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Changes in adrenal capacity contribute to a decline in the stress response with age in a long-lived seabird

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ABSTRACT

In response to stressors, most vertebrates elevate secretion of glucocorticoids (CORT) to produce a 'stress response' that enhances survival, but simultaneously inhibits reproduction. Circumstances in which the value of current reproduction is high relative to the value of future reproduction and survival, often lead to suppression of the stress response thus ensuring that critical resources are not diverted away from reproduction. Consistent with this expectation, we have previously reported that the magnitude of the stress response (maximum levels of CORT) declines with age in breeding adults of a long-lived seabird, the common tern (*Sterna hirundo*). While age-related changes in the stress response may be common in vertebrates, the mechanisms that underlie them are poorly understood. The glucocorticoid stress response is regulated by the hypothalamic–pituitary–adrenal (HPA) axis, and one mechanism that may contribute to an age-related decline in the stress response is changes in adrenal capacity (adrenal sensitivity to ACTH and/or an ability to secrete CORT in response to ACTH). To test this hypothesis, we captured and injected 92 known-aged adult common terns (*Sterna hirundo*) ranging in age from 3 to 29 years with either a control saline or an experimental adrenocorticotropin (ACTH) solution and measured the effects on stress-induced CORT after 30 min of restraint. In both treatment groups, stress-induced CORT significantly declined with age, suggesting that a decrease in adrenal capacity contributes to a reduction in the stress response in older adults.

1. Introduction

In vertebrates, natural stressors such as inclement weather, reduced food availability, and predators stimulate a rise in glucocorticoid (CORT) levels (Sapolsky et al., 2000). Elevated CORT is expected to enhance survival by promoting gluconeogenesis (Sapolsky et al., 2000), foraging, and escape behavior (Wingfield, 2003), while simultaneously suppressing reproduction and parental care behavior (Silverin, 1987; Wingfield et al., 1995; Kitaysky et al., 2001; Wingfield, 2003). Consequently, when the value of current reproduction is high relative to the value of survival and future reproduction, the stress response is often attenuated (reviewed in Wingfield and Sapolsky, 2003). The value of current reproduction is predicted to increase with age because future reproductive opportunities are expected to decline (Stearns, 1992). In terms of the stress response, older parents are therefore predicted to respond less strongly to stressors than younger parents to ensure that reproduction is not inhibited (Wingfield and Sapolsky, 2003; Heidinger et al., 2006).

Consistent with this expectation, we have previously reported that maximum CORT levels produced in response to an experimentally imposed stressor—capture and restraint stress—decrease with age in both sexes of breeding adults of a long-lived seabird, the common tern (*Sterna hirundo*) (Heidinger et al., 2006). An age-related attenuation of the stress response has also been reported in turtles (Jessop and Hamann, 2005), rats (Hess and Riegle, 1970, 1972; Britton et al., 1975; Brett et al., 1983), and in some studies of humans (reviewed in Otte et al., 2005).

Age-related changes in the stress response may also occur as a result of senescence, which may involve an increase as opposed to a decrease in the magnitude and duration of the stress response (Stein-Behrens and Sapolsky, 1992). Some human studies have reported an increase in the magnitude and the duration of the stress response in elderly adults, which may be linked to several age-related health disorders including Alzheimer's disease, depression, diabetes, metabolic syndrome, and hypertension (reviewed in Otte et al., 2005). The duration of the stress response has also been reported to increase with age in rats (Sapolsky et al., 1983a;

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Meaney et al., 1988, 1990). Although age-related changes in the glucocorticoid stress response may be common, we currently have little information about the physiological mechanisms involved in mediating these changes in natural populations of long-lived vertebrates (Stein-Behrens and Sapolsky, 1992).

