A peer-reviewed version of this preprint was published in PeerJ on 23 October 2018.

View the peer-reviewed version (peerj.com/articles/5842), which is the preferred citable publication unless you specifically need to cite this preprint.

Gott A, Andrews C, Larriva Hormigos M, Spencer K, Bateson M, Nettle D. 2018. Chronological age, biological age, and individual variation in the stress response in the European starling: a follow-up study. PeerJ 6:e5842 https://doi.org/10.7717/peerj.5842

Chronological age, biological age, and individual variation in the stress response in the European starling: A follow-up study

Annie Gott [1], **Clare Andrews** [1], **Maria Larriva Hormigos** [2], **Karen Spencer** [2], **Melissa Bateson** [1], **Daniel Nettle** [1]
Corresp. 1

[1] Centre for Behaviour and Evolution, Newcastle University
[2] School of Psychology and Neuroscience, University of St. Andrews, United Kingdom

Corresponding Author: Daniel Nettle
Email address: daniel.nettle@ncl.ac.uk

The responsiveness of the avian stress system declines with age. A recently published study of European starlings (*Sturnus vulgaris*) found that a marker of biological age predicted stress responsiveness even in individuals of the same chronological age. Specifically, birds that had experienced greater developmental telomere attrition showed a lower peak corticosterone response to an acute stressor, and more rapid recovery of corticosterone levels towards baseline. Here, we performed a follow-up study using the same capture-restraint-handling stressor in a separate cohort of 27 starlings. Unlike the original study, we measured the response at two different age points (4 and 18 months). We did not replicate the associations with developmental telomere attrition observed in the previous study at either age point. However, a meta-analysis of the present results combined with those of the earlier study still lent some support to the conclusions of the earlier paper. Estimates of familial influence on stress responsiveness differed across the two age points. We found little evidence of individual consistency in stress responsiveness between 4 and 18 months. Peak corticosterone was significantly lower at the second age point than the first, though interpretation of this as age-related decline is problematic due to the samples having been analysed at different times.

Chronological age, biological age, and individual variation in the stress response in the European starling: A follow-up study

Annie Gott[1, a], Clare Andrews[1, a], Maria Larriva Hormigos[2], Karen Spencer[2], Melissa Bateson[1, b], Daniel Nettle[1, b]

1. Centre for Behaviour and Evolution & Institute of Neuroscience, Newcastle University, Newcastle upon Tyne, UK
2. School of Psychology and Neuroscience, University of St. Andrews, St. Andrews, UK

a. Joint first authors
b. Joint senior authors

Corresponding author:

Daniel Nettle[1]

Email address: daniel.nettle@ncl.ac.uk

Abstract

The responsiveness of the avian stress system declines with age. A recently published study of European starlings (*Sturnus vulgaris*) found that a marker of biological age predicted stress responsiveness even in individuals of the same chronological age. Specifically, birds that had experienced greater developmental telomere attrition showed a lower peak corticosterone response to an acute stressor, and more rapid recovery of corticosterone levels towards baseline. Here, we performed a follow-up study using the same capture-restraint-handling stressor in a separate cohort of 27 starlings. Unlike the original study, we measured the response at two different age points (4 and 18 months). We did not replicate the associations with developmental telomere attrition observed in the previous study at either age point. However, a meta-analysis of the present results combined with those of the earlier study still lent some support to the conclusions of the earlier paper. Estimates of familial influence on stress responsiveness differed across the two age points. We found little evidence of individual consistency in stress responsiveness between 4 and 18 months. Peak corticosterone was significantly lower at the second age point than the first, though interpretation of this as age-related decline is problematic due to the samples having been analysed at different times.

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27057v1 | CC BY 4.0 Open Access | rec: 26 Jul 2018, publ: 26 Jul 2018

Introduction

The hypothalamic-pituitary-adrenal (HPA) axis is a highly conserved, integrated system in vertebrates that functions to prioritize immediate survival over non-essential activities in the face of acute threats. In birds, the responsiveness of the HPA system generally declines with chronological age (Heidinger, Nisbet & Ketterson, 2006; Heidinger et al., 2010; Wilcoxen et al., 2011; Elliott et al., 2014; Lendvai, Giraudeau & Bo, 2015; López-Jiménez et al., 2017), possibly reflecting adaptive shifts in behavioural allocation as expected future lifespan reduces. However, individuals do not all age at the same rate: an individual's biological age can be either older or younger than their chronological age would suggest (Belsky et al., 2015). Biological age is by definition a better predictor of future lifespan than chronological age is. Hence, we should expect markers of individual biological age to explain variation in stress responsiveness that cannot be explained by chronological age alone. A possible reason that early-life conditions have often been observed to influence the functioning of the adult stress response may be that early-life conditions can speed up or slow down the biological ageing process, and consequently advance or retard age-related shifts in the functioning of the stress response system. A potential marker of biological age is the attrition of telomeres, the DNA caps at the ends of linear chromosomes (Bize et al., 2009; Bauer et al., 2018), with more lifetime attrition indicating greater biological age. The rate of telomere attrition is much higher during the developmental period than in adulthood, and has been shown to be accelerated by early-life adversity (Boonekamp et al., 2014; Nettle et al., 2015, 2017).

