

Article

Telomere shortening is associated with corticosterone stress response in adult barn swallows

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Handling editor: David Swanson

Received on 28 July 2020; accepted on 3 March 2021

Abstract

When vertebrates face stressful events, the hypothalamic–pituitary–adrenal (HPA) axis is activated, generating a rapid increase in circulating glucocorticoid (GC) stress hormones followed by a return to baseline levels. However, repeated activation of HPA axis may lead to increase in oxidative stress. One target of oxidative stress is telomeres, nucleoprotein complexes at the end of chromosomes that shorten at each cell division. The susceptibility of telomeres to oxidizing molecules has led to the hypothesis that increased GC levels boost telomere shortening, but studies on this link are scanty. We studied if, in barn swallows *Hirundo rustica*, changes in adult erythrocyte telomere length between 2 consecutive breeding seasons are related to corticosterone (CORT) (the main avian GC) stress response induced by a standard capture-restraint protocol. Within-individual telomere length did not significantly change between consecutive breeding seasons. Second-year individuals showed the highest increase in circulating CORT concentrations following restraint. Moreover, we found a decline in female stress response along the breeding season. In addition, telomere shortening covaried with the stress response: a delayed activation of the negative feedback loop terminating the stress response was associated with greater telomere attrition. Hence, among-individual variation in stress response may affect telomere dynamics.

Key words: allostasis, corticosterone, *Hirundo rustica*, stress response, telomere

Organisms are adapted to face predictable and unpredictable events with different physiological and behavioral responses. Endocrine regulatory systems have evolved to mediate slow and long-term physiological changes related to predictable, demanding transitions between life history stages. In addition, hormones are involved in the rapid response to occasional short-term stressors and in the

maintenance of allostasis (Goymann and Wingfield 2004; McEwen 2010). An adequate physiological response to stressors is essential for survival in life-threatening situations or sudden environmental challenges. In vertebrates, the endocrine stress response is mainly mediated by the activation of the hypothalamic–pituitary–adrenal (HPA) axis (Sapolsky et al. 2000). Exposure to a source of stress is

accompanied by a rapid increase in the synthesis of glucocorticoids (GCs), resulting in a temporary inhibition of some body functions such as digestion, reproduction, immune response, and pain perception, and in a stimulation of essential ones such as energy mobilization, cognitive capacity, and cardiovascular tone (Sapolsky et al. 2000; McEwen 2010). The termination of the response to a stressful condition typically results in the activation of a GC-mediated negative feedback loop and in the subsequent return of GC to baseline levels. However, a prolonged or repeated exposure to stressors can result in chronic stress (Dickens and Romero 2013). In turn, this may lead to an increase of baseline GC levels, an increase in the intensity of the hormonal response to acute stressors, and an altered recovery dynamic of GC levels after the acute stress response (McEwen 1998).

In general, relatively high stress-induced GC levels potentially shift allostatic load to overload (McEwen and Wingfield 2003; Romero et al. 2009), with multiple physiological consequences, including increased metabolism (Jimeno et al. 2018). Increased metabolism, in turn, may lead to an overproduction of oxidizing reactive oxygen species (Finkel and Holbrook 2000), although the link between oxidative stress and metabolic rate has been questioned by some authors (Speakman and Garratt 2014; Alonso-Alvarez et al. 2017). If not balanced by antioxidant mechanisms, reactive oxygen species can damage biological molecules, including DNA, lipids, and proteins (Falnes et al. 2007). A potentially sensitive target for pro-oxidant molecules are telomeres, repeated DNA sequences rich in guanine that are particularly vulnerable to damaging strand breaks (Chatelain et al. 2020). Terminal telomeres are noncoding nucleoprotein complexes located at the end of eukaryotic chromosomes, where they mainly accomplish the function of stabilizing genomic material and preventing chromosome end fusion (Palm and de Lange 2008). In normal somatic cells, telomeres shorten at each cell division until a lower threshold length is reached; the cell then enters replication senescence or undergoes apoptosis (Blackburn 1991). Telomere attrition is thus part of physiological aging, which may be accompanied by progressive organismal loss of function (Monaghan and Haussmann 2006). However, the rate at which telomeres shorten depends on a number of extrinsic factors. These may include social environment (Costanzo et al. 2017), nutritional conditions (Young et al. 2017), environmental factors such as traffic noise (Injaian et al. 2019), and exposure to elevated GC levels (Haussmann et al. 2012; Herborn et al. 2014). In addition, telomere attrition does not proceed at a stable rate across life stages; in birds for example telomere shortening can be more pronounced in early life (Parolini et al. 2015, 2019) compared with adulthood (Hall et al. 2004; Pauliny et al. 2012).

The effects of GC on the production of pro-oxidants combined with the susceptibility of telomere dynamics to the action of oxidizing molecules have led to the hypothesis that increased GC levels can boost telomere shortening (Haussmann and Marchetto 2010; Monaghan 2014; Haussmann and Heidinger 2015; Bateson 2016; Angelier et al. 2018). Accordingly, negative covariation between the circulating concentration of the main avian GC, corticosterone (CORT), and telomere length has been observed in studies on nestlings during the pre-independence period and in adult birds (Angelier et al. 2018, but see Cerchiara et al. 2017). Most of the studies carried out so far have focused on baseline CORT levels, thus whether telomere dynamics can be associated to the responsiveness of the HPA axis during acute stress remains to be addressed (Angelier et al. 2018).

In this study, we investigated whether in adult barn swallows *Hirundo rustica* telomere length decreased from 1 year to the

following one. This analysis was not confounded by selective mortality since the same individuals were assayed in consecutive years. Moreover, in the second year, we analyzed the CORT stress response to a standard capture-restraint protocol, likely simulating predation (Wingfield and Ramenofsky 1999; Jones et al. 2016; Costanzo et al. 2018). Since inter-individual responses to stressful events may greatly vary in timing and intensity among individuals, we characterized the time course of the stress-induced CORT response by determining circulating CORT at 0 (baseline), 20, and 30 min after capture. Finally, we tested if CORT concentrations in the second year explained change in telomere length between the first and second year. Individuals with higher baseline and stress-induced CORT were predicted to have a larger decline in telomere between years. Studies of temporal consistency in stress response in other bird species showed repeatability levels of $R = 0.16$ for baseline and $R = 0.32$ for stress-induced CORT concentration (Schoenemann and Bonier 2018). In the barn swallow, the only study that investigated CORT levels repeatability was conducted within 1 breeding season and showed a repeatability of $R = 0.48$ for baseline and $R = 0.57$ for stress-induced CORT levels (Vitousek et al. 2017). Therefore, despite a large fraction of individual variation in CORT concentration remains unexplained, our analyses are based on the assumption that individuals may maintain similar patterns of stress responses across their lifetime.