The glucocorticoid stress response is regulated by the hypothalamic–pituitary–adrenal (HPA) axis (Sapolsky et al., 2000) and changes in stress-induced CORT secretion may be modulated at several different, non-mutually exclusive levels (reviewed in Romero, 2006). For example, age-related attenuation of the stress response may be due to reductions in any or all of the following: (i) the release of corticotropin releasing hormone (CRH) and/or arginine vasotocin (AVT) from the hypothalamus (Astheimer et al., 1995; Romero et al., 1998b,c), (ii) pituitary sensitivity to CRH and/or AVT and/or an ability of the pituitary to produce adrenocorticotropin (ACTH) (Romero et al., 1998a,b), (iii) adrenal sensitivity to ACTH and/or an ability of the adrenal to produce CORT (Romero and Wingfield, 1998), (iv) negative feedback regulation of CORT secretion (Stein-Behrens and Sapolsky, 1992), or an increase in (v) the rate at which CORT is cleared from the body (Purnell et al., 2004).

As a first step towards elucidating which aspects of the HPA axis are involved in mediating age-related changes in the stress response in a natural population of a long-lived vertebrate, we captured known-aged, breeding adult common terns (*Sterna hirundo*) and exposed them to either an experimental ACTH injection (which stimulates the adrenal gland to produce CORT) or a control saline injection and measured the effects on stress-induced CORT levels after 30 min of restraint. If suppression of the stress response with age occurs largely because of a decrease in adrenal capacity (sensitivity to ACTH and/or an ability to produce CORT, iii above), we predicted that stress-induced CORT levels would decrease with age in adults given either a saline or an ACTH injection. Alternatively, if age-related attenuation of the stress response occurs not at the level of the adrenal gland, but rather higher up the HPA axis at the level of the pituitary, hypothalamus, or perception of the stressor, we predicated that the stress response would decrease with age in adult common terns (*Sterna hirundo)* given saline injections, but not experimental ACTH injections.

2. Materials and Methods

2.1. Study site and species

Between May and July 2005 we studied a large breeding population of common terns (*Sterna hirundo*) on Bird Island, in Buzzards Bay, MA, USA (41°40′N, 70°43′W; for a detailed description of this study site see Nisbet et al., 1984). The reproductive biology of this population has been studied for over 30 years and many of the chicks have been banded soon after hatching each year. Because chicks often return to Bird Island to breed as adults, many of the terns in this population are of known age. We have previously reported that the stress response (maximum elevation of CORT in response to capture and restraint) is attenuated with age, as well as varying with body mass and date in male and females in this population (Heidinger et al., 2006). We included known-aged adults of both sexes in this study and conducted all research in accordance with the Bloomington Institutional Animal Care and Use Committee.

2.2. Measuring adrenal sensitivity to adrenocorticotropin hormone (ACTH)

As in our previous study, we located and marked nests as eggs were laid, and sampled adults between days 9 and 15 of the incubation period (Heidinger et al., 2006). To measure baseline CORT levels we captured terns on the nest using walk-in treadle traps and collected a 200 μl blood sample from the jugular vein within 3 min of capture. Terns were then randomly assigned to a treatment group and given either a 40 μl intra-muscular injection of 100 IU kg^{-1} of average body weight (0.12 kg for this population) porcine ACTH (Sigma Chemical Co. # A6303) in the experimental group or saline in the control group within 5 min of capture. This ACTH dosage was chosen based on what has previously been reported in the literature for several species of birds (Romero et al., 1998c; Meddle et al., 2003; Romero, 2006) as well as on a pilot study conducted on this population of terns earlier in the season. In the pilot study, individuals that were given either 100 or 200 IU kg^{-1} of average body weight porcine ACTH had significantly higher stress-induced CORT levels than adults given saline injections, however there was no significant differences between the two doses employed. Therefore, we chose the lower dose to be consistent with other previously reported studies (Romero et al., 1998c; Meddle et al., 2003; Romero, 2006) and because extremely elevated ACTH levels are expected to enhance the duration of the stress response rather than elicit greater CORT secretion (Stein-Behrens and Sapolsky, 1992).

