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Repeated exposure to stressful conditions can have beneficial effects on survival

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Abstract

Repeated exposure to stressful circumstances is generally thought to be associated with increased pathology and reduced longevity. However, growing lines of evidence suggest that the effects of environmental stressors on survival and longevity depend on a multitude of factors and, under some circumstances, might be positive rather than negative. Here, using the zebra finch (*Taeniopygia guttata*), we show that repeated exposure to stressful conditions (i.e. unpredictable food availability), which induced no changes in body mass, was associated with a decrease in mortality rate and an increase in the age of death. As expected, the treated birds responded to the unpredictable food supply by increasing baseline glucocorticoid stress hormone secretion and there were no signs of habituation of this hormonal response to the treatment across time. Importantly, and consistent with previous literature, the magnitude of hormone increase induced by the treatment was significant, but relatively mild, since the baseline glucocorticoid concentrations in the treated birds were substantially lower than the peak levels that occur during an acute stress response in this species. Taken together, these data demonstrate that protracted exposure to relatively mild stressful circumstances can have beneficial lifespan effects.

Key-words. Chronic stress, unpredictable food availability, glucocorticoids, corticosterone, HPA axis, hormesis.

1.1 Introduction

Protracted or repeated exposure to stressful stimuli, such as those experienced by individuals living in uncontrollable or highly unstable environments, can have wide ranging effects on animal physiology, but the extent to which these effects are adaptive is the subject of considerate debate (Broonstra 2013). In vertebrates, one of the main systems mediating responses to stressful environmental conditions is the Hypothalamic-Pituitary-Adrenal axis (HPA axis), which regulates both basal production and transient surges of circulating glucocorticoid stress hormones. Transient increases in circulating glucocorticoids are a highly conserved component of the vertebrate stress response and play a key role in initiating an array of metabolic changes intended to mobilise energy, including hepatic gluconeogenesis and inhibition of glucose uptake by peripheral tissues (Sapolsky et al. 2000). These changes are thought to be vital for promoting short-term survival (Wingfield et al. 1998; Sapolsky 2000). At the same time, dynamic changes in basal level of glucocorticoids, such as those occurring across differing seasons within the same year in a variety of free-living vertebrate species, are also thought to be critical for survival (Romero 2002). Over a longer time scale, however, the repeated activation of the HPA axis may lead to a dysregulation and dysfunction of the stress axis (Sapolsky 2000).

The predominant view is that repeated stress exposure, and the consequent prolonged elevation of glucocorticoid levels, is harmful since it can induce a large variety of downstream negative effects, including impairment of brain functioning and immune responses (Sapolsky 1992; deKloet et al. 2005). As a consequence, it is often predicted that living in chronically stressful environments should result in long-term adverse health effects, and, therefore, in reduced longevity (McEwen & Wingfield 2003). Support for this idea comes primarily from the biomedical field, but as recently noted, it does not quite hold up in some well-studied ecological systems (Broonstra 2013). For example, snowshoe hares (*Lepus*

americanus) show evidence of chronic stress during the cyclic population declines associated with high predation risk (Sheriff et al. 2011), but during these periods they show no sign of increased pathology or relevant dysfunction of the stress axis (Cary & Keith 1979). It has been argued that, at least in these populations, animal responses to protracted or repeated stress exposure represent an evolved strategy that enables individuals to respond in a manner that gives the best fitness outcome when stressful conditions prevail (Broonstra 2013).

Experimental studies of the link between stress exposure and longevity have had conflicting results. For example, in birds, experimental elevation of glucocorticoids by twice daily oral dosing during the nestling period in zebra finches, which increases the strength of the acute stress response (Spencer et al. 2009), causes a marked reduction in adult longevity (Monaghan et al. 2012). In contrast, exposure to an unpredictable food supply in juvenile grey partridges, presumed to be associated with increased glucocorticoid levels, was found to improve survival when the animals were released in the wild at adulthood (Homberger et al. 2014). Such differences might depend on the type and degree of stress exposure (Costantini et al. 2010), the stage at which the stressful environment was experienced, and for how long the exposure occurred. In both of the aforementioned studies, the manipulations were carried out in young individuals and the stressful circumstances were imposed over a relatively short-time scale (2-3 weeks). We do not know if the same effects could occur with stress exposure in adulthood, and importantly, we do not know how survival might be altered when stress exposure is experienced over a much longer time period. Furthermore, the level and pattern of increase in glucocorticoids induced by the stressor may be very important (Costantini et al. 2010). In the zebra finch study by Spencer et al. 2009, the oral dosing with hormones caused a rapid rise in circulating levels similar to that induced by an acute stressor and the apparently permanent increase in stress reactivity means that these animals are then exposed to higher glucocorticoids whenever stressors are encountered. It is possible that the elevation of

hormone levels induced by an unpredictable food supply, which generally elevate baseline glucocorticoids (e.g. Pravosudov et al. 2001; Jenni-Eiermann et al. 2008), is less severe.

In this study, we examined the effects of prolonged and repeated exposure to stressful conditions in adulthood on long term survival in the zebra finch (*Taeniopygia guttata*). The stress exposure that we used was random withdrawal of food, mimicking a natural environmental stressor in unpredictable environments. Previous studies in birds that have used similar intermittent food withdrawal protocols have shown that they simulate stressful conditions since they often elevate endogenous glucocorticoid levels within the natural range (e.g. Pravosudov et al. 2001; Jenni-Eiermann et al. 2008). We exposed young adult females to unpredictable food availability. We monitored survival in both the experimental and control birds kept on *ad libitum* food until they were 3 years of age, a time window within which survival differences have been measured in this species in other studies (Monaghan et al. 2012, Costantini et al. 2014).

