Pituitary hormone secretion in familial longevity : The Switchbox Study

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CHAPTER 8

Physiological Responding to Stress in Middle-Aged Males Enriched for Longevity: A Social Stress Study

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ABSTRACT

Individuals enriched for familial longevity display a lower prevalence of age-related diseases, such as cardiovascular- and metabolic diseases. Since these diseases are associated with stress and increased cortisol levels, one of the underlying mechanisms that may contribute to healthy longevity might be a more adaptive response to stress. To investigate this, male middle-aged offspring from long-lived families (n=31) and male non-offspring (with no familial history of longevity) (n=26) were randomly allocated to the Trier Social Stress Test or a control condition in an experimental design. Physiological (cortisol, blood pressure, heart rate) and subjective responses were measured during the entire procedure. The results showed that Offspring had lower overall cortisol levels compared to Non-offspring regardless of condition, and lower absolute cortisol output (AUCg) during stress compared to Non-Offspring, while the increase (AUC_i) did not differ between groups. In addition, systolic blood pressure in Offspring was lower compared to Non-offspring during the entire procedure. At baseline, Offspring had significantly lower systolic bloodpressure and reported less subjective stress than Non-offspring and showed a trend towards lower heart rate. Offspring from long-lived families might thus be less stressed prior to potentially stressful events and consequently show overall lower levels in physiological responses. Although attenuated physiological responding cannot be ruled out, lower starting points and a lower peak level in physiological responding when confronted with an actual stressor, might already limit damage due to stress over a lifetime. Lower physiological responding may also contribute to the lower prevalence of cardiovascular diseases and other stress-related diseases in healthy longevity.

INTRODUCTION

With the expansion of the aging population, the prevalence of all major age related diseases is increasing. Studies investigating individuals who have the propensity to reach old age in good health are important to disentangle mechanisms that lead to healthy human longevity. For instance, individuals from long-lived families display lower prevalence of age-related diseases, such as cardiovascular and metabolic diseases(1-4). Since cardiovascular diseases, diabetes mellitus, metabolic syndrome have been associated with stress and increased cortisol levels(5-9), one of the underlying mechanisms that may contribute to healthy human familial longevity might be a more adaptive stress response.

Evidence from animal and human aging studies suggest that activity of the hypothalamic-pituitary-adrenal (HPA) axis contributes to biological aging, for instance through elevated cortisol release(10). Aging has been associated with increased basal cortisol levels(11-13), and high basal cortisol levels have been associated with e.g., physical frailty, insulin resistance, high blood pressure, impaired memory, which are all hallmarks of aging(5, 14-17). Interestingly, middle-aged individuals from long-lived families show lower basal HPA-activity(18), lower circulating glucose levels(3), increased insulin sensitivity(19), a lower prevalence of cardiovascular disease(4), metabolic syndrome(2) and mortality(1) than individuals who are not "enriched for longevity". Therefore, one of the underlying mechanism of healthy longevity may be an altered HPA-axis reactivity.

With regard to HPA-axis reactivity in aging, older individuals generally respond with an increased cortisol response to pharmacological challenge compared to young individuals(20). Studies using psychological challenges, such as the Trier Social Stress Test (TSST), showed that stress-induced cortisol elevations in aged individuals were higher than in young individuals(20, 21), especially in male participants(22-26). Aging thus appears to be related to an increased salivary cortisol response to social stress, particularly in aged males.

Given that individuals enriched for familial longevity are thought to be biologically younger than age-matched peers(4), their physiological response to stress might be more reflective of that of younger individuals, with a lower cortisol response to stress. This lower stress responsiveness could be one of the underlying mechanisms of their healthy phenotype. To investigate this, we randomly allocated male offspring from long-lived families ("Offspring"), and males who did not meet our criteria for familial longevity ("Non-offspring") to acute stress using the TSST or a non-stressful control condition in an experimental design. We hypothesized that offspring would respond with lower cortisol levels in response to social stress than their matched peers. As aging has been shown to be related to higher blood pressure (Bp) in response to challenges (e.g. cold water stress) than younger individuals(27, 28), we also expected

that offspring would respond with lower Bp to social stress than non-offspring. We had no clear expectation on the heart rate (HR) response to the TSST, given that findings on heart rate response to stress are contradictory(21, 23, 26-29).

