RESEARCH NOTE

Patterns of variation of serum oxidative stress markers in two seabird species

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Abstract

Variation in oxidative stress markers in natural populations may provide a useful background for understanding variation in life history strategies. In this study, we seek to evaluate patterns of variation in levels of reactive oxygen metabolites (markers of oxidative damage), serum antioxidant capacity, and serum concentration of thiols (antioxidants endogenously synthesized) in nestling and breeding blue petrels (*Halobaena caerulea*) and in breeding Antarctic prions (*Pachyptila desolata*). Male and female prions and nestling petrels did not differ in any of the oxidative stress markers. The serum antioxidant capacity positively correlated with the sample time in nestling blue petrels. Breeding petrels with higher body condition index had higher serum antioxidant capacity and circulating thiols. Finally, both seabird species showed lower levels of reactive oxygen metabolites and higher levels of serum antioxidant capacity than previously studied bird species.

Stressful conditions are an important ecological and evolutionary force, modulating adaptive responses of natural populations (von Schantz et al. 1999; Romero 2004; Korte et al. 2005; Costantini 2008). Individuals in a population are continually challenged with stressors of a different nature, which may jeopardize individual fitness. Stressful challenges may cause a disturbance in the balance between pro-oxidants and antioxidants, leading to oxidative damage to biomolecules. This gives rise to oxidative stress (Sies 1991; Halliwell & Gutteridge 2007; Costantini 2008; Monaghan et al. 2009), which may lead to degenerative pathologies, cell senescence and cell death (Beckman & Ames 1998; Finkel & Holbrook 2000).

In birds, several factors have been shown to affect the oxidative stress state of an individual. For example, immune responses (Costantini & Dell'Omo 2006) or stress hormones (Costantini, Fanfani et al. 2008) may cause changes in oxidative stress markers. The identification of the factors underlying the oxidative stress response is important to identify adaptations evolved by animal species to cope with oxidative stress, and to understand interspecific variation in life histories.

In this study, we seek to evaluate patterns of variation in levels of reactive oxygen metabolites (ROMs; markers of oxidative damage), serum antioxidant capacity (OXY) and serum concentration of thiols (a class of endogenously synthesized antioxidants, such as thioredoxin and glutathione) in nestling and breeding blue petrels (*Halobaena caerulea* Gmelin, 1789) and in breeding Antarctic prions (*Pachyptila desolata* Gmelin, 1789).

Blue petrels are small, slow-breeding and long-lived seabirds that undergo long fasts several times per year during breeding (Brooke 2004). Blue petrels lay a single egg in a nest burrow, and both parents incubate the egg for around 44 days. During this phase, partners alternate incubation shifts, relieving each other from the nest every 9–12 days (Brooke 2004). Antarctic prions, closely related to blue petrels, and with similar life-history traits, are also small, slow-breeding and long-lived seabirds. Prions also lay a single egg and incubate it for around 44 days (Brooke 2004).

Materials and methods

Study area and sample collection

Blood samples were collected on a small sub-Antarctic island (Ile Verte, 49°51'S, 70°05'E) in the Kerguelen

Archipelago between January and March 2005 for adult Antarctic prions and blue petrel chicks, and in November and January 2007 for adult blue petrels. A study colony of 180 burrows of both species has been followed since 2001 on this island. Burrows were fitted with a window over the incubating chamber to facilitate the capture of birds. In 2005, 15 blue petrel chicks (five females and 10 males), and 15 incubating Antarctic prions (nine females and six males) were sampled, and in 2007 eight incubating blue petrels (one male, six females and one unknown) were sampled. All breeders were in roughly the same phase of incubation (15–25 days), and chicks, given the high synchrony of hatching in this species, were all around 45–50 days old. The tarsus length, wing length and body mass of each bird were recorded. Blood samples were collected from the brachial vein, and blood was immediately centrifuged and serum stored at −20°C while in the field. Samples were then transported to the laboratory and stored at −80°C until the analysis.