In a recent study of two cohorts of adult European starlings (*Sturnus vulgaris*), Andrews et al. (2017) showed that developmental telomere attrition (DTA; the extent of shortening of erythrocyte telomeres over the course of development) explained variation in individuals' stress responses. Specifically, individuals that were biologically older by this measure (all individuals were approximately the same chronological age) showed a lower peak level of corticosterone (CORT) in response to an acute capture-handling-restraint stressor, and also showed stronger recovery of CORT levels towards baseline between 15 and 30 minutes after the onset of the stressor. DTA was unrelated to baseline CORT. The birds studied by Andrews et al. (2017) had been subjected to experimental manipulations of early-life conditions: a manipulation of brood size for one cohort (Nettle et al., 2013), and of the focal individual's size relative to its competitors in the other cohort (Nettle et al., 2015). These manipulations affected DTA, with the more adverse treatment (having more or larger competitors respectively for the two cohorts) leading to greater telomere shortening in early life. However, it was DTA, rather than the early-life treatments themselves, that significantly predicted stress responsiveness. This suggests that DTA captures both the adversity due to the experimental manipulation, and other sources of adversity, and also incorporates the fact that individuals are differentially affected by the same external conditions. Thus, DTA is a better marker of biological ageing than any of the individual environmental or genetic factors that may influence it. The cohorts of birds were made up of quartets of siblings, which allowed the researchers to find evidence consistent with modest familial effects on baseline and peak CORT, but not on the change in CORT between 15 and 30 minutes after the onset of the stressor.

In light of recent focus on reproducibility in bioscience (e.g. Fidler et al., 2017), the results of Andrews et al. (2017) require replication. Moreover, the CORT response was only measured at one age point (around one year of age on average). Thus, it is not clear whether associations between DTA and stress responsiveness appear before, or persist after, this stage of life. Longitudinal studies have often found individual birds not to be consistent in their CORT measures from one year to the next (Ouyang, Hau & Bonier, 2011; Baugh et al., 2014; Lendvai, Giraudeau & Bo, 2015) (though see (Angelier et al., 2010)). One study concluded that the response to an acute stressor showed moderate individual consistency, but baseline CORT did not (Cockrem & Silverin, 2002). Thus, it is important to establish whether there is

individual consistency over time in the CORT parameters studied in Andrews et al. (2017), as well as whether the associations of these parameters with DTA are robust.

Here, we report a follow-up study conducted with a different cohort of birds. The quartets of siblings in the current cohort were hand-reared according to a two-by-two factorial design that varied early food amount and begging effort, to simulate different patterns of naturally-occurring nestling adversity (Nettle et al., 2017). Reduced food amount and increased begging effort separately and additively increased DTA over the first 56 days of life (Nettle et al., 2017). In the present experiments, we measured the CORT response to an acute capture-restraint-handling stressor using the same protocol as Andrews et al. (2017), but did so twice, at approximately 4 months (age point 1) and 18 months (age point 2) of age.

Our main aim was to replicate at each age point separately the key findings of Andrews et al. (2017), namely that DTA is associated with peak CORT and change in CORT between 15 and 30 minutes, but not baseline CORT; that DTA is a better predictor of CORT parameters than the early-life conditions to which birds have been exposed; and that there is modest familial resemblance in baseline and peak CORT. In addition, our study, though having two age points, gave us the opportunity to characterise individual consistency in CORT variables. The second age point sample was not planned at the time the first was carried out. We therefore performed the laboratory assays on the blood samples from the first age point separately to the second, and with a small variation in the laboratory protocol. Although common standard samples were run on both occasions, this sharply limits any inferences about absolute within-individual change, since age and laboratory batch are completely confounded. However, we can still ask whether an individual with a relatively high peak CORT at age point 1 also has a relatively high peak CORT at age point 2.

Materials & Methods

Study subjects and husbandry

This research was approved by the Animal Welfare and Ethics Review Board, Newcastle University, and carried out under UK Home Office project licence 70/8089. Study subjects were a cohort of 32 starlings (16 male) hatched in 2014 (hence the '2014 cohort') described in detail elsewhere (Nettle et al., 2017). Briefly, quartets of natural nest-mates were taken from the wild on day 5 post-hatch and hand-reared to day 15 under controlled conditions. One sibling was assigned to each combination of food Amount ('Plenty' vs. 'Lean') and begging Effort ('Easy' vs. 'Hard'), thus creating four experimental groups. The Amount manipulation was achieved by feeding each Plenty group to satiation on each feed and measuring the quantity consumed, then restricting the intake of the corresponding Lean group to 73% of this quantity. The Effort manipulation was achieved by interspersing, for the Hard groups, each true feed with another nest visit of similar duration where the nestlings were stimulated to beg but no food was delivered. After day 15, all birds were hand-fed ad libitum until independence. Birds were subsequently kept in mixed-treatment flocks in indoor aviaries (215x340x220 cm; 18^{o}C, 40% humidity), with ad libitum access to food and water. Birds were maintained in non-breeding conditions by a constant 15 hours light: 9 hours dark cycle.

Developmental telomere attrition

Telomere length was measured in erythrocyte DNA from blood samples taken on day 5 and day 56 post-hatch using a real-time PCR amplification method (for details see Nettle et al., 2017). We used the two T/S ratio values to gain a single-number summary of telomere shortening (henceforth ΔTL). ΔTL was corrected for the expected regression to the mean in imperfectly correlated repeated measurements using the method of Verhulst et al. (2013). Consequently, 0 represents the average amount of change for the

cohort, and a negative number more dramatic shortening. Similar results are obtained using the raw difference in T/S ratios instead.

Blood sampling and corticosterone assays

Birds were aged 127-134 days at age point 1, and 584-601 days at age point 2. All 32 birds were sampled at age point 1, and 30 at age point 2, as two had died in the interim. For the period of sampling, birds were individually caged (75x45x45cm) whilst maintaining full acoustic and visual contact with others for a period of 3-27 days (time 1: mean, 5 days; time 2: mean, 10 days). All birds had access to two wooden perches, two drinking bottles, a water bath (removed approximately four hours prior to sampling) and a bowl containing ad libitum food. The birds were maintained in environmental conditions identical to the free-flight aviaries. Habituation to the cages occurred over a minimum of three nights prior to blood sampling.