Materials and Methods

Animal handling

We studied barn swallows at 8 colonies located close to Milan (Northern Italy) in 2017 (mean sampling date = May 22 [10.3 d SD]) and 2018 (mean sampling date = June 1 [9.7 d SD]). In 2017, we captured (using mist-nets), sexed, and individually marked all breeding adults; in addition, a blood sample was collected for telomere length analyses. In 2018, we focused our attention only on returning individuals that had already been sampled in 2017 (see Supplementary Material S1 for sample sizes). Since barn swallows of our study population have extremely high breeding philopatry (Møller 1994), we can assume that all the individuals which had not been already caught in the year preceding the experiments (2016) were 1-year-old individuals at their first breeding season. Thanks to our annual ringing activity at these colonies, we could therefore assess the age of all breeding individuals. However, due to a local annual mortality of 0.6–0.7 (Møller and De Lope 1999), the number of individuals older than 3 years of age in 2018 was low. Thus, we classified individuals in the following 3 age classes: class 1 (individuals of 1 year of age in 2017), 2 (individuals of 2 years of age in 2017), and 3 (individuals of 3 or more years of age in 2017).

In 2018, 75 recaptured individuals were subjected to a standard capture-restraint protocol (Wingfield and Ramenofsky 1999). We decided against a design with the stress protocol in the first year because this design would have required enlarging the sample to more than 200 birds in order to obtain enough recaptures the following year. At our study sites, birds spend the night inside the farms where they nest. Before dawn, we placed mist-nets at all the exits and we caught the birds as they left their colonies between 4:00 and 6:00 AM. This way, we could trap all the individuals soon after they had resumed their activities and presumably before they had encountered any other stressor. We took a blood sample within 3 min after capture to determine baseline CORT levels (see Romero and Reed 2005). Birds were then placed into a cloth bag until a second and a third blood sample was taken at 20 and 30 min after capture

(C_{20} and C_{30} , respectively) to measure stress-induced CORT levels. The C_{30} sample could be collected only from 25 out of the 75 sampled birds and comprised only 1 female of age class 3; therefore, we excluded this data point from further analysis and the final sample size for C_{30} was 24 (see [Supplementary Material S1](#) for sample sizes).

Blood samples were collected by puncturing the brachial vein and collecting blood (ca. 60 μ L) in heparinized capillary tubes. Upon capture, females were inspected for brood patch to obtain a proxy of the breeding stage. For the capture-restraint protocol, we used only females with incubation patches that could be unambiguously attributed either to the egg incubation stage (brood patch completely featherless, breast muscle not visible because of opaque skin, and broad wrinkles) or the nestling stage (brood patch featherless, breast muscle visible, and markedly thinner wrinkles) (own personal observation), indicative of females that were actively reproducing. Blood samples were kept on ice and then centrifuged within 12 h after collection in order to separate plasma (used for CORT analyses) from red blood cells (used for telomere length analyses) and finally stored at -80°C until laboratory analyses.

Telomere length analyses

Genomic DNA was extracted starting from 5 to 10 μ L of red blood cells using the Wizard DNA extraction kit (Promega, WI, USA). The amount and purity of the extracted DNA were measured using a Nanophotometer (IMPLEN). Telomere length was measured by monochrome multiplex quantitative PCR method (MMQPCR) ([Cawthon 2009](#)) on an iQ5 real-time PCR detection system (BioRad). According to this method, telomere length was measured as the ratio (T/S) between the amount of telomeric repeats (T) and the amount of a single-copy gene, CTCF (S), relative to a reference sample, and expressed as relative telomere length (RTL). Full methodological details of RTL measurement in the barn swallow are reported in [Parolini et al. \(2015\)](#). All reactions were run in triplicate. Samples were randomly assigned to the plates. Two samples were replicated in each plate to assess repeatability of telomere measurements. The mean intra- and inter-plate coefficient of variation (\pm SD) of RTL measures was $2.8 \pm 2.3\%$ and $2.3 \pm 1.6\%$, respectively.

CORT assay

We used a commercially available CORT ^{125}I radioimmunoassay kit (catalog no. 07-120102; MP Biomedicals, Solon, OH, USA) to quantify plasma levels of total CORT. We followed the protocol of the manufacturer with modifications as described in [Washburn and Millsbaugh \(2002\)](#). Briefly, the volume of all reagents was halved, and the dilution of the samples was 1:50 instead of 1:200. Furthermore, the standard curve was extended of 2 points below the lowest concentration to increase sensitivity. This assay has been validated and used for a number of avian species ([Soldatini et al. 2015](#); [Huber et al. 2017](#)). All samples were analyzed in duplicate in a total of 10 assays. The inter- and intra-assay coefficients of variation were below 6% and 10%, respectively.

Statistical analyses

A linear mixed model (LMM) was used to investigate the change of RTL between 2017 and 2018 with the bird ID as a random grouping factor and the fixed effects of sex, age class, year of sampling together with all two-way interactions. The same approach was used to investigate the effects of the capture-restraint protocol on baseline

C_0 , C_{20} , and C_{30} CORT concentrations by including the fixed effects of sex, age class, sampling date, sampling time, and all the two-way interactions. Pearson's correlation tests were performed to investigate the covariation between time of day when blood samplings were performed and CORT levels. In addition, to test the three-way interaction effects of sex, sampling time, and sampling date, we fitted a separate LMM to the C_0 and C_{20} data only (which were available for all individuals). To investigate the effects of female breeding stage (two-level factor indicating either incubation or nestling stage) on CORT concentrations, we fitted a LMM that included only females and tested additionally the fixed effects of age class, sampling date, sampling time, together with all the two-way interactions. Breeding stage and sampling date could be simultaneously included in the analyses because they were only weakly associated (binomial generalized linear model of breeding stage in relation to sampling date: $z = 0.24$, $P = 0.81$). The effects of sex, age class, sampling date, year, and CORT response (C_0 , C_{20} , C_{30} levels; change in CORT concentrations between C_0 and C_{20} ; change in CORT concentrations between C_{20} and C_{30}), and the interaction between year and CORT response on change in RTL were investigated by LMMs, including bird ID as a random grouping factor. In addition, the same approach was applied to investigate whether change in RTL could be predicted by the integral of CORT concentration from baseline to C_{30} (area under the curve, AUC_1), computed according to [Pruessner et al. \(2003\)](#) as area under the curve with respect to the baseline CORT concentration. In order to avoid the risk of inflating type-I error rate, two- and three-way interaction effects were included in the analyses only when an a priori hypotheses existed ([Whittingham et al. 2006](#)). Tukey's post-hoc tests were used to evaluate pairwise differences between levels of statistically significant factors. Since the number of observations for age classes 2 and 3 was low, we re-ran all the analyses pooling individuals of age class 2 and 3 and considering age as a two-level factor (age 1 versus older); the results were qualitatively unchanged (details not shown for brevity), so we reported in the main text only the analyses considering age as a three-level factor. All the analyses were run using R 3.5.2 statistical package ([R Core Team 2018](#)). LMMs were fitted using the lmer function from the lme4 ([Bates et al. 2015](#)) and the lmerTest package ([Kuznetsova et al. 2017](#)).