METHODS

Participants

For the present study, 59 male volunteers who were eligible and willing to participate were included. Only males were included given the sex dependency of the cortisol response to stress in both the young(30, 31) and aged individuals, showing a significantly higher free salivary cortisol response to stress in aged men than in aged women (22-24). Participants were recruited via letters from the participant-pool of the Leiden Longevity Study (LLS). The LLS is a study consisting of 421 Caucasian families, enrolled between 2002 and 2006(1, 4). The LLS families comprise nonagenarians, their male or female offspring and the offspring's partners. Criteria for familial longevity are that at least 2 long-lived siblings are still alive and meet the age criteria of ≥ 89 yrs for men and ≥ 91 yrs for women. Only male volunteers from the LLS were screened for inclusion, half of them were included because their parents met the above described criteria for familial longevity (Offspring), the other half consisted of males whose parents did not meet the criteria for familial longevity (male Non-offspring, who are partners of female Offspring). Besides being male, other inclusion criteria were: being middle-aged (55-77 years) and having a stable body mass index (BMI) between $19kg/m^2<BMI<33kg/m^2$. The following exclusion criteria were used: the use of any hormone medication (including oral, nasal and inhalation corticosteroids) or having a current depression or other psychiatric disease influencing the HPA-axis as assessed with the MINI(32) and the Geriatric depression scale (GDS)(33). In addition, participants were excluded if their fasting plasma glucose was above 7 mmol/L, or if they had any significant chronic, renal, hepatic or endocrine disease, or if they used any medication known to influence lipolysis, thyroid function, glucose metabolism, GH/IGF-1 secretion or any other hormonal axis. Other exclusion criteria were smoking- and alcohol addiction, and extreme diet therapies. Based on the exclusion criteria, one male (Non-offspring) who scored > 11 points on the 30 items geriatric depression scale (GDS-30), indicating a mild depression was excluded. In addition, 1 participant (Offspring) was excluded for analysis due to abnormally high saliva cortisol levels (> 92 nmol/L). The final sample thus consisted of 57 male participants (31 Offspring and 26 Non-offspring). Upon inclusion, participants of each Group (Offspring vs Non-offspring) were randomly allocated to the Stress or Control condition in an experimental design.

Table 1 Participant characteristics of Group (Offspring vs Non-offspring) by Condition (Control vs Stress)

	Offspring		Non-offspring	
	Control (n=15)	Stress (n=16)	Control (n=15)	Stress (n=11)
Age mother (yrs)	87.6 ± 11.2*	93.6 ± 4.7*	77.2 ± 17.8*	80.1 ±15.1*
Age father (yrs)	88.7 ± 11.0*	81.6 ± 19.5*	74.8 ± 9.1*	71.3 ±10*
Age (yrs)	67.1 ± 5.0	66.1 ± 8.3	67.1 ± 5.4	64.7 ± 3.3
BMI (kg/m^2)	27.2 ± 4.0	26.6 ± 3.2	26.0 ± 3.1	25.9 ± 3.0
STAI trait	32.3 ± 5.9	28.8 ± 5.2	30.2 ± 6.3	28.8 ± 4.4
SCL-90	114.8 ± 12.2	109.4 ± 12	116.7 ±14.7	115.4 ±15.7
Cardiovascular disease (%)	2 (15)	1 (6.3)	3 (20)	1 (9.1)
Hypertension (%)	4 (26.7) [a]	3 (18.8) [b]	5 (33.3) [c]	2 (18.2)[d]

BMI = Body Mass Index; STAI-trait = Trait version of the State-trait anxiety inventory; SCL-90 = symptom checklist 90.

[a.] 1 offspring used a combination of AT2-antagonist, β-blocker and diuretic; 1 offspring used a combination of diuretics, a calcium channel blocker and an ACE-inhibitor; 1 offspring used an ACE-inhibitor; 1 Offspring used diuretics;

[b.] 1 Offspring used a combination of β-blocker, ACE-inhibitor and diuretics, 2 Offspring used a combination of β-blocker and diuretic;

[c.] 1 Non-offspring used a combination of diuretics and ACE-inhibitor, 1 used a combination of diuretic, calcium channel blocker and AT2-antagonist, 3 used an AT2-antagonist;

[d.] 1 non-offspring used a combination of diuretics and ACE-inhibitor; 1 used an ACE-inhibitor.

ACE-inhibitor = angiotensin-converting-enzyme inhibitor; AT2-antagonist= angiotensin 2 receptor antagonist;

* = Offspring ≠ Non-offspring, $p = .001$

Means and standard deviations (*SD*s) of subject characteristics of the study population are presented in Table 1. As we selected the Offspring group on the age of the parents, both the age of the mother and father were significantly higher in the Offspring group compared to the Non-Offspring (Age mother: $F(1, 57) = 11.97$, $p = .001$; Age father: $F(1, 57) = 11.22$, $p = .001$). There were no differences between groups or condition in age, BMI, prevalence of cardiovascular disease or hypertension. Only in the Offspring group beta blockers were used in combination with other anti-hypertensive medication, whereas the Non-offspring used different types of anti-hypertensive medication without additional beta blockers. Mean total scores on psychoneuroticism (symptom checklist 90; SCL-90(34)) and trait anxiety (STAI(35)) were within the normal range according to norm scores for a healthy population.

The study was approved by the Medical Ethics Committee of the Leiden University Medical Centre and was performed according to the Helsinki declaration(36). All participants gave written informed consent before participation.