1.2 Materials and Methods

1.2.1 Experimental design

We used females produced from two breeding events in a stock population of zebra finches maintained at the University of Glasgow (replicate 1 produced: April-June 2011; replicate 2 produced: August-September 2011- full details in Supplementary material). The experiment started when the birds were fully adult and age was 152 ± 15 days (average ± SD - all ages are estimated from recorded hatching date of the first chick hatched in each clutch). They were moved into treatment-specific cages (n = 7-10 per 120x50x50cm cage) and randomly assigned to two experimental groups: unpredictable food (replicate 1 = 49, replicate 2 = 66) or control (replicate 1 = 49, replicate 2 = 64). Females that hatched in the same nest were counterbalanced between the two treatment groups, and family of origin was taken into

account in the analyses. Before the treatment started (when the birds were on average 152 days old), there were no differences in body mass (measured to the nearest 0.01g) or structural size (measured to the nearest 0.01mm using a digital calliper - Dial Max, Wiha, Switzerland) between treatment groups (mean ± SEM - body mass: Control: 17.01g ± 0.18, Unpredictable food: 16.84g ± 0.16; tarsus length: Control = 14.81mm ± 0.06, Unpredictable food: 14.77mm ± 0.05 - full statistics in Tables S1 and S3, Supplementary Material). Adult tarsus length was measured in 204 out of 228 birds (Control = 103; Unpredictable food = 101). Tarsus was always measured by one experimenter; within experimenter error was tested in a subset of 30 birds and measurement repeatability for tarsus was very high (repeatability coefficient = 0.95, $p < 0.0001$, Lessels & Boag 1987). Photoperiod was 14 hours:10 hours light:dark cycle and temperature was maintained between 20-24°C.

Females in the unpredictable food treatment were denied access to food for a continuous period of 4.9 hours a day (i.e. approximately one third of the daylight hours), 4 days per week on a random schedule (full details in Supplementary Material). For the remaining two thirds of the day they had *ad libitum* food**.** Birds in the unpredictable food treatment were always maintained on this regime other than when breeding (breeding events, n = 3 at 188 ± 13, 408 ± 12 days, and 653 ± 11 days of age – mean ± SD for all) when they received *ad libitum* food continuously for approximately 2 months. Birds in the control treatment were always provided with *ad libitum* food and experienced exactly the same breeding regime as the unpredictable food birds. The unpredictable food treatment employed here was not designed to induce caloric restriction since the treated females had 65% of daylight hours to replenish their daily energy requirements. Females were weighed at regular intervals during the experiment. The difference in the time available for feeding did not have a significant influence on body mass dynamics since (1) average body mass tended to increase over time in the birds in both treatments (descriptive statistics in Table S2,

Supplementary Material), (2) there were no overall significant effects of the treatment on this variable (full details in Table S3, Supplementary Material). There is a suggestion in the latter analysis of a slight reduction in body mass in the unpredictable-food birds compared with the controls at the 1 year sampling point, but this difference was very small (unpredictable food birds on average 3.98% lighter than controls), and there was no significant treatment difference at any other point. In a subset of birds (n = 21 from each experimental group in replicate 1 birds) we also measured fat scores one month after the treatment regime had started (age birds, mean ± SD: 188 ± 13 days) on a scale ranging from 0 (no fat) to 5 (furcula and abdomen bulging with fat) following Busse (1974). There was no significant effect of the treatment on this variable (Pearson Chi-square = 0.38, df = 2, p = 0.83). Our treatment, therefore, primarily altered the temporal predictability of food resources rather than the daily overall food intake, mimicking an environmental stressor experienced by animals living in highly unstable environments, such as those with frequent inclement weather conditions (Wingfield & Kitaysky 2002).

We monitored the survival of the birds for the same time period in the two replicates, three years (i.e. 1096 days of age). All procedures were carried out under Home Office Project Licence 60/4109.

1.2.2 The effects of unpredictable food on baseline corticosterone

To check the effects of the food treatment on baseline corticosterone (the main glucocorticoid in birds), we sampled subsets of randomly selected birds from both replicates two weeks after the first treatment exposure period (which occurred between the start of the treatment at 152 ± 15 days of age until the first interruption of the treatment at 188 ± 13 days of age – mean ± SD for all; replicate 1: n = 12 control and 14 unpredictable food birds; replicate 2: n = 14 control and 15 unpredictable food birds). Then, during the second treatment exposure period

(which occurred between 283 ± 14days of age until the second interruption of the treatment at 408 ± 12 days of age - mean ± SD for all), we sampled a random subset of birds from replicate 2 (n = 29 control and 30 unpredictable food birds - for logistic reasons we could not sample replicate 1 birds) after two weeks of treatment; the majority of these birds (n = 25 control and n = 28 unpredictable food birds) were blood sampled again at six weeks into the treatment period (n = 27 control and 28 unpredictable food birds). At the end of a period of food withdrawal in the experimental birds, birds in both treatment groups were blood sampled (~75µl) within 3 minutes of entering the room to obtain a baseline blood sample (Wingfield et al. 1982). We recorded bleed time from each individual bird. Blood samples were stored on ice, centrifuged, separated and frozen at -80 ºC until analyses. Blood samples were collected between 13:00-17:00hours.

