Title

Paternal line effects of early experiences persist across three generations in rhesus macaques

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Three Generations of Paternal Line Transgenerational Effects of Early Experience in Rhesus Macaques

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Abstract

The effects of early stress may not be limited to the exposed generation, but are sometimes passed on to subsequent generations. Such non-genetic transgenerational inheritance is a potentially important developmental and evolutionary force. We compared the transgenerational effects of maternal and paternal line early stress on anxiety- and health-related traits in three non-exposed generations (F1, F2 and F3) of semi-naturalistically raised rhesus macaques. As infants, F0 macaques were exposed to nursery rearing (NR) or semi-naturalistic social conditions (CONTROL). Three hundred forty non-exposed F1–F3 descendants were CONTROL reared and physiological and behavioral measures were collected during standardized assessment at 3–4 months of age. Paternal line NR was significantly associated with greater nervousness in F1–F3 and lower immune cell counts in F1–F2. Maternal-line NR effects were not observed. This study suggests that acquired stress-related traits may be “inherited” across generations in primates, through complex social or germ-line mechanisms.

Keywords

paternal line; early life stress; rhesus macaque; nursery rearing

Early stress re-organizes physical and mental development, and can lead to poorer physical and mental health (Wegman and Stetler, 2009; Conti, Hansman, Heckman, Novak, Ruggiero, & Suomi, 2012; Danese, Moffitt, Harrington, Milne, Polanczyk, Pariante, Poulton, & Caspi, 2009; Bowlby, 1954; Miller & Cole, 2012). It has long been known that the effects of early stress are not limited to the exposed generation, but may impact subsequent generations as well (Dodge, Bates, & Pettit, 1990; Spinetta & Rigler, 1972; Francis, Diorio, Liu, & Meaney, 1990). Transgenerational influences may occur through the maternal and/or paternal line in mammals (Fairbanks and McGuire, 1988; Kinnally et al., 2013; Kinnally and Capitanio, 2015), although potentially through distinct mechanisms. For example, there is ample comparative evidence to suggest that maternal early stress affects anxiety-related traits in the next generation in rodents (poor maternal care: Francis et al., 1999), non-human primates (poor maternal care: Fairbanks, 1989; variable foraging demand stress: Kinnally et

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al., 2013) and humans (trauma: Yehuda et al., 2012). The effects of paternal early stress are less well known.

There is recent significant evidence that paternal periconceptional experiences may impact subsequent generations. In rodents, fathers' exposure to social defeat, footshock, and fear conditioning in the immediate period preceding conception affect offspring behavioral and physiological development (Saavedra-Rodríguez and Feig, 2013; Franklin, Russig, Weiss, Gräff, Linder, Michalon, Vizi, & Mansuy, 2010; Dias & Ressler, 2014; Mychasiuk, Harker, Ilnytskyy, & Gibb, 2013; Rodgers, Morgan, Bronson, Revello, & Bale, 2013; Hoyer, Richter, Brandwein, Riva, & Gass 2013). Less is known about paternal early life stress, although some evidence suggests that paternal exposure to early stress shapes health in subsequent generations in humans: cardiovascular risk is greater in offspring of males that experienced temporary separations from parents as children (Alastalo et al., 2012), and paternal grandparent exposure to famine impacts mortality ratios in a sex-specific manner in humans (Kaati et al., 2005).

Rhesus macaques (*Macaca mulatta*) are an ideal species for disentangling the multigenerational effects of early stress on anxiety-related outcomes. They show genetic, neural, and social complexity that is more directly comparable to humans than other mammalian species widely available for study (Capitanio & Emborg, 2009; Phillips, Bales, Capitanio, Conley, Czoty, Hart, et al., 2014). In the present study, we investigated whether the effects of early life stress in one F0 generation would be observed in three subsequent generations (F1, F2 and F3) of semi-naturalistically raised rhesus macaques, and whether transmission patterns could be identified. One of the best-characterized early life stressors in macaques is maternal deprivation, called peer-rearing or nursery -rearing, (NR, Higley, Suomi, & Linnoila, 1991; Kinnally, Lyons, Abel, Mendoza, & Capitanio, 2008). NR monkeys can be compared with subjects that are raised by their mothers in extended social groups (CONTROL rearing), as is typical in macaque life history. Infants reared in a nursery are removed from their mothers typically in the first few days of life, although occasionally later in infancy, and then pair-housed with peers. One of the important consequences of NR is reorganization of the immune, endocrine, and nervous systems. Compared with CONTROL animals, NR macaques are characterized by widespread dysregulation of stress pathway genes (Kinnally et al., 2008; Ichise et al., 2006; Spinelli et al., 2010), altered regulation of the hypothalamic-pituitary-adrenal (HPA) axis (Capitanio et al., 2005; Higley et al., 1992), production of fewer immune cells, which may indicate poorer immunity (Lubach, Coe, & Ershler, 1995), poorer health (Conti et al., 2012) and exaggerated emotional reactivity to stress, comparable to anxiety (Gottlieb and Capitanio, 2013; Corcoran et al., 2012; Kinnally et al., 2010; Suomi, 1991). We previously observed an effect of paternal NR on physiology, immunity, and behavior in the F1 generation in rhesus macaques (Kinnally and Capitanio, 2015). No primate study has yet compared the relative effects of maternal and paternal line ancestral stress across multiple generations.

In the present report, male and female infant macaques in F0 experienced nursery or control rearing, and once they reached adolescence, most were relocated to one of six new social groups in large outdoor enclosures. New individuals born in these groups

were CONTROL reared, as they were born and raised in semi-naturalistic conditions. These groups remained together for seven to seventeen years, generating heterogeneous groups of NR and CONTROL reared individuals and their offspring (F1), grand-offspring (F2) and great-grand-offspring (F3). We compared the biobehavioral development of these CONTROL reared descendants, who were reared in the same six social groups, and differed only in the patterns of their ancestral NR. We measured multiple behavioral, physiological, and immunological traits related to anxiety in these three subsequent generations, and compared the relative effects of maternal and paternal line early NR.

Materials and Methods

Subjects

Male (n= 158) and female (n=182) infant (90–120 days of age) rhesus macaques were included in this study as F1–F3 descendants of NR or CONTROL reared ancestors. All subjects were derived from the CNPRC specific pathogen free (SPF) colony, meaning that animals were free of several pathogens. Prior to testing, all animals were housed in one of six half-acre outdoor enclosures at the California National Primate Center (CNPRC) between the years 2001–2012. Animals were fed monkey chow (LabDiet, 5045) twice per day, once in the morning and once in the afternoon. Average pairwise relatedness amongst our 340 subjects was less than 2.0%.