Stress-induction

To induce psychological stress the Trier Social Stress Test (TSST) was used(37). This is a widely used laboratory protocol that reliably stimulates biomarkers of stress, and consists of a 10-min speech preparation period in anticipation of a 5-minute free speech and a 5-min arithmetic task (counting backwards from 1033 to zero, in steps of 13) performed in front of a selection committee of three alleged experts in non-verbal signs of stress, a camera and band recorder. During the arithmetic task, one committee member responded to incorrect answers by saying out loud "incorrect, please start again from 1033", while keeping up the participants performance by means of a clearly visible score sheet.

In the control condition, participants used the same anticipation period of 10-min to prepare a speech about a book or movie of their own choice. Thereafter they had to speak out loud about this book or movie for 5 minutes followed by a 5 min period of arithmetics (counting backwards from 50 to zero at their own pace)(38). The presentation and arithmetics were performed in the same laboratory room as in the stress condition, but without an audience, camera or other recording devices.

Physiological assessments

Salivary cortisol was assessed using Salivettes (Sarstedt, Nümbrecht, Germany). All saliva samples were immediately stored after the experiment at -20°C.

Systolic blood pressure (SBP, mmHg), diastolic blood pressure (DBP, mmHg) and heart rate (HR, bpm) were recorded using an automatic blood pressure monitor (OMRON, R5-I). BP and HR were measured in the same arm during the whole study period.

Subjective stress

Subjective stress was measured using a visual analogue scale (VAS) score during the whole study period at the same time points when the physiological measurements were taken. Participants were asked to rate their current level of stress on a scale from 0 to 10, ("0" *not stressed at all*, to "10" *highly stressed)*.

Procedure

Screening. Volunteers first underwent a standardized screening by telephone. Participants were asked about their present medication use and medical history, and the given information was then checked with data records obtained from the pharmacy and general practitioner (see Table 1 for medication use). Past and present

psychiatric symptoms were also assessed using a shortened version of the MINI international neuropsychiatric interview (MINI)(32).

Experiment. All participants started the stress or control procedure at 08.00h, or at 09.15h. The timing of stress and control condition were balanced over participants, to reduce variation in morning cortisol levels due to differences in clock time as much as possible between groups. Participants were asked to refrain from taking medication, eating and drinking caffeine-, sugar- or alcohol-containing beverages starting at 22.00h the evening before the experiment. After arrival, participants were seated in a quiet room where information was given about the study day and written informed consent was obtained. Next, each participant was brought to the dressing room to change into the obligatory hospital clothing. Subsequently the TSST protocol started with instructions (i.e., to prepare a presentation). Saliva samples were obtained at four times: immediately before TSST instructions (T0 "baseline"), after the preparation phase of the TSST (T1 "pre-speech"), at the end of the TSST (T2 "post-TSST"), and 50 minutes later (T3 "post-experiment"). Between the last two samples, tasks were performed inside a MRI-scanner (to be published elsewhere). Blood pressure, heart rate and subjective stress were sampled at the four sample time points. After the final sample time point, participants were administered questionnaires and weight and height were measured using the same weighing scale and height meter for all participant. Thereafter participants continued with a protocol unrelated to the present experiment.

Chemical analysis

All saliva cortisol samples were assayed at Professor Kirschbaum`s laboratory (http://biopsychologie.tu-dresde.de). Cortisol concentrations in saliva were measured using a commercially available chemiluminescence-immunoassay kit with high sensitivity (IBL, Hamburg, Germany). Inter-and intra-assay coefficients of variation were below 10%.

Data analysis

Raw cortisol levels, SBP, DBP and heart rate measurements were checked for outliers. Outliers were defined as values beyond 3 standard deviations (*SD*) below or above the mean and were replaced by the mean plus 3 *SDs*. Missing cortisol values, due to limited amount of saliva, were replaced by the mean per group (Stress vs Control). The dependent variables cortisol, BP, HR and subjective measurements of stress were analyzed using repeated measures (RM) ANOVAs, with Group (Offspring/Non-offspring) and Condition (Stress/No-Stress) as between-subjects factors and Time (T0-T3) as within-subject factors and followed up by *t*-tests. Greenhouse-Geisser corrections were applied when appropriate.

For analyses the Statistical Package for the Social Sciences program for Windows, version 20 (SPSS, Chicago, IL) was used. $p < .05$ was considered statistically significant.