To measure the effect of treatment on stress-induced CORT levels, we placed terns in individual holding tubes, which held the birds loosely but prevented them from struggling and opening their wings. We then collected a subsequent 200 μl blood sample from the alar vein after 30 min (the time when CORT levels are typically maximized in response to handling and restraint stress) (Heidinger et al., 2006). Adults were weighed and measured before release. Sexes were determined based on head length; birds in the zone of overlap were treated as indeterminate (Nisbet et al., 2007). Blood samples were kept on ice for <6 h before they were centrifuged and separated. Plasma was stored at −20 °C until hormone analysis.

2.3. CORT assay

Plasma CORT levels were measured using standard radioimmunoassay techniques (Wingfield and Farner, 1975; Ketterson et al., 1991). Samples were equilibrated with 2000 cpm ^{3}H-B for 24 h so that extraction recoveries could be measured. The samples were then extracted with 4 ml of diethyl ether and re-suspended in 550 μl of phosphate buffer. Finally, the samples were measured in duplicate using a competitive binding assay. We corrected final values for plasma volumes and individual recoveries after extraction. The average recovery value was 77% and the average intra and inter-assay coefficients of variation were 8% and 9%, respectively.

2.4. Statistical analysis

We sampled 92 known-aged terns, 41 in the experimental treatment group whose ages ranged from 4 to 21 years, and 51 in the control treatment group whose ages ranged from 3 to 29 years. *T*-tests were used to verify that treatment groups did not differ with respect to age, body mass, date, or baseline CORT levels (initial sample collected within 0–3 min of capture prior to injection) and a repeated-measures ANOVA was used to confirm that restraint elevated CORT levels. We used an ANCOVA to examine the potential effects of injection treatment, sex, age, body mass, date, and baseline CORT on stress-induced CORT levels (sample collected 30 min post-capture). CORT levels were log-transformed to improve normality. We used a backward elimination process and independent variables and interactions with ($p > 0.1$) were excluded from the final model. All statistical analyses were performed in SPSS (16.0 for Mac OS X).

Sex could be assigned with greater than 90% confidence for 50 adults (9 females and 13 males in the experimental group and 12 females and 16 males in the control group). There was no significant effect of sex (ANCOVA, $F_{3,46} = 0.025$, $p = 0.874$) or interaction between sex and injection treatment (ANCOVA, $F_{3,46} = 2.320$, $p = 0.153$) on stress-induced CORT levels. Consequently, sex was not included as a factor in subsequent analyses.

3. Results

There were no significant differences between treatment groups with respect to age ($F_{1,90} = 0.255$, $p = 0.514$), body mass ($F_{1,90} = 1.387$, $p = 0.900$), date ($F_{1,90} = 0.546$, $p = 0.269$), or baseline CORT levels ($F_{1,90} = 1.172$, $p = 0.381$) and CORT levels significantly increased in response to restraint stress in both treatment groups (repeated-measures ANOVA, $F_{1,91} = 466.4$, $p < 0.001$).

Adults that received ACTH injections had significantly higher stress-induced CORT levels than controls (ANCOVA, $F_{4,87} = 6.605$, $p = 0.012$). Importantly, however, stress-induced CORT levels negatively covaried with age regardless of treatment (ANCOVA, $F_{4,87} = 7.531$, $p = 0.007$) and there was no significant interaction effect between treatment and age (ANCOVA, $F_{5,86} = 2.441$, $p = 0.122$). There was a trend for stress-induced CORT levels to increase with baseline CORT levels (ANCOVA, $F_{4,87} = 3.613$, $p = 0.061$) and to decrease with date (ANCOVA, $F_{4,87} = 2.772$, $p = 0.100$) in both treatment groups. However, there were no significant effects of body mass (ANCOVA, $F_{6,85} = 2.088$, $p = 0.152$) or any interaction effects between treatment and body mass (ANCOVA, $F_{8,83} = 0.015$, $p = 0.903$), or date (ANCOVA, $F_{7,84} = 0.600$, $p = 0.441$) on stress-induced CORT levels.