Rearing Conditions

F1, F2, and F3 infant subjects were reared in CONTROL conditions at the CNPRC between 2001 and 2012 (details in Golub et al., 2008). These conditions include housing in one of six half-acre outdoor enclosures containing social groups (34–160 macaques) with individuals from at least 6 genetically distinct matrilines with extended kin networks. They were born into groups that had been founded by NR individuals. In the intervening years since group formation (ranging from 7–17 years among the six social groups), new group members were born into the group and thus were reared in CONTROL conditions, which contributed to heterogeneous rearing ancestry among social group members.

Our subjects were reared by their biological mothers within these groups, as is typical in macaque life history. Infant subjects did not yet have an established rank in the social hierarchy but infants' mothers' and fathers' rank were considered for analysis. In the majority of cases, dams (n = 340) and sires (n = 309), resided in the social group with the infant prior to biobehavioral testing. Maternal grandmothers were present in the social group during the infant's first 4 months of life in 46% of cases, maternal grandfathers were present in 23% of cases, paternal grandmothers were present in 39% of cases, and paternal grandfathers were present in 21% of cases. Great-grandfathers were never present in the social group with great-grandoffspring.

Ancestral Rearing

Parents, grandparents, and great-grandparents of our subjects had been reared in either CONTROL conditions or nursery rearing (NR) conditions (details of rearing procedures in Kinnally et al., 2010). Rearing histories were available for all parents and grandparents of

our 340 subjects. Rearing data were only available for 200 paternal great grandfathers of our 340 subjects. NR individuals were separated from their mothers before independence (most commonly on the day of birth, but up to 9 months of age in 12/149 NR mothers and 7/94 NR fathers) and were relocated indoors. NR infants were housed in an incubator for the first month of life with *ad libitum* access to formula. Following this month, infants were housed in indoor individual cages (.46 × .61 × .69 m) with continuous access to a sex- and age-matched pair-mate. At this time, animals were weaned onto monkey chow (LabDiet). At approximately one year of age, juveniles were relocated to new social groups made up of other NR animals in outdoor enclosures.

Our subjects included 191 offspring of CONTROL-reared females (102 F, 89 M), and 149 offspring of NR females (69 F, 80 M). Of these, 246 were offspring of CONTROL reared males (137 F, 109 M) and 94 were offspring of NR males (45 F, 49 M). One hundred twenty eight infants (60 F, 68 M) were paternal grand-offspring of CONTROL reared males and 212 were grand-offspring of NR males (122 F, 90 M). Figure 1 depicts the sample sizes of paternal line NR and CONTROL rearing for each ancestral generation. Our subjects included multiple offspring and grand-offspring of the same individuals: offspring were born to one of 20 different NR males or 58 CONTROL males, to one of 89 different NR females or one of 98 different CONTROL reared females. These subjects were grand-offspring to one of 23 different NR males or 27 different CONTROL males. Seven NR and six CONTROL males had both grand-offspring and offspring in our study. Despite this redundancy, because these animals were spread throughout six distinct social groups representing distinct lineages, subjects were considerably genetically diverse, showing low pairwise relatedness amongst subjects (< 2.0%). We also controlled for pedigree in our statistical analyses (see data analysis below).

Pedigree Determination

Microsatellite analysis is used to determine the individual's place in the colony pedigree, i.e. the identity of their mothers, fathers, grandparents and great-grandparents (Kanthaswamy, Von Dollen, Kurushima, Alminas, Rogers, et al., 2006).

Social Rank

Rank of the mother and father at the time of infant birth was determined and included in the analysis. Social hierarchy rank is determined in a standardized fashion on a monthly basis at the CNPRC by trained behavioral management staff. Data are collected in all field enclosures for at least 30 minutes on a bi-weekly basis, totaling at least one hour of data collection per month. Data are collected with scan sampling to record dyadic displacement interactions between individuals. These data are entered into a hierarchy grid to analyze for the best hierarchical configuration in each cage. Separate rank hierarchies are determined for males and females. We calculated social rank as a continuous variable, calculated as the absolute rank/number of animals of the same sex in the social group. Paternal ranks averaged 23% (ie, 77% of animals are lower ranked than focal), and ranged from the second percentile (higher ranked animals, 98% of animals are lower ranked than focal) to the 89th percentile (lower ranked animals, 11% of animal are lower ranked than focal). Maternal ranks averaged 49% (51% of animals were lower ranked than focal), and ranged from 1%

(higher ranked, 99% of animals were lower ranked than focal) to 100% (lower ranked, 0% of animals were lower ranked than focal). There was no association between paternal or maternal rearing and social rank.

Biobehavioral Assessment

At 90–120 days of age, subjects were assessed during a standardized biobehavioral assessment (BBA) at the CNPRC. During a 25-hour relocation and separation from mothers and social groups, multiple behavioral (activity, emotionality, novel object interaction, temperament ratings), physiological (plasma cortisol at four time points), and immune (white blood cell count, CD 4 + and CD 8 + cell counts) measures were collected from infant subjects. Standardized procedures were designed to ensure that each subject had comparable experiences to all other subjects who underwent assessment. These procedures have been described in detail elsewhere (Golub et al., 2008; Kinnally et al., 2010), and not all measures collected were considered in present study; rather we chose to test specific hypotheses based on our previous work (Kinnally & Capitanio, 2015).

Subjects were relocated from their outdoor enclosures to indoor individual housing (.81 m × .61 m × .66 m) in a temperature-controlled room under a 12:12 hr light/dark cycle. For plasma cortisol measures, blood was sampled via femoral venipuncture four times over the 25-hour period, and each sample was decanted into EDTA-treated collection vials. The first sample was collected at 1100 h (AM sample), approximately 2 hrs following social separation/relocation. The second blood sample was collected approximately 5.0 hours after the first sample at 1600 h (PM sample). Subjects were then immediately injected I.M. with dexamethasone (500 ug/kg, American Regent Laboratories, Shirley, NY). The next blood sample was taken via femoral venipuncture 16.5 hours later, at 0830 h (DEX sample). Following this sample, animals were injected I.M. with 2.5 IU Adrenocorticotopic hormone and blood was sampled 30 minutes later (ACTH sample). Cortisol is measured in each of these samples using radioimmunassay as described previously (3). Immune cells counts (white blood cells, lymphocytes, CD4+ and CD8+ cells) were counted from AM samples as previously described (Capitanio, Mendoza and Cole, 2011).

