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STABLE BEHAVIORAL INHIBITION AND GLUCOCORTICOID PRODUCTION AS PREDICTORS OF LONGEVITY

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Abstract

Several personality/temperament traits have been linked to health outcomes in humans and animals but underlying physiological mechanisms for these differential outcomes are minimally understood. In this paper, we compared the strength of a behavioral trait (behavioral inhibition) and an associated physiological trait (glucocorticoid production) in predicting life span. In addition, we examined the relative stability of both the behavioral and physiological trait within individuals over a significant portion of adulthood, and tested the hypothesis that a stable behavioral trait is linked with a stable physiological bias. In a sample of 60 Sprague-Dawley male rats, we found that stable inhibition/neophobia was a stronger predictor of life span than stably elevated glucocorticoid production. In addition, these predictors appeared to have an additive influence on life span in that males with both risk factors (stable inhibition *and* consistently high glucocorticoid production) had the shortest life spans of all, suggesting both traits are important predictors of life span. Across a 4-month period in young adulthood, inhibition and glucocorticoid reactivity were relatively stable traits, however these two traits were not highly correlated with one another. Interestingly, baseline glucocorticoid production was a better predictor of life span than reactivity levels. The results indicate that glucocorticoid production in young adulthood is an important predictor of life span, although not as strong a predictor as inhibition, and that other physiological processes may further explain the shortened life span in behaviorally-inhibited individuals.

Keywords

behavioral inhibition; neophobia; temperament; glucocorticoids; corticosterone; stress; mortality; life span

INTRODUCTION

Several behavioral traits have been associated with longevity in humans and rodents (e.g. [1-5]). Despite strong indications that temperament is associated with life span, there is limited examination of the role that correlated physiological profiles play in these differential life spans

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[6,7]. In the current paper, we compared the relative strength of a behavioral trait and an associated physiological trait in predicting life span in a rodent model.

A behavioral trait recently associated with shortened life span in humans and rats is lack of curiosity or neophobia. Specifically, in older humans 'cautious' and 'aggressive/hostile Type A' men have been shown to have shorter lives than 'curious' and 'Type B' individuals [8,9], and in rodents, those identified as neophobic or low-exploratory die younger than neophilic/exploratory ones [10,11]. Interestingly, the shorter-lived inhibited rodents also have elevated hypothalamic-pituitary-adrenal axis activity relative to their longer-lived non-inhibited counterparts, a physiological trait that has also been observed in inhibited children [12]. Shorter-lived inhibited rodents had baseline and reactivity corticosterone levels approximately 30% higher than non-inhibited mice and rats [10,11,13]. Given the allostatic load hypothesis, this physiological difference in glucocorticoid production may be one mechanism leading to differential life span between inhibited and non-inhibited individuals [14,15].

Elevated glucocorticoid production has been associated with several behavioral traits in humans and other animals (e.g. behavioral inhibition, novelty-seeking [4,12,16-20]). Assuming that these are stable traits, it can be hypothesized that individuals with these traits naturally experience consistently elevated glucocorticoids over a portion of life. Given that long-term exposure to elevated glucocorticoid levels is associated with altered immune function, altered memory and neurogenesis, increased health problems and possibly accelerated aging [14,15,21-24], it can be hypothesized that consistent natural differences in glucocorticoid production may be one physiological mechanism influencing health and aging trajectories.

One important assumption underlying this possible mechanism is that differential glucocorticoid production is a stable trait in individuals over time. However, the stability of glucocorticoid production profiles has not been extensively tested in rodents (c.f. [25,26]), and there is only limited evidence to support the assumption that naturally-elevated glucocorticoid production is a stable trait over a significant portion of the life span in humans and other species [27-29]. Most estimates of glucocorticoid stability are from repeat measures taken over a relatively short portion of the organisms' life span (from a few days/weeks to, at best, approximately 3% of the life span). Given the cumulative stress argument, it is important to determine the relative stability of behavioral and physiological profiles that may place individuals at risk of certain health outcomes. Many studies assume the stability of behavioral and physiological traits based on one or a few tests at a given age, however, true measures of trait stability require behavioral or physiological measures across conditions and over time [30-33].

Recently, biomarker measures in humans have been used to assess the predictive nature of glucocorticoid production and mortality in elderly subjects [34]. However, there is no evidence that elevated glucocorticoid production is a stable trait in young adulthood, or that stably elevated glucocorticoid production is associated with temperament, or that it provides a biological mechanism to explain temperament-associated shortened life spans. In fact, some evidence suggests that elevated glucocorticoid production is associated with lengthened life span. For example, caloric restriction causes long-term, pronounced elevations in basal glucocorticoid production - approximately twice as high as control animals during early and late adulthood - yet caloric restriction leads to lengthened life spans [35]. In free-ranging animals, elevated glucocorticoid production (baseline and in response to challenge) has been associated with both increased and decreased survival probabilities (lizards [36], rabbits [37], iguanas [38]). Thus, the relationship between glucocorticoid production and life span is not clear at this point. In addition, it is not clear whether baseline or reactivity levels of glucocorticoid production should have differential influences on longevity. Theoretically,

resting levels of glucocorticoid production may be more important than short-term reactivity levels since elevated resting levels are high over a longer period and thus should have a longer cumulative influence on other physiological processes than elevated reactivity levels that last a limited time.