Laboratory analyses

Reactive oxygen metabolites (ROMs) and total serum antioxidant capacity were measured using the d-ROMs test (Diacron International, Grosseto, Italy) and the OXY-Adsorbent test (Diacron International), respectively, in accordance with methods reported for previous studies (e.g., Costantini et al. 2006; Costantini & Dell'Omo 2006). Repeatability was significantly high for both markers of oxidative stress (intra-assay repeatability: ROMs, $r = 0.89$, $P < 0.001$; OXY, $r = 0.86$, $P < 0.001$).

Thiols were quantified using the -SHp test (Diacron International). Thiols are compounds that contain a functional group composed of a sulfur atom and a hydrogen atom (-SH). Therefore, they are also known as sulfhydryl groups. Thioredoxin and glutathione are three of the major thiols synthesized by animals. These molecules play important roles in antioxidant systems (Dickinson & Forman 2002; Bindoli et al. 2008). Firstly, 25 µL of 5,5-dithiobys-2-nitrobenzoyc acid (DTNB; chromogen) was diluted with 500 µL of a buffer phosphate solution (pH 7.6). Then, serum (30 µL) was diluted with this solution and incubated at room temperature for 5 min. Serum thiols react with DTNB, producing a coloured complex directly correlated with thiol concentration (Ellman 1959; Carratelli et al. 2001). The absorbance was read with a Beckman DU 7400 spectrophotometer at a wavelength of 405 nm (repeatability: $r = 0.98$, $P < 0.001$). A blank zero adjustment was always performed. Concentrations were calculated using a standard solution of L-cysteine (496 µM of -SH groups) purchased with the kit (Diacron International).

Statistical analyses

Statistical analyses were performed with STATISTICA 7.0 (StatSoft, 2004, Tulsa, OK, USA). The variation in levels of serum oxidative stress markers in nestling blue petrels, adult blue petrels and adult prions was analysed by one-way ANOVA, including the species as an independent variable, and ROMs, OXY or thiol concentration as dependent variables. Post-hoc comparisons with Fisher's least significant difference (LSD) test were computed when results were significant. Sexes were compared using a Mann–Whitney U-test because of the small sample size. Bivariate correlations were computed using Pearson correlation.

The correlation between body mass and each physiological variable was computed using partial correlation. Body mass and metric measures (wing length and tarsus length for adult blue petrels and prions, tarsus length for nestling blue petrels) were always included in the partial correlation model as independent variables. This approach allowed us to correct the correlation between body mass and the dependent variable for variation in body size, so as to estimate the correlation between body condition (body energy reserves, such as fat or proteins) and oxidative stress markers (García-Berthou 2001). Values are shown as means ± standard errors.

Results

Differences between the three groups in ROMs ($F_{2,35} = 3.89$, $P = 0.03$), serum antioxidant capacity ($F_{2,35} = 10.17$, $P < 0.001$) and circulating thiols ($F_{2,29} = 6.44$, $P = 0.005$) were significant. Nestling (0.40 ± 0.06 mM H_2O_2 equivalents) and adult (0.39 ± 0.11 mM H_2O_2 equivalents) blue petrels had similar levels of ROMs, whereas Antarctic prions had lower ROM levels (0.20 ± 0.01 mM H_2O_2 equivalents) than nestling and blue petrels (Fig. 1a). Serum antioxidant capacity did not differ between nestling petrels (291.4 ± 18.5 mM HOCl neutralized) and prions (305.7 ± 15.1 mM HOCl neutralized), whereas adult petrels (413.0 ± 21.0 mM HOCl neutralized) had higher levels than nestling petrels and prions (Fig. 1b). Circulating thiols did not differ between nestling (205.3 ± 22.3 µM) and adult (200.5 ± 48.5 µM) petrels, whereas prions (326.8 ± 22.6 µM) had higher levels than nestling and blue petrels (Fig. 1c). Sexes did not differ in ROMs, serum antioxidant capacity or circulating thiols, nor in body condition index in both nestling petrels ($U = 1$–21, $P \geq 0.08$) and adult prions ($U = 9$–26, $P \geq 0.11$).