Corticosterone levels were measured using an enzyme-immunoassay (EIA) (Assay Designs Corticosterone Kit 901-097, Enzo Life Sciences, Exeter UK) as described in Herborn et al. (2014). Briefly, corticosterone was extracted two times in 1ml of diethyl ether (Rathburn Chemicals,Walkerburn, UK) from plasma aliquots (~16µl). Tracer amounts (~1500 c.p.m.) of [1, 2, 6, 7-3H] corticosterone label (NET 399, PerkinElmer, Waltham, MA, USA) were added to each sample to estimate extraction efficiencies. After extraction, corticosterone concentrations (ng/ml) were measured following the EIA kit manufacturer instructions. A total of 175 samples were run in 7 assays and the average extraction efficiency was 84%, the average intra-assay variation was 10%, and the inter-assay variation was 12%. Eight samples fell below the detection limit of the assay and were assigned the minimum detectable values (i.e. 0.2 and 0.1ng/ml).

1.2.3 Data analysis

We used Linear Mixed Effects models (LMEs) to monitor the effects of unpredictable food on baseline corticosterone levels. We first examined the potential effects of the treatment during the first exposure period after two weeks of unpredictable food regime. In this model fixed factors were: treatment, replicate, and their interaction; while family identity was entered as random factor. We then performed a second LME to examine whether the birds were still responding to the treatment over a longer time period (two compared with six weeks using only replicate 2 birds). Here, fixed factors were: treatment, time, and also their interaction; other than family identity, we also included bird identity in the random structure since the majority of the birds were sampled at both 2 and 6 weeks. Although all baseline samples were taken within the recommended time of 3 min of entering the room (Romero & Reed 2005), bleed time positively correlated with corticosterone levels during the second exposure period and this covariate was included in the LME. In both LMEs, corticosterone levels were natural log- transformed to improve normality. To analyse the effect of unpredictable treatment on long-term survival, we only included in the analyses experimental females that died naturally (or were culled on welfare grounds after veterinary assessment verified that their death was imminent due to age-related degenerative disease; 3 controls, 4 unpredictable food birds excluded from the survival analyses). We used Mixed Effects Cox Models fitted by maximum likelihood in R v3.1.2 (R package "coxme"; R core team, 2014) to assess the effect of treatment on survival. Data were right-censored to allow inclusions of birds still alive at the completion of the survival monitoring period (71.5%; 158 out of 221 birds). In preliminary analyses, we performed separate models to assess whether the risk of dying was affected by the following covariates: body mass at 1 year (i.e. 380 ± 12 days, mean ± SD) or the percentage in body mass change between 1 year and the start of the experiment (day 152). Body mass at 1 year was chosen to allow inclusion of the majority of the birds in the model as mortality increased when the birds were around 1.6 years. There were no effects

of these covariates as main factors or in their two- and three-way interactions with treatment and replicate ($p > 0.05$ for all) and they were subsequently excluded from the final model. In the final survival model we entered: treatment and replicate as fixed factors, and included also their interaction; family id was included as a random factor. We then performed a separate analysis using a General Linear Model (GLM) only for those birds that died (up to three years of age) to examine whether differences in body mass could have contributed to explaining differences in the age at which death occurred between the two treatment groups. Factors entered in the model were: treatment, the change (%) in body mass between day 152 and 1 year of age, and their interaction; we also included in the model replicate as main factor and its interaction with treatment and the continuous covariate to check for consistency of the effect of the treatment between the two experimental replicates. Eight females (4 control and 4 unpredictable food birds; equally spread between the two replicates) did not have a body mass measurement at 1 year and were excluded from this model (final sample size, n = 35 control and 20 unpredictable food birds). In preliminary analyses in which we used LME with family identity as random factor the correlation across observations from birds sharing the same family was very low (<0.0001) and family was consequently removed from the final model due to the limited degrees of freedom. In all analyses, minimum adequate models were achieved by using a backward stepwise procedure, starting from the interaction terms, to exclude non-significant terms ($p > 0.05$). Assumption of homogeneity of variance and independence in the GLM/LMEs performed were upheld. Unless otherwise specified, all analyses were conducted using the software SPSS v19. Unless otherwise specified, values are given as means ± SE.

1.3 Results

1.3.1 The effects of unpredictable food on baseline corticosterone

As expected from other studies, the food withdrawal was accompanied by a rise in baseline corticosterone ($F_{1, 47.30} = 7.42$, $p = 0.009$; Figure 1), clearly evident in both replicates ($F_{1, 46.05} = 0.03$, $p = 0.864$; Figure 1), though there was a slight difference in the average corticosterone values in the birds in the two replicates ($F_{1, 45.82} = 4.39$, $p = 0.042$; Figure 1; full model details in Table S4a in Supplementary Material). When we compared the effect of the duration of the treatment exposure on the responsiveness of the birds during the second exposure period (i.e. sampling at 2 weeks and 6 weeks of unpredictable food regime using replicate 2 birds), we found that, birds in the experimental treatment still responded to the food withdrawal with higher corticosterone concentrations than controls ($F_{1, 58.31} = 6.37$, $p = 0.014$; Figure 2), and the response to the treatment did not differ after two and six weeks of unpredictable food ($F_{1, 65.70} = 0.28$, $p = 0.600$; Figure 2; full model details in Table S4b in Supplementary Material).