In the current paper, we determine: (a) the stability of behavior and glucocorticoid production in young male rats across a 4-month interval, (b) the association between stable neophobia and stably elevated glucocorticoid production during this age, and (c) the extent to which stable behavioral and glucocorticoid profiles in young adulthood predict life span. We repeatedly tested male rats to identify consistently inhibited individuals across two novel situations (one non-social and one social) and over time (from early to middle adulthood). To identify individuals with stably elevated glucocorticoid levels, we measured baseline and reactivity levels at both ages. We expected that individuals that were repeatedly inhibited across situations and time would have stably elevated basal and/or reactivity corticosterone levels. If stable inhibition is associated with a variety of physiological biases that have an additive influence on life span, we expected stable inhibition to be a better predictor of shortened life span than elevated glucocorticoid production alone. Alternatively, if elevated glucocorticoid production is a key physiological mechanism underlying the relationship between neophobia and life span, we expected stably elevated glucocorticoid production (baseline or reactivity) to be a more powerful predictor of life span than stable neophobia alone. In particular, we hypothesized that in a low stress (laboratory) environment, elevated *baseline* levels of glucocorticoid production would be more predictive of a shortened life span than elevated reactivity levels since baseline elevations would lead to greater cumulative influences over the life span than short term elevations that may occur infrequently in the low stress environment.

METHODS

Overall Design & Sample

Sprague-Dawley male rats (60 males, 60 days of age) from Charles River Laboratories (Wilmington, MA) were individually housed in solid bottom plastic cages (43.5 × 23.5 × 20.5 cm). They were maintained on a 14L:10D lighting schedule (lights on at 1900h EST) with food and water available *ad libitum*. Cages were cleaned once a week and the colony room maintained at 22° C with ~50% humidity. Rats were allowed to acclimate to the laboratory/housing for 2 weeks prior to testing and were handled and weighed every other day to habituate them to handling. Plastic cages and handling were also used to minimize possible isolation stress caused by individual housing [39]. Methods detailed below were approved by the Pennsylvania State University Institute for Animal Care and Use Committee.

A timeline of the study is given in Figure 1. To measure general neophobia in response to different kinds of novelty – i.e. behavioral inhibition, rats were tested twice on each of two novel environments: a non-social and a social arena (described below). These two arenas were used because we have previously found that consistently long approach latencies in both arenas provides a relatively strong predictor of glucocorticoid production in Sprague-Dawley rats [13]. To ascertain stability of behavior over time, males were tested on both arenas at two different ages: early adulthood (2.5–4 months of age) and middle adulthood (8 months). These ages were chosen because they represent a significant portion of the healthy adult period (18% of median life span) while avoiding the beginning of the aging period which may influence behavioral or corticosterone responses [40]. To estimate individual stability in glucocorticoid production and to identify individuals with consistently high or low glucocorticoid production, we measured baseline and reactive corticosterone levels. These measures were collected at 4 and 8 months of age (again, well before we might expect any differential aging of the HPA axis between more and less inhibited rats [11,40]. Because of the highly pulsatile release of

hormones into the blood stream, an additional measure of baseline corticosterone was collected at 6 months of age.

Behavioral Response to Novelty

Behavioral testing was conducted in the middle of the rats' active period (starting between 1300h and 1500h, 4–6 hrs after lights off). Rats were tested in a non-colony room illuminated with two 25-watt red bulbs reflected off the walls of the room providing approximately 6 lux of light at the center of the novelty test arenas. Males were tested on both test arenas at both ages - half on the non-social arena first, half on the social arena first. Order of testing within a day was reversed for the two arenas (e.g. if a male was the first male tested on the novel non-social arena in a given day, he was the last tested on the novel social arena). Order of testing was additionally balanced across test ages, switching the order in which males were tested on the two arenas and the order in which males were tested within each arena.

Novel Non-Social Arena—This arena was designed to be minimally anxiety-provoking [11]. The square test arena (120cm x 120cm) included 46cm-high white polypropylene walls and a clear plastic cover. Three of the four corners contained a novel rat-sized object (a plastic tube, an inverted bowl, or a wire tunnel), placed 13cm from the arena walls. The floor was covered with clean corn cob bedding and sprinkled with soiled bedding (feces removed) from all cages in the colony room to provide rat odors. During testing, males were placed into a clean ceramic bowl with 5-cm high walls and lowered into the empty arena corner. Rats were video recorded for 5 minutes in the arena then removed after testing and the ceramic bowl rinsed with water and dried for the next subject. On the rare occasion that a rat defecated in the arena during testing, feces were removed, but the arena floor was not cleaned any further so as to maintain conspecific odors for the next rats tested.

