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Oxidative status and stress during highly energetic life-history stages of Chinstrap Penguins: breeding versus molting

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Oxidative status and stress during highly energetic life-history stages of Chinstrap
Penguins: breeding versus molting

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ABSTRACT. Life-history stages such as reproduction and molt are energetically costly. Reproductive costs include those associated not only with offspring production, but also protecting and provisioning young. Costs typically associated with molting include decreased thermoregulatory and locomotive performance and/or increased metabolic and nutritional costs. Energetic demands may disrupt homeostasis, particularly in terms of its maintenance (e.g., oxidative stress and immunity). Few investigators have explored the relationship between effort (increased metabolic rate) and oxidative status and stress by comparing life-history stages that have different energetic demands. However, comparative studies are crucial for understanding the processes of energy allocation and their consequences for different physiological functions. Our objective was to determine how two highly demanding life-history stages, breeding and molting, affected oxidative balance in Chinstrap Penguins (*Pygoscelis antarcticus*), a species where these two activities do not overlap. We found that the heterophil/lymphocyte (H/L) ratio was significantly higher during breeding than molting; oxidative damage was also higher during breeding, but the difference was only marginally significant. In contrast, we found no significant differences between these stages in total antioxidant capacity. We also found sex differences, with males showing higher oxidative damage (ROM) than females. Our results suggest that breeding is more stressful and more demanding for Chinstrap Penguins than molting, and provide further support for the relationship between effort, in terms of increased metabolic rate, and oxidative balance.

Key words: Antarctica, *Pygoscelis antarcticus*, H/L ratio, oxidative stress, reactive oxygen species

Basic metabolic activities generate pro-oxidative by-products, i.e., reactive oxygen species, or ROS (Finkel and Holbrook 2000, Metcalfe and Alonso-Alvarez 2010, but see Speakman and Garratt 2014 and Salin et al. 2015 for recent results showing how complex the link might be). According to the free radical theory (Harman 1956), free radicals (ROS) damage cellular components (e.g., lipids, proteins, and DNA) and this damage, accumulated over time, is thought to be a causal agent of ageing (Finkel and Holbrook 2000, Selman et al. 2012). Protection and repair systems that mitigate oxidative damage exist, but are not always completely efficient (Beckman and Ames 1998, Selman et al. 2012), thus leading to oxidative stress, which is defined as the negative effect on homeostasis when normal cellular redox signalling processes are disrupted (Jones 2006). Nevertheless, the contrasting results of recent studies suggest that the relationship between increased metabolic rates and oxidative stress is still not well defined (Speakman and Garratt 2014, Salin et al. 2015).

Reproduction is one of the most energy-demanding life-history stages (Green et al. 2009, Costantini 2014, but see Williams 2018). Several investigators have examined how reproduction causes oxidative stress in both captive (birds: Alonso-Alvarez et al. 2004, Wiersma et al. 2004, mammals: Oldakowski et al. 2012) and wild (Christe et al. 2012, Wegmann et al. 2015) animals. However, the results of these studies have been contradictory, with some showing a positive (Alonso-Alvarez et al. 2004), negative (Garratt et al. 2011), or no (Oldakowski et al. 2012) association between reproductive effort and oxidative stress. Therefore, additional studies of the possible association between effort and increased metabolic rate/oxidative stress are needed (Metcalfe and Monaghan 2013, but see Costantini et al. 2014, Xu et al. 2014, Colominas-Ciuró et al. 2017b). Comparing life stages (e.g., reproduction) with different energetic demands may not only provide key insights into this complex relationship, but also a better

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3 understanding of the processes of energy allocation and their physiological
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5 consequences. However, to date, only a few comparative studies have been conducted.
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7 Indeed, the results of these studies suggest that incubation is a more demanding phase of
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9 reproduction in terms of oxidative balance than chick rearing (Colominas-Ciuró et al.
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11 2017a, Kulaszewicz et al. 2018), although molt as an energetic life stage was not
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13 examined.
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17 Molting is another costly life-history event for birds due to its high energy
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19 demand. Costs of molting include impaired thermoregulation and flight performance
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21 and increased metabolic and nutritional costs (Payne 1972, Lindström et al. 1993, Jenni
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23 and Winkler 1994, Bridge 2009). Most species of penguins molt once a year, usually
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25 after breeding, over a period of two to four weeks (Williams 1995). Unlike other
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27 species, penguins renew their entire plumage during molt. They also fast during this
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29 time because they cannot go into the water to forage until their plumage is completely
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31 restored. The relationship between fasting and oxidative status has been little studied in
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33 seabirds. The results of previous studies have revealed that antioxidant defenses
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35 shielded individuals that were experimentally starved (~ 2 weeks), with levels of
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37 oxidative damage constant in King Penguins (*Aptenodytes patagonicus*; PII group,
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39 Schull et al. 2016), but decreasing in Mallards (*Anas platyrhynchos*; Geiger et al. 2012).
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41 These results might be linked to a potential reduction in metabolism of some fasting
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43 birds that would attenuate oxidative damage (Geiger et al. 2012). Energy expenditure
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45 also increases when penguins are molting due to growth of new feathers and the
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47 increased cost of thermoregulation (Adams and Brown 1990). Penguins, therefore, are
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49 ideal organisms for exploring the relationship between energy expenditure and oxidative
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51 stress by comparing different, non-overlapping life-history stages, i.e., breeding and
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53 molting.
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Elevated energetic costs during certain life-history stages may also contribute to an increase in glucocorticoid levels (stress hormones) in the blood, triggering changes in leukocyte components (Davis et al. 2008). Stress causes increased production of heterophils and decreased production of lymphocytes and, as a result, the heterophil/lymphocyte (H/L) ratio has been used as an indicator of stress in birds because it is positively related to the magnitude of the stressor and to levels of circulating glucocorticoids (Moreno et al. 2002, Davis et al. 2008, Ochs and Dawson 2008, Barbosa et al. 2013).