RESULTS

Cortisol

A RM-ANOVA was performed with Group (Offspring vs. Non-offspring) and Condition (Control vs Stress) as between-subjects factors and Time (T0, T1, T2, T3) as within-subject factor, and cortisol levels as dependent variable. There was a significant effect of Time (F[2.25; 119.46] = 4.15, p = .02, = 0.07), and Condition (F(1, 53) = 5.93, p = .02, = 0.101), with higher cortisol levels in the Stress condition ($M \pm SE$: 15.8 ± 1.0) compared to the Control condition ($M \pm SE$: 12.4 ± 0.9), and a significant Condition by Time interaction (F(3, 159) = 4.06, p = .008, = 0.07), showing that the stress manipulation was successful with no difference between Stress and Control at baseline (T0) (t_{55} = -0.13, p = .89) or after the preparation phase (T1) (t_{55} = -1.47, p = .15), and significantly higher cortisol levels in the Stress- than in the Control condition immediately after the TSST (T2) (t_{55} = 2.25, p = .03), and 50 minutes after the TSST (T3) (t_{55} = 2.31, p = .03) (see Figure 1).

In addition, there was a significant between-subjects effect of Group (F[1, 53] = 5.52, p =.02, = 0.094), indicating that overall mean cortisol levels, regardless of Condition, were lower in Offspring ($M \pm SE$: 12.5 ± 0.9) than in Non-offspring ($M \pm SE$: 15.7 ± 1.0) (see Figure 2A). There was no Group by Time interaction (F[3, 159] = 1.37,

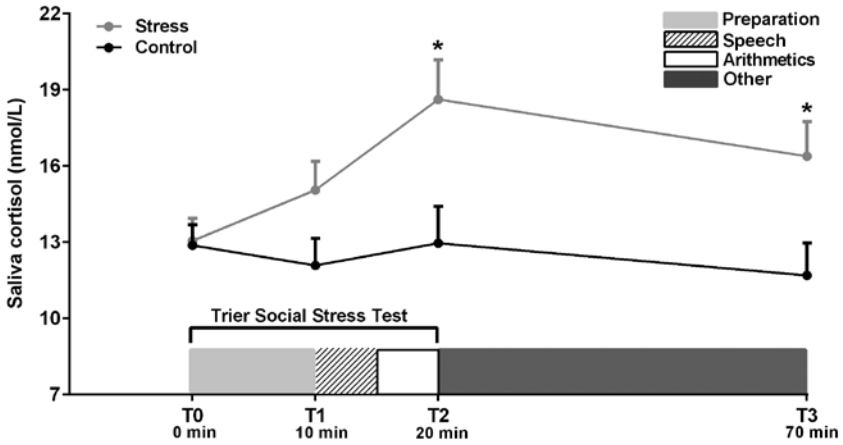

Figure 1 Mean saliva cortisol levels and standard error in the Stress- and in the Control condition

Cortisol levels rise significantly in response to the Trier Social Stress Test in the stress condition compared to the control condition. *$p \leq .05$

p = .25), but there was a significant quadratic contrast of Group by Time (F[1, 53] = 5.84, p = .019, = 0.099. Follow-up t-tests showed that there was no difference between Offspring ($M \pm SE$ = 11.97 ± 0.77) and Non-Offspring ($M \pm SE$ = 13.81 ± 0.93) at T0 (t_{55} =1,55, p = .13), a significantly lower mean cortisol in Offspring than in Non-Offspring ($M \pm SE$, Offspring= 11.45 ± 0.77; Non-Offspring =15.34 ± 1.47) after the anticipation phase, T1 ($t_{38,18}$ = 2.35, p = .024, equal variances not assumed) and a trend towards lower cortisol levels at T2 in Offspring ($M \pm SE$ =13.52 ± 1.45) compared to Non-offspring ($M \pm SE$ =17.43 ± 1.73) (t_{55} = 1.75, p = .086). Finally, there was no significant Group by Condition interaction (F(1, 53) = 2.46, p = .12, = 0.044) and no three-way interaction between Group, Condition and Time (F[3, 159] = 0.30, p = .83).

A univariate ANOVA with baseline cortisol level as dependent variable showed that cortisol levels at baseline were not significantly different between Groups (F[1, 56] = 2.65, p = .11) or Condition (F[1, 56] = 0.02, p = .89). There was also no Group by Condition interaction at baseline (F[1, 56] = 1.89, p = .18), which suggested that overall lower mean cortisol levels in the Offspring group were not indicative of pre-existing differences, but of lower responses to stress over time (see figure 3). We therefore additionally calculated the area under the curve with respect to the ground (AUC_g) to assess the differences in total cortisol output between the groups, and the area under the curve with respect to increase (AUC_i), to assess the differences in the rate of change(39). An ANOVA with Group as fixed factor and AUC_g as dependent variable showed that under control conditions, AUC_g did not differ between groups (F[1, 30] = 0.61, p = .44), nor did the AUC_i differ between groups (F[1, 30] = 0.09, p = .76). In the stress condition, Offspring had a significantly smaller AUC_g in comparison to the non-Offspring (F[1, 27] = 5.37, p =.029, = 0.177), but there was no difference in AUC_i (F[1, 27] = 0.86, p = .36).