In nestling petrels, ROM levels did not correlate with the sample time ($r = -0.08$, $n = 15$, $P = 0.77$), nor with the

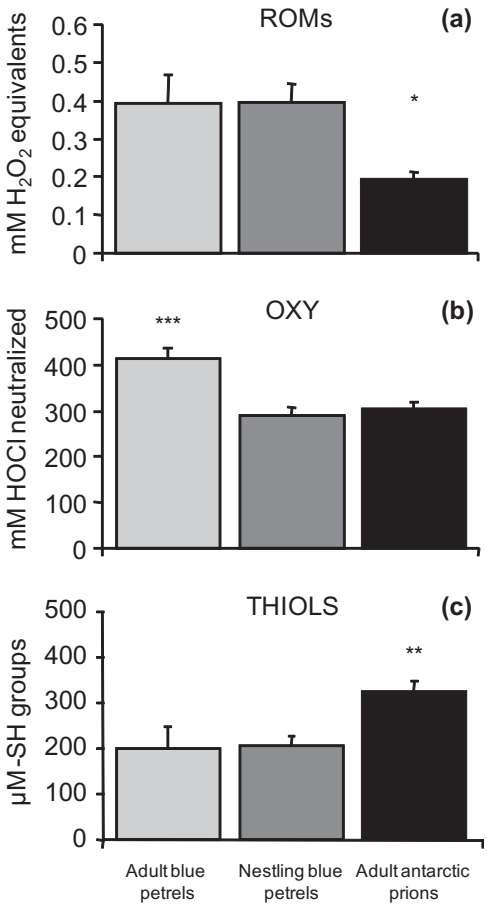

Fig. 1 (a) Antarctic prions had lower levels of reactive oxygen metabolites (ROMs) than both nestling and adult blue petrels (*$P < 0.05$); (b) adult blue petrels had higher levels of serum antioxidant capacity (OXY) than both nestling blue petrels and adult Antarctic prions (***$P < 0.001$); (c) Antarctic prions had higher levels of circulating thiols than both nestling and adult blue petrels (**$P < 0.01$). Means + standard errors are shown.

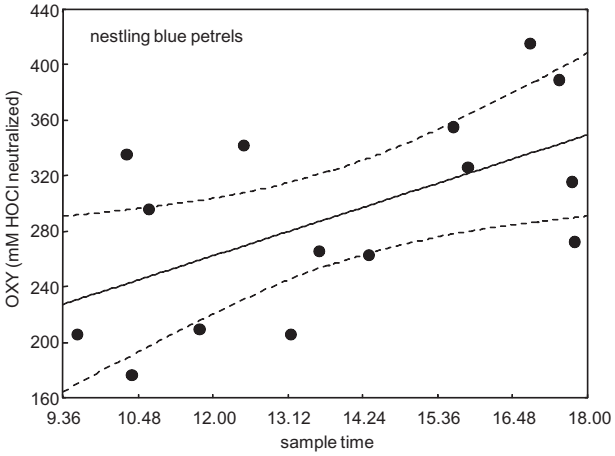

Fig. 2 In nestling blue petrels ($n = 15$), the serum antioxidant capacity (OXY) negatively correlated with the sample time (0.58, $n = 15$, $P = 0.023$). Dashed lines indicate the 95% confidence interval.

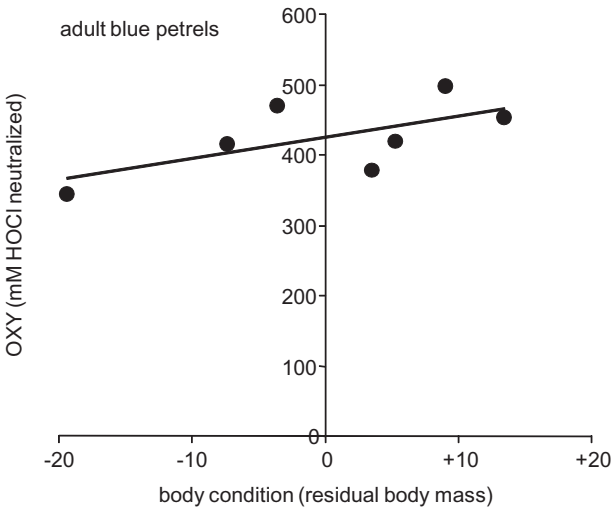

Fig. 3 Incubating blue petrels ($n = 7$) in better body condition (values shown as residuals of a multiple regression of body mass onto wing length and tarsus length) had higher serum antioxidant capacity (OXY).