Novel Social Arena—The novel social arena was the same size and height as the non-social arena, but instead of novel physical objects, the arena contained two wire cages – an empty cage and one with an unfamiliar male rat of similar size and age as the test rat [13]. To minimize the impact of the stimulus animal's behavior on test animals, the stimulus remained in the cage. This is slightly different than the 'social interaction' test [41] and the test used to identify behavioral inhibition in young rats [42]. The cage had ventilation slits allowing test animals to come in contact with and smell the novel stimulus animal, but the two rats could not injure one another through the cage walls. Test rats were introduced to the arena and video recorded for 5 minutes then removed and the start bowl cleaned in the same way as for the novel non-social arena.

To assess neophobia a trained coder, unaware of each rat's identity, recorded each rat's latency to approach the first novel object in the non-social arena and the social partner in the social arena from video recordings. Approach was defined as coming within one centimeter of a novel object/rat with the nose facing the object. In these arenas, we have found that approach latencies are a significant predictor of overall corticosterone production immediately following arena exposure and predict corticosterone values better than time spent interacting with objects (Cavigelli & Michael, unpublished data). In addition, latency to approach a novel object or a novel social partner were correlated with the number of times a rat approached the objects/social partner (non-social arena: $r_{60} = -0.46$, $p < .001$; social arena: $r_{60} = -0.35$, $p < .01$), but not necessarily with the amount of time a rat spent investigating novelty (nose to object/social partner; non-social: $r_{60} = -0.07$, $p = .58$; social: $r_{60} = -0.16$, $p = .22$) or interacting with novelty (i.e. touching object/social partner; non-social, $r_{60} = -0.13$, $p = 0.33$; social $r_{60} = -0.07$, $p = .61$). This lack of relationship between approach latencies and the duration of investigations and interactions may be because the duration of investigation may not necessarily reflect neophilia. For example, a fearful rat may spend a great deal of time slowly investigating and habituating

to one novel object, whereas a more neophilic rat may spend an equal amount of time investigating objects but distribute this time across multiple novel objects, indicative of decreased neophobia. Given these basic behavioral correlations, we use latency to approach novelty, but not time spent investigating or interacting with novel objects/partner, to estimate neophobia/neophilia.

Corticosterone: Baseline and Reactivity to Novelty

To identify individual rats in which glucocorticoid production was stably elevated across situations and time, we measured the change in circulating corticosterone levels after novelty exposure at 4 and 8 months of age as well as baseline levels at 4, 6 and 8 months of age. To measure the dynamics of the corticosterone response to novelty at 4 and 8 months, serial blood samples were collected at 10, 40, 80 and 120 minutes following novel arena testing and then placement into a novel cage in a novel test room - experiences known to lead to significant changes in circulating corticosterone levels (e.g. [13,43]). Sampling times were selected to capture the acute rise (10 and 40 minutes) and later decline in corticosterone production (80 and 120 minutes) during novelty exposure. To measure baseline levels, blood samples were collected 24-hrs following novelty testing at both ages. These baseline measures were collected after reactivity measures to avoid sampling prior to behavioral testing which may have altered behavioral and/or corticosterone responses to novelty. Because of pulsatile hormone secretion and the relative lability of corticosterone production in response to slight alterations in environmental conditions [43,44], an additional blood sample was collected at 6 months of age to provide an additional measure of baseline corticosterone levels. To control for the glucocorticoid circadian rhythm (e.g. [45,46]), we began all blood collections at the same time of day: 1300–1500h. This corresponds to a time of day when baseline glucocorticoid levels are at intermediate levels – a time at which individual differences in production may be most salient [47] – and a time of day that mimics afternoon cortisol measures frequently used in human studies.

To measure corticosterone reactivity, each rat was carried to a separate room immediately after novel arena testing and placed into a cage comparable to their home cage. Repeat blood samples were collected at 10, 40, 80 and 120 minutes after the rat was first placed in the novel test arena. For each sample, the rat was removed from the holding cage, a blood sample collected within 3 minutes using the tail clip method, and the rat was returned to the holding cage. The tail clip method was selected over the tail nick method because it allows for serial blood collection without repeatedly cutting the rats and the two methods have been shown to produce comparable corticosterone measures [48]. Because of the longitudinal and behavioral measures in the study, we did not place indwelling catheters for blood collection for corticosterone measures (e.g. [47]) to avoid any influence on rats' behavioral response to novelty and/or their health trajectories. Corticosterone reactivity does not differ between tail nick and catheter collected blood samples, however baseline levels during the light phase are significantly greater when blood is collected by tail nick vs. catheter [48]. For baseline corticosterone levels, rats were transported in their home cage to the collection room, removed from their home cage and a blood sample collected within 3 minutes of the cage being moved from the colony room. To avoid blood odors in the colony room, sampled rats were not returned to their colony room until all rats had been sampled.

Blood samples were collected into EDTA tubes (Becton Dickinson and Company) and kept on ice for 40 min until centrifuged and plasma collected. Plasma was frozen at -80°C until assayed, and corticosterone was measured using a commercial radioimmunoassay kit (Rat & Mice Corticosterone kit, MP Biomedicals, Solon, OH). All samples were run in duplicate across six assays, with males balanced across assays according to their behavioral responses

to novelty. Intra-assay variability for a low and high control was 11.8 and 7.3%; inter-assay variability for these controls was 14.0 and 11.5%.