Heart rate

The RM-ANOVA with heart rate (HR) as dependent variable showed an effect of Condition at trend levels (F[1, 51] = 3.11, p = .08, = 0.058), with slightly higher HR in the Stress condition ($M \pm SE$: 67.9 ± 1.8) compared to the Control condition ($M \pm SE$: 63.7 ± 1.6), no effect of Time, F(3, 153) = 1.93, p = .13, but a significant Condition by Time interaction (F[3, 153] = 10.11, $p < .0005$, = 0.165). Follow-up t-tests showed that at baseline (T0) (t_{54} = -1.11, p = .91), and at T1 ($t_{42.8}$ = 0.71, p = .48), there were no differences between the groups. Directly after stress (T2) participants in the Stress condition had significantly higher HR compared to the Control condition ($t_{37.6}$ = 2.42, p = .02) , and no differences 50 minutes after the TSST (T3) (t_{53} = 0.79, p = .43). Only right after the TSST (T2), HR was significantly higher in the Stress condition ($M \pm SE$: 69.3 ± 2.6) compared to the Control condition ($M \pm SE$: 62.1 ± 1.4, $t_{37,6}$ = 2.42, p = .02). There was a significant between-subjects effect of Group F(1, 51) = 4.58,

$p = .037$, $= 0.082$, with lower overall HR in Offspring ($M \pm SE$: 63.3 ± 1.6) compared to the Non-offspring ($M \pm SE$: 68.3 ± 1.8). There was no significant Group by Time interaction ($F[3, 153] = 0.93$, $p = .43$), no Group by Condition interaction $F(1, 51) = 0.72$, $p = .40$ and no significant three-way interaction between Group, Condition and Time $F(3, 153) = 1.02$, $p = .39$.

Univariate ANOVA´s showed that HR at baseline was lower in the Offspring group ($M \pm SE$: 62.3 ± 1.6) compared to the Non-offspring group ($M \pm SE$: 67.8 ± 1.8) ($F[1, 56] = 4.75$, $p = .034$, $= 0.081$), but there were no other pre-stress differences in HR (Condition: ($F[1, 56] = 0.012$, $p = .91$; Group by Condition: ($F[1, 56] = 1.06$, $p = .31$, $= 0.020$).

Blood pressure

Systolic blood pressure (SBP). The RM ANOVA with SBP as dependent variable showed a significant between-subjects effect of Condition $F(1, 51) = 7.52$, $p = .008$, $= 0.128$, with higher mean SBP in the stress condition ($M \pm SE$: 162 ± 2.8) compared

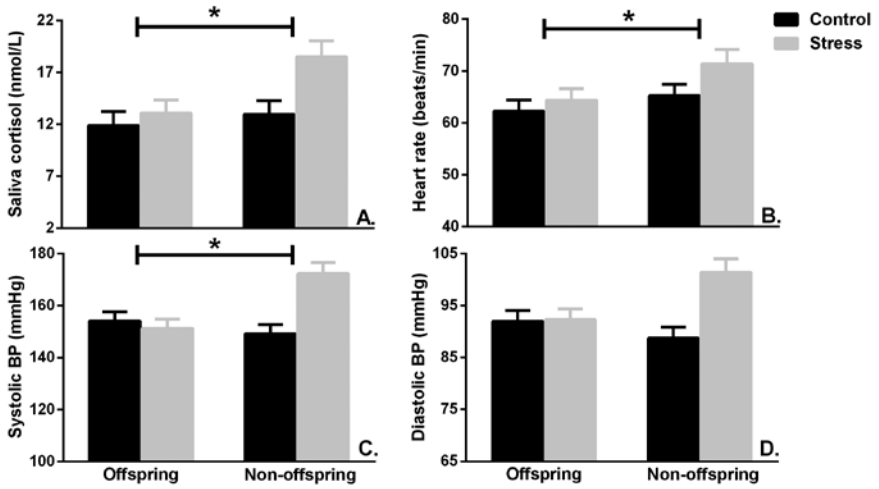

Figure 2 Overall mean physiological responses of Group (Offspring vs Non-offspring) by Condition (Control vs Stress)

Mean cortisol, heart rate and systolic blood pressure are significantly lower in Offspring compared to Non-offspring during the experiment regardless of stress or control condition.
A. Cortisol; **B**. Heart rate; **C**. Systolic blood pressure; **D**. Diastolic blood pressure.
Error bars represent the standard error of the mean.
* = Significant difference between Offspring and Non-offspring: cortisol ($p = .02$), heart rate ($p = .037$) and systolic blood pressure ($p = .03$).

to the control condition ($M \pm SE$: 152 ± 2.5). We found a significant effect of Time, $F(2.38; 121.46) = 3.27, p = .03,$ $= 0.06$, and a significant Condition by Time interaction $F(3, 153) = 7.92, p < .0005,$ $= 0.134$. Follow-up *t*-tests showed no difference between Stress and Control condition, before the start of the TSST, at baseline (T0) ($t_{54} = 0.63$, $p = .53$) and at T1 ($t_{42.1} = 1.43$, $p = .16$), while right after the TSST (T2), SBP was significantly higher in the Stress- compared to the Control condition ($t_{54} = 4.13$, $p < .0005$), a difference that had disappeared 50 minutes after TSST ($t_{39.7} = 0.64$, $p = .52$).