Two of the data points were statistically significant outliers at $p = 0.05$ (Sokal and Rohlf, 1995), the oldest individual and the individual with the highest stress-induced CORT level. However, when

both of these data points were excluded we obtained qualitatively similar results. ACTH injections significantly elevated stress-induced CORT levels relative to controls (ANCOVA, $F_{3,86} = 4.820$, $p = 0.031$; Fig. 1). Stress-induced CORT levels significantly declined with age in both treatment groups (ANCOVA, $F_{3,86} = 4.046$, $p = 0.047$; Fig. 2) and there was no significant treatment × age interaction effect (ANCOVA, $F_{6,83} = 0.246$, $p = 0.0.621$). There were no significant effects of baseline CORT levels (ANCOVA, $F_{4,85} = 2.680$, $p = 0.105$), date (ANCOVA, $F_{5,84} = 1.325$, $p = 0.253$), or any interaction effects between treatment and body mass (ANCOVA, $F_{7,82} = 0.015$, $p = 0.904$), or date (ANCOVA, $F_{8,81} = 0.016$, $p = 0.899$) on stress-induced CORT levels. However, in contrast to the analysis that included the outliers, stress-induced CORT significantly declined with body mass in both treatment groups (ANCOVA, $F_{3,86} = 6.130$, $p = 0.015$).

4. Discussion

Age-related changes in the stress response may be common among vertebrates, but we currently have little information from natural populations about the mechanisms that underlie these changes. Here we report the first evidence that a change in adrenal capacity contributes to the age-related decline in the stress response previously reported in a free-living population of a long-lived seabird. In common terns, stress-induced CORT levels significantly declined with age irrespective of ACTH exposure. This finding suggests that older terns produce lower stress-induced CORT levels than younger terns at least in part because of a reduction in adrenal sensitivity to ACTH and/or the ability to secrete CORT in response to a stressor.

Although few studies have examined the mechanisms that underlie age-related changes in the stress response in non-mammalian vertebrates, our results are generally consistent with what has been found in rats. Several studies have reported that the adrenal glands of older rats are hypo-responsive to ACTH both *in vivo* and *in vitro* (Hess and Riegle, 1970, 1972; Britton et al., 1975; Tang and Phillips, 1978; Malamed and Carisa, 1983; Popplewell et al., 1986, 1987; although see Riegle, 1973; Sonntag et al., 1987; Scaccianoce et al., 1995; Kasckow et al., 2005).

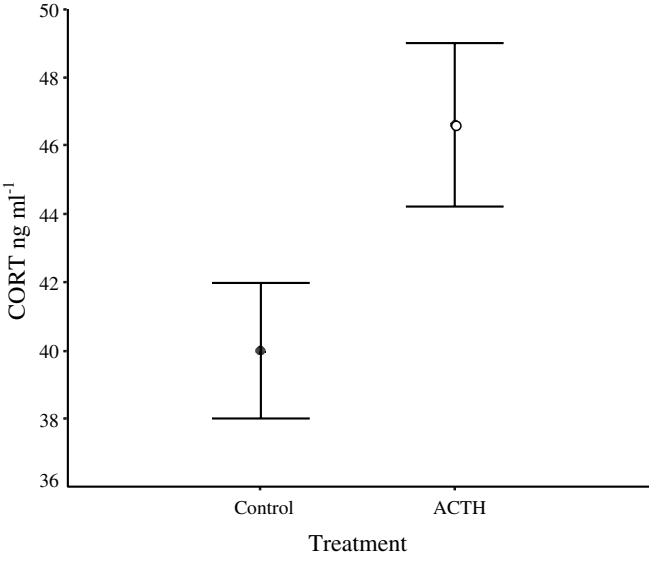

Fig. 1. Changes in plasma CORT levels (mean ± sem) in response to capture and restraint stress of adults that received either control saline (closed circle, $n = 50$) or ACTH (open circle, $n = 40$). Adults that received ACTH injections had significantly higher stress-induced CORT levels 30 min post-capture than adults that received control saline injections (ANCOVA, $F_{3,86} = 4.820$, $p = 0.031$).