1.3.2 The effects of unpredictable food on survival

We found that females exposed to the unpredictable food regime during adulthood showed improved life expectancy compared to control females ($z = -2.42$, $p = 0.016$). The survival curves of the unpredictable food and control females started to diverge within approximately a year from the start of the experiment (Figure 3). This effect became gradually more pronounced as the birds increased further in age (Figure 3). The effect of the treatment on survival was consistent between the two replicates (treatment x replicate interaction: $z = 1.32$, $p = 0.19$); and there was no significant effect of replicate as main factor ($z = -0.24$, $p = 0.81$).

Amongst those birds that died, age of death was not affected by the treatment ($F_{1, 50} = 0.022$, $p = 0.883$) or body mass change between day 152 and 1 year of age as main factor ($F_{1, 50} = 0.180$, $p = 0.673$), but the interaction between the two was significant ($F_{1, 50} = 6.095$, $p =$

0.017). Such significant effect was consistent across the two experimental replicates (treatment x replicate x body mass change: $F_{2, 48} = 1.286$, $p = 0.286$; replicate as main factor was also not significant: $F_{1, 50} = 2.074$, $p = 0.156$). As can be seen in Figure 4, birds in the unpredictable food group that gained more mass between 1 year and day 152 lived longer, whereas birds in the control group showed the opposite trend, with those birds that gained more mass dying at a younger age.

1.4 Discussion

In this study we showed that the prolonged and repeated exposure to an unpredictable stressor in adulthood (random withdrawal of food) reduced the probability of death in female zebra finches as compared to females having a predictable and constant supply of food. This result was consistent across two replicates of the experiment. Our data from subsets of birds randomly selected from this same population showed that the females subjected to the unpredictable food regime showed increased baseline levels of plasma corticosterone as compared to control females. Importantly, our corticosterone sampling monitoring showed that the treated birds continued exhibiting significant increases in basal glucocorticoid secretion during the food withdrawal period compared to control birds even after a prolonged exposure to the treatment (up to six weeks), suggesting that there was no habituation to the treatment in these birds. Taken together, therefore, these results suggest that prolonged exposure to stressful environmental conditions (involving a repeated and unpredictable activation/stimulation of the HPA axis) may trigger beneficial effects on long-term health and longevity. These data support the hypothesis of an evolved adaptive - or at least not necessarily detrimental - role of protracted stress exposure in natural populations (Broonstra 2013). This is the first longitudinal study in a vertebrate species in which the positive effects on risk of death were associated with protracted stressful circumstances imposed in adult life

since the exposure to the stressful conditions started when the birds were sexually mature individuals.

Unpredictable food availability is an almost ubiquitous stressor in natural conditions and can induce behavioural and physiological responses in individuals in order to regulate energy storage and expenditure (Bednekoff & Houston 1994). There is large variation in the results from studies investigating such responses, likely to be due to differences in life-history traits among species, but also to variation in the unpredictable food protocols and the severity of the stress response that it induces (see Fokidis et al. 2012 for a discussion of this point). For example, temporal variability in food supply has been associated with reduced (Fokidis et al. 2012), increased or stabilised body mass (Witter et al. 2000; Buchanan et al. 2003). In our study, while a very small difference in mass was detectable at one year, overall the treatment had no effect on body mass, and both the treated and control females gained weight with age. This suggests that the stress experienced was relatively mild, as does the effect on baseline corticosterone (discussed in the paragraph below). Thus our results do not seem attributable to any caloric restriction effect since the treated birds could replenish their energy requirements by consuming more food than the control birds when the food was available. Compensatory hyperphagia is common in animals experiencing episodes of food scarcity followed by abundance (Bull & Metcalfe 1997), and this change in feeding behaviour as a result of unpredictable food supply has been confirmed in a previous study where food consumption was carefully monitored (Fokidis et al. 2012). It is possible that body composition might have differed between the groups, and body mass is a relatively crude proxy of body composition and may not correlate well with variation in organ mass (e.g. Fokidis et al. 2012). Indeed, unpredictable access to food has been shown to alter body composition, for example by increasing fat stores (Witter and Swaddle, 1997), which have been linked with elevated corticosterone (Wingfield et al. 1997), or by decreasing pectoral

muscles (Fokidis et al. 2012). The "adaptive fattening strategy" that has been observed across several bird species when the risk of resource unpredictability is high as an "insurance" against the perceived risk of starvation (Witter and Swaddle, 1995; Rogers and Reed, 2003; see also Smith and Metcalfe, 1997 for a mini-review on this aspect) does not appear likely in our treated individuals, since we did not observe any significant effect of the unpredictable food regime on subcutaneous fat reserves after one month since the start of the treatment.

In our study, the risk of dying during the three year monitoring period was not related to body mass changes. Interestingly, however, for those birds that died, the birds subjected to random withdrawal of food that gained more body weight from the start of the treatment tended to die at older ages than those that stabilised or lost body mass within the same period of time. In the control birds, the opposite pattern was seen; amongst the control birds that died, higher body mass gain was associated with an earlier age of death. Such differences might reflect different behavioural responses for example, with the unpredictable-food treated individuals that gained more mass consuming more food and/or being more efficient in extracting food energy and thus better able to cope with food restriction (Wingfield et al. 1997; Wingfield & Kitaysky, 2002).