Contradictory results have been published regarding sex differences in the oxidative status of penguins. Indeed, whereas previous studies of *Pygoscelis* penguins have revealed no differences between the sexes in oxidative status (Beaulieu et al. 2010, 2011), a recent study has shown that differences between the sexes in reproductive effort differentially affects oxidative status, suggesting the existence of sex-specific strategies to cope with reproductive costs (Colominas-Ciuró et al. 2017b). Also, sex differences in H/L ratios have been reported in other species of birds (Schmidt et al. 2007) and other species of *Pygoscelid* penguins during the guard phase (Colominas-Ciuró et al. 2017b, Palacios et al. 2018).

Chinstrap Penguins (*Pygoscelis antarcticus*) have a circumpolar distribution, breeding mainly on the Antarctic Peninsula, the South Shetlands, and the sub-Antarctic islands in the South Atlantic. From late September to early November, adults return to nest colonially at high densities, with females typically laying two eggs (Williams 1995, Barbosa et al. 1997). Parents then alternate shifts during the incubation period (33–35 days) until the semi-altricial nidicolous chicks hatch (from December to mid-January) (Barbosa et al. 1997). Chicks are fed alternately by the two parents during both the guard phase, when adults protect and feed chicks in the nest, and the crèching phase,

when parents are no longer guarding and chicks aggregate in crèches (Moreno et al. 1994, Viñuela et al. 1996). After breeding, penguins go to sea to forage for ~15 days to create a thick layer of fat that provides energy for molting. They then return to land (>75% to their breeding colony) to molt, fasting for 13 days while their new plumage develops (Williams 1995). After molting, they return to the ocean until the next breeding season.

Among penguins, metabolic rates increase 4–4.7 times above the basal rate during breeding (Moreno and Sanz 1996) and about two times above the basal rate during molting (Croxall 1982, Gales and Green 1990). Also, an energetic high ceiling was found in Macaroni Penguins (*Eudyptes chrysolophus*; mean daily energy expenditure / resting metabolic rate = 3.6–4) during breeding and the pre-molt trip, although it decreased during molt (2.9–3.1; Green et al. 2009). Higher metabolic rates may increase ROS production, resulting in more oxidative damage unless levels of antioxidant defenses also increase (Alonso-Alvarez et al. 2004, Costantini et al. 2008, Costantini 2008, but see Salin et al. 2015 and Speakman and Garratt 2014 for contrasting results), as might the H/L ratio (Ilmonen et al. 2003). Thus, reproduction and molt likely trigger changes in oxidative balance and stress. For example, when the reproductive effort of Adélie Penguins (*Pygoscelis adeliae*) was experimentally manipulated (either by reducing their hydrodynamic properties or by decreasing brood size), the resulting antioxidant capacity of experimental birds was lower than that of control penguins (Beaulieu et al. 2011, Colominas-Ciuró et al. 2017b).

Our main objective was to explore the relationship between effort and both oxidative status and stress by comparing these factors during two different life-history stages (breeding and molting) of Chinstrap Penguins. Because metabolism may be related to oxidative status, we hypothesized that breeding penguins, due to the high

energy expenditures associated with breeding (e.g., increased parental activity to feed young), would exhibit greater oxidative damage, lower antioxidant defenses, and higher H/L ratios than molting penguins (Croxall 1982, Moreno and Sanz 1996, Green et al. 2009). In addition, because male penguins engage in more agonistic interactions (for nest protection against intruders by flipper hits and pecks) during breeding than females (Moreno et al. 1995), which could lead to a metabolic increment triggering more ROS production and greater oxidative damage (Alonso-Alvarez et al. 2004), as well as higher glucocorticoid levels (Davis et al. 2008, but see Thierry et al. 2014), we predicted that breeding males would exhibit greater oxidative damage and higher H/L ratios, but lower antioxidant defenses, than breeding females.