At a set time during the afternoon (the period of minimal diurnal CORT variation, Romero & Remage-Healey, 2000), lights were extinguished and two birds caught from their cages and transferred immediately to an adjacent procedure room. Approximately 120µl of a baseline blood sample was collected within 3 minutes of the lights being extinguished (age 1: mean time to baseline sample: 94.9 ± 23.6s; age 2: 108.4 ± 29.7s). One bird was removed from analysis at age 1 as the time to baseline sample was at the 180 second limit and visual analysis of the radioimmunoassay data confirmed a very high level of baseline CORT. Bleeding was stemmed using cotton wool and birds were placed in drawstring cloth bags. Further samples were taken at 15 and 30 minutes after the initial disturbance. Blood sampling was by puncture of an alar or metatarsal vein, and collection by heparinised micro-capillary tubes. After the final sample, birds were weighed and returned to the cage under observation. Each experimental room was disturbed for sampling only once per day, and no-one entered the room for at least 2 hours prior to a sample being taken.

Blood samples were centrifuged (10 minutes at 3000 rpm) to separate plasma from erythrocytes and stored at -80^{o}C until radioimmunoassay analysis. Samples from age point 1 and age point 2 were analysed at different times. Batch 1 (age point 1) consisted of two separate assays, and batch 2 (age point 2) consisted of four assays. The same protocol was followed for both batches, and two standard chicken plasma samples (P3266-1ML, Sigma Aldrich) were run in all assays in both batches. All samples were run in duplicate within the same assay. Freezer storage times were approximately 120 days (batch 1) and 30 days (batch 2).

CORT levels in plasma extracts were quantified using a radioimmunoassay previously validated in European starlings (Buchanan et al., 2003). CORT concentrations were measured after extraction of up to 35µl aliquots of plasma in 1ml diethyl ether (24004-2.5L-M, Sigma Aldrich) by a Dextran 70-coated charcoal radioimmunoassay method. The anti-CORT serum was ABIN880 (Antibodies Online) for batch 1 and 07120016 (MP Biomedical) for batch 2. Extraction efficiencies per sample were estimated at 61-100% (mean 97.8%) for batch 1, and 77-100% (mean 96.1%) for batch 2. Final CORT concentration values were corrected accordingly.

The average intra-assay coefficient of variation was 8.7% for batch 1 and 13.5% for batch 2. The inter-assay coefficient of variation (collapsing across batches) was 21.9%. The batch 2 average concentration values for the two control samples were 99.84% and 78.15% of the batch 1 values respectively.

Data analysis

As in (Andrews et al., 2017), the dynamics of the stress response were characterised by three dependent variables: baseline CORT (the first sample value); peak CORT (higher of second and third sample values); and ΔCORT (the change in CORT value between 15- and 30-minute samples, with a negative number indicating a reduction from 15 to 30 minutes).

To estimate familial effects, we fitted linear mixed models with a random effect of natal family, and no fixed predictors, to estimate the family and residual variances. To examine individual consistency, we calculated intra-class correlation coefficients for the three CORT variables at age point 1 and age point 2.

For the main objective, replication of findings of Andrews et al (2017), we analysed the data from the two age points separately, in order to keep our analysis as similar as possible to those in the previous study and avoid any issues caused by combining the two laboratory batches. Conclusions were unchanged if we analysed the data from the two age points combined and added age point as an additional term. For each outcome, we fitted comparable models to the previous paper (each CORT parameter in turn predicted by ΔTL, with a random effect of natal family, plus the inclusion of baseline CORT as a covariate in the model of peak CORT, and CORT at 15 minutes as a covariate in the model of ΔCORT). We did not include additional covariates (sex, body weight, time elapsed before baseline sample) that we might otherwise have considered, since these were not included in the analyses of the previous paper. We can report that including these does not substantively alter the results presented here.

In order to establish the current balance of evidence on associations between ΔTL and CORT variables, we also performed fixed-effects meta-analyses on all three cohorts of birds (the two from the previous paper plus the present one; the cohorts from the earlier paper are referred to as 2012 and 2013). Separate meta-analyses were performed using age point 1 to represent the 2014 cohort, and using age point 2. Meta-analyses were carried out using R package 'metafor' (Viechtbauer, 2010), and all parameter estimates were recalculated as standardized βs for this purpose.

To investigate effects of developmental treatments, we fitted models of each CORT variable at each age point with Amount, Effort and their interaction as fixed predictors, plus a random effect of natal family. Again, baseline CORT was included as a covariate for the model of peak CORT, and CORT at 15 minutes for the model of ΔCORT.

All analyses were performed in R version 3.5.0 (R Core Development Team, 2018), using the contributed packages 'irr' (Gamer et al., 2012) for intra-class correlation coefficients, 'afex' (Singmann et al., 2018) for linear mixed models, and 'metafor' (Viechtbauer, 2010) for meta-analysis. Type-III significance tests for linear mixed models were by likelihood ratio test (LRT) with a significance threshold of 0.05, and hence parameter estimation was by maximum likelihood. We also created a simple R simulation tool to simulate the chances of finding a significant ($p < 0.05$) association between two variables in small samples, for a given true strength of association. This tool generates 10,000 samples of a specified size from datasets where the true strength of association is as specified, and tabulates how many of them find a significant effect, how many a non-significant effect but in the predicted direction, and how many an effect in the opposite direction. Raw data and R scripts are available for download from: https://doi.org/10.5281/zenodo.1317793.

Results

Descriptive statistics

Descriptive statistics for CORT values at all time points and both age points are shown in table 1. At both age points, the baseline blood sample gave the lowest CORT value for all birds (figure 1). Some birds'

CORT values were highest at 15 minutes and then declined by 30 minutes, giving ΔCORT values less than zero (16/31 at age point 1, 16/30 at age point 2). The remainder continued to show an increase between 15 minutes and 30 minutes. CORT concentrations were significantly lower at age point 2 than age point 1 for 15 and 30 minutes, and hence peak CORT, but not for baseline CORT. Average ΔCORT was not significantly different at the two age points.