What are the potential mechanisms that could be driving the beneficial effects of prolonged environmental stressors on survival and resilience? Obviously such mechanisms are likely to be very complex and act at many levels. We showed that the unpredictable food regime produced the expected effect of increasing baseline corticosterone levels. Consistent with previous work (Pravosudov et al. 2001), the magnitude of hormone increase induced by the treatment was much lower than that which occurs during an acute stress response in this species (~10-20ng/ml). This suggests that the environmental stressor imposed on the birds by the temporary withdrawal of food was mild overall. Although we were unable to examine statistically interactions between corticosterone and the treatment on the likelihood of

survival, since we measured hormone levels in a relatively small number of birds compared to the total sample size, we speculate that such moderate, but repeated and protracted, HPA axis stimulation generated by unpredictable food supply had a key role in driving the beneficial effects on longevity. Glucocorticoids are known to exert permissive and preparative actions that can altogether aid organisms in adapting to a chronic stressor (Sapolski et al. 2000; Romero et al. 2009). There may be U-shaped relationships between glucocorticoids and survival, with a threshold below which HPA axis stimulation can promote health and longevity, and above which detrimental effects may arise (Pravosudov et al. 2001; Brown et al. 2005). For example, in the Swainson's thrush (*Catharus ustulatus*), individuals with naturally high baseline corticosterone levels during the nestling stages showed increased survival rate post-fledging compared to birds showing naturally low basal corticosterone concentrations (Rivers et al. 2012). On the other hand, during severe naturally occurring stressful events, such as during the El Niño, corticosterone levels have been observed to increase disproportionately in Galapagos marine iguanas (*Amblyrhynchus cristatus*) and were negatively correlated with likelihood of survival (Romero & Wikelski 2001).

In a recent study by Costantini and collaborators (2014) (see also Costantini et al. 2013), adult zebra finches exposed to short episodes of heat stress in adulthood showed increased long-term survival and resilience only if they had been previously exposed to thermal stress before reaching sexual maturity. Since in our study, the birds continued to experience the food shortage throughout the period when survival was monitored, it seems likely that the protracted exposure to unpredictable food may have triggered hormetic responses, behavioural and/or physiological that increased likelihood of survival and, at least to some extent, prolonged lifespan. This may have come about through a reduction in reproductive effort, and we are currently investigating this possibility. However, the birds

bred only 3 times during the study period, and other studies have shown that the cost of reproduction for zebra finches rearing an un-manipulated brood size are small, even for birds breeding several times per year (Heidinger et al. 2012). As a final note, although we did not induce caloric restriction in the experimental birds, it is interesting to remark that the effects of unpredictability of food on survival observed in our study resemble lifespan extension found in caloric-restricted animals in several taxa (Masoro 2005). Since caloric restriction does increase baseline stress levels (Patel & Finch 2002), we could hypothesise that in a range of nutritional stressors or other mild and unpredictable environmental stressor, hormetic responses via moderate stimulation of the HPA axis may represent a conserved phenomenon that promotes survival and counteracts anti-ageing effects, even in the absence of body mass changes. Future studies in which the degree of exposure to an environmental stressor is experimentally manipulated are needed to test this hypothesis.

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Figure Captions

Figure 1. The effects of the unpredictable food treatment during the first treatment exposure periods (between 152 ± 15 days and 188 ± 13 days of age, mean ± SD for both - see Materials and Methods for details on experimental design) on baseline corticosterone levels in female zebra finches produced from both experimental replicates. Birds were sampled after two weeks of unpredictable food treatment. Females in the unpredictable food treatment are represented by red and females in the control treatment are represented by black. There was a significant effect of the treatment ($p = 0.009$; un-transformed means ±SE: control: 2.32 ± 0.21ng/ml, unpredictable food: 3.78 ± 0.48ng/ml), and replicate as main factor ($p = 0.042$) but no significant interaction between the treatment and replicate ($p = 0.864$) on baseline corticosterone levels.

Figure 2. The effects of unpredictable food treatment within the second treatment exposure period (between 283 ± 14 days and 408 ± 12 days of age, mean ± SD for both - see Materials and Methods for details on experimental design) on baseline corticosterone levels in female zebra finches **(**replicate 2 only birds). The birds were sampled after two and six weeks of unpredictable food treatment (indicated in the graph as 2 and 3, respectively); represented in red unpredictable food females and in black control females. There was a significant effect of the treatment ($p = 0.014$; un-transformed means ± SE - control: 2.02 ± 0.21ng/ml, unpredictable food: 2.25 ± 0.17ng/ml), but no significant effect of sampling time ($p = 0.447$), or an interaction between the treatment and sampling time ($p = 0.600$) on baseline corticosterone levels.

Figure 3. Survival up to three years of age (i.e. 1096 days) of experimental zebra finch females exposed to unpredictable food treatment (red) or control females (black). The arrow indicates the start of the experiment (on average at 152 days of age). Survival in the unpredictable food birds was higher than the control birds ($p = 0.016$).

Figure 4. Graphical representation of the significant interaction effect ($p = 0.017$) between treatment (in red: females exposed to unpredictable food, in black: control females) and % of body mass change between the start of the experiment (pre-treatment measure, average age of the birds was 152 days) and 365 days on age at death.