Life Span

Males were allowed to live their natural life span or until they displayed pre-defined symptoms indicative of non-recoverable health problems or prolonged pain (n=20 and 40) (i.e. 'endpoints': rapid excessive weight loss, dehydration, inability to ambulate and obtain food and water, labored respiration, infected or necrotic tumors, tumors that impair ability to walk with normal gait). Decisions to sacrifice were made by a veterinarian that did not know the hypotheses of the study or individual rat's behavioral or corticosterone profiles. The study was terminated when the rats were 695 days of age (23 months), at which point all surviving males were sacrificed whether they exhibited health problems or not (n=7). For these 7 males, age of death was identified as the sacrifice day and classified as 'censored' data in survival analyses.

Analyses

Prior to conducting the above analyses, we analyzed whether the following factors had a significant effect on behavioral responses or glucocorticoid levels: (a) time of day tested, (b) order of testing within day, and (c) order in which males were tested on the two arenas (i.e. either tested on the novel non-social or the novel social first). These analyses were done using regression analyses (time of day) or t-tests (test order). Because corticosterone and latency to approach values were not normally distributed (according to Kolmogorov-Smirnov tests), we used log-transformed values to satisfy distribution requirements for parametric statistical analyses.

To determine if behavioral responses to different kinds of novelty were stable within individuals over time (from 4 to 8 months), for each rat we calculated a mean approach latency on the two novelty arenas at each test age (4 and 8 months), and then used Pearson's correlation analyses to determine the stability of mean latencies from 4 to 8 months of age. For further comparisons of behavior to glucocorticoid production and life span, we categorized males into three groups: (1) 'Inhibited' males, with longer-than-median approach latencies in all 4 novelty tests (2 non-social, 2 social), (2) 'Non-Inhibited' males, with faster-than-median latencies in all 4 tests, and (3) 'Mixed' males, with longer-than-median latencies on 1, 2, or 3 of the 4 novelty tests.

Corticosterone production was divided into two basic measures, one that provided an estimate of minimally stimulated corticosterone production (baseline) and one that provided an estimate of overall corticosterone reactivity to novelty. An integrative measure of glucocorticoid reactivity - area-under-the-curve (AUC) – was used to provide an overall estimate of reactivity to novelty. To compare reactivity specifically, without confounding this measure with starting basal levels, we used a recently described estimate of corticosterone reactivity that measures the relative response from baseline without incorporating baseline levels into the estimate. This measure, named "area-under-the-curve with respect to increase" (AUCi), was recently described by Pruessner and colleagues [49] and provides a measure of corticosterone reactivity that is not influenced by starting baseline levels of corticosterone. To determine if glucocorticoid production was stable within individuals over time, we compared glucocorticoid levels across test ages using Pearson's correlation analyses. We compared AUCi at 4 and 8 months, and baseline levels at 4, 6 and 8 months. To identify males that had consistently elevated glucocorticoid levels across a majority of the sampling periods, we grouped males into three categories based on their glucocorticoid levels at the 11 sampling times: (1) 'High' if they had greater-than-median levels during 8 or more samples, (2) 'Low' if they had lower-than-median levels during 8 or more samples, and (3) 'Mixed' for all other males. These criteria were selected because it represented a number of 'hits' close to that required to achieve

statistical significance in a binomial test (i.e. > 8 hits). We chose the slightly less stringent criterion because it allowed us to identify 10 individuals in each of the 'high' and 'low' groups to provide enough individuals for meaningful statistical analyses. In addition, when examining the distribution of the data, there was a marked step-like drop in the number of rats that had corticosterone values above the median at ≤ 3 or ≥ 8 of the 11 samples. These criteria also meant that to be considered stably 'high' corticosterone, a rat had to have elevated glucocorticoid levels for several samples at both the 4 and 8 month sampling periods. We further grouped males according to their baseline corticosterone production and their reactivity production in response to novelty. Specifically, males were identified as having either higher-than-median levels at all three baseline sampling times (4, 6 & 8 months) or lower-than-median levels at all three points ('High' vs. 'Low'). Additionally, males were identified as having either greater-than-median AUC_i levels at both test ages (4 & 8 months) or lower-than-median levels at both ages ('High' vs. 'Low'). We used χ^2 analyses to determine if Inhibited males were also those with High glucocorticoid levels. Survival analyses (log-rank χ^2) were used to determine if stable neophobia and/or stably elevated glucocorticoid levels were significantly associated with shortened life spans.

One male had to be sacrificed prior to the second behavioral tests at 8 months, thus, for behavioral analyses of stability, there were data from 59 males. For glucocorticoid analyses, samples greater than 3 standard deviations from the mean were removed as outliers. Based on this criterion, there was a full set of repeat blood samples at both test ages for 53 males. Statistical significance was set at 0.05 and all results are reported as means $\pm$ SEM, unless otherwise stated.

RESULTS

Time of testing and order of testing (within each test day, or across the two novel arenas) was not associated with male approach latencies or glucocorticoid levels (all p-values > 0.10), therefore all males were analyzed together, regardless of time of day or order of testing.