METHODS

Our study was conducted at the Vapour Col Chinstrap Penguin Rookery (63°00'S 60°40'W) on Deception Island, South Shetlands Archipelago (Antarctic Peninsula), during the 2012-2013 breeding season. Breeding samples were collected during the guard phase at the beginning of January when chicks were 2-15 days old; molting samples were collected in February. We captured 56 Chinstrap Penguins, including 27 breeding adults by hand at their nest sites during the guard stage and 29 different molting adults using a long-handled net. Young of breeding adults were kept safe while adults were sampled. After sampling, adults and their young were returned to nests where all adults were observed to resume care of their young. Molting adults were captured near their breeding colonies where they gather to molt and, after sampling, were returned to the same area. All birds were individually marked using waterproof paint to avoid resampling. The large proportion of feathers shed indicated that the birds sampled were in a late-molting phase so they had likely been fasting for at least one

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3 week prior to sampling. We did not have information about the breeding status of
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5 molting birds, but they may have been non-breeders or failed breeders because there had
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7 likely not been enough time for breeders to start molting. However, we assume that the
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9 condition was similar at the time of sampling for all molting individuals captured,
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11 regardless of breeding or not because they molt early in the season before
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13 environmental conditions deteriorate and negatively affect their condition, thus
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15 compensating for their potential low-quality status. Ideally, we would have sampled the
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17 same individuals during breeding and molt, but this was not possible because penguins
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19 may molt at locations other than at their nest sites. In addition, we did not have a control
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21 group of real non-breeders, which should show a baseline for the oxidative status,
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23 because we could not distinguish between them and failed breeders as we arrived to the
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25 breeding colony at hatching. Birds that were not breeding during sampling could belong
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27 to both groups which differ in the initial energy investment and probably in their
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29 oxidative status and thus, is not appropriate as a control group. Moreover, even arriving
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31 at the beginning of the breeding season to have a control group of enough sample size
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33 for statistical analyses, it would be highly unpredictable and would require to band a
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35 huge number of individuals and follow them during at least one month which is a highly
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37 difficult task in a colony of several thousands of individuals.
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45 Blood samples were taken within 5 min of capture from a foot vein of captured
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47 adults using heparinized capillary tubes. For each sample, one drop was smeared on
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49 individually marked microscope slides, air-dried, and later fixed in 96% ethanol for 5
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51 min. The remaining blood was later centrifuged (Centrolit II-BL, J. P. Selecta S. A.,
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53 Barcelona, Spain) at 12,000 rpm for 15 min to separate plasma from red blood cells.
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55 Both components were frozen and stored at -20°C until subsequent processing. Blood
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57 smears were stained with Giemsa pH 7.2 for 40 min for microscopic analyses.
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Laboratory analyses. We examined 100 leukocytes per smear under a 100x oil immersion objective. Only cells arranged in a monolayer were counted to avoid differences in thickness (all counts performed by RCC). The H/L ratio was then calculated (see Barbosa et al. 2013 and references therein for method details).

To assess the oxidative balance in blood plasma, both oxidant and antioxidant components were measured using d-reactive oxygen metabolites (d-ROMs) and OXY-adsorbent tests (Diacron International, Grosseto, Italy), respectively. The OXY-adsorbent test measures the total plasma antioxidant capacity (OXY) by quantifying the ability of a large section of exogenously and endogenously synthesized antioxidants to counter oxidation by treating with hypochlorous acid (HClO), a powerful oxidant. The unreacted HClO radicals react with a chromogenic substrate that can then be measured photometrically (Beaulieu et al. 2010, Van de Crommenacker et al. 2012). The d-ROMs test measures plasmatic hydroperoxides, a reactive oxygen metabolite (ROM) resulting from oxidative damage (attack of ROS on organic substrates such as amino acids, proteins, and nucleotides). The OXY-adsorbent and d-ROMs test procedures were carried out following Beaulieu et al. (2010) with minor modifications, such as using a vortex machine (15 sec), short spins for thawed plasma and mixtures (plasma–reagent, calibrator–reagent, and blank–reagent), and incubation with shaking (level 6 of 10) in a hybridization oven/shaker (Amersham Biosciences, Little Chalfont, UK) for better homogenization. Absorbance was determined with a spectrophotometer at 37°C; the microplate was shaken (medium level) for 1 min before reading at a wavelength of 490 nm (Multi-Mode Microplate Reader, Synergy HT, BioTek, Winooski, VT). Measurements are expressed in $\mu\text{mol HClO/ml}$ sample and $\text{mg H}_2\text{O}_2/\text{dl}$ for the OXY-adsorbent and d-ROMs tests, respectively. Both d-ROMs and OXY-adsorbent tests were performed using microplates where the treatment (i.e., life-history stage) was

evenly distributed together with duplicates for each sample, blank, and calibrator. Intra-assay coefficients of variation for OXY and d-ROMs from plates I to V were 9.76%, 3.38%, 5.77%, 13.53%, and 29.89%, and 8.17%, 21.30%, 14