There was a significant between-subjects effect of Group $F(1, 51) = 4.72, p = .03,$ $= 0.085$ with lower mean SBP in Offspring ($M \pm SE$: 153 ± 2.5) compared to Non-offspring ($M \pm SE$: 161 ± 2.9). There was no significant Group by Time interaction $F(3, 153) = 0.81$, $p = .49$, and no significant three-way interaction between Group, Condition and Time ($F[3, 153] = 0.85$, $p = .47$). There was, however, a significant Group by Condition interaction $F(1, 51) = 12.18, p = .001,$ $= 0.193$. Follow-up *t*-tests showed that during the Control condition Offspring and Non-offspring did not differ in SBP ($t_{28} = 1.10$, $p = .28$), whereas in the stress condition Non-Offspring had significantly higher mean SBP than Offspring ($t_{24} = -3.60$, $p = .001$) (see Figure 3C). However, although SBP at baseline did not differ between Offspring ($M \pm SE$: 153.6 ± 3.3) and Non-offspring ($M \pm SE$: 160.1 ± 3.7, $F[1, 56] = 1.71$, $p = .20$), and also not between Control ($M \pm SE$: 155 ± 3.4) and Stress condition ($M \pm SE$: 158 ± 3.7) ($F(1, 56) = 0.40$, $p = .53$), an interaction of Condition by Group showed that the Non-Offspring in the stress condition already had higher SBP at baseline ($F[1, 56] = 4.59$, $p = .037,$ $= 0.081$). To check whether the significant RM ANOVA Condition by Group interaction was driven by pre-stress baseline differences, we calculated difference scores by subtracting baseline (T0) mean from T1, T2 and T3. A RM ANOVA performed with these new variables showed that the Condition by Group interaction was not significant when taking these baseline differences into account ($F[1, 51] = 1.15$, $p = .29$).

Diastolic blood pressure (DBP). The RM ANOVA with DBP as dependent variable showed a significant between-subjects effect of Condition $F(1, 51) = 8.27$, $p = .006$, $= 0.140$, with higher mean DBP in the stress condition ($M \pm SE$: 96.8 ± 1.7) compared to the control condition ($M \pm SE$: 90.3 ± 1.5), but no significant within-subjects effect of Time ($F[3, 153] = 1.08$, $p = .36$) and a trend for a Condition by Time interaction ($F[3, 153] = 2.53$, $p = .06$).

There was no significant between-subjects effect of Group $F(1, 51) = 1.71$, $p = .20$, no Group by Time interaction $F(3, 153) = 1.06$, $p = .37$, and no three-way interaction between Group, Condition and Time $F(3, 153) = 0.28$, $p = .84$. However, there was a significant Group by Condition interaction $F(1, 51) = 7.48$, $p = .009,$ $= 0.128$, showing a similar pattern to SBP (see Figure 3D), with significantly higher mean DBP in the Stress condition in the Non-offspring compared to the Offspring ($t_{24} = -2.90$, $p = .008$), and no differences between Offspring and Non-offspring in the Control

condition (t_{28} =1.08, p = .29). However, again, to check whether baseline differences were not driving this interaction, we ran a univariate ANOVA on the baseline means. DBP at baseline did not differ between Offspring ($M \pm SE$: 92.7 ± 1.56) and Non-offspring ($M \pm SE$: 93.6 ± 1.77, F(1, 56) = 0.15, p =.69), and also not between Control ($M \pm SE$: 91.4 ± 1.58) and Stress condition ($M \pm SE$: 94.9 ± 1.75) (F[1, 56] = 2.23, p = .14), but an interaction of Condition by Group showed that the Non-Offspring in the stress condition already had higher DBP at baseline (F[1, 56] = 4.60, p = .037, = 0.081). To investigate whether the overall Condition by Group interaction would still stand when baseline was taken into account, another RM ANOVA was performed with difference scores, which showed that the interaction was not significant anymore (F[1, 51] = 0.69, p =.41).