Results

Annual variation in RTL

Forty out of the 68 individuals (58.8%) for which RTL was measured in 2017 and 2018 showed a decline in RTL with age, whereas change in RTL was positive for the remaining individuals. A non-significant trend for a decrease in RTL between the 2 years was found (mean RTL in 2017: 1.02 [0.07 SD]; 2018: 1.00 [0.07]; $F_{1,64} = 2.29$, $P = 0.14$), and no significant effects of sex, age class, or their interaction on change in RTL emerged ($F_{1,62} \leq 2.14$, $P \geq 0.15$).

Variation in CORT concentrations

Individuals showed a classical stress response following a restraint ([Table 1](#)) with low baseline CORT levels [C_0 , mean \pm SE (ng/mL): 34 ± 19.2] and an increase of CORT concentrations after 20 (C_{20} : 73 ± 32.8) and 30 (C_{30} : 73 ± 31.5) min of restraint (Tukey's post hoc test: C_0 – C_{20} : $t_{89,2} = 10.10$, $P < 0.001$; C_0 – C_{30} : $t_{112,9} = 3.96$, $P < 0.001$). In 14 out of the 24 individuals for which we had both C_{20} and C_{30} CORT levels, CORT concentrations were higher at C_{20} than at C_{30} ([Figure 1](#)); however, the difference in CORT

Table 1. LMM of CORT concentrations according to sex, age class, time after capture, sampling date, and all their two-way interactions on all individuals and LMM of CORT concentrations according to the same predictors plus breeding stage on females only

	<i>F</i>	<i>df</i>	<i>P</i>
Males and females (<i>n</i> = 75)			
Sex	5.65	1, 66.41	0.020
Age class	2.27	2, 65.39	0.11
Time	90.58	2, 92.54	<0.001
Sampling date	3.37	1, 70.09	0.07
Sex × age class	0.73	2, 66.54	0.49
Sex × time	0.76	2, 94.90	0.47
Sex × sampling date	4.52	1, 70.08	0.037
Age class × time	4.08	4, 94.88	0.004
Age class × sampling date	0.31	2, 69.96	0.74
Time × sampling date	1.42	2, 100.89	0.25
Females only (<i>n</i> = 36)			
Age class	0.09	2, 27.17	0.91
Time	30.97	2, 40.97	<0.001
Sampling date	6.17	1, 29.53	0.019
Breeding stage	0.05	1, 26.93	0.82
Age class × time	2.37	3, 39.88	0.09
Age class × sampling date	0.78	2, 29.84	0.47
Age class × breeding stage	1.24	2, 30.11	0.30
Time × sampling date	2.29	2, 47.00	0.11
Time × breeding stage	0.40	2, 42.76	0.68
Sampling date × breeding stage	0.17	1, 29.80	0.69

Breeding stage (incubating versus brooding) was assessed inspecting female brood patches. Bold typeface indicates statistically significant effects.

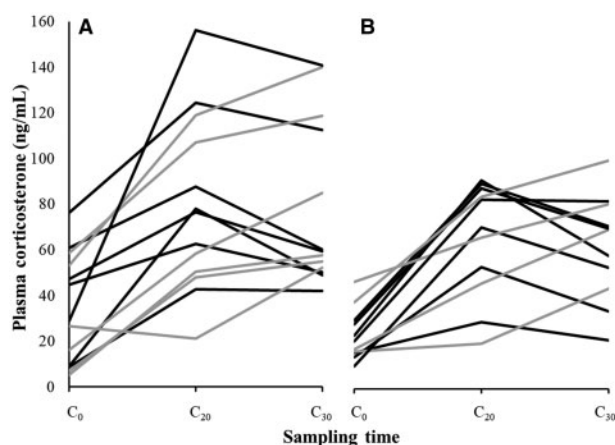


Figure 1. Change in CORT concentration measured after 20 (C_{20}) and 30 (C_{30}) min of restraint stress. Black lines represent those individuals whose CORT concentrations decreased after C_{20} ($n = 14$), while gray lines represent those whose CORT concentrations kept rising after that time point ($n = 10$). (A) Males ($n = 13$); (B) females ($n = 11$).

concentrations between C_{20} and C_{30} was statistically non-significant (Tukey's post hoc test: C_{20} – C_{30} : $t_{112.9} = 1.57$, $P = 0.26$). These results were not influenced by the time of day when blood samplings were performed (covariation between time of day and CORT response [C_0 , C_{20} , C_{30} levels; change in CORT concentrations between C_0 and C_{20} ; change in CORT concentrations between C_{20} and C_{30}]: $|r| < 0.34$, $P > 0.10$). CORT concentrations of individuals in age class 1 were higher than those of age class 3 at C_{20} only ($t_{115} = 2.82$, $P = 0.016$) while no other significant pairwise differences were detected ($t_{133} \leq |1.99|$, $P \geq 0.12$). Post-hoc tests

revealed that individuals of age class 1 had the most pronounced increase in CORT levels 20 min after capture, when compared with individuals of age classes 2 ($t_{89.2} \geq 2.82$, $P < 0.006$) and 3 ($t_{89.2} \geq 2.89$, $P < 0.005$), whereas no other differences in stress response among age classes and time intervals were found ($t_{105.1} \leq 0.78$, $P \geq 0.44$). CORT concentrations varied differently with sampling date according to sex (Table 1). In particular, CORT concentrations were on average higher in males than in females ($t_{102} = 2.02$, $P = 0.046$) and decreased during the breeding season in females ($t_{86.53} = 2.15$, $P = 0.034$) but not in males ($t_{88.03} = 0.16$, $P = 0.88$). When we restricted the analyses to C_0 and C_{20} time points, the three-way interaction effect showed that baseline CORT levels did not change during the breeding season in both sexes (males: slope estimate = -0.31 ± 0.48 [SE], $t_{100.18} = 0.65$, $P = 0.52$; female: -0.68 ± 0.51 , $t_{99.33} = 1.34$, $P = 0.18$), while C_{20} CORT levels showed a statistically significant decline in females (-1.70 ± 0.51 , $t_{99.33} = 3.33$, $P = 0.001$) but not in males (0.22 ± 0.48 , $t_{100.18} = 0.45$, $P = 0.66$) (Figure 2). Finally, female breeding stage did not significantly affect CORT levels and the seasonal decrease of CORT (Table 1).

Covariation between RTL and CORT concentrations

C_0 , C_{20} , and C_{30} CORT concentrations, and AUC_1 , were separately tested as predictors of change in RTL between 2017 and 2018; however, none of these factors had significant effects on RTL (Supplementary Material S2).