Familial effects and individual consistency

To estimate the proportions of variation in CORT measures explained by natal family, we performed variance partition analyses on the two age point data sets (figure 2). Estimates of familial effects differed substantially between the two time points. At age point 1, the familial estimates were 68% for baseline CORT; 33% for peak CORT; and 0% for ΔCORT, whereas for age point 2, the familial estimates were 2% for baseline CORT; 22% for peak CORT, and 40% for ΔCORT.

The intra-class correlation coefficients between age points 1 and 2 were 0.13 (95% CI -0.24 to 0.46) for baseline CORT; -0.03 (95% CI -0.38 to 0.33) for peak CORT; and 0.32 (95% CI -0.04 to 0.61) for ΔCORT. Thus, the CORT variables were not individually consistent from the first age point to the second, with only ΔCORT providing any suggestion of stability.

Replication of previous study and meta-analysis

An exact replication of the findings of Andrews et al (2017) would produce significant positive associations between ΔTL and peak CORT (after controlling for baseline CORT) and ΔTL and ΔCORT (after controlling for CORT at 15 minutes). There would be no significant association between ΔTL and baseline CORT. Table 2 summarises the relevant models for both age points. We found non-significant associations between ΔTL and baseline CORT at both age points. Associations between ΔTL and peak CORT after controlling for baseline CORT were also non-significant. As expected, higher CORT at 15 minutes predicted more negative ΔCORT values, but, contrary to the previous study, there were no significant associations between ΔTL and ΔCORT after controlling for CORT at 15 minutes.

To evaluate the balance of evidence from the three cohorts of birds combined (the present 2014 cohort, plus the 2012 and 2013 cohorts reported in Andrews et al (2017)), we performed fixed-effects meta-analyses (figure 3). As the figure shows, the summary association between ΔTL and baseline CORT was not significantly different from zero, regardless of whether the age point 1 or age point 2 results were used for the 2014 cohort. The association between ΔTL and peak CORT after controlling for baseline CORT was significantly positive (that is, more telomere loss associated with a lower peak CORT concentration) if the age point 1 data were used to represent the 2014 cohort ($\beta = 0.30$, 95% CI 0.01 to 0.41, $p = 0.04$), but not significantly different from zero if the age point 2 data were used ($\beta = 0.16$, 95% CI -0.04 to 0.36, $p = 0.12$). The summary association between ΔTL and ΔCORT after controlling for CORT at 15 minutes was significantly positive (that is, more telomere loss, more CORT return towards baseline between 15 and 30 minutes after onset of stressor). This was true using either the age point 1 ($\beta = 0.30$, 95% CI 0.11 to 0.49, $p < 0.01$) or age point 2 data ($\beta = 0.30$, 95% CI 0.11 to 0.49, $p < 0.01$).

Effects of developmental treatment

Table 3 summarises the models using the two developmental treatments (Amount and Effort) and their interaction as the predictors. At age point 1, there were no significant effects of the developmental treatments on baseline or peak CORT. However, there was a significant effect of Effort in predicting ΔCORT. Birds who experienced 'Hard' begging effort had more negative ΔCORT values; that is, their CORT concentrations return more towards baseline between 15 and 30 minutes after onset (-2.27±6.24

for 'Hard' vs. -0.08±7.69 for 'Easy' begging effort). At age point 2, again there were no significant developmental treatment effects on baseline or peak CORT. There was however a significant Amount by Effort interaction in predicting ΔCORT. This was driven by the birds from the combination of 'Hard' begging effort and 'Plenty' amount having more positive ΔCORT values (Mean ± SD, 1.51 ± 5.20) than any of the other three groups ('Lean-Easy': -0.69 ± 5.69; 'Lean-Hard': -0.98 ± 5.13; 'Plenty-Easy': -2.48 ± 4.19). That is, the CORT concentrations of the 'Plenty-Hard' group returned less towards baseline between 15 and 30 minutes after onset than those of any other group.

Power simulation

To set our failure to replicate the significant patterns reported in the previous paper into context, we simulated 10,000 samples of 27 individuals from populations where the 'true' association between ΔTL and peak CORT was 0.28, which is the pooled estimate from the two cohorts of birds reported in Andrews et al (2017). Of these 10,000 samples, 30% produced 'significant' estimates of association with $p < 0.05$; 63% produced non-significant associations but with an estimate in the positive direction; and 7% produced estimates of association in the other direction. We repeated the same exercise for the estimate of association between ΔTL and ΔCORT (0.43). This produced 'significant' associations 64% of the time, non-significant associations in the same direction 35% of the time, and estimated associations in the opposite direction 1% of the time.

Discussion

The main aim of our experiment was to replicate the findings of Andrews et al.'s (2017) study in a different cohort of birds measured at two time points. We did not replicate the main patterns observed in the previous paper. ΔTL, the change in erythrocyte telomere length over development, did not significantly predict any of the CORT parameters in the present cohort at either age point. However, meta-analysis of the data from all of the experiments combined gave some grounds for believing that ΔTL may be related to stress responsiveness in the starling in general. We also found some significant effects of the developmental treatments to which the birds had been exposed on ΔCORT.

Our failure clearly to replicate the patterns of the previous study does not support the conclusions of that paper. However, we should not necessarily infer that those conclusions were spurious, either. Meta-analysis of all the extant evidence supports a moderate association between DTA and ΔCORT ($\beta = 0.30$), with starlings that have experienced more DTA showing more rapid recovery of CORT towards baseline; and possibly between DTA and peak CORT, with starlings that have experienced greater DTA having a lower peak. The former conclusion is supported whether age point 1 or age point 2 is used to represent the present 2014 cohort of birds. The latter conclusion is only supported if the age point 1 data are used. Age point 2 (584-601 days) was more similar to the age at which the birds' stress responsiveness was measured in the previous study (2012 cohort, 208–432 days; 2013 cohort, 428–456 days). Our simulations showed that if the associations between DTA and CORT variables do in fact exist, and have the strength estimated in (Andrews et al., 2017), then we should not expect them to be statistically 'significant' (i.e. have $p < 0.05$) in every small-*n* sample considered individually. For example, if the estimated parameters in Andrews et al (2017) are correct, then we should only expect a 'significant' finding about one experiment in three for peak CORT, and two in three for ΔCORT.