Temperament measures were collected by a trained observer at the end of 25-hour testing. The rater recorded their perception of each subject with regard to 16 adjectives describing temperament (eg, confident, nervous, tense, timid), on a Likert scale of 1 −7 (Golub et al., 2008). Exploratory and confirmatory factor analyses were applied to scores on each of these adjectives to detect underlying dimensions of temperament, which include Nervousness (including high scores on nervous, fearful, timid, and low scores on calm, confident), Confident (including high scores on confident, bold, active, curious, playful), Gentle (including high scores on gentle, calm, flexible, curious) and Vigilant (including high scores on vigilant, and low scores on depressed, tense, timid).

Data Analysis

All analyses were conducted using SPSS version 26. Two separate factor analyses were conducted to describe the common factors underlying 1.) parameters of the HPA system (plasma cortisol from AM, PM, DEX and ACTH sampling), and 2.) immune cell counts

(white blood cells, lymphocytes, CD 4 + and CD 8 + cells). Factor loadings of 0.4 or greater were considered to contribute significantly to the model, and factor scores were generated using the regression method. For the cortisol factor, all sample measures loaded on this factor, meaning that animals with higher AM, PM, DEX, and ACTH had higher cortisol factor scores. The immune cell factor included high count of lymphocytes, white blood cells, CD4+ and CD8+ cells.

To determine the relative contributions of maternal and paternal line ancestral rearing history on infant cortisol, immune, and Nervousness factor scores, multivariate analyses were used in three separate models: 1.) paternal and maternal rearing as predictors, 2.) all grandparental rearing as predictors, and 3.) paternal great-grandfather's rearing as a predictor. Infant sex, social group identification number, group size at infant birth, cohort (testing year), paternal and maternal age at infant birth, and paternal and maternal social rank at infant birth, were included as covariates in each analysis, as well as the ancestral rearing histories of relatives not being tested as the main predictors. We controlled for genetic relatedness by entering the identification numbers of mothers, fathers, all grandparents, and in the final model, the paternal great grandfather, as covariates. We tested all two-way interactions between significant factors. Significance level was set at $p \leq .05$.

Results

We first investigated whether maternal and paternal rearing in F0 would influence immune, physiological, and behavioral outcomes in their CONTROL-reared offspring (F1). In the analysis, we controlled for potential genetic and environmental influences, including pedigree, infant sex, social group, group size, cohort (year of testing), paternal and maternal age and social rank at infant birth, and each grandparent's rearing history. Paternal rearing history significantly predicted male and female infant measures in F1 ($F (3, 316) = 3.906$, $p = .009$, partial $eta^2 = .036$; See Figure 2). The effects were such that infants with NR fathers exhibited significantly greater nervous factor scores ($F(1, 318) = 6.896$, $p = .009$, partial $eta^2 = .021$) and lower immune factor scores ($F(1, 318) = 3.938$, $p = .048$, partial $eta^2 = .012$). Maternal rearing history did not significantly predict anxiety-related traits ($F(3, 316) = 1.072$ $p = .166$, partial $eta^2 = .016$), nor did pedigree, paternal or maternal age, maternal or paternal rank, cohort, or social enclosure (all $p > .05$). Group size predicted F1–F3 infant outcomes infant outcomes ($F(3, 316) = 2.648$, $p = .049$, partial $eta^2 = .025$). The effects were such that smaller group size predicted lower cortisol ($F(3, 316) = 4.282$, $p = .039$, partial $eta^2 = .013$), but immunity and nervousness were not significantly influenced by group size.

The effects of F0 NR were also observed in F2, but only through the paternal line. Grand-offspring of NR males showed similar biobehavioral organization to offspring of NR males (($F(3, 316) = 6.298$, $p < .001$, partial $eta^2 = .056$; See Figure 3). The effects were such that male and female infants with NR paternal grandfathers exhibited greater nervousness factor scores ($F (1, 316) = 5.884$, $p = .016$, partial $eta^2 = .018$), lower immune factor scores ($F (1, 316) = 6.247$, $p = .013$, partial $eta^2 = .019$), and lower cortisol factor scores ($F (1, 316) = 5.059$, $p = .025$. partial $eta^2 = .016$). No other grandparental rearing history measure predicted infant biobehavioral scores (all $p > .05$). In a *post-hoc* interaction analysis, we

observed no significant interaction in the effects of paternal and grandpaternal NR ($F(3, 316) = .225$, $p = .879$, partial $eta^2 = .002$).

The effects of paternal great-grandfather rearing in F3 were significant ($F(1, 177) = 2.907$, $p = .036$, partial $eta^2 = .047$; See Figure 4). The effect was such that infants with NR paternal great grandfathers exhibited significantly higher nervousness scores ($F(1, 316) = 4.193$, $p = .042$, partial $eta^2 = .023$). There was only one significant two-way interaction in ancestral NR effects, between paternal great-grandfather NR and paternal grandfather NR ($F(3,170) = 2.701$, $p = .047$, partial $eta^2 = .046$). The effect was such that only immune cell counts differed ($F(1,177) = 8.172$, $p = .005$, partial $eta^2 = .046$). Immune cell counts were highest in individuals that had a CONTROL reared paternal great-grandfather and grandfather.

Sex was a significant predictor of biobehavioral organization ($F(3, 316) = 2.795$, $p = .040$, partial $eta^2 = .026$). The effect was such that females exhibited significantly higher plasma cortisol than males ($F(1, 316) = 6.934$, $p = .009$, partial $eta^2 = .021$), but there were no significant differences in nervousness or immune factor scores based on sex (both $p > .50$). Post hoc interaction analyses revealed no significant sex differences in the effects of paternal, maternal, grandpaternal or great-grandpaternal NR (all $p > .05$).