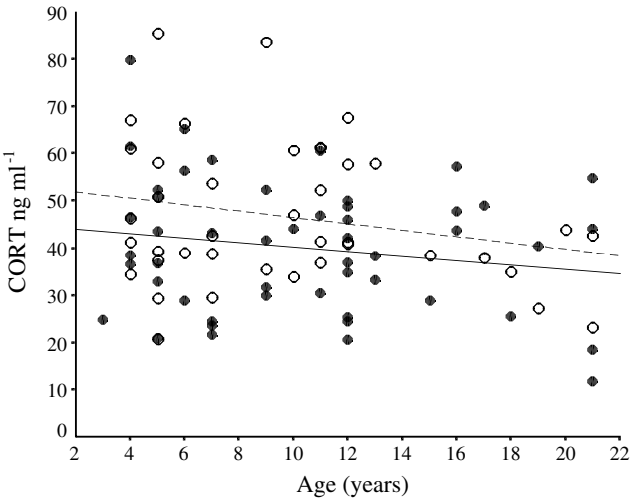

Fig. 2. The relationship between age and stress-induced CORT levels (30 min post-capture) in adults that received either control saline (closed circles, solid line) or experimental ACTH injections (open circle, dotted line). Stress-induced CORT levels significantly decreased with age regardless of treatment (ANCOVA, $F_{3,86} = 4.046$, $p = 0.047$).

This decreased adrenal responsiveness in rats does not appear to be due to a reduction in ACTH receptor densities or ACTH activation of cAMP (Popplewell et al., 1986). Instead, it is most likely caused by a decrease in the delivery of cholesterol for steroid synthesis (Popplewell et al., 1987; Popplewell and Azhar, 1987). There is evidence that the activity of cholesterol esterase, an enzyme that converts stored cholesterol esters to cholesterol for steroidogenesis, declines with age (Popplewell et al., 1987; Popplewell and Azhar, 1987). Similarly, the activity of hydroxymethylglutaryl (HMG) CoA reductase, an enzyme essential for steroid synthesis, has also been reported to be lower in older adults (Popplewell et al., 1987; Popplewell and Azhar, 1987). Together these age-related decreases in enzyme activity may play an important role in reducing the ability of the adrenocortical cells to synthesize and secrete CORT in response to ACTH (Popplewell et al., 1987; Popplewell and Azhar, 1987).

Age-related changes in the stress response may also occur upstream of the adrenal gland at the level of the pituitary, hypothalamus, or perception as well as downstream of the adrenal gland at the level of glucocorticoid binding globulins (CBG), glucocorticoid receptors, or clearance rates. The hypothalamus has been reported to produce higher stress-induced CRH levels with age (Hauger et al., 1994), while the pituitaries of older rats have been reported to be hypo-responsive to CRH *in vivo* (Hylka et al., 1984; Hauger et al., 1994), to have fewer CRH receptors (Heroux et al., 1991), and to produce lower stress-induced ACTH levels (Hauger et al., 1994) than the pituitaries of younger rats. In female rats, it has been suggested that declining estrogen levels may also contribute to a decrease in stress-induced CORT levels with age (Stein-Behrens and Sapolsky, 1992). Estrogen has been reported to induce CBG (Sandberg and Slaunwhite, 1959); consequently declining estrogen may lead to a concomitant reduction in CBG. If CBG levels decrease, CORT production may also decline to compensate for this change so that the amount of free CORT that is able to bind to receptors remains unchanged (Stein-Behrens and Sapolsky, 1992). It is unlikely, however, that this mechanism is involved in mediating an age-related modulation of the stress response in common terns, because neither estrogen (Nisbet et al., 1999) nor CBG levels (Heidinger, unpublished data) declines with age in this population.