We acknowledge the low power of our experiment. However, there are strong logistical constraints involved in capturing, keeping, in our case hand-rearing, and sampling live wild animals. For example, to detect an association of $\beta = 0.30$ with the conventional 80% power requires over 80 birds, which is

beyond the population size of our starling breeding colony or our ability to hand-rear nestlings. This means that modest sample sizes are difficult to avoid, especially in the early stages of exploration of certain questions. With small samples, one can attempt to minimize variability due to variation in developmental history or genetic background; this precision is often lost if we attempt to scale operations up. The evidential value of this or other small cohorts of birds is not zero, even if it is modest. Given that we cannot rear very large cohorts of birds, we have to turn to replication and cumulative meta-analysis, rather than individual-experiment *p*-values, as a way of ensuring robustness of conclusions.

The three CORT parameters were not individually consistent across the two age points (only ΔCORT showed any suggestion of individual consistency across the two age points, and this was still low). This must again be interpreted cautiously given the separate laboratory batches. It does however concur with previous findings in the avian literature that CORT parameters tend not to be very individually consistent from year to year (Ouyang, Hau & Bonier, 2011; Baugh et al., 2014; Lendvai, Giraudeau & Bo, 2015), and that, if there is any consistency, it is in responsiveness rather than baseline CORT (Cockrem & Silverin, 2002). Our estimates of familial components were very different between the two age points. Averaging our two age points together produced very similar to conclusions to the earlier study (i.e. around 25% for baseline and peak, and closer to zero for ΔCORT). However, the variability between the age points within this study does highlight the limitations of estimating these components in small samples.

We found peak CORT to be considerably lower at age point 2 compared to age point 1. This should be interpreted cautiously, as chronological age was completely confounded with laboratory batch in our study. Nonetheless, it may at least partly represent within-individual biological change with age. There were common control samples run in both batches; the average concentration for these standards at age point 2 was 99.84% and 78.15% of their age point 1 values, whereas peak CORT was 71.47% of its average age 1 value. A decline in peak CORT but not baseline CORT with age is what previous literature would lead us to expect (Lendvai, Giraudeau & Bo, 2015), and chronological age-related decline in CORT responsiveness is one of the key assumptions on which Andrews et al. (2017) based their hypotheses, but which they did not directly test.

We found some evidence here that the developmental manipulations to which the birds were subjected may have affected their adult stress responses, specifically ΔCORT. The finding at age point 1–Hard effort birds had more negative values of ΔCORT—is consistent with the thesis advanced by Andrews et al. (2017). Increased begging effort accelerates biological ageing, and thus it makes sense that it would do the same thing to the HPA axis as increased DTA, namely increase the tendency of CORT to return towards baseline between 15 and 30 minutes. Thus, this finding is in a way a corroboration of the claim that whatever ages an individual also ages their stress response. However, the finding at age point 2—that the combination of Plenty food amount and Hard begging effort is associated with more positive ΔCORT values—is not interpretable from this perspective. The Plenty-Hard combination is intermediate in the treatment groups in terms of the overall amount of adverse developmental experience (as measured for example by the amount of telomere attrition), so there is no obvious reason this group should have a 'younger' stress response that the others. Given the amount of multiple testing and the difficulty interpreting these developmental treatment findings, they should be viewed with caution.

Conclusions

In conclusion, we did not confirm any significant associations between biological age, as measured by DTA, and stress responsiveness, as measured by CORT response to an acute capture-restraint-handling stressor, in a cohort of hand-reared European starlings. However, the overall evidence is still consistent

with biological age being associated with aspects of the stress response in starlings. Moreover, our data are consistent with there being a substantial decline in stress responsiveness with chronological age within birds, as the biological age hypothesis requires.

Acknowledgements

We thank Pat Monaghan and Sophie Reichert, who collaborated with us on the telomere attrition of the birds studied in this paper; and Tom Bedford and Michelle Waddle, who assisted with us in the care of the birds.

References

Andrews C., Nettle D., Larriva M., Gillespie R., Reichert S., Brilot BO., Bedford T., Monaghan P., Spencer K., Bateson M. 2017. A marker of biological age explains individual variation in the strength of the adult stress response. *Royal Society Open Science* 4:171208.

Angelier F., Wingfield JC., Weimerskirch H., Chastel O. 2010. Hormonal correlates of individual quality in a long-lived bird: a test of the "corticosterone-fitness hypothesis." *Biology Letters* 6:846–849.

Bauer CM., Graham JL., Abolins-Abols M., Heidinger BJ., Ketterson ED., Greives TJ. 2018. Chronological and Biological Age Predict Seasonal Reproductive Timing: An Investigation of Clutch Initiation and Telomeres in Birds of Known Age. *The American Naturalist* 191.

Baugh AT., Oers K van., Dingemanse NJ., Hau M. 2014. Baseline and stress-induced glucocorticoid concentrations are not repeatable but covary within individual great tits (Parus major). *General and Comparative Endocrinology* 208:154–163.

Belsky DW., Caspi A., Houts R., Cohen HJ., Corcoran DL., Danese A., Harrington H., Israel S., Levine ME., Schaefer JD., Sugden K., Williams B., Yashin AI., Poulton R., Moffitt TE. 2015. Quantification of biological aging in young adults. *Proceedings of the National Academy of Sciences* 112:E4104-4110.