sample date ($r = 0.39$, $n = 15$, $P = 0.15$). Conversely, serum antioxidant capacity correlated positively with the sample time ($r = 0.58$, $n = 15$, $P = 0.023$; Fig. 2), but was not correlated with the sample date ($r = -0.27$, $n = 15$, $P = 0.33$). Levels of circulating thiols did not correlate with the sample date ($r = 0.42$, $n = 9$, $P = 0.26$), nor with the sample time ($r = 0.005$, $n = 9$, $P = 0.99$).

In adult petrels, the body condition index was not correlated with ROMs (partial $r = 0.06$, $P = 0.93$), nor with circulating thiols (partial $r = 0.62$, $p = 0.27$), whereas it was marginally correlated with serum antioxidant capacity (partial $r = 0.88$, $p = 0.051$; Fig. 3).

In nestling petrels and adult prions, the body condition index was not correlated with any of the physiological variables (partial $r = -0.21$ to 0.24, $P \geq 0.45$). In nestling petrels, the correlation between the body condition index and serum antioxidant capacity also remained non-significant when controlling for sample time.

Overall, circulating thiols negatively correlated with ROMs ($r = -0.40$, $n = 32$, $P = 0.023$; intragroup correlation, –0.30 in nestling blue petrels, –0.34 in adult blue petrels, –0.35 in prions, all not significant) and OXY ($r = -0.45$, $n = 32$, $P = 0.010$; intragroup correlation, –0.27 in adult blue petrels, –0.28 in nestling blue petrels, –0.46 in prions, all not significant), whereas ROMs and OXY were not correlated ($r = 0.20$, $n = 38$, $P = 0.22$). This latter result was the effect of the absence of a correlation between ROMs and OXY in nestling petrels ($r = -0.02$).

After excluding those nestlings from the model, the correlation was positive and significant ($r = 0.58$, $P = 0.004$; intragroup correlation, 0.65 in adult petrels and 0.47 in prions, both with $P = 0.07$).

Discussion

In this study, we sought to evaluate patterns of variation in three serum oxidative stress markers in two seabird species. As a correlative investigation, our study does not allow us to draw definitive conclusions on what factors actually cause variation of the oxidative state in blue petrels and Antarctic prions. Also, some of the non-significant correlations should be interpreted cautiously because of the small sample size. Nevertheless, our results provide some indication that nestling and adult blue petrels differ in their serum antioxidant capacity, that the oxidative state does not differ between sexes, and that long-lived bird species have lower levels of ROMs and higher levels of OXY than short-lived bird species.

In detail, our data show that: nestling and adult blue petrels had similar levels of oxidative damage (ROMs), whereas Antarctic prions had 50% lower ROM levels; serum antioxidant capacity did not differ between nestling petrels and adult prions, whereas adult petrels had higher levels than both other groups; circulating thiols did not differ between nestling and adult blue petrels, whereas they were higher in prions; prion males and females did not differ for any of the physiological variables measured, nor for body condition index; nestling petrels did not show any gender-related differences in oxidative stress markers, nor in body condition index; breeding petrels with higher body condition index had higher serum antioxidant capacity.

The lower serum antioxidant capacity of nestling blue petrels compared with adults can be interpreted in at least two ways: nestlings depleted antioxidants to mitigate the oxidative damage caused by growth-related intense metabolic activity, or their antioxidant capacity was still immature compared with that of adults (Alonso-Alvarez et al. 2007; Costantini et al. 2006; Costantini, Fanfani et al. 2007). Regarding the comparison between adult petrels and prions, we are aware that comparison between two species limits conclusions concerning adaptive differences (Garland & Adolph 1994). However, we would like to highlight that although living in the same habitats and with similar life-history traits, adult blue petrels and prions significantly differed in each of the oxidative stress markers measured. Such differences could be explained by a number of factors, such as environmental conditions of the sampling year (e.g., food availability and ambient temperature), age of the specimens or different physiological adaptations to oxidative stress. Future studies will therefore be needed to tease apart the potential effects of such factors.