Change in CORT concentrations between C_0 and C_{20} did not predict change in RTL between the 2 breeding seasons (Table 2). Conversely, change in CORT concentrations between C_{20} and C_{30} predicted change in RTL between the 2 breeding seasons. In particular, individuals showing a prolonged increase in CORT concentration between the 2 breeding seasons ($t_{21} = 2.24$, $P = 0.023$) (Figure 3), independently of any sex, age class, or sampling date effect. All the results remained qualitatively unchanged when the analyses were restricted to the 24 individuals for which we had C_{30} CORT concentrations (details not shown for brevity).

Discussion

In the present study, we investigated the association between the endocrine stress response and telomere dynamics in individuals of our long-term study population of barn swallows. To this aim, we compared telomere attrition within adult barn swallows between 2 consecutive years and subjected them to a standard capture-restraint protocol to evaluate the temporal pattern of increase in circulating CORT levels.

We found only a marginal decline in RTL during the 2 consecutive breeding seasons, with approximately 40% of the individuals experiencing an increase in RTL, which, in some cases, was non-negligible. It is unlikely that these results are due to systematic measurement errors, since the same analytical methods highlighted a significant decline in telomere length during the nestling period in a companion study (A. Costanzo, unpublished data; see also Parolini et al. 2015). Telomere elongation had been previously attributed to measurement errors, in particular in qPCR-based studies, however recent work showed that telomeres may recover in wild populations (Spurgin et al. 2018; van Lieshout et al. 2019), although statistical tests for detecting true positive change in RTL are still under development (Nettle and Bateson 2017). The present results are therefore consistent with other studies of avian species that failed to find

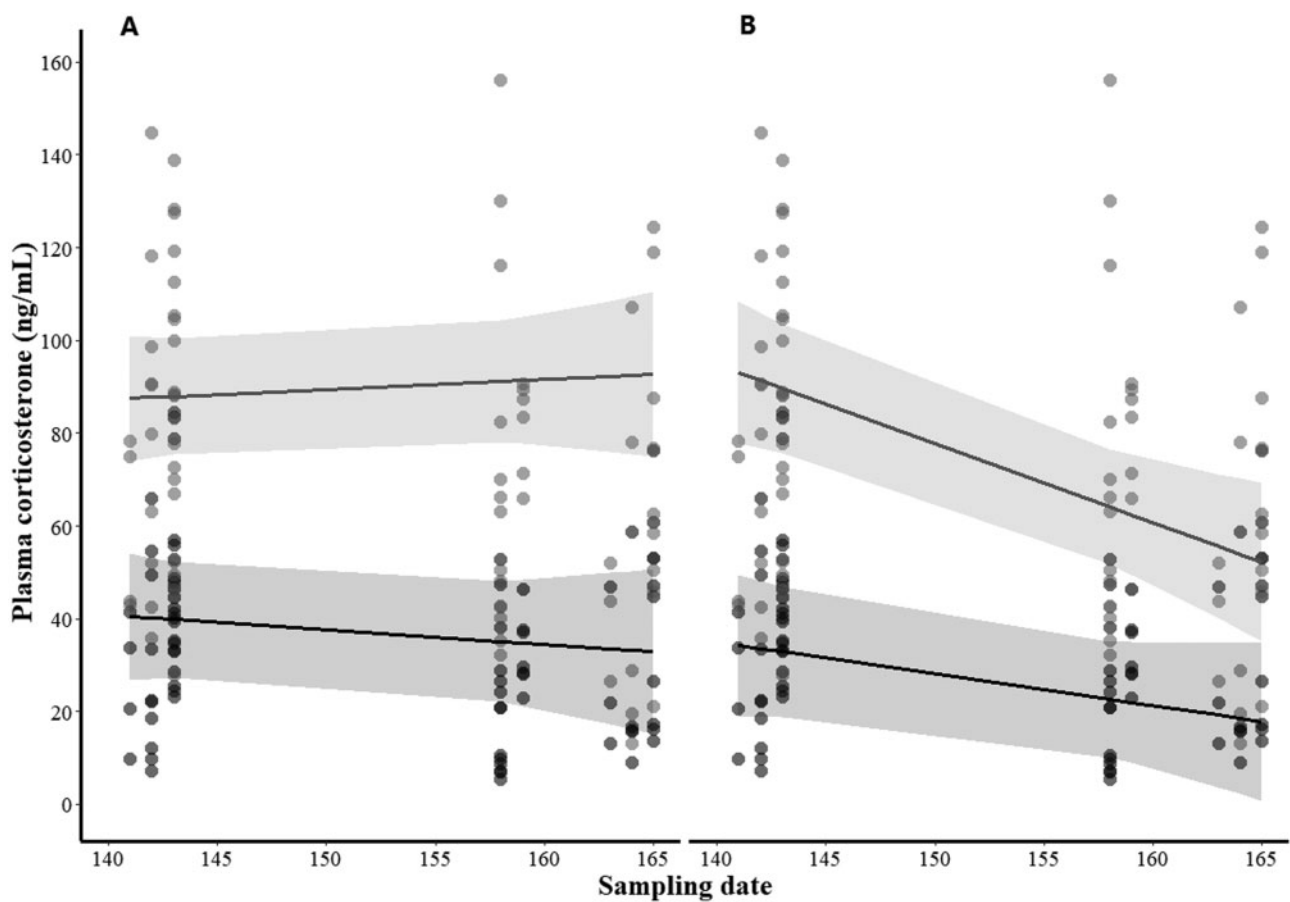


Figure 2. Change in C₀ (black line) and C₂₀ (gray line) CORT concentrations along the breeding season (Julian date, 1 = 1st January) in (A) males and (B) females.

Table 2. LMM of change in telomere length in relation to sex, age class, year, sampling date and change in CORT concentration between C₀ and C₂₀ and between C₂₀ and C₃₀

	<i>F</i>	<i>Df</i>	<i>P</i>
Change in RTL (<i>n</i> = 68)			
Sex	2.08	1, 61.99	0.16
Age class	0.33	2, 61.99	0.72
Sampling date	0.06	1, 61.99	0.82
Year	1.57	1, 65.99	0.22
Change in CORT concentration between C ₀ and C ₂₀	0.01	1, 61.99	0.93
Year × Change in CORT concentration between C ₀ and C ₂₀	0.18	1, 65.99	0.67
Change in RTL (<i>n</i> = 23)			
Sex	0.18	1, 17	0.68
Age class	0.32	2, 17	0.55
Sampling date	0.01	1, 17	0.92
Year	0.59	1, 21	0.45
Change in CORT concentration between C ₀ and C ₂₀	0.10	1, 17	0.75
Year × Change in CORT concentration between C ₀ and C ₂₀	6.06	1, 21	0.023

Bold typeface indicates statistically significant effects.

evidence for telomere attrition in adulthood (Hall et al. 2004; Pauliny et al. 2012), and further suggest that telomere dynamics may be life stage-specific (Young 2018).