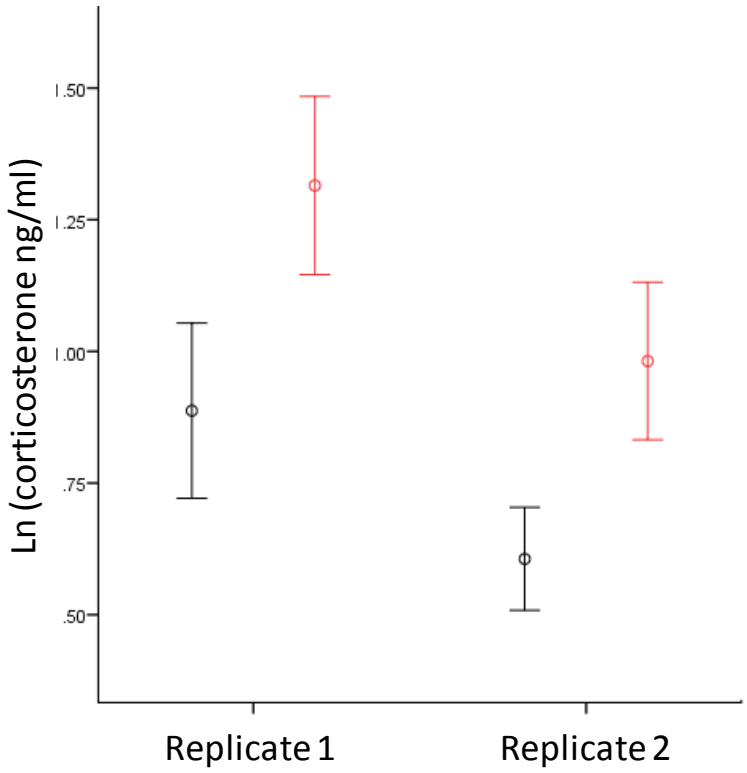
Ln (corticosterone ng/ml)
1.50
1.25
1.00
.75
.50
Replicate 1
Replicate 2