Although not always highly comparable because of the different nature of the studies (e.g., different ages of specimens and life-history phases, captive versus field study), levels of serum ROMs (as measured using the d-ROMs test) and of serum antioxidant capacity (as measured using the d-OXY test) of both petrels and prions, were, respectively, the lowest and the highest ever measured in birds (e.g., Costantini, Cardinale et al. 2007; Costantini, Coluzza et al. 2007; Costantini, Dell'Ariccia et al. 2008; Costantini, Fanfani et al. 2008; Costantini unpubl. data). Levels of both markers are comparable with those measured in another long-lived bird species, the pigeon (*Columba livia*), with a maximum lifespan of 35 years (Costantini, Dell'Ariccia et al. 2008). Birds have evolved several adaptations to slow down aging, and seabirds, such as Procellariiformes, represent a relevant example. For example, recent results showed that the great longevity of seabirds may be explained by a reduced peroxidation index of cell membranes (Buttemer et al. 2008), which exposes them to low levels of oxidative damage.

The high serum antioxidant capacities of petrels and prions may be an adaptation to their fasting periods. These birds experience five or six long fasting periods during each breeding season, which could increase oxidative stress and, possibly, reduce their fitness. Recent studies on birds suggest that fasting has a negative impact on the oxidative state (Milinkovic-Tur et al. 2007; Rey et al. 2008). Considering the body condition index as a proxy for fasting (where higher values indicate larger nutrient reserves), our study shows that the antioxidant defences in breeding petrels were lower in specimens of worse body condition, suggesting that they were depleted to prevent severe oxidative stress related to fasting. Thus, there emerged no correlation between body condition index and ROMs, and a positive correlation between body condition index and OXY and circulating thiols, respectively. Our explanation is, however, fairly hypothetical, and deserves further investigation with a larger sample size and taking into account the nutritional status of each bird, and possibly other markers of oxidative damage.

Sex appeared not to explain variation in oxidative stress markers in both seabird species. Similar correlative results were found in previous studies on kestrels (*Falco tinnunculus*) (Costantini et al. 2006; Costantini, Coluzza et al. 2007; Costantini, Fanfani et al. 2008) and barn swallows (*Hirundo rustica*) (Costantini, Cardinale et al. 2007). However, other studies suggested that sexes could have evolved different strategies to cope with oxidative

stress (Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Costantini, Fanfani et al. 2008).

The negative correlation between serum antioxidant capacity and sample time in nestling petrels is open to several explanations. One of these warranting future investigations is whether antioxidants show circadian and ultradian rhythmicity, as hormones do, and, if so, what factors modulate such changes. To address these points, it will be necessary to study the rhythmicity of antioxidants in the same individuals.

Data on circulating thiols in free-living birds are lacking, so no comparisons could be performed in this study. Compared with humans, petrels and prions have two- to three-fold higher absolute levels (i.e., not corrected for body size differences) of circulating thiols (Carratelli et al. 2001), whereas rats (*Rattus* spp.) showed thiol levels comparable with those of petrels (Kayali et al. 2007).

Thiol concentration was negatively correlated with both oxidative damage and total serum antioxidant capacity. On the one hand, it could be suggested that thiols were depleted to cope with oxidative stress, acting as a first antioxidant barrier. On the other hand, thiol concentration could have increased in response to the decrease of dietary antioxidants (for a similar correlation between circulating antioxidants and glutathione, see Galván & Alonso-Alvarez 2008). The regulation of antioxidant levels is complex, and different classes of antioxidants may detoxify the biological system, acting at different stages in the oxidative sequence (Costantini 2008). Such regulation may result in an increase of some antioxidants and a concomitant decrease of others (Halliwell & Gutteridge 2007; Costantini 2008), suggesting biochemical integration between the components of the overall oxidative system.

In conclusion, our study shows that blue petrels and Antarctic prions have lower levels of ROMs and higher levels of serum antioxidant capacity than previously studied bird species. Our correlative data also warrant future studies on relationships between fasting and oxidative stress. Finally, given that micromolecular antioxidant levels may reflect differences in life histories (Cohen et al. 2008), our study provides baseline values that might be useful for future comparative studies on links between oxidative stress and life history traits.

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