Williamson et al., 2016 for a more detailed
247 description). To compare the frequency of wins/hour and losses/hour between animals of

248 different social ranks in high and low despotism cohorts, we performed unpaired Wilcoxon rank
249 sum tests.

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

261 The relationship between social status and body weight or body weight change in paired-
262 housed animals was assessed using a paired Wilcoxon Signed Rank Test. In group housed
263 individuals, social rank effects on body weight were examined by running a GLMM with initial
264 body weight or body weight change as the outcome variable, social status as a fixed effect and
265 cohort as a random effect. To examine the relationship between body weight and circulating
266 hormone levels, we ran GLMMs with hormones as outcome variables and initial body weight or
267 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 ± 0.43 g vs Subordinates = 31.51 ± 0.53 g; $V = 42$, $p = 0.160$, $r = 0.33$) nor body weight change over the housing period (Dominants = 5.50 ± 0.62 g vs Subordinates = 5.80 ± 0.60 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 ± 2.5 fights per pair were observed (range 5-40). Dominant males won an average of 2.27 ± 1.84 wins/hour compared to subordinates winning 0.35 ± 0.57 wins/hour. No clear relationship existed between social status and plasma testosterone levels (**Figure 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 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 $p > 0.200$).

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**), and verification of stable Glicko scores across the final three days. Further, no

307 alpha male lost a fight in the final week, verifying the stability of our alpha males. The average
 308 number of aggressive interactions per group over the housing period was 993.2, with a
 309 standard deviation of 295.5. As each hierarchy was linear, we determined individual ranks and
 310 the normalized Glicko scores of each social status group (**Table 4**). We determined that 9/20
 311 alpha males had despotism scores >0.5 and were considered as having high despotism. The
 312 remaining 11/20 alpha males had despotism scores <0.5 and were considered as having low
 313 despotism. Highly despotic alpha males won significantly more fights per hour than low
 314 despotism alpha males (**Figure 3A**, $W = 87.5$, $p = 0.004$, $N = 20$, $r = 0.64$). Subordinate males in
 315 low despotism groups won significantly more fights per hour than subordinate males in high
 316 despotism groups (**Figure 3A**, $W = 114$, $p = 0.028$, $N = 38$, $r = 0.36$). There is also a trend towards
 317 beta males in low despotism groups winning more fights per hour than beta males in high
 318 despotism groups ($W = 21$, $p = 0.053$, $N = 19$, $r = 0.45$). There was no statistically significant
 319 difference in the frequency of losses per hour for alpha or beta males between the high and
 320 low despotism groups (alphas: $W = 35$, $p\text{-value} = 0.287$, $N = 20$, $r = 0.25$; betas: $W = 51$, $p\text{-value}$
 321 $= 0.661$, $N = 19$, $r = 0.11$). There is a trend for subordinate males in highly despotic groups to
 322 experience fewer losses per hour than subordinate males in low despotism groups (**Figure**
 323 **3B**, $W = 114$, $p\text{-value} = 0.060$, $N = 38$, $r = 0.31$). There were no significant differences between
 324 social ranks in initial body weight, though subordinate males had a trend towards lower initial
 325 body weight than alpha and beta males (Alphas = $34.06 \pm 0.49\text{g}$, Betas = $34.16 \pm 0.46\text{g}$,
 326 Subordinates = $33.23 \pm 0.30\text{g}$; GLMM: alphas vs. betas: $\beta = 0.105 \pm 0.556$, $N = 80$, $p = 0.851$;
 327 alphas vs. subordinates: $\beta = -0.823 \pm 0.481$, $N = 80$, $p = 0.093$; betas vs. subordinates: $\beta = -0.928$
 328 ± 0.481 , $N = 80$, $p = 0.059$). Change in body weight over the housing period was not different

between ranks (Alphas = $3.43 \pm 0.39g$, Betas = $3.50 \pm 0.34g$, Subordinates = $2.96 \pm 0.41g$;
 GLMM: alphas vs. betas: $\beta = 0.070 \pm 0.634$, $N = 80$, $p = 0.913$; alphas vs. subordinates: $\beta = -$
 0.470 ± 0.553 , $N = 80$, $p = 0.399$; betas vs. subordinates: $\beta = -0.540 \pm 0.553$, $N = 80$, $p = 0.333$).

Table 3 – Variation in Measures of Social Hierarchy Dynamics

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

Table 4 – Normalized Glicko scores

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

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 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 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 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 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 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 2B**).

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 ± 13.261 ng/ul, $N = 9$; groups: 149.769 ± 10.131 ng/ul, $N = 38$; GLM: $F_{1,47} = 4.923$, $p = 0.032$, $N = 47$).

Alphamales had equivalent levels of corticosterone regardless of housing condition (pairs: 124.901 ± 9.904 ng/ul; groups: 136.660 ± 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 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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FIGURE LEGENDS

Figure 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 ± 0.6 . The average total hours of observation per group was 37.5 hours.

Figure 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 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 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 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 - *** $p \leq 0.001$, * $p < 0.05$, \$ $p < 0.10$.

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Pair-Housing

Group-Housing

Figure 1

Figure 2

Figure 2

Figure 3

Figure 3

Figure 4

Figure 5