Bize P., Criscuolo F., Metcalfe NB., Nasir L., Monaghan P. 2009. Telomere dynamics rather than age predict life expectancy in the wild. *Proceedings of the Royal Society B: Biological Sciences* 276:1679–83.

Boonekamp JJ., Mulder GA., Salomons HM., Dijkstra C., Verhulst S. 2014. Nestling telomere shortening, but not telomere length, reflects developmental stress and predicts survival in wild birds. *Proceedings Of The Royal Society : Biological Sciences* 281:20133287.

Buchanan KL., Spencer KA., Goldsmith AR., Catchpole CK. 2003. Song as an honest signal of past developmental stress in the European starling (Sturnus vulgaris). *Proceedings of the Royal Society B: Biological Sciences* 270:1149–1156.

Cockrem JF., Silverin B. 2002. Variation within and between birds in corticosterone responses of great tits (Parus major). *General and Comparative Endocrinology* 125:197–206.

Elliott KH., O'Reilly KM., Hatch SA., Gaston AJ., Hare JF., Anderson WG. 2014. The prudent parent meets old age: A high stress response in very old seabirds supports the terminal restraint hypothesis. *Hormones and Behavior* 66:828–837.

Fidler F., En Chee Y., Wintle BC., Burgman MA., McCarthy MA., Gordon A. 2017. Metaresearch for evaluating reproducibility in ecology and evolution. *BioScience* 67:282–289.

Gamer M., Lemon J., Fellows I., Singh P. 2012. irr: Various coefficients of interrater reliability and agreement.

Heidinger BJ., Chastel O., Nisbet ICT., Ketterson ED. 2010. Mellowing with age: Older parents are less responsive to a stressor in a long-lived seabird. *Functional Ecology* 24:1037–1044.

Heidinger BJ., Nisbet ICT., Ketterson ED. 2006. Older parents are less responsive to a stressor in a long-lived seabird: a mechanism for increased reproductive performance with age? *Proceedings of the Royal Society B: Biological Sciences* 273:2227–2231.

Lendvai Z., Giraudeau M., Bo V. 2015. Within-individual plasticity explains age-related decrease in stress response in a short-lived bird. *Biology Letters* 11:20150272.

López-Jiménez L., Blas J., Tanferna A., Cabezas S., Marchant T., Hiraldo F., Sergio F. 2017. Lifetime variation in feather corticosterone levels in a long-lived raptor. *Oecologia* 183:315–326.

Nettle D., Andrews C., Reichert S., Bed T., Kolenda C., Parker C., Martin-Ruiz C., Monaghan P., Bateson M. 2017. Early-life adversity accelerates cellular ageing and affects adult inflammation : Experimental evidence from the European starling. *Scientific Reports* 7:40794.

Nettle D., Monaghan P., Boner W., Gillespie R., Bateson M. 2013. Bottom of the Heap: Having Heavier Competitors Accelerates Early-Life Telomere Loss in the European Starling, Sturnus vulgaris. *PLoS ONE* 8:e83617.

Nettle D., Monaghan P., Gillespie R., Bedford T., Brilot B., Bateson M. 2015. An experimental demonstration that early-life competitive disadvantage accelerates telomere loss. *Proceedings Of The Royal Society B-Biological Sciences* 282:20141610.

Ouyang JQ., Hau M., Bonier F. 2011. Within seasons and among years: When are corticosterone levels repeatable? *Hormones and Behavior* 60:559–564.

R Core Development Team. 2018. R: A Language and Environment for Statistical Computing.

Romero LM., Remage-Healey L. 2000. Daily and seasonal variation in response to stress in captive starlings (Sturnus vulgaris): corticosterone. *General and comparative endocrinology* 119:52–9.

Singmann H., Bolker B., Westfall J., Aust F. 2018. Analysis of factorial experiments.

Verhulst S., Aviv A., Benetos A., Berenson GS., Kark JD. 2013. Do leukocyte telomere length dynamics depend on baseline telomere length? An analysis that corrects for "regression to the mean." *European Journal of Epidemiology* 28:859–866.

Viechtbauer W. 2010. Conducting Meta-Analyses in R with the metafor Package. *Journal of Statistical Software* 36:1–48.

Wilcoxen TE., Boughton RK., Bridge ES., Rensel MA., Schoech SJ. 2011. Age-related differences in baseline and stress-induced corticosterone in Florida scrub-jays. *General and Comparative Endocrinology* 173:461–466.

Figure captions

Figure 1. CORT values for individual birds at baseline, 15 minutes after onset of stressor, and 30 minutes after onset of stressor, at the two age points.

Figure 2. Estimated familial components of variation for each of the CORT measures at the two age points (age point 1: 127-134 days; age point 2 584-601 days).

Figure 3. Forest plot of associations between developmental telomere change and CORT variables in the present cohort of birds (2014) and the two cohorts described previously (2012 and 2013). The points and whiskers show standardized parameter estimates and their 95% confidence intervals. The lozenges show summary effects from meta-analytically combining the three datasets (upper lozenges use the age 1 point data for the present cohort, lower lozenges use the age point 2 data).

Table 1(on next page)

Descriptive statistics (means ± sd) for CORT variables (ng/ml) at age point 1 and age point 2.

Peak CORT represents the higher of CORT 15 minutes and CORT 30 minutes; ΔCORT represents CORT 30 minutes minus CORT 15 minutes.

Table 1. Descriptive statistics (means ± sd) for CORT variables (ng/ml) at age point 1 and age point 2. Peak CORT represents the higher of CORT 15 minutes and CORT 30 minutes; ΔCORT represents CORT 30 minutes minus CORT 15 minutes.