General Description of Behavior and Glucocorticoid Production at Two Test Ages

Males approached novelty slightly slower at the later test age (8 months) compared to the earlier age (2.5–4 months) but these differences were not statistically significant (non-social median latencies: 23.0 vs. 25.5s paired $t_{58}=1.59$, ns; social median latencies: 43.0 vs. 33.0s, paired $t_{58}=1.69$, $p=.10$). Corticosterone reactivity (AUC_i) and baseline levels were comparable across test ages (AUC_i: 189 ± 21 vs. 209 ± 18 ng/ml*hr; paired $t_{52}=1.20$, ns; baseline: 133 ± 7 vs. 137 ± 8 vs. 118 ± 7 ng/ml; paired $t_{52}=0.49, 1.99, 0.81$, ns). Baseline corticosterone values were in the range expected for the sampling method (tail clip) and the time of day (middle of the dark phase) [50,51].

Stability of Behavioral and Glucocorticoid Responses to Novelty

Individual rat mean approach latencies in the two arenas were relatively stable across the 4-month test-retest interval; males that had slow mean approach latencies in the novel social and non-social arenas at 2.5–4 months were again slow to approach at 8 months (Figure 2; $r_{59}=0.39$, $p<.01$), suggesting latency to approach novelty is a stable trait.

Individual rat corticosterone responses to novelty (AUC_i) were very consistent across the 4-month test-retest interval (Figure 3a; $r_{53}=0.66$, $p<.0001$), and individual baseline levels were relatively stable over a 2-month test-retest interval, although not highly stable across 4 months (Figure 3b; 4 vs. 6 month: $r_{53}=0.37$, $p<.01$; 6 vs. 8 months: $r_{53}=0.23$, $p<.10$; 4 vs. 8 months: $r_{53}=0.19$, ns). In other words, a rat that produced high levels of corticosterone at 4 months was highly likely to produce high levels at 6 and 8 months as well.

Relation between Neophobia and Glucocorticoid Production Over Time

Group Assignment—Of the 53 males that had both behavioral and corticosterone data, 10 were categorized as Inhibited and 6 as Non-Inhibited. Of the remaining 38 males, 10 were slower than median on 1 of the 4 tests, 18 were slower on 2 of the 4 tests, and 10 were slower on 3 of the 4 tests (all categorized as Mixed). When categorized according to their corticosterone production at both ages, 10 males had High corticosterone production, 10 had Low production, and 33 had Mixed levels.

Unexpectedly, the Inhibited males were not necessarily the High corticosterone males (Table 1; $\chi^2=0.90$, $df=4$, ns). In addition, mean latency across the 4 novelty arenas was not linearly related to mean baseline or AUCi corticosterone values across ages ($r_{51}=0.05$, ns; $r_{51}=-0.09$, ns), and mean latency on either novel arena across time was not related to these corticosterone values (non-social: $r_{51}=-0.15$, ns; $r_{51}=-0.02$, ns; social: $r_{51}=0.03$, ns; $r_{51}=0.13$, ns). Finally, Inhibited males did not have significantly higher baseline or AUCi corticosterone levels than Non-Inhibited males (Table 2a: baseline: $t_{13}=0.85$, ns; AUCi: $t_{13}=0.58$, ns), and High corticosterone males were not slower to approach novelty than Low corticosterone males (Table 2b; $t_{18}=0.34$, ns).

Behavioral and Glucocorticoid Stability as Predictors of Life Span

Stable neophobia and elevated glucocorticoid levels over time were not highly related in this population. Not all Inhibited males had High corticosterone baseline or reactivity levels across time and not all Non-Inhibited males had Low corticosterone levels over time (Table 1). These results suggest that stable inhibition as defined here does not necessarily associate with stably elevated glucocorticoid production over time or across situations.

Inhibited males died significantly earlier than Mixed or Non-Inhibited males (Figure 4; $\chi^2=15.13$, $df=2$, $p<.001$). The median life span for Inhibited males was 32% shorter than for Non-Inhibited males (Table 2a; 471 vs. 695 days). The magnitude of this difference was further accentuated by the number of males of each category that were able to survive to the end of the study (695 days). None of the 10 Inhibited males survived to the end of the study whereas 5/9 (56%) of the Non-Inhibited survived to the end. Median life spans for Mixed males was intermediate to that of the Inhibited or Non-Inhibited (578 days). Interestingly, males that had consistently longer-than-median latencies on the non-social arena across test ages had a shorter life span than those that had consistently shorter-than-median latencies ($\chi^2=12.80$, $df=2$, $p<.01$; median life spans: Inhibited=561 vs. Non-Inhibited=605 days). On the other hand, males with consistently longer-than-median latencies on the social arena did not have a significantly shorter life span than those with shorter-than-median latencies on this test at both ages ($\chi^2=3.89$, $df=2$, $p=0.14$; median life spans: Inhibited=578 vs. Non-Inhibited=585 days).