Discussion

Early life stress reorganizes the physiology (Meaney, 2001; Capitanio et al., 2005) health (Danese et al., 2009; Brown, Anda, Tiemeier, Felitti, Edwards, et al., 2009; Kinnally, 2014), and behavior (Bowlby, 1951; Francis et al., 1999) of the exposed individual, with lifelong consequences. NR macaques experience a number of different types of exposures compared with CONTROL reared monkeys, including diet (formula vs. breastmilk), social contact (peer vs. mother and elaborate social connections with others), physical environment (indoor housing vs. large outdoor enclosures), and human contact (extensive in the nursery vs. minimal in CONTROL conditions). As such, NR is species atypical, and likely stressful, for infant monkeys. Our data extend the results of previous studies demonstrating that NR is associated with biobehavioral differences including reduced numbers of immune cells in circulation (Lubach et al., 2004), lower plasma cortisol (Capitanio et al., 2005), and greater anxiety-related behavior (Suomi, 1991) in rhesus macaques. We demonstrate that some of these effects are also observed in offspring (F1), paternal grand-offspring (F2), and paternal great-grandoffspring (F3) of NR males. F1 offspring and F2 grand-offspring of NR males exhibited greater trait nervousness and lower immune cell counts compared with descendants of CONTROL males residing in the same social groups. F3 great grand-offspring of NR males showed greater trait nervousness only. It is unlikely that genetic inheritance explains these results. NR was conducted independent of genetic considerations, we controlled for pedigree in our analysis, and our subjects were largely unrelated. Further, paternal line NR effects were not explained by any potentially confounding factors that we considered, including maternal or paternal age, maternal or paternal social rank, or social group demographics. Maternal line effects of NR were not observed, nor were there effects of female paternal relatives' rearing. Our work suggests that vestiges of early NR stress may not be limited to exposed individuals in primates, but may be observed in three subsequent generations, and specifically through the paternal line.

The most consistent effect of paternal line NR was to predict greater trait nervousness. This finding is consistent with studies demonstrating paternal line effects of stress on anxiety- and health-related traits, including our own (Alastalo et al., 2012; Kaati et al., 2005; Kinnally and Capitanio, 2015; Saavedra-Rodríguez and Feig, 2013; Franklin, et al., 2010; Dias & Ressler, 2014; Mychasiuk, et al., 2013; Rodgers et al., 2013; Hoyer, et al., 2013). Our other measures were not so consistently affected: plasma cortisol was affected by grandpaternal NR, consistent with studies demonstrating a similar effect in NR-exposed macaques (Capitanio et al., 2005), but not paternal or great-grandpaternal NR. Paternal line NR was linked with lower immune cell counts in F1 and F2, but not F3. As trait anxiety has been previously linked with indicators of poor immunity and disease (Capitanio et al., 2011) as well as glucocorticoid production (Meaney et al., 2001), it is possible that trait nervousness is primarily influenced by NR ancestry, while related outcomes such as immunity and plasma cortisol are reorganized with each generation in response to an anxious temperament and other moderating factors. We might expect that other, yet unmeasured traits related to anxiety may be similarly affected by paternal line NR.

While other studies have demonstrated sex differences in susceptibility to stress (Mueller and Bale, 2008; Trainor, Pride, Landeros, Knoblauch, Takahashi et al., 2011) and sex differences in the effects of parental or grandparental stress exposure (Saavedra-Rodríguez and Feig, 2013; Franklin et al., 2010), this was not the case in our population. F1–F3 males and females were both affected by paternal line NR in our study. However, while both males and females were comparably susceptible to the paternal and grandpaternal effects of NR, only males transmitted the effects of paternal NR to their offspring, consistent with some studies in rodents and humans (Saavedra-Rodríguez and Feig, 2013; Kaati et al., 2007; Pembrey et al., 2006). This suggests that males may be more susceptible to transmitting the effects of adversity across generations.

Social or environmental mechanisms for the effects of paternal line NR are possible. We investigated the possibility that specific social confounds explained the effects of ancestral NR, but neither maternal nor paternal rank or age, nor social group identity or size, explained the effects of paternal stress. Other social mechanisms, such as paternal interactions with the infant, cannot yet be eliminated. In macaque life history, as in this study, many fathers remained part of the social group during the early stages of infant development. While fathers are not thought to play as large a role in macaque development as mothers, and exhibit little paternal behavior (Brandt, Irons, and Mitchell 1970), it is possible that even minimal interactions with the father affect infant development. Several studies have demonstrated that, though adult male macaques interact relatively little with infants or young juveniles, they spend more time with their own offspring than with non-offspring, and these interactions tend to be affiliative (Suomi, 1977; Langos, Kulik, Mundry, and Widdig, 2013). If NR fathers differ from CONTROL fathers in their interactions with offspring, this could partly explain our finding. There is precedent to suspect differences between NR and CONTROL fathers: disruption of early attachment relationships can change how fathers parent their own children in biparental species (Spinetta and Rigler, 1972; Dodge et al., 1990), changing offspring trajectories (Birnie, Taylor, Cavanaugh, and French, 2013; Perkeybile, Griffin, and Bales, 2013). Another social mechanism that would explain our findings is that mothers may adjust their investment based on qualities of

the father, which may be related to their experience of NR. In support of this idea, a previous study suggested that mouse offspring of recently stressed fathers displayed greater anxiety related traits, but that a lack of maternal exposure to the father diminished the effect (Dietz et al., 2011). In this study, if mothers were implanted with an egg fertilized with sperm from a stressed male using in vitro fertilization, the effects of paternal stress on offspring development were weaker than if the mother had been exposed to the male during mating (Dietz et al., 2011). Another group demonstrated that some effects of paternal stress were specifically mediated by aspects of maternal behavior (Mashoodh, Franks, Curley, and Champagne, 2012). Female mice mated with males from impoverished lifelong conditions negatively adjusted their maternal investment toward offspring, which corresponded in a linear fashion with the rate of anxiety-related behaviors exhibited by the father. This reduction in maternal care, in turn, predicted offspring growth (Mashoodh et al., 2012). This study was consistent with the ethological literature that suggests that females may invest differently in offspring based on perceived characteristics of the male mate (Cunningham and Russell, 2000). It is not yet known if rhesus macaque females adjust maternal investment based on qualities of the biological father, but because mothers had ample opportunity to interact with fathers in the social group, this is a plausible route of influence. A related maternally mediated effect of paternal NR could be that mothers that choose to mate with NR males may differ on a host of characteristics, genetic and behavioral, that may impact infant development. Undermining the potential explanatory value of these maternally-mediated mechanisms for paternal NR effects is the fact that a feature of macaque polygynous life history is that adult males and females breed with multiple members of the social group during the breeding season. It is not known if females have the capacity to “select” or even have knowledge regarding which male ultimately fathers her offspring. Nonetheless, these remain possible explanations.