Variable	Age 1 (127-134 days)	Age 2 (584-601 days)	Difference between age points
Baseline CORT	2.21 ± 1.99	2.76 ± 1.53	$t_{28} = 1.39$, $p = 0.17$
CORT 15 minutes	19.58 ± 8.89	13.89 ± 6.23	$t_{28} = -3.02$, $p = 0.005$
CORT 30 minutes	18.44 ± 6.41	13.25 ± 5.27	$t_{28} = -3.54$, $p = 0.001$
Peak CORT	21.66 ± 8.50	15.48 ± 5.77	$t_{28} = -3.11$, $p = 0.004$
ΔCORT	-1.14 ± 7.00	-0.64 ± 5.01	$t_{28} = -0.001$, $p = 0.996$

Table 2(on next page)

Summaries of statistical models testing for effects of developmental telomere attrition (DTA) on stress response (CORT) variable.

All models contain random effects of natal family. LRT: Likelihood ratio test. Age point 1: 127-134 days; age point 2 584-601 days.

Table 2. Summaries of statistical models testing for effects of developmental telomere attrition (DTA) on stress response (CORT) variables. All models contain random effects of natal family. LRT: Likelihood ratio test. Age point 1: 127-134 days; age point 2 584-601 days.

Age point	Outcome	Predictors	B	s.e. (B)	LRT	p-value
1	Baseline CORT	DTA	-0.48	0.98	0.23	0.63
	Peak CORT	Baseline CORT	1.10	0.93	1.36	0.24
		DTA	2.75	5.79	0.23	0.64
	ΔCORT	CORT 15 mins	-0.57	0.12	15.75	<0.001
		DTA	1.45	3.72	0.15	0.70
2	Baseline CORT	DTA	1.58	1.22	1.55	0.21
	Peak CORT	Baseline CORT	0.70	0.63	1.00	0.32
		DTA	-1.99	3.98	0.22	0.64
	ΔCORT	CORT 15 mins	-0.56	0.12	15.60	<0.001
		DTA	0.91	2.62	0.11	0.74

Table 3(on next page)

Summaries of statistical models testing for effects of developmental treatments on stress response (CORT) variables.

All models contain random effects of natal family. Age point 1: 127-134 days; age point 2 584-601 day.

Table 3. Summaries of statistical models testing for effects of developmental treatments on stress response (CORT) variables. All models contain random effects of natal family. Age point 1: 127-134 days; age point 2 584-601 days

Age point	Outcome	Predictors	B	s.e. (B)	LRT	p-value
1	Baseline CORT	Amount	-0.00	0.21	0.00	0.99
		Effort	0.09	0.21	0.20	0.65
		Amount * Effort	0.02	0.21	0.01	0.92
	Peak CORT	Baseline CORT	1.82	0.81	4.54	0.03
		Amount	1.34	1.10	1.48	0.22
		Effort	1.19	1.10	1.15	0.28
		Amount * Effort	-1.26	1.10	1.30	0.25
	ΔCORT	CORT 15 mins	-0.69	0.09	25.84	<0.001
		Amount	-0.20	0.68	0.08	0.77
		Effort	1.51	0.65	4.75	0.03
		Amount * Effort	-0.87	0.66	1.60	0.21
2	Baseline CORT	Amount	0.08	0.27	0.02	0.89
		Effort	0.13	0.27	0.06	0.81
		Amount * Effort	1.03	0.27	0.88	0.35
	Peak CORT	Baseline CORT	0.95	0.64	1.85	0.17
		Amount	-0.00	0.91	0.00	0.99
		Effort	-0.90	0.91	0.95	0.33
		Amount * Effort	0.63	0.92	0.45	0.50
	ΔCORT	CORT 15 mins	-0.43	0.10	14.35	<0.001
		Amount	-0.09	0.52	0.03	0.86
		Effort	-1.10	0.52	4.08	0.04
		Amount * Effort	1.09	0.52	4.08	0.04

Figure 1

CORT values for individual birds at baseline, 15 minutes after onset of stressor, and 30 minutes after onset of stressor, at the two age points.