High corticosterone males died significantly earlier than Low males (Figure 5a; $\chi^2=9.10$, $df=2$, $p<.01$). Median life span for High males was 23% shorter compared to Low or Mixed males (Table 2b; 475 vs. 596 or 586 days). The median life span for High males (475 days) was comparable to that of the Inhibited males (471 days). When the corticosterone-life span relationship was more closely evaluated, we found that baseline corticosterone production was a better predictor of life span than corticosterone reactivity (AUCi). Specifically, males with consistently elevated *baseline* corticosterone levels died approximately one month earlier than males with consistently low levels (Figure 5b; $\chi^2=3.16$, $df=1$, $p=.076$), whereas males with consistently elevated corticosterone *reactivity* levels did not die any earlier than those with consistently low reactivity levels (Figure 5c; $\chi^2=0.21$, $df=1$, $p=.65$).

To determine if there was an additive influence of stable neophobia and elevated glucocorticoid production, males were classified as having 0, 1, or 2 of these risk factors (i.e. Inhibited or

High corticosterone levels). Males that were both Inhibited and had High corticosterone levels across time (2 risk factors) had a 30% reduction in life span compared to males with neither (0) risk factor (408 vs. 592 days; Figure 6 & Table 2c; $\chi^2=22.40$, $df=2$, $p<.0001$), and median life span was 50 days shorter than males that were *either* Inhibited or High in corticosterone production. There were only 3 males with both risk factors and they were all dead before 460 days of age, whereas only 12% (6/50) of the remaining males had died by this age. Furthermore, Inhibited males with High corticosterone production died, on average, more than 140 days before Non-Inhibited males with High corticosterone levels.

Overall, males that were stably inhibited across four novelty testing sessions during young to middle adulthood had a median life span approximately 7.5 months shorter than stably non-inhibited males. In addition, males with consistently elevated corticosterone production during this time had a life span comparable to that of stably inhibited males, and on average about 4 months shorter than males without consistently elevated corticosterone production.

Interestingly, males with elevated corticosterone production during young to middle adulthood were not necessarily the same males that were stably inhibited during this age. However, there appeared to be an additive influence of neophobia and elevated corticosterone production, in that males that were both stably inhibited and had stably high glucocorticoid production had the shortest life span of all – more than 2 months shorter than stably inhibited males or males with stably elevated corticosterone production. Finally, considering corticosterone dynamics, stably elevated *baseline* glucocorticoid levels were more closely associated with a shortened life span than stably elevated *reactivity* levels.

DISCUSSION

In the present study, behavioral and glucocorticoid responses to novelty were relatively stable traits in male rats during young adulthood (4 to 8 months of age). However, these two traits were not closely related to one another – i.e. stably inhibited rats did not necessarily have stably elevated glucocorticoid levels. Interestingly, both stable neophobia and stably elevated glucocorticoid production were significant predictors of a shortened life span. The median life span for rats consistently inhibited across time and situations (non-social and social) was approximately 7.5 months shorter than for consistently non-inhibited rats. The magnitude of this difference in median life span is approximately 2-times that found in earlier studies in which rodents that were identified as neophobic/non-exploratory or neophilic/exploratory based on their response to one novel (non-social) environment [10,11]. Additionally, in comparison to an earlier report with group-housed Sprague-Dawley rats, in the current study we found a shorter median life span overall with individually-housed Sprague-Dawley rats (590 days relative to 650 days), which may indicate that individual housing may have been one factor that accelerated mortality in the current study. This suggests isolation stress may have existed in the current study, although a series of other factors (e.g. different laboratory facility, etc.) may have also contributed to this difference. Consistently elevated glucocorticoid levels during young to middle adulthood also predicted a shortened life span in male rats. In particular, baseline corticosterone levels were a better predictor of life span than reactivity levels. These results suggest that glucocorticoid overproduction may be an important mechanism of shortened life span in behaviorally-inhibited rodents, but that multiple other physiological processes must also underlie this relationship between temperament and life span.

Behavioral & Glucocorticoid Stability

Mean approach latencies to non-social and social novelty were relatively stable over a 4-month period in young adulthood. The degree of stability in this behavioral response ($r=0.39$) is slightly lower than the degree of stability observed in young children ($r=0.52$) [12], although

the period between test and retest in the current study with rats represents a longer proportion (0.13) of the average life span than is true in most studies of human behavioral inhibition (0.05) (e.g. [12]). In addition, behavioral correlation coefficients for children were calculated based on children pre-selected to fall into the two ends of the behavioral inhibition spectrum. The importance of trait stability has been shown in a study of behaviorally-inhibited children in which stably inhibited children were at increased risk of developing anxiety disorders whereas those that were inhibited at some times but not at others were at no greater risk than uninhibited children [52]. Behavioral stability has been documented previously in rats using different behavioral tests [25,26]. The current work supports these prior findings of behavioral stability over a significant portion of the life span, and shows this stability in a novel behavioral domain. It should be noted that the individual-housing used in the current study may have provided less dynamic social conditions for the rats in this study, and may therefore have heightened individual stability in behavior and glucocorticoid production over time. However, importantly, not all males that were inhibited at 2.5–4 months remained inhibited at 8 months. This behavioral flexibility or rigidity is important to take into account when considering how behavior may relate to underlying physiology and future health and aging trajectories. Only through repeated testing can we identify the most stably inhibited individuals and develop more accurate predictors of health outcomes [52,53].