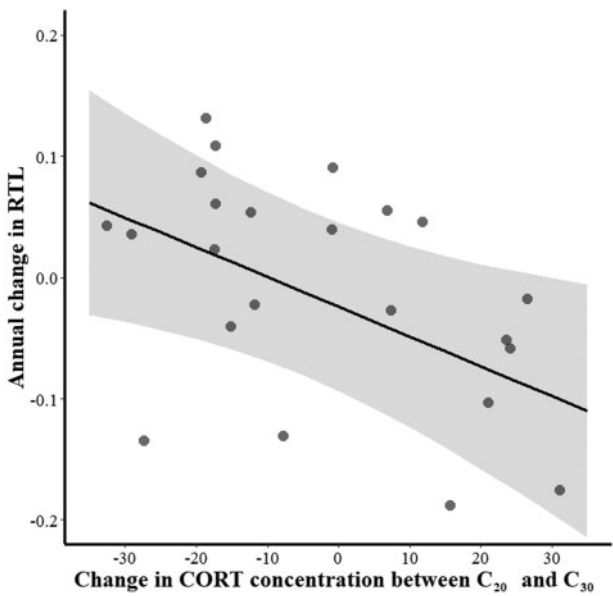


Figure 3. Change in RTL between 2017 and 2018 in relation to change in CORT concentration measured in 2018 after 20 (C₂₀) and 30 (C₃₀) min of restraint stress. Negative values of telomere change represent telomere shortening between the 2 breeding seasons.

When subjected to the capture-restraint protocol developed by Wingfield and Ramenofsky (1999), individuals showed a typical endocrine stress response, with an increase of CORT secretion

20 min after capture (Table 1). Despite intense experimental effort, sample size for some tests was limited and the relative results should be taken cautiously. CORT baseline values found in the present study (34 ± 19.2 ng/mL) are overall higher compared with other studies of the same species (e.g., 6.2 ± 0.7 ng/mL; Vitousek et al. 2014). This difference might be due to different reasons. Populations may differ in baseline levels due to unknown environmental factors. A more plausible reason is the timing of sampling. In the present study, blood sampling was performed between 04:00 and 06:00 a.m. Considering the circadian rhythm of CORT concentration, we probably caught the birds during a time window in which CORT levels are decreasing from the peak reached before sunrise but are still elevated compared with day hours (Breuner et al. 1999; Rich and Romero 2001; Turriani et al. 2016). Because some studies have shown that during this time window also the stress response might vary (see Carere et al. 2003), the lack of a covariation between time of day and CORT response strongly suggests that the narrow sampling time window allowed us to minimize potentially confounding inter-individual differences due to circadian variation in hormone concentrations (Saino 2002). In both sexes, baseline CORT levels did not vary across the breeding season, whereas CORT levels at C_{20} declined with sampling date in females only (Figure 2). Studies conducted on other avian species disclosed sex differences in adrenal activity in breeding birds (Bokony et al. 2009), while no sex differences were observed in other life history stages (Cornelius et al. 2012; Krause et al. 2014). The sensitivity of the HPA axis to a stressor may reflect adaptations linked to sex roles and facilitate sex-specific reproductive behaviors (Romero and Wingfield 1999) related to parental care (Adkins-Regan 2005). In species in which females provide more parental care than males, the former sex usually shows lower baseline and stress-induced CORT levels during the breeding season than the latter (Holberton and Wingfield 2003; Meddle et al. 2003). Indeed, it has been demonstrated that elevated CORT levels are associated with a higher likelihood of abandoning the clutch (Silverin 1986; Wingfield and Silverin 1986). In contrast, higher stress-induced CORT concentrations in males are associated with increased aggressive behavior (Kitaysky et al. 2003), potentially promoting successful nest and partner defense.

Individuals belonging to different age classes showed clear differences in stress response (Table 1). In particular, individuals in age class 1 showed the highest increase in CORT concentrations following restraint. Individuals in age class 3, on the other side, had the lowest baseline CORT levels and a dampened stress response. The observation of age differences in CORT concentrations does not necessarily contradict our assumption of consistency in individual stress response both within and among breeding seasons, since we assumed that individuals tend to have CORT levels higher or lower than other individuals of the same age. The stress response is among the key hormonal mechanisms mediating the trade-off between investment in current reproduction and future survival and reproduction, as predicted by the “brood value hypothesis” (Heidinger et al. 2006; Bokony et al. 2009). According to this theory, as an organism ages, the value of its current reproductive effort increases due to the reduction of future reproductive opportunities. While immediate survival is favored by a fast elevation of CORT levels (Sapolsky et al. 2000) that promotes gluconeogenesis and enhances the respiratory and the cardiovascular system following a stressful event (Sapolsky et al. 2000), the same CORT response inhibits processes that are not immediately vital, such as reproduction, parental care, and immune functions. Thus, in accordance with the “brood value

hypothesis,” individuals in age class 1 showed the strongest GC response in their stress response to our standardized stress protocol. However, it remains unclear whether the reduced stress response in individuals in age classes 2 and 3 was due to aging effects or if these individuals were less sensitive to capture-restraint procedures due to habituation to previous captures (Rabdeau et al. 2019). It is important to note that these results are not confounded by differences in timing of sampling relative to breeding stage among individuals of different age classes (Table 1). Indeed, in males, baseline and stress-induced CORT levels did not change along the breeding season, suggesting that variation in CORT levels among individuals of different ages was not related to differences in reproductive conditions. Similarly, in females the observed decrease in stress-induced CORT levels during the breeding season was not related to differences in their breeding stage, as no differences in CORT levels were found between incubating and brooding individuals.

Although CORT responses are often discussed at population level in terms of mean response, considerable variation in magnitude and time course of CORT synthesis exists among individuals (Cockrem 2007). This applies also to our results. Indeed, the analyses of the mean responses showed no significant differences in CORT levels between C_{20} and C_{30} . However, one of the most peculiar result of this study is that a closer inspection of the CORT stress response profiles revealed the existence of 2 distinct patterns of stress response (Figure 1). Some individuals showed a decline in CORT levels between C_{20} and C_{30} , probably due to an earlier activation of the negative feedback loop terminating the stress response. Other individuals showed a prolonged CORT response, with a further increase in CORT levels between C_{20} and C_{30} , which suggests a delayed activation of the negative feedback. This result was independent of any age and sex effect (see Figure 1). Intraspecific variation in patterns of CORT secretions has been linked to personality in other species (Carere et al. 2010). Personalities vary along a continuum, but individuals are usually classified as “proactive” or “reactive,” with the former being generally bolder and more aggressive than the latter (Dingemanse and Réale 2005). Studies carried out on birds selected for divergent CORT responses (Cockrem 2007; Baugh et al. 2012) showed that proactive, bolder individuals exhibit a lower CORT response following a given stressor (Baugh et al. 2012). Unfortunately, to date little research on personality has been conducted in barn swallows (Corti et al. 2017; Costanzo et al. 2018), therefore this aspect is at present speculative and deserves further investigations.