Figure 1

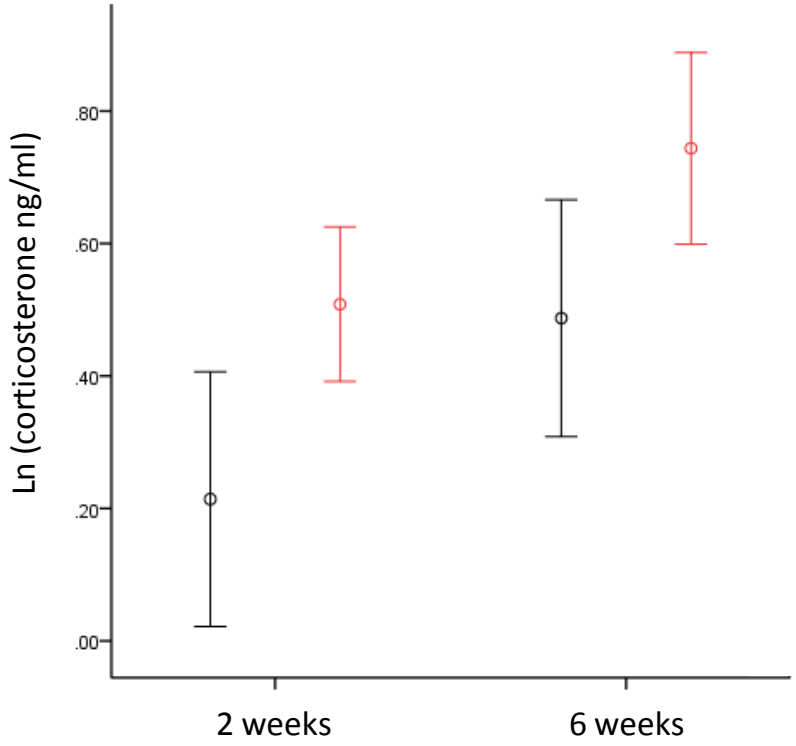

Figure 2

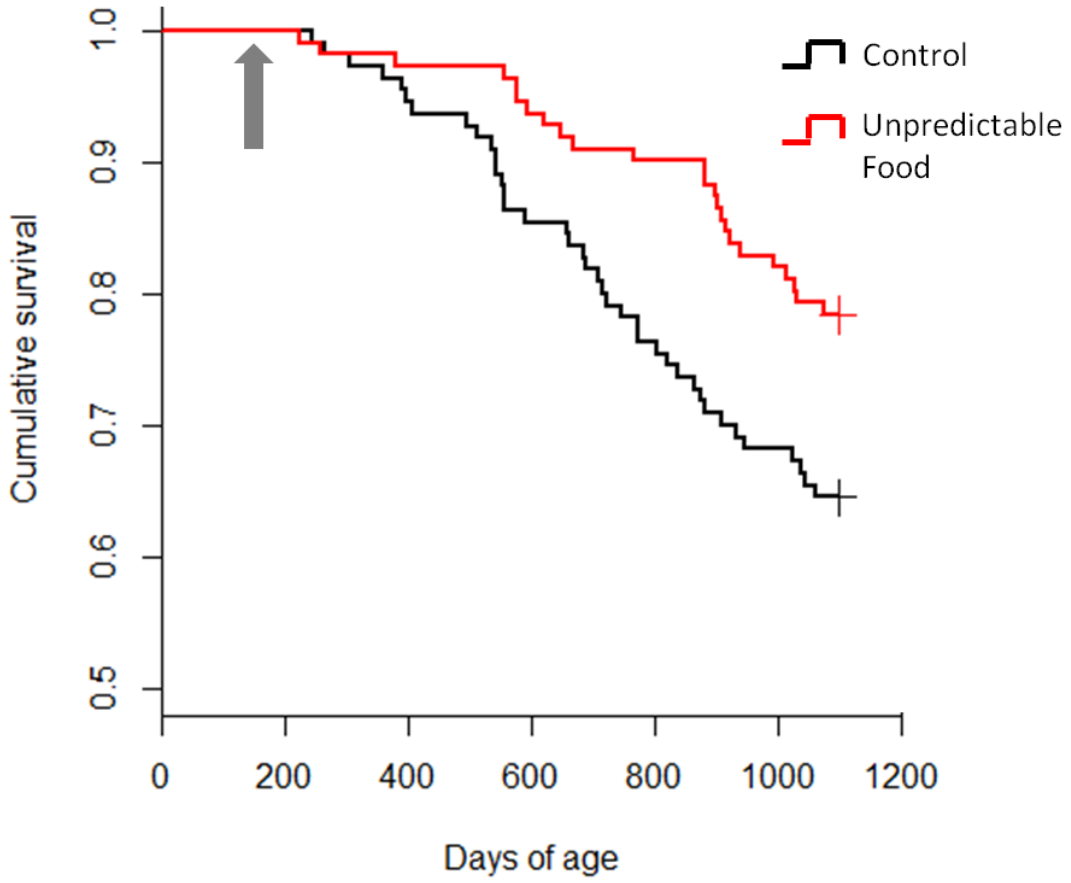
Cumulative survival
1.0
0.9
0.8
0.7
0.6
0.5
0
200
400
600
800
1000
1200
Days of age
Control
Unpredictable
Food

Figure 3

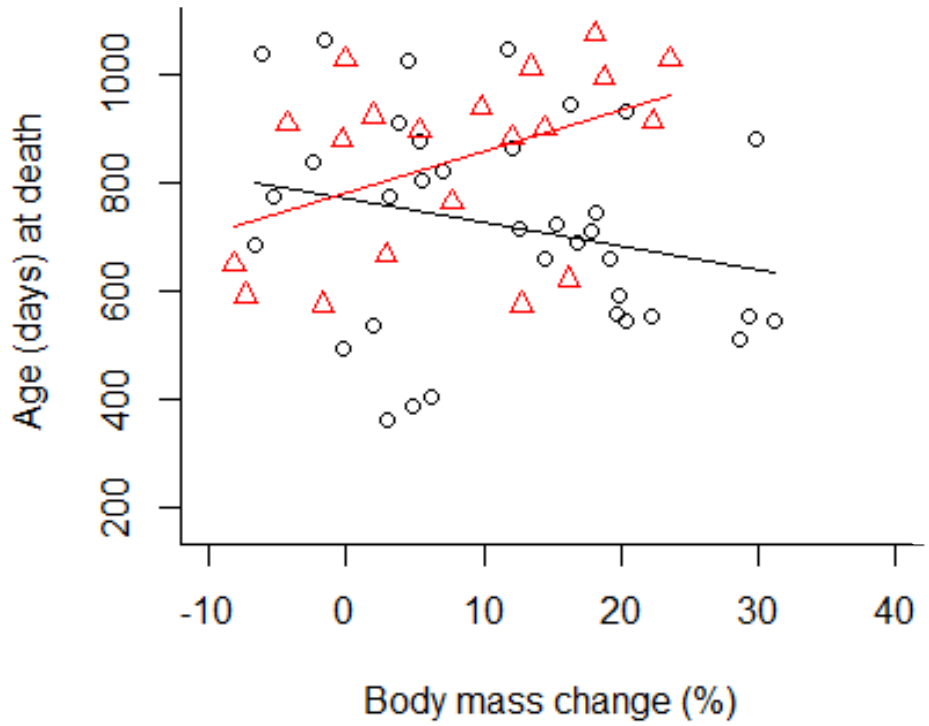
Age (days) at death
200
400
600
800
1000
-10
0
10
20
30
40
Body mass change (%)

Figure 4

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Supplementary Material

Repeated exposure to stressful conditions can have beneficial effects on survival

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Material and Methods

Breeding stock

The parent birds used to obtain the experimental females used in this study (from both replicate 1 and replicate 2) were randomly paired and housed in 60x50x50cm cages equipped with nest boxes and coconut fibres as nesting material. Food consisted with an *ad libitum* diet of seed (Haiths Ltd, Grimsby, UK), shell grit and cuttlefish bone. Birds were also supplemented two times per week with rearing and conditioning food (Haiths Ltd), and spinach until chick hatching. At hatching, the chicks were individually marked with unique colour combinations applied on the feathers until approximately 30 days of age when they could be individually ringed. At this time, the birds were separated from their parents, but were maintained in family groups until 50 days of age when they could be sexed by colour plumage and moved in sex-specific aviaries (180x180x200cm).

Unpredictable food treatment

Access to food was prevented by placing a shelf in the bottom of the cage in order to assure complete coverage of the food bowls and of any seed food scattered on the floor cage. At the termination of each food withdrawal period, *ad libitum* food was restored for the rest of the day (same diet regime as described above for the breeding stock).

Table S1. Model output from Linear Mixed Effect model fitted by restricted maximum likelihood (REML) with a normal error distribution examining potential differences of tarsus length between unpredictable food and control zebra finch adult females; family id was included as random factor; *indicates rejected terms from the models. Estimates ± SE are given for Treatment = Control and Replicate = 1.