Comparable to behavioral stability, the stability of glucocorticoid production within individuals over time and across situations has been infrequently tested. Evidence from prior studies suggests that glucocorticoid responses to challenges are relatively stable within individuals across a relatively short time interval (e.g. [26,28,29]). Márquez and colleagues documented individually stable glucocorticoid responses to a variety of challenges in male rats across several days in young adulthood [26,54], and Cockrem and colleagues showed individually stable glucocorticoid responses to handling procedures over several weeks in captive birds, although basal levels were not stable [28]. Similar stability was found in older women tested twice in a standardized stressor task administered at a one year interval: individual baseline and reactivity levels were relatively stable over time [29]. Furthermore, basal plasma cortisol levels were relatively stable in older men and women repeat tested 2.5 years apart [27], and this physiological stability has been documented in other stress-related physiological systems (e.g. [55]). Findings from the current study are comparable to the above findings – i.e. reactivity and baseline glucocorticoid levels were relatively stable over time and across situations. However, in contrast to prior studies, the present study spanned a significantly longer portion of the relative life span (18% of median life span) whereas prior reports showed stability over a much shorter portion of the life span (less than 3% of median life span). Interestingly, some stability was shown in baseline glucocorticoid levels, even with the relative noise involved in these measures. This stability in glucocorticoid production indicates that basal and reactivity glucocorticoid production represents a stable trait over a significant amount of time in both humans and rats.

In the current study, low correlation coefficients for individual baseline corticosterone levels across time may not reflect a true lack of physiological stability, but rather the fact that resting glucocorticoid measures are relatively ‘noisy’. Steroid hormones are produced in a pulsatile manner [56], therefore making a single measure a rough estimate of average production over time. Furthermore, in the current study, baseline samples were collected in the middle of the active period (4–6 hrs after lights off) when the amplitude of corticosterone pulses are still relatively high; this may have further added to the ‘noise’ in the baseline corticosterone measures [44]. In addition, glucocorticoid production is highly sensitive to environmental conditions, and can rise quite rapidly following a challenge, which means baseline measures can occur after exposure to uncontrolled low-grade husbandry room challenges (e.g. noise, movement, etc.) whereas reactivity samples are usually conducted after a standardized stressor protocol (e.g. novelty testing, restraint, etc.). Also, human baseline levels may appear more

stable over time than rodent levels, because true basal measures may be easier to collect from informed human participants compared to uninformed and vigilant rodents for whom circulating glucocorticoid levels increase rapidly during the sampling protocol. Finally, the tail clip method used for baseline measures in the current paper are associated with additional 'noise' in that they must have involved an early corticosterone reactivity response; they are approximately 10 times higher than levels measured at a similar time of day from an indwelling catheter (e.g. [47,57]). Interestingly, even with all the noise of the baseline levels, we still found that these baseline measures of corticosterone were a better predictor of life span than reactivity levels.

Few studies have compared behavioral stability to stability of physiological processes. In the current study, with a relatively strict criterion of stability, we found that stable neophobia over time and across situations was not strongly associated with consistently elevated glucocorticoid production across time and situations (either baseline or reactivity levels). This is an important finding because without repeated testing, we would assume that stable inhibition is associated with stably elevated glucocorticoid production [11,13]. Only through repeated testing can we identify those individuals that are the most stable in both of these traits, and only through this repeat testing is it possible to determine that stable behavioral differences are not associated with stable glucocorticoid over-production. This lack of association may be because we used relatively stringent criteria for behavioral and glucocorticoid stability in the current study. Alternatively, more frequent and diverse measures of behavioral and physiological profiles may better reveal possible biases in physiology that are associated with behavioral traits. However, the current results may indicate that physiological processes, other than glucocorticoid production, or a suite of physiological biases are more closely associated with stable neophobia. For example, in humans behavioral inhibition is also associated with cardiovascular function and amygdala activity [12,58-61]. An area of future investigation is to determine if different forms of behavioral inhibition exist with distinct underlying physiological profiles. Differential underlying profiles could predict different health and aging trajectories for individuals similarly identified as behaviorally-inhibited according to external behavioral traits.

Two predictors of life span

In the current study, we found that both stable neophobia and stably elevated glucocorticoid production during young adulthood were significant predictors of male rat life span. In addition, these variables seemed to have an additive influence such that stably inhibited individuals that also had stably elevated glucocorticoid production died at the earliest ages. (It should be noted that only three rats met this dual criterion, and thus cautionary interpretation of these findings is necessary.) Overall, the above results indicate that glucocorticoid production is a stable trait and is an important predictor of life span, regardless of behavior [34]. However, stable inhibition in young adulthood was a more powerful predictor of life span than glucocorticoid production, suggesting that glucocorticoid production may be one factor leading inhibited individuals to have a shortened life span, but that there are other physiological processes influencing this relationship (e.g. sympathetic activity, immune function, cardiovascular function, etc.[62]). Finally, for young adult male rats, stable inhibition in a physically novel situation may be a better predictor of life span than stable inhibition in a novel social situation. This difference in predictive power does not reflect the fact that inhibition in a non-social arena is a better predictor of elevated corticosterone production than inhibition in a social arena, but rather may reflect the fact that in a novel situation male rodents tend to engage social novelty less readily than physical novelty and that variance in individual latencies to approach non-social novelty is greater than to approach social novelty (Cavigelli & Michael, unpublished data). This basic difference in male approach latencies in a novel situation may make their

response to novel physical environments a more powerful predictor of both physiological and aging processes.