There are several pieces of evidence that point to non-social mechanisms for the effects of paternal NR. If the transgenerational effects of NR were mediated purely by social mechanisms, we would expect a stronger effect of maternal NR, because mothers are the primary caregivers for macaque infants. We did not observe such an effect. This does not mean that maternal NR has no effect on offspring development, but its effects may be more complex to detect. Traits inherited via the maternal line arise from maternal genetics, prenatal factors, and/or postnatal investment. One recent report using this study population demonstrated this complexity, showing that between mother-infant dyads, similarly in temperament depended on “sensitivity” genotypes that the infant possessed (Sullivan, Mendoza, and Capitanio, 2011). While there may be some interactive effects of maternal NR with other factors, a main effect of maternal NR is simply not detectable as is paternal NR, undermining somewhat a purely social explanation for this finding. Additionally, the potential explanatory value of social mechanism is weakened by the fact that grandpaternal and great-grandpaternal NR effects cannot easily be explained with social mechanisms. In the present study, as in macaque life history, paternal grandfathers were the least likely grandparent to be present in F2 infants’ early life, yet they were the only grandparent whose rearing significantly influenced infant development. Great-grandfathers were never present in the social groups of the F3 generation, yet the vestiges of their NR experience were evident in F3 infants. Even more notably, the same traits were influenced by paternal,

grandpaternal, and great-grandpaternal NR and in the same direction. Finally, and perhaps most tellingly, these results were consistent with our previous study that showed an effect of paternal NR on offspring that had no social interaction with mothers, fathers, or social groups after the first days of life because *the offspring themselves were NR*. These prior results suggested that paternal NR influenced F1 offspring anxiety and immunity independent of social mechanisms. Taken altogether, these results hint strongly at non-social mechanisms in the inheritance of the effects of paternal line NR.

The fact that the effects are transmitted specifically through the paternal line may point to differential susceptibility of the male germ line to the effects of NR. Although some previous studies identified sex differences in macaque response to deprivation conditions like isolation, where males appeared more sensitive to isolation than females (Sackett, 1972), we believe differential germ line susceptibility explains our results better than potential differences in behavioral susceptibility in males. First, our previous data comparing NR with CONTROL animals have shown no sex-dependent effects of NR on the measures we describe here (Capitanio et al., 2005; Kinnally et al., 2009; Karere et al., 2009; Kinnally et al., 2012). Additionally, if behavioral changes were more pronounced in males, these differences would likely only indirectly impact the offspring, as we discussed in the previous section. If, in contrast, germ line (sperm) elements were changed specifically in males following early NR stress and were stably inherited by progeny, this could explain the specific transgenerational effects of paternal line NR. If so, this might also explain why maternal line differences were not observed – female eggs are largely protected from exposures until the months immediately preceding follicular development and ovulation (Golub, Hogrefe and Vandevoort, 2014), while spermatogonia and sperm may be more susceptible to exposures throughout the lifespan. The germ line elements involved could include epigenetic signals, such as histone modifications, microRNAs, hormones/transcription factors, and DNA methylation patterns factors, which are all present in paternal germ cells and may be inherited by the developing infant (Saavedra-Rodríguez and Feig, 2013, Dietz et al., 2010). While several recent studies in rodents have demonstrated such transgenerational inheritance of stress-induced DNA methylation patterns and micro RNA expression via sperm (Saavedra-Rodríguez and Feig, 2013; Franklin, et al., 2010; Dias & Ressler, 2014; Mychasiuk, et al., 2013; Rodgers et al., 2013; Hoyer, et al., 2013), it is not yet known if stress-induced germ-line changes are inherited in primates, including humans. Evolutionary theory suggests we might expect such a phenomenon. Because most mammalian fathers (95% of mammals that do not engage in biparental care) contribute little more than germ cells to progeny, it has been theorized that we would expect such germ-line mediated mechanisms to evolve, to allow for intergenerational transmission of acquired information between males and their descendants in the absence of social contact (Curley and Mashoodh, 2010; Haig, 1997), in order to enhance males' fitness. We would not necessarily expect such germ line mechanisms for transmission to arise between mother and offspring, because there are other opportunities for transmission of acquired information between mother and infant in mammals, including prenatal and postnatal signaling.

Whether the mechanisms for paternal line NR effects are socially or germ-line mediated, the persistence of effects of stress across three subsequent generations after the exposed generation has important evolutionary implications. When early stress in a previous

generation puts individuals at risk for an anxious temperament and poorer immunity in subsequent generations, this ancestral programming may influence the fitness and reproductive success of future generations. Initially, it would seem that the transgenerational programming of anxiety-related traits may be disadvantageous to descendants' survival and reproductive success, especially if it leads to poor mental and physical health. One might expect that a compensatory effect of parental and grandparental environmental exposures on the health of their descendants would be a more adaptive strategy in an evolutionary context. Such a compensatory effect has been observed in humans (Kaati et al., 2007; Pembrey et al., 2006). However, it may, in fact, be adaptive if stress in a previous generation facilitates stress coping in the next generation. For this to be an advantageous strategy, one might expect that stress programming should optimize fitness under ancestral stress conditions. To our knowledge, this has not yet been investigated, but several studies have demonstrated that early stress actually may optimize survival skills, energy expenditure and reproductive strategies in the exposed generation (Cameron et al., 2008, Champagne et al., 2008, Sangenstedt, Szardenings, Sachser, & Kaiser, 2018). Rats that received poor maternal care as infants reach puberty earlier and engage in higher rates of sexual behavior as adults, perhaps ensuring reproductive success in uncertain conditions (Cameron et al., 2008). Additionally, while rats that received lower maternal care in infancy perform worse on a learning task than infants that received better care, these low care infants perform better than high care rats under stressful conditions, suggesting that early stress may have optimized performance for future stressful conditions (Champagne et al., 2008). While the persistence of the effects of early stress across generations may seem maladaptive, transgenerational inheritance of stress coping strategies may actually optimize stress adaptation in the next generation. Future studies will examine whether transgenerational stress programming may confer an advantage in subsequent generations in primates.