Changes in CORT levels between C_{20} and C_{30} were found to be related to telomere shortening (Table 2 and Figure 3). Individuals showing a further increase in CORT 20 min after capture-restraint showed a larger telomere attrition between 2 consecutive years when compared with those showing decreasing CORT after 20 min. CORT concentrations at C_{20} and C_{30} were overall higher in individuals of age class 1 (see above); however, it is important to remember that in this analysis we did not consider the absolute values of CORT concentration but the relative differences between time points, which are not influenced by any sex or age effect. Individual differences in the activation of the negative feedback that terminates the stress response have been related to the ability to cope with challenges, and different studies demonstrated an association between the strength of the negative feedback and several major physiological and fitness traits such as oxidative stress and telomere shortening (Rich and Romero 2005; Haussmann et al. 2012; Monaghan 2014; Casagrande and Hau 2018), sexual and parental behavior (Vitousek et al. 2019; Zimmer et al. 2019), and survival (Romero and Wikelski 2010). An

additional explanation for the association between telomere shortening and stress response may be related to variability in personality traits, which is known to covary with life-history and physiological differences. Indeed, proactive and reactive personalities are associated to higher or lower responsiveness of the HPA axis, respectively; a stronger reactivity of the HPA axis is, in turn, associated with an increase in metabolism (Angelier et al. 2018), with potentially negative effects on telomere length via an imbalance of redox status in favor of pro-oxidants (Finkel and Holbrook 2000; Falnes et al. 2007). Therefore, an association between telomere dynamics and slower or faster activation of the negative feedback could arise. In addition, different CORT levels may affect the process restoring telomere length by acting on the enzyme telomerase (Choi et al. 2008). Despite no telomerase activity is commonly considered to occur in normal somatic cells of adult birds, some studies have shown telomere elongation with age, even when controlling for mortality related to telomere length (Haussmann et al. 2007). Yet, an alternative interpretation of the present correlational results is that both telomere length and stress response strategies covary with an underlying causative factor, and that the relationship between CORT concentration and telomere length is not directly causal in nature. For example, individuals exposed to specific ecological or social conditions could retain relatively high baseline CORT levels and undergo more rapid telomere shortening if extrinsic factors have effects on telomere dynamics that are not mediated by the HPA axis. Therefore, in the barn swallow, future studies should be aimed at disentangling whether the association between telomere dynamics and the activity of the HPA axis is causal or, conversely, results from an underlying causative factor.

In conclusion, we showed that in our barn swallow population a capture-restraint protocol caused an increase in circulating CORT concentrations that varied according to sex and age of individuals. In particular, females but not males showed a decrease in stress-induced CORT concentrations during the breeding season, suggesting a reduced probability of nest abandonment. In addition, in agreement with the “brood value hypothesis,” individuals in age class 1 showed the strongest stress response, favoring the immediate survival over the inhibition of non-vital processes such as parental care. However, the alternative explanation of a physiological aging effect cannot be ruled out, and future experimental studies should be carried out to determine the relative importance of these 2 non-mutually exclusive hypotheses. Moreover, our data suggested the existence of 2 different patterns of stress response, with some individuals showing further increase and others decline of CORT concentration after 20 min of restraint (C_{20}). Finally, we identified a relationship between CORT stress response and telomere shortening between 2 breeding seasons, whereby individuals with a probably delayed termination of the CORT response showed a larger annual decrease in telomere length. Therefore, our results broaden the understanding of the mechanistic links between the stress response and aging, revealing that among-individual differences in the time course of the stress response, likely reflecting personality differences, may impact telomere dynamics. Further experimental studies are needed to establish the direction of causation in the associations between functioning of the HPA axis, as reflected by the strength of the negative feedback that terminates the CORT stress response, and telomere dynamics.

Author Contributions

A.C., R.A., M.P., D.R., L.F., and V.C. designed the study. A.C., R.A., M.P., M.C., and D.R. collected the data. A.C., M.P., M.C., S.S., and V.C. performed the laboratory analyses. A.C., R.A., L.F.,

and V.C. analyzed the data. A.C. and V.C. wrote the article. All authors read and approved the final manuscript.

Acknowledgments

The authors wish to thank Fondazione Fratelli Confalonieri (Milan, Italy) for funding the postdoctoral fellowship of A.C., and the anonymous reviewers for constructive criticism that improved previous drafts of the manuscript. The hormone analyses were supported by start-up funds of the University of Vienna to L.F. This work was inspired by the ideas of the late Prof. Nicola Saino, who significantly contributed to planning, data collection, and initial analyses conducted for this study.

Supplementary Material

Supplementary material can be found at <https://academic.oup.com/cz>.

Competing Interests

The authors have no competing interests to declare.

References

- Adkins-Regan E, 2005. *Hormones and Animal Social Behavior*. Princeton: Princeton University Press.
- Alonso-Alvarez C, Canelo T, Romero-Haro A, 2017. The oxidative cost of reproduction: theoretical questions and alternative mechanisms. *BioScience* 67: 258–270.
- Angelier F, Costantini D, Blévin P, Chastel O, 2018. Do glucocorticoids mediate the link between environmental conditions and telomere dynamics in wild vertebrates? A review. *Gen Comp Endocrinol* 256: 99–111.
- Bates D, Sarkar D, Bates MD, Matrix L, 2015. Fitting Linear Mixed-Effects Models Using lme4. *Journal of Statistical Software* 67:1–48.
- Bateson M, 2016. Cumulative stress in research animals: telomere attrition as a biomarker in a welfare context? *BioEssays* 38: 201–212.
- Baugh AT, Schaper SV, Hau M, Cockrem JF, de Goede P et al., 2012. Corticosterone responses differ between lines of great tits *Parus major* selected for divergent personalities. *Gen Comp Endocrinol* 175: 488–494.
- Blackburn EH, 1991. Structure and function of telomeres. *Nature* 350: 569–573.
- Bokony V, Lendvai AZ, Liker A, Angelier F, Wingfield JC et al., 2009. Stress response and the value of reproduction: are birds prudent parents? *Am Nat* 173: 589–598.
- Breuner CW, Wingfield JC, Romero LM, 1999. Diel rhythms of basal and stress-induced corticosterone in a wild, seasonal vertebrate, Gambel's white-crowned sparrow. *J Exp Zool* 284: 334–342.
- Carere C, Caramaschi D, Fawcett TW, 2010. Covariation between personalities and individual differences in coping with stress: converging evidence and hypotheses. *Curr Zool* 56: 728–740.
- Carere C, Groothuis TGG, Möstl E, Daan S, Koolhaas JM, 2003. Fecal corticosteroids in a territorial bird selected for different personalities: daily rhythm and the response to social stress. *Horm Behav* 43: 540–548.
- Cawthon RM, 2009. Telomere length measurement by a novel monochrome multiplex quantitative PCR method. *Nucleic Acids Res* 37: 1–7.
- Casagrande S, Hau M, 2018. Enzymatic antioxidants but not baseline glucocorticoids mediate the reproduction-survival trade-off in a wild bird. *Proc R Soc B Biol Sci* 285: 20182141.
- Cerchiara JA, Risques RA, Prunkard D, Smith JR, Kane OJ et al., 2017. Magellanic penguin telomeres do not shorten with age with increased reproductive effort, investment, and basal corticosterone. *Ecol Evol* 7: 5682–5691.
- Chatelain M, Drobniak SM, Szulkin M, 2020. The association between stressors and telomeres in non-human vertebrates: a meta-analysis. *Ecol Lett* 23: 381–398.