Interestingly, in terms of glucocorticoid production, the current results indicate that specific aspects of glucocorticoid production may be more important than others. Specifically, baseline glucocorticoid levels may be more important in influencing life span than reactivity levels. This supports the hypothesis that long-term elevations in basal production may pose greater cumulative threat to other physiological systems than elevated short-term reactivity levels [63,64]. Overall, it appears that a complex profile of physiological function will provide the most accurate predictor of a shortened life span in inhibited individuals. Some of these traits may be related to glucocorticoid production [65], while others may not. Physiological biases associated with behavioral traits are important mechanisms by which temperament may influence health outcomes; this complements work showing that differential health behaviors across temperaments influence health and longevity [6].

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Figure 1.
Study timeline.

Figure 2.
Comparison of male mean approach latencies on two novel arenas (one non-social and one social) from 2.5–4 to 8 months of age.

a. **b.**

Figure 3. Comparison of (a) male corticosterone reactivity (AUCi) at 4 and 8 months of age, and (b) male baseline corticosterone at 4 and 6 months of age.

Figure 4.

Life span for males that were stably inhibited (black) was significantly shorter than for stably non-inhibited (white) and mixed (grey).

Figure 5.

(a) Males with consistently high *overall* corticosterone production (black) lived shorter than males with consistently low (white) and mixed levels (grey). (b) Males with consistently high *baseline* corticosterone production (black) lived shorter than males with consistently low levels (white). (c) Males with consistently high *reactivity* (AUCi) corticosterone levels (black) did not have a shorter life span than males with consistently low levels (white).

Figure 6.

Males that were stably inhibited and had stably elevated corticosterone production (black) lived significantly shorter than males that were *either* inhibited or had high corticosterone (grey) or males that were *neither* inhibited nor had high corticosterone (white).

Tables 1 a, b, c

Males cross-tabulated according to their behavioral and glucocorticoid stability group assignments. Stably inhibited males were not more likely to have stably high glucocorticoid levels, either at baseline (a), in response to novelty (b), or across all 11 repeat blood samples (c) (n=53).

a			
BEHAVIOR	<i>Baseline</i> CORTICOSTERONE		
	Stably HIGH	Mixed	Stably LOW
Stably INHIBITED	3	4	2
Mixed	9	20	9
Stably NON-INHIBITED	1	4	1

b			
BEHAVIOR	<i>AUC_i</i> CORTICOSTERONE		
	Stably HIGH	Mixed	Stably LOW
Stably INHIBITED	3	2	4
Mixed	17	6	15
Stably NON-INHIBITED	1	2	3

c			
BEHAVIOR	<i>Overall</i> CORTICOSTERONE		
	Stably HIGH	Mixed	Stably LOW
Stably INHIBITED	3	4	2
Mixed	7	24	7
Stably NON-INHIBITED	0	5	1

TABLE 2 a, b, c

Median (and range) of approach latencies, corticosterone production, and life span for males according to their assigned behavioral (a) corticosterone (b) and risk factor (c) categories. T-test results compare inhibited vs. non-inhibited males and high vs. low corticosterone males.

a	BEHAVIOR			t
	INHIBITED (n=9)	Mixed (n=38)	NON-INHIBITED (n=6)	
Approach Latency (s)	106 (44–168)	38 (12–132)	15 (10–27)	7.15***
Baseline CORT (ng/ml)	116 (68–214)	124 (38–205)	118 (90–140)	0.85
AUCi CORT (ng/ml * hr)	153 (9–346)	229 (–88–475)	220 (77–322)	0.58
Life Span (days)	471 (319–600)	578 (284–669)	695 (562–695)	2.96*

b	CORTICOSTERONE			t
	HIGH (n=10)	Mixed (n=33)	LOW (n=10)	
Approach latency (s)	51 (23–168)	40 (10–143)	34 (18–116)	0.34
Baseline CORT (ng/ml)	171 (113–203)	116 (65–214)	104 (38–145)	4.67***
AUCi CORT (ng/ml * hr)	212 (–47–330)	262 (–88–475)	183 (28–322)	0.22
Life Span (days)	475 (284–621)	586 (319–695)	596 (410–695)	2.59*

c	BEHAVIOR & CORTICOSTERONE			t
	INHIBITED and HIGH CORT (n= 3)	INHIBITED or HIGH CORT (n= 13)	NON-INHIBITED and/or LOW CORT (n= 37)	
Approach latency (s)	55 (46–162)	52 (16–108)	35 (10–133)	3.16**
Baseline CORT (ng/ml)	155 (113–201)	167 (68–214)	116 (38–205)	1.85 [†]
AUCi CORT (ng/ml * hr)	260 (111–268)	153 (–47–346)	237 (–88–475)	0.02
Life Span (days)	408 (335–457)	561 (284–621)	592 (352–695)	4.31***

[†]
p<.10

*
p<.05

**
p<.01

p<.001