There were several important limitations of this study. First, because we were not powered to test three-way interactions between paternal, grandpaternal and great-grandpaternal effects, we cannot estimate whether the effects of NR accumulate or wane across more than two generations. We present one piece of data to support waning effects across generations. The F1 offspring of NR males exhibit approximately double the magnitude of nervousness factor scores and immune cell deficits compared with F2 grand-offspring of NR males (F3 was made up of a subset of the subject pool, so we cannot accurately compare the magnitudes of differences in F3 traits with F1 and F2). These data suggest that there may be a general decline in magnitude of effect of NR across generations, but future studies will bear this out. Another limitation of this study is that while the majority of NR individuals had been removed from their mothers on the first day of life, a small subset of males had been separated from mothers later in development (7% of NR fathers). However, when we compared descendants of NR individuals separated on postnatal day one vs. later in development, there were no significant differences, and so we included the entire population of NR ancestors in our analysis. Another potential limitation of our design was that all six social groups were formed with NR animals, and subsequent generations were tracked for biobehavioral development. This means that depending on the year of assessment, subjects from the same social group may have experienced slightly different social experiences because the proportions of NR subjects in the social group declined over our 12-year study,

as more animals were born into these CONTROL conditions. To address this potential limitation and to control for changes over successive years (both within groups and within parents), we statistically controlled for group identifier, each parent's age at offspring birth, infant testing cohort, and group size at infant birth. The advantage of this approach is that we have avoided the confound that may arise in comparing NR founded groups with CONTROL founded groups. Groups founded by differently reared populations would likely have had significant social or cultural differences that could influence infant development, but with our present design, this is less of a concern.

Finally, because we assessed anxiety-related traits at only one time point (3–4 months of age), the lifetime effects of NR ancestry on behavior, physiology, and health, and the relevance to fitness outcomes, are not yet known. We believe that these measures are indicators of future outcomes, however, as previous work has linked these biobehavioral measures with critical health and social outcomes later in life. For example, macaques with biobehavioral profiles suggesting greater anxiety display airway hyperresponsiveness, a predictor of asthma, in adolescence (Capitanio, Miller, Schelegle, Mendoza, Mason, and Hyde, 2011; Chun, Miller, Schelegle, Hyde, and Capitanio, 2013). There are also social consequences associated with these measures: in adolescence, macaques tend to prefer to spend time with individuals with similar biobehavioral profiles (Weinstein and Capitanio, 2008; Weinstein and Capitanio, 2012), indicating that these traits measured early in life may influence social relationships and even mating preferences. These studies hint that the transgenerational inheritance of anxiety-related traits may impact important aspects of macaque social life and health, but future studies will be needed to investigate the continuity of effects into adulthood and ultimate effects on longevity and reproductive success.

In conclusion, stress related traits and diseases, such as cardiovascular disease, metabolic disease, inflammation, and psychiatric illness, have long been known to run in families. This phenomenon has often been attributed to genetic causes. Our finding that a stress exposure influences three subsequent generations through the paternal line contributes to a growing literature that suggests that *acquired* stress-related traits may also be "inherited" in mammals, including primates. Future studies will focus on understanding the likely complex social and germ line mechanisms of the effects of paternal-line early stress in primates, and their consequences for mental and physical health, reproductive success, and natural selection.

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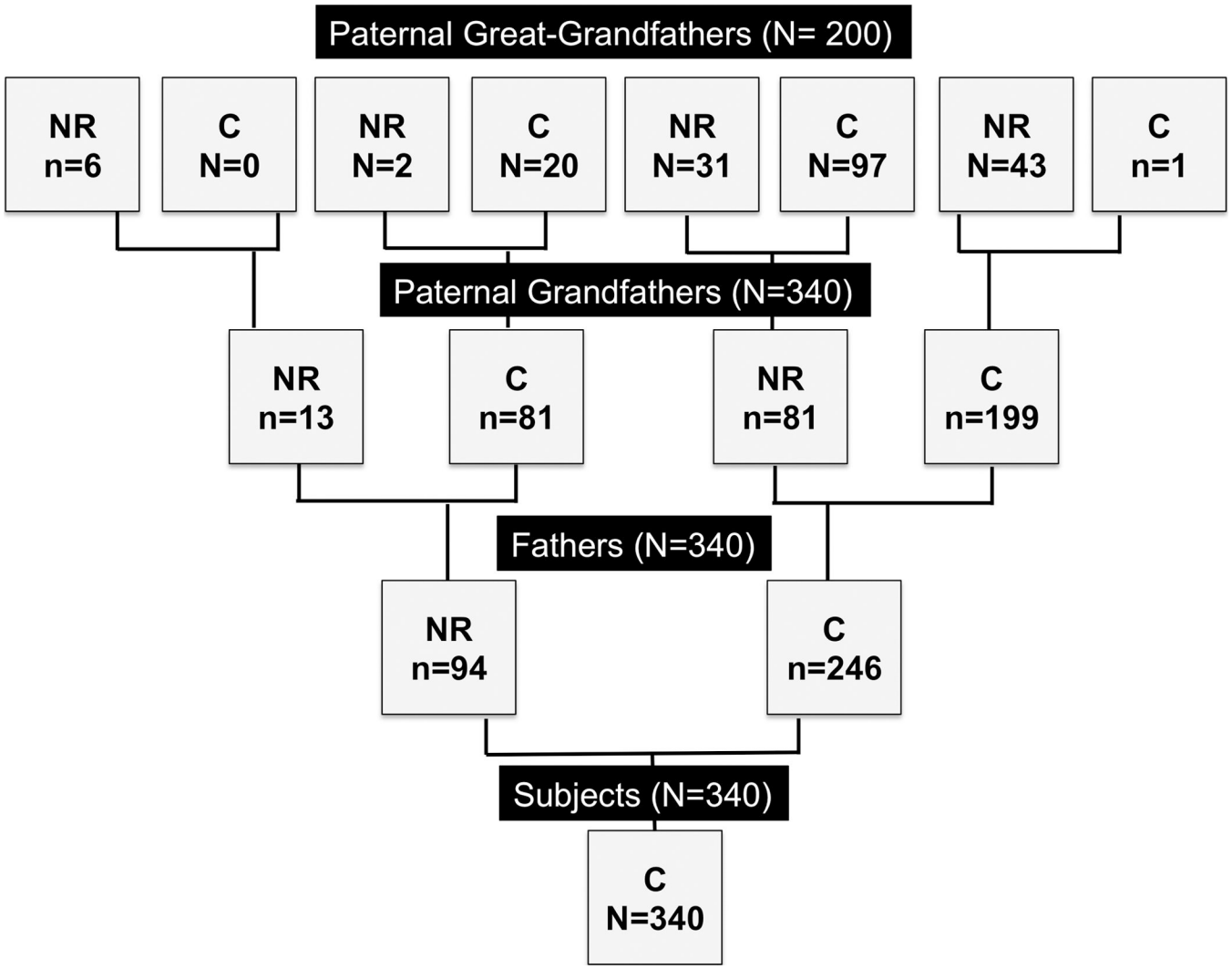

Figure 1.
Schematic of sample size in each paternal line NR and CONTROL ancestral line.