Although older terns have lower stress-induced CORT levels than younger terns, baseline CORT levels do not change with age

in this species (Heidinger et al., 2006). In contrast, aging in rats is often accompanied by an increase in baseline CORT levels (Tang and Phillips, 1978; Sapolsky et al., 1983a; Brett et al., 1983) as well as the duration of the stress response (Sapolsky et al., 1983a; Meaney et al., 1988, 1990). One mechanism that is thought to contribute to this hyper-secretion of CORT is a senescent degeneration of the hippocampus (reviewed in Stein-Behrens and Sapolsky, 1992; Wang et al., 1997). The hippocampus is an area in the brain with large concentrations of both high-affinity Type I and lower-affinity Type II corticosterone receptors that are involved in the negative feedback regulation of CORT release (McEwen et al., 1986). Both types of corticosterone receptors have been reported to decline in the hippocampus of older rats (Meaney et al., 1990; Sapolsky et al., 1983b; van Eekelen et al., 1991), and this reduction is thought to contribute to an increase in baseline CORT levels and the duration of the stress response in old age.

This study focused on the mechanisms underlying an attenuation of the stress response with age in breeding adults; however, there is also evidence that the stress response changes with age during early development (reviewed in Wada et al., 2007). In rats (Haltmeyer et al., 1966), trout (Barry et al., 1995), and several species of birds (Sims and Holberton, 2000; Love et al., 2003; Walker et al., 2005; Blas et al., 2006; Wada et al., 2007) the stress response is suppressed soon after hatching or birth and increases throughout maturation. A suppression of the stress response during this time is thought to be adaptive because exposure to elevated CORT levels during development can have several negative, long-term consequences including suppressed growth (Morici et al., 1997), immunity (reviewed in McEwen et al., 1997), and neuronal cell numbers (Howard and Benjamins, 1975). Few studies have looked at the mechanisms that regulate a suppression of the stress response early in life. In the white-crowned sparrow (*Zonotrichia leucophrys nuttalli*), an altricial songbird, the adrenal gland does appear to be involved in this early suppression. Sparrow chicks injected with exogenous ACTH within 3 days of hatching were not capable of secreting CORT levels as high as those produced by adults (Wada et al., 2007). In contrast, in the Magellanic penguin (*Spheniscus magellanicus*), a semi-altricial seabird, the adrenal gland does not seem to play a role in regulating an early suppression of the stress response as penguin chicks exposed to exogenous ACTH within 5 days of hatching were capable of producing adult-like CORT levels (Walker et al., 2005).

In addition to modulation with age, the stress response has also been reported to vary seasonally (Romero and Wingfield, 1998; Romero et al., 1998a,c; Carsia and John-Alder, 2003; Romero, 2006) and between the sexes (Meddle et al., 2003; Carsia and John-Alder, 2003). Changes in adrenal sensitivity to ACTH have been reported to be involved in seasonal variation in CORT secretion during molt in many species of songbirds (Romero and Wingfield, 1998; Romero et al., 1998a,c; Romero, 2006) and following the reproductive period in eastern fence lizards (*Sceloporus undulates*) (Carsia and John-Alder, 2003). Changes in adrenal sensitivity to ACTH have also been reported to play a role in sex-related differences in the stress response in Smith's longspur (*Calcarius pictus*) (Meddle et al., 2003) and eastern fence lizards (Carsia and John-Alder, 2003).

In summary, our results suggest that terns produce lower maximum CORT levels in response to a stressor when they are older than when they are younger due at least in part to reduced adrenal capacity. Because this study was necessarily cross-sectional in design the decline we observed might be attributable to population level processes such as selection for individuals with lower adrenal capacity, longitudinal changes within individuals, or both. In addition, age-related modifications of the stress response may also involve changes upstream of the adrenal gland at the level of the pituitary, hypothalamus, or perception, as well as downstream of the adrenal gland at the level of CBG, glucocorticoid receptors, or clearance rates. Our results highlight the need for future studies on a wide range of species that vary in lifespan to gain a full understanding of the mechanisms that underlie age-related changes in the stress response.

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