Subjective stress

The RM ANOVA with VAS-scores of subjective stress as dependent variable showed a significant effect of Time (F[2.60; 132.38] = 10.96, p < .0005, = 0.177), a significant between-subjects effect of Condition F(1, 51) = 4.00, p = .05, = 0.073, with higher VAS score in the Stress condition ($M \pm SE$: 3.2 ± 0.3) compared to the Control condition ($M \pm SE$: 2.3 ± 0.3), and a significant Condition by Time interaction F(3, 153) = 7.43, p < .0005, = 0.127. Follow-up t-tests showed that there was a significant

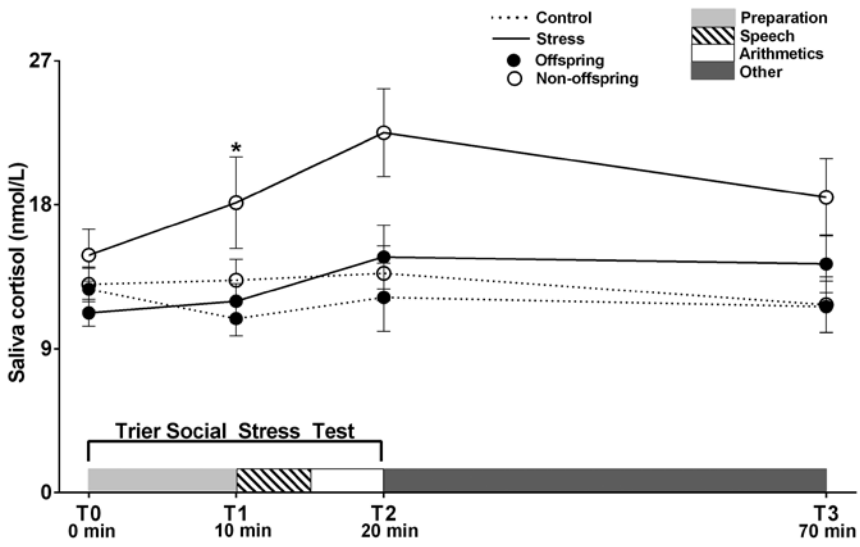

Figure 3 Mean cortisol levels on all time points in Offspring and Non-Offspring in the Control and Stress condition.

rise in VAS-scores due to the TSST in the Stress condition. At baseline (T0) (t_{54} = -0.80, p = .43) and at T1 (t_{54} = 0.71, p = .48) mean VAS-scored did not differ between the Stress and Control condition, while mean VAS-scores right after the TSST (T2) (t_{54} = 3.13, p = .003) and 50 minutes after TSST (T3) (t_{53} = 2.4, p = .02) were significantly higher in the Stress condition than in the Control condition. There was no between-subjects effect of Group $F(1, 51) = 0.92$, p = .34, no Group by Time interaction $F(3, 153) = 2.10$, p =.10, no three-way interaction between Group, Condition and Time $F(3, 153) = 0.34$, p =.80, and no Group by Condition interaction $F(1, 51) = 0.52$, p = .48

A univariate ANOVA showed that Offspring ($M \pm SE$: 1.6 ± 0.3) initially had lower VAS-scores at baseline than Non-offspring ($M \pm SE$: 2.8 ± 0.3) ($F[1, 56] = 7.39$, p = .009, = 0.124), but there were no differences between groups at all other time points in VAS assessments (see supplementary Table 1 for means and *SDs* at all time points).

Analyses without beta blocker users

Although the participants were fasted from 22h the day before and did not take their standard medication the morning of the experiment, the lower baseline HR in the Offspring might have been caused by beta blocker users (3 Offspring in the Stress- and 1 Offspring in the Control condition). Beta blockers are known to interfere with the sympathetic nervous system and the HPA-axis stress response, by decreasing adrenergic indices such as HR and Bp, and increasing cortisol levels(40-42). When the analyses were rerun without the 4 beta blocker users, the Group difference in HR at baseline was only a trend ($F(1, 52) = 3.43$, p = .07) and the RM ANOVA between-subjects effect of Group was not significant anymore ($F[1, 47] = 2.58$, p = .12). Exclusion of the beta blocker users, however, did not significantly change the results on cortisol or Bp (see Supplementary material for the analyses without the 4 beta blocker users).

DISCUSSION

Because physiological responses to stress, in particular increases in cortisol and blood pressure, have shown to increase with age(20-23, 27, 28), it was hypothesized that individuals who come from long-lived families would display attenuated physiological responses to stress compared to individuals who do not come from long-lived families. To investigate this, we exposed middle-aged males from long-lived families (“offspring”) and age-matched controls (“non-offspring”) to acute social stress using the TSST or a non-stressful condition. The stress manipulation increased cortisol, heart rate (HR), blood pressure (Bp) and subjective stress. However, during the entire procedure offspring had lower cortisol levels and systolic blood pressure

compared to non-offspring. Although the relative cortisol *increase* between groups did not differ, offspring had smaller absolute overall cortisol output in response to stress compared to non-offspring, specifically during stress anticipation.