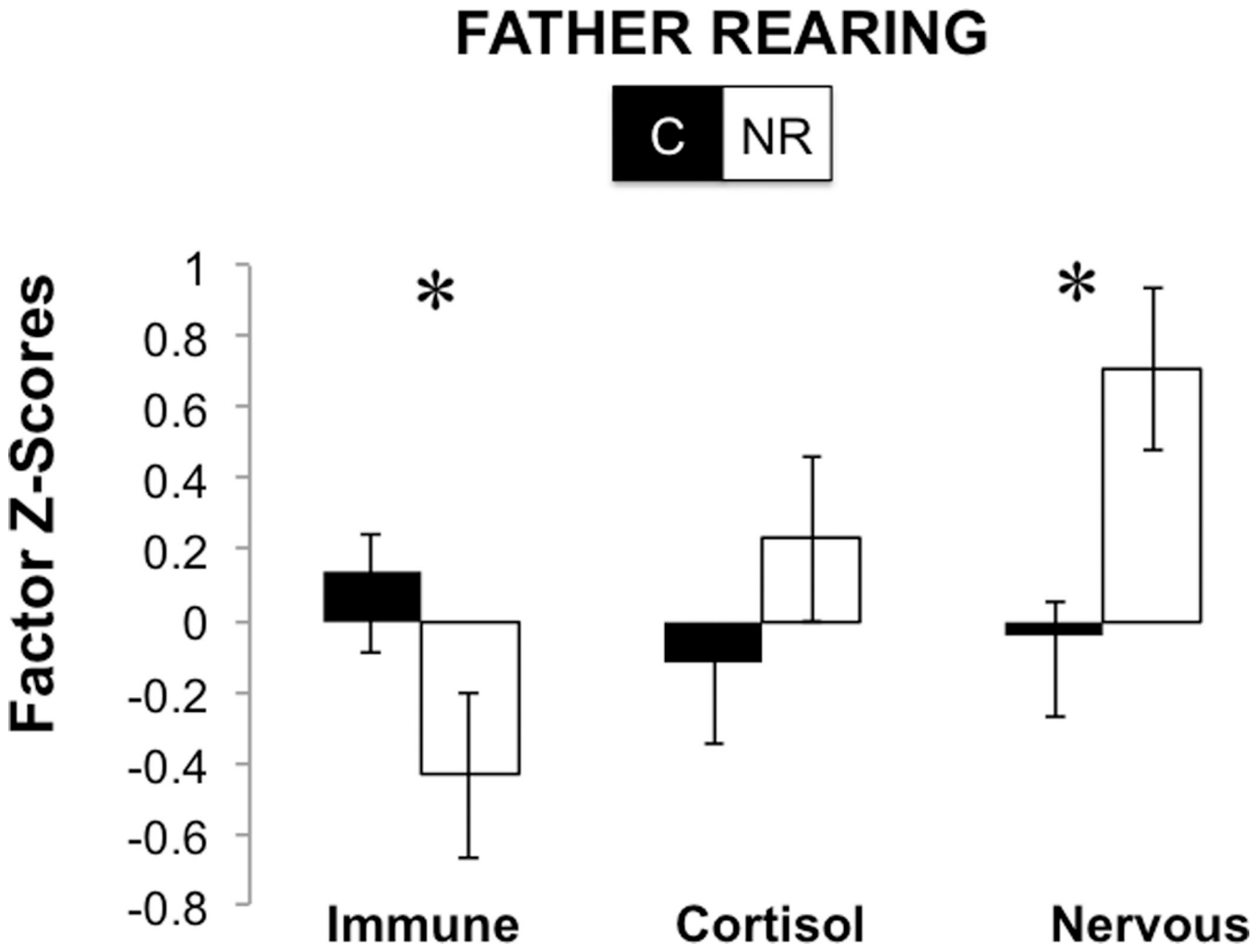

Figure 2.
Paternal NR is associated with reduced immune cell count and higher trait nervousness factor scores. Weighted means are presented ± standard error of the mean. * $p < .05$