- Choi J, Fauce SR, Effros RB, 2008. Reduced telomerase activity in human T lymphocytes exposed to cortisol. *Brain Behav Immun* 22: 600–605.
- Cockrem JF, 2007. Stress, corticosterone responses and avian personalities. *J Ornithol* 148: 169–178.
- Cornelius JM, Breuner CW, Hahn TP, 2012. Coping with the extremes: stress physiology varies between winter and summer in breeding opportunists. *Biol Lett* 8: 312–315.
- Corti M, Bazzi G, Costanzo A, Podofillini S, Saino N et al., 2017. Behavioural stress response and melanin-based plumage colouration in barn swallow nestlings. *Behaviour* 154: 853–874.
- Costanzo A, Parolini M, Bazzi G, Khoraiuli L, Santagostino M et al., 2017. Brood size, telomere length, and parent–offspring color signaling in barn swallows. *Behav Ecol* 28: 204–211.
- Costanzo A, Romano A, Ambrosini R, Parolini M, Rubolini D et al., 2018. Barn swallow antipredator behavior covaries with melanin coloration and predicts survival. *Behav Ecol* 29: 1472–1480.
- Dickens MJ, Romero LM, 2013. A consensus endocrine profile for chronically stressed wild animals does not exist. *Gen Comp Endocrinol* 191: 177–189.
- Dingemanse NJ, Réale D, 2005. Natural selection and animal personality. *Behaviour* 142: 1159–1184.
- Falnes P, Klungland A, Alseth I, 2007. Repair of methyl lesions in DNA and RNA by oxidative demethylation. *Neuroscience* 145: 1222–1232.
- Finkel T, Holbrook NJ, 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239–247.
- Goymann W, Wingfield JC, 2004. Allostatic load, social status and stress hormones: the costs of social status matter. *Anim Behav* 67: 591–602.
- Hall ME, Nasir L, Daunt F, Gault EA, Croxall JP et al., 2004. Telomere loss in relation to age and early environment in long-lived birds. *Proc R Soc B Biol Sci* 271: 1571–1576.
- Hausmann MF, Heidinger BJ, 2015. Telomere dynamics may link stress exposure and ageing across generations. *Biol Lett* 11: 20150396.
- Hausmann MF, Longenecker AS, Marchetto NM, Juliano SA, Bowden RM, 2012. Embryonic exposure to corticosterone modifies the juvenile stress response, oxidative stress and telomere length. *Proc R Soc B Biol Sci* 279: 1447–1456.
- Hausmann MF, Marchetto NM, 2010. Telomeres: linking stress and survival, ecology and evolution. *Curr Zool* 56: 714–727.
- Hausmann MF, Winkler DW, Huntington CE, Nisbet ICT, Vleck CM, 2007. Telomerase activity is maintained throughout the lifespan of long-lived birds. *Exp Gerontol* 42: 610–618.
- 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? *Proc R Soc B Biol Sci* 273: 2227–2231.
- Herborn KA, Heidinger BJ, Boner W, Noguera JC, Adam A et al., 2014. Stress exposure in early post-natal life reduces telomere length: an experimental demonstration in a long-lived seabird. *Proc R Soc B Biol Sci* 281: 20133151.
- Holberton RL, Wingfield JC, 2003. Modulating the corticosterone stress response: a mechanism for balancing individual risk and reproductive success in arctic-breeding sparrows? *Auk* 120: 1140–1150.
- Huber N, Fusani L, Ferretti A, Mahr K, Canoine V, 2017. Measuring short-term stress in birds: comparing different endpoints of the endocrine–immune interface. *Physiol Behav* 182: 46–53.
- Injaian AS, Gonzalez-Gomez PL, Taff CC, Bird AK, Ziur AD et al., 2019. Traffic noise exposure alters nestling physiology and telomere attrition through direct, but not maternal, effects in a free-living bird. *Gen Comp Endocrinol* 276: 14–21.
- Jimeno B, Hau M, Verhulst S, 2018. Corticosterone levels reflect variation in metabolic rate, independent of ‘stress.’ *Sci Rep* 8: 1–8.
- Jones BC, Smith AD, Bebus SE, Schoech SJ, 2016. Two seconds is all it takes: European starlings *Sturnus vulgaris* increase levels of circulating glucocorticoids after witnessing a brief raptor attack. *Horm Behav* 78: 72–78.
- Kitaysky AS, Kitaishkaia EV, Piatt JF, Wingfield JC, 2003. Benefits and costs of increased levels of corticosterone in seabird chicks. *Horm Behav* 43: 140–149.
- Krause JS, Dorsa D, Wingfield JC, 2014. Changes in plasma concentrations of progesterone, dehydroepiandrosterone and corticosterone in response to acute stress of capture, handling and restraint in two subspecies of white-crowned sparrows. *Comp Biochem Physiol Part A Mol Integr Physiol* 177: 35–40.
- Kuznetsova A, Brockhoff PB, Christensen RHB, 2017. lmerTest package: tests in linear mixed effects models. *J Stat Softw* 82: 1–26.
- McEwen BS, 1998. Stress, adaptation, and disease: allostasis and allostatic load. *Ann N Y Acad Sci* 840: 33–44.
- McEwen BS, 2010. Allostasis and allostatic overload in the context of aging. In: Fillit HM, Rockwood K, Woodhouse K, editors. *Brocklehurst's Textbook of Geriatric Medicine and Gerontology*. Elsevier. 158–162.
- McEwen BS, Wingfield JC, 2003. The concept of allostasis in biology and biomedicine. *Horm Behav* 43: 2–15.
- Meddle SL, Owen-Ashley NT, Richardson MI, Wingfield JC, 2003. Modulation of the hypothalamic–pituitary–adrenal axis of an Arctic-breeding polygynandrous songbird, the Smith's longspur *Calcarius pictus*. *Proc R Soc B Biol Sci* 270: 1849–1856.
- Møller AP, 1994. *Sexual Selection and the Barn Swallow* Oxford: Oxford University Press.
- Møller AP, De Lope F, 1999. Senescence in a short-lived migratory bird: age-dependent morphology, migration, reproduction and parasitism. *J Anim Ecol* 68: 163–171.
- Monaghan P, 2014. Organismal stress, telomeres and life histories. *J Exp Biol* 217: 57–66.
- Monaghan P, Hausmann MF, 2006. Do telomere dynamics link lifestyle and lifespan? *Trends Ecol Evol* 21: 47–53.
- Nettle D, Bateson M, 2017. Detecting telomere elongation in longitudinal datasets: analysis of a proposal by Simons, Stulp and Nakagawa. *PeerJ* 5: e3265.
- Palm W, de Lange T, 2008. How shelterin protects mammalian telomeres. *Annu Rev Genet* 42: 301–334.
- Parolini M, Possenti CD, Romano A, Caprioli M, Rubolini D et al., 2019. Perinatal variation and covariation of oxidative status and telomere length in yellow-legged gull chicks. *Curr Zool* 65: 509–516.
- Parolini M, Romano A, Khoraiuli L, Nergadze SG, Caprioli M et al., 2015. Early-life telomere dynamics differ between the sexes and predict growth in the barn swallow *Hirundo rustica*. *PLoS ONE* 10: 1–21.
- Pauliny A, Larsson K, Blomqvist D, 2012. Telomere dynamics in a long-lived bird, the barnacle goose. *BMC Evol Biol* 12: 1–8.
- Pruessner JC, Kirschbaum C, Meinlschmid G, Hellhammer DH, 2003. Two formulas for computation of the area under the curve represent measures of total hormone concentration versus time-dependent change. *Psychoneuroendocrinology* 28: 916–931.
- Rabreau J, Badenhauer I, Moreau J, Bretagnolle V, Monceau K, 2019. To change or not to change experimenters: caveats for repeated behavioural and physiological measures in Montagu's harrier. *J Avian Biol* 50: e02160.
- Rich EL, Romero LM, 2001. Daily and photoperiod variations of basal and stress-induced corticosterone concentrations in house sparrows *Passer domesticus*. *J Comp Physiol B* 171: 543–547.
- Rich EL, Romero LM, 2005. Exposure to chronic stress downregulates corticosterone responses to acute stressors. *Am J Physiol Integr Comp Physiol* 288: 1628–1636.
- Romero LM, Dickens MJ, Cyr NE, 2009. The reactive scope model—a new model integrating homeostasis, allostasis, and stress. *Horm Behav* 55: 375–389.
- Romero LM, Wingfield JC, 1999. Alterations in hypothalamic–pituitary–adrenal function associated with captivity in Gambel's white-crowned sparrows *Zonotrichia leucophrys gambelii*. *Comp Biochem Physiol Part B: Biochem Mol Biol* 122: 13–20.
- Romero LM, Reed JM, 2005. Collecting baseline corticosterone samples in the field: is under 3 min good enough? *Comp Biochem Physiol Part A Mol Integr Physiol* 140: 73–79.
- Romero LM, Wikelski M, 2010. Stress physiology as a predictor of survival in Galapagos marine iguanas. *Proc R Soc B Biol Sci* 277: 3157–3162.
- Saino N, 2002. Immune response of male barn swallows in relation to parental effort, corticosterone plasma levels, and sexual ornamentation. *Behav Ecol* 13: 169–174.