Tarsus length

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	0.030 ± 0.069	1, 142.805	0.191	0.663
Replicate*	0.011 ± 0.089	1, 103.438	0.015	0.903
Treatment x Replicate*	0.098 ± 0.142	1, 146.518	0.471	0.493

Family id – variance ± SE: 0.080 ± 0.032, Wald Z = 2.504, $p = 0.012$.

Table S2. Descriptive statistics (mean ± SEM) of body mass values recorded at day 152 ± 15 (pre-treatment measurement), and year 1 (i.e. 380 ± 12 days), year 2 (727 ± 14 days), and year 3 (i.e. 1101 ± 12 days) of age in the Control and Unpredictable food birds. All age intervals are provided as mean ± SD.

Replicate 1

Age	Control	Unpredictable food
Day 152	17.37 ± 0.29	17.09 ± 0.26
Year 1	18.53 ± 0.39	17.97 ± 0.33
Year 2	17.66 ± 0.40	16.82 ± 0.32
Y ear 3	18.22 ± 0.54	17.78 ± 0.35

Replicate 2

Age	Control	Unpredictable food
Day 152	16.78 ± 0.22	16.69 ± 0.20
Year 1	19.23 ± 0.31	18.36 ± 0.25
Year 2	18.82 ± 0.36	18.89 ± 0.29
Year 3	18.84 ± 0.36	18.27 ± 0.37

Table S3. Model output from Linear Mixed Effect model fitted by restricted maximum likelihood (REML) with a normal error distribution examining potential treatment differences of body mass at (a) day 152; (b) year 1 (c) year 2, and (d) year 3 of age between unpredictable food and control adult zebra finch females; family id was included as random factor in all models; *indicates rejected terms from the models. We used a Bonferroni correction to account for multiple comparisons and regarded variables as significant at $\alpha = 0.013$. Estimates ± SE are given for Treatment = Control and Replicate = 1.

(a) Body mass day 152 (pre-treatment)

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	0.160 ± 0.191	1, 136.910	0.697	0.405
Replicate	0.641 ± 0.298	1, 112.302	4.628	0.034
Treatment x Replicate*	-0.068 ± 0.388	1, 136.922	0.031	0.861

Family id – variance ± SE: 1.562 ± 0.374, Wald Z = 4.176, $p < 0.0001$

(b) Body mass year 1

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	0.657 ± 0.280	1, 157.078	5.506	0.020
Replicate*	-0.511 ± 0.364	1, 117.326	1.970	0.163
Treatment x Replicate*	-0.331 ± 0.570	1, 157.656	0.336	0.563

Family id – variance ± SE: 1.524 ± 0.503, Wald Z = 3.029, $p = 0.002$

(c) Body mass year 2

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	0.269 ± 0.312	1, 132.010	0.747	0.389
Replicate	-1.595 ± 0.386	1, 97.561	17.099	0.0001
Treatment x Replicate*	0.777 ± 0.634	1, 133.715	1.501	0.223

Family id – variance ± SE: 1.374 ± 0.623, Wald Z = 2.204, $p = 0.028$

(d) Body mass year 3

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	0.604 ± 0.343	1, 113.348	3.109	0.081
Replicate*	-0.579 ± 0.445	1, 101.227	1.697	0.196
Treatment x Replicate*	-0.284 ± 0.697	1, 112.640	0.166	0.685

Family id – variance ± SE: 2.160 ± 0.706, Wald Z = 3.061, $p = 0.002$

Table S4. Model output from Linear Mixed Effect model fitted by restricted maximum likelihood (REML) with a normal error distribution examining potential treatment differences on baseline corticosterone levels between zebra finch females exposed to repeated and protracted unpredictable food regime and control females during (a) treatment exposure 1 after 2 weeks of unpredictable food regime, and (b) treatment exposure 2 after 2 weeks and 6 weeks of unpredictable food regime (using replicate 2 birds); family id was included as random factor in both models (a, b) and bird id was an additional random factor in the second model (b) to control for repeated-measures from the same individuals; *indicates rejected terms from the models. In (a) estimates ± SE are given for Treatment = Control and Replicate = 1; in (b) estimates ± SE are given for Treatment = Control and Time = 2 weeks.

(a) Treatment exposure 1

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	-0.385 ± 0.141	1, 47.304	7.420	0.009
Replicate	0.307 ± 0.147	1, 45.817	4.394	0.042
Treatment x Replicate*	-0.052 ± 0.300	1, 46.053	0.030	0.864

Family id – variance ± SE: 0.010 ± 0.023, Wald Z = 0.434, $p = 0.664$

(b) Treatment exposure 2

Factor	**Estimate ± SE**	**DF (num, den)**	**F**	***p*-value**
Treatment	-0.393 ± 0.156	1, 58.313	6.368	0.014
Bleed time	0.011 ± 0.002	1, 99.537	20.810	<0.0001
Time*	-0.104± 0.136	1, 60.835	0.585	0.447
Treatment x Time*	-0.142 ± 0.270	1, 65.699	0.278	0.600

Family id – variance ± SE: 0.095 ± 0.134, Wald Z = 0.709, $p = 0.478$; Bird id - variance ± SE: 0.047 ± 0.134, Wald Z = 0.348, $p = 0.727$.