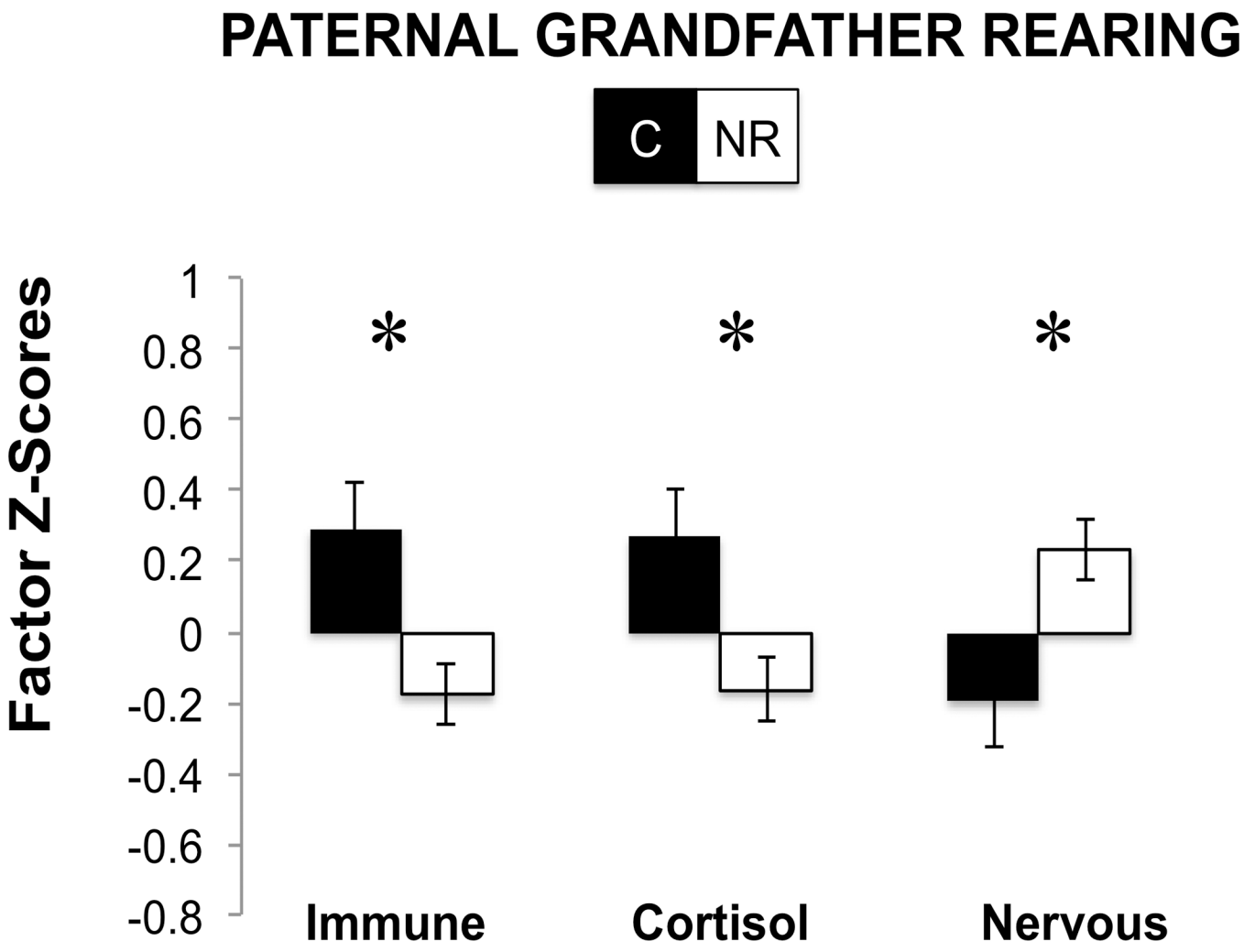

Figure 3.

Paternal grandfather NR is associated with reduced immune cell counts, lower cortisol and higher trait nervousness factor scores. Weighted means are presented ± standard error of the mean. * $p < .05$

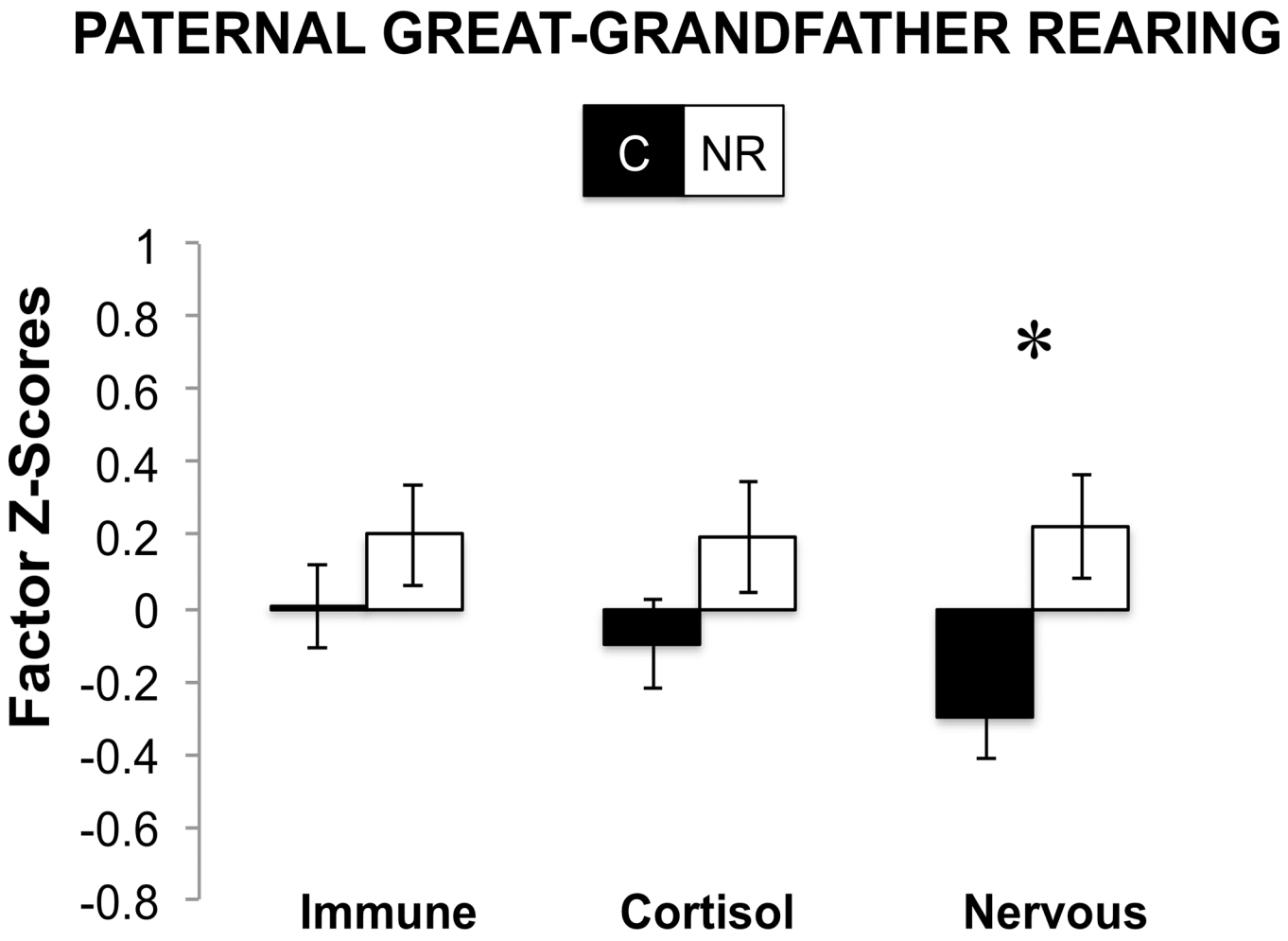

Figure 4.

Paternal great-grandfather NR is associated with higher trait nervousness factor scores. Weighted means are presented ± standard error of the mean. *p < .05