- Sapolsky RM, Romero LM, Munck AU, 2000. How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. *Endocr Rev* 21: 55–89.
- Silverin B, 1986. Corticosterone-binding proteins and behavioral effects of high plasma levels of corticosterone during the breeding period in the pied flycatcher. *Gen Comp Endocrinol* 64: 67–74.
- Soldatini C, Albores-Barajas YV, Tagliavia M, Massa B, Fusani L et al., 2015. Effects of human disturbance on cave-nesting seabirds: the case of the storm petrel. *Conserv Physiol* 3: cov041.
- Schoenemann KL, Bonier F, 2018. Repeatability of glucocorticoid hormones in vertebrates: a meta-analysis. *PeerJ* 6: e4398.
- Speakman JR, Garratt M, 2014. Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model. *BioEssays* 36: 93–106.
- Spurgin LG, Bebbington K, Fairfield EA, Hammers M, Komdeur J et al., 2018. Spatio-temporal variation in lifelong telomere dynamics in a long-term ecological study. *J Anim Ecol* 87: 187–198.
- R Core Team, 2018. R: A Language and Environment for Statistical Computing. Version 3.5.2. Vienna: R Foundation for Statistical Computing.
- Turriani M, Bernabò N, Barboni B, Todisco G, Montini L et al., 2016. Circadian rhythm and stress response in droppings of *Serinus canaria*. *Vet Med Int* 2016: 3086353.
- van Lieshout SHJ, Bretman A, Newman C, Buesching CD, Macdonald DW et al., 2019. Individual variation in early-life telomere length and survival in a wild mammal. *Mol Ecol* 28: 4152–4165.
- Vitousek MN, Jenkins BR, Hubbard JK, Kaiser SA, Safran RJ, 2017. An experimental test of the effect of brood size on glucocorticoid responses, parental investment, and offspring phenotype. *Gen Comp Endocrinol* 247: 97–106.
- Vitousek MN, Jenkins BR, Safran RJ, 2014. Stress and success: individual differences in the glucocorticoid stress response predict behavior and reproductive success under high predation risk. *Horm Behav* 66: 812–819.
- Vitousek MN, Taff CC, Ryan TA, Zimmer C, 2019. Stress resilience and the dynamic regulation of glucocorticoids. *Integr Comp Biol* 59: 251–263.
- Washburn BE, Millspaugh JJ, 2002. Effects of simulated environmental conditions on glucocorticoid metabolite measurements in white-tailed deer feces. *Gen Comp Endocrinol* 127: 217–222.
- Wingfield JC, Ramenofsky M, 1999. Hormones and the behavioral ecology of stress. In: Balm PHMS, editor. *Stress Physiology in Animals*. Sheffield, UK: Sheffield Academic Press. pp. 1–51.
- Whittingham MJ, Stephens PA, Bradbury RB, Freckleton RP, 2006. Why do we still use stepwise modelling in ecology and behaviour? *J Anim Ecol* 75: 1182–1189.
- Young AJ, 2018. The role of telomeres in the mechanisms and evolution of life-history trade-offs and ageing. *Philos Trans R Soc B Biol Sci* 373: 20160452.
- Young RC, Welcker J, Barger CP, Hatch SA, Merkle T et al., 2017. Effects of developmental conditions on growth, stress and telomeres in black-legged kittiwake chicks. *Mol Ecol* 26: 3572–3584.
- Zimmer C, Taff CC, Ardia DR, Ryan TA, Winkler DW et al., 2019. On again, off again: acute stress response and negative feedback together predict resilience to experimental challenges. *Funct Ecol* 33: 619–628.