Finding lower overall cortisol levels in offspring is consistent with earlier studies showing a tendency towards lower cortisol levels in offspring from long-lived families in the cortisol awakening response and in evening cortisol levels(18). In times of stress, cortisol levels may increase at the same rate in offspring as in non-offspring, however, the net result would be a lower lifetime exposure to cortisol. Given the strong associations between, for instance, cortisol exposure and declining cognitive function(43, 44), one of the consequences of lower exposure to cortisol could be a better maintained cognitive function, as was previously found in middle-aged offspring(45). It may be too soon, however, to conclude that offspring do not respond with attenuated cortisol to stress. First, baseline cortisol levels in offspring were not significantly lower than those of the non-offspring before stress was induced, although they were slightly lower on a descriptive level. This suggests that the overall lower cortisol levels in offspring were not specifically due to pre-existing lower cortisol levels, but - at least partly - a consequence of a smaller cortisol response to stress. Indeed, the area under the curve with respect to the ground showed significantly smaller absolute cortisol output in offspring only during stress, but not during the control condition. Because the magnitude, or relative rise, in stress-induced cortisol levels did not differ significantly between offspring and non-offspring, our results point at the importance of subtle – non-significant- baseline differences in cortisol level. Furthermore, individual differences may have prevented finding a robust attenuated cortisol response to stress in offspring, as some individuals within this group did respond with high cortisol to stress. As longevity is an inheritable phenotype, not every offspring from long-lived siblings may be enriched with familial longevity. Finally, the effect size of the interaction was between small and medium, which, together with our small sample size, indicates that the chance of a Type 2 error is not negligible. In sum, on the basis of the current results we cannot exclude the possibility that offspring actually do have an attenuated cortisol response to stress.

Similar to cortisol, systolic Bp (but not diastolic Bp) was lower in offspring than in non-offspring throughout the entire procedure, also when taking beta blocker use into account. Although Bp appeared to be specifically attenuated due to stress in the offspring, this was not the case. Regretfully, the non-offspring in the stress condition already had significantly higher Bp compared to all others, regardless of group or condition, before the stress procedure had even started. This was despite random allocation, and despite the fact that both experimenters and stress committee were blind to the offspring or non-offspring status of the participants. Still, an overall lower Bp, with a standard rise due to stress, would consistently lead to less pressure on the arteries compared to individuals with higher overall Bp. A Bp response with lower

peaks to life's stressors reduces atherosclerotic risk factors and might thus be an important protective factor with regard to the development of cardiovascular disease(46-49). Whether offspring actually has an attenuated response to stress, apart from a lower general baseline, should be studied in preferably a larger group than used in the present study to minimize unfortunate Bp distributions.

HR was also lower in offspring during the entire procedure compared to non-offspring, and the relative HR increase in response to stress did not appear to be attenuated. We, however, did not have a clear hypothesis on HR response after psychological stress, due to the inconsistent and limited amount of literature in relation to ageing(21, 23, 29), and when taking beta blocker use into account, this effect was abolished. Still, baseline heart rate tended to be lower in offspring. As lower HR at rest is associated with longer lifespan across all species(50), lower HR in offspring may be a reflection of their longevity phenotype, however, this should be investigated in a larger sample.

With regard to subjective stress, offspring were significantly more relaxed than non-offspring in the run-up to participating in the experiment but as apprehensive as non-offspring during the stress experiment itself. In addition, offspring responded with lower cortisol than non-offspring, specifically after the anticipation phase of the procedure. Maybe offspring from long-lived families have less anticipatory stress, subjectively and physiologically, prior to stressful events because they worry less. Worry in anticipation of what might be stressful in the near future and ruminations prior to actual stressful events are related to enhanced activation of cardiovascular, immune, endocrine and neurovisceral systems(51), which might contribute to diseases related to these systems(52).

A limitation of this study is the family-based study recruitment strategy. Although we selected both groups on the age of the parents, the non-offspring may also become long-lived, whereas the offspring group may very well consist of some individuals who will not live up to a high age. This might explain the individual differences in responses, such as a few offspring with a high cortisol response to stress or non-offspring with low cortisol response to stress. Another limitation of this study was the medication use of the aged participants, although these are commonly prescribed in this age category. Apart from beta blockers, that were used by 4 offspring, the use of anti-hypertensive medication was comparable between groups. All participants were fasted from 22h the day before and thus did not take their standard medication the evening before, and morning of the experiment. Another limitation was our small sample size. Given the small to medium effect sizes, this study was probably underpowered. Finally, this study cannot be generalized to females and conclusions are thus limited to males.

In sum, male offspring from long-lived families compared to male non-offspring feel less stressed before encountering a potentially stressful situation, and have

overall lower cortisol and systolic Bp compared to non-offspring during the entire event, whether it was stressful or not. Consequently, offspring reach lower peak physiological levels than non-offspring regardless of the situation they find themselves in. Offspring might actually have an attenuated physiological response to stress, as we did find indications of reduced responding to stress, such as a lower total cortisol output. As psychological stress in daily life is common up to a high age, lower physiological output may delay the onset of cardiovascular disease, and cognitive deterioration, and might therefore contribute to a healthy long-living phenotype.

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