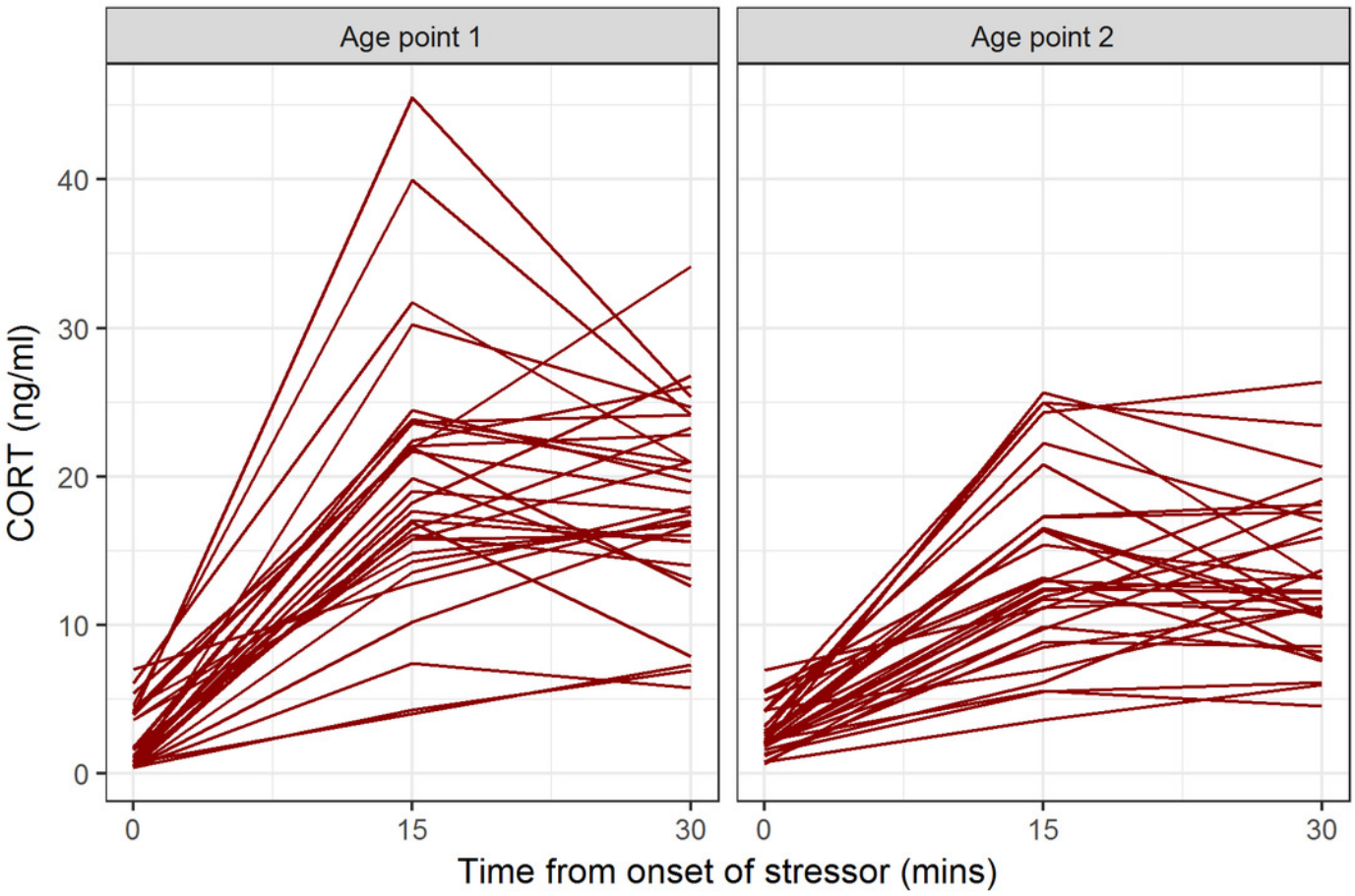

Figure 2

Estimated familial components of variation for each of the CORT measures at the two age points (age point 1: 127-134 days; age point 2 584-601 days).

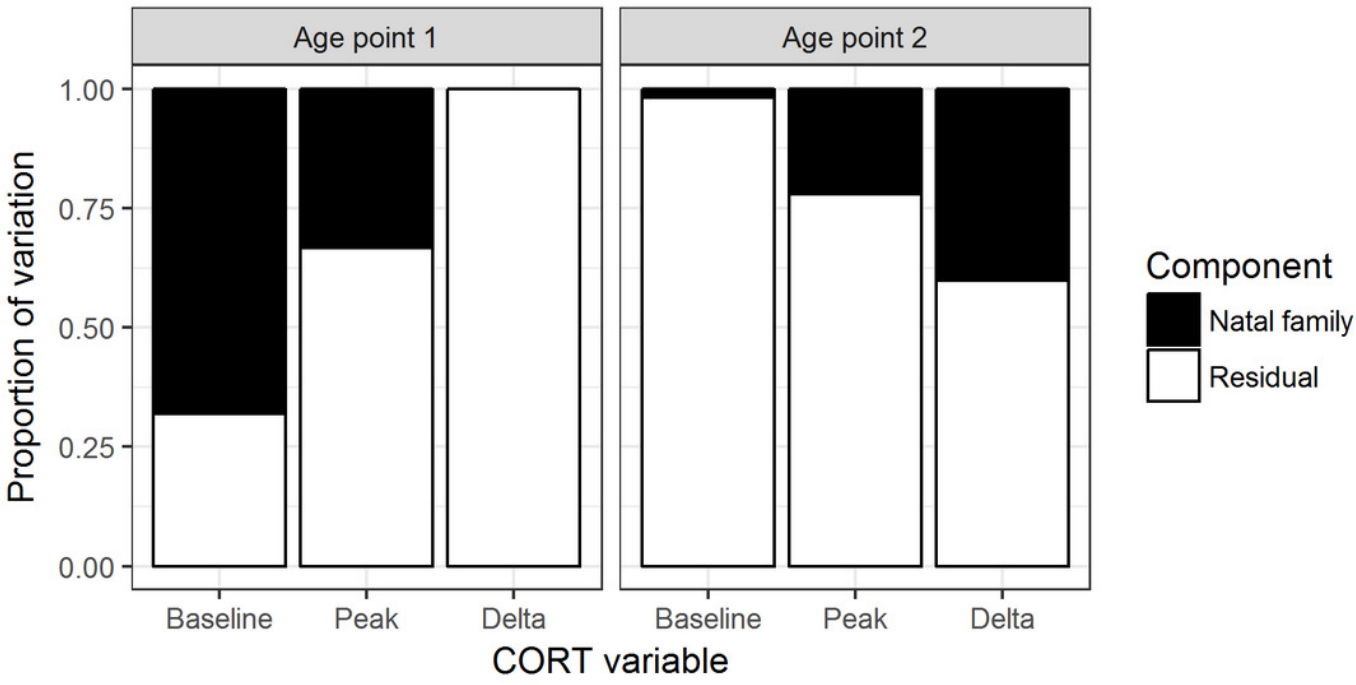

Figure 3

Forest plot of associations between developmental telomere change and CORT variables in the present cohort of birds (2014) and the two cohorts described previously (2012 and 2013).

The points and whiskers show standardized parameter estimates and their 95% confidence intervals. The lozenges show summary effects from meta-analytically combining the three datasets (upper lozenges use the age 1 point data for the present cohort, lower lozenges use the age point 2 data).

PeerJ Preprints | https://doi.org/10.7287/peerj.preprints.27057v1 | CC BY 4.0 Open Access | rec: 26 Jul 2018, publ: 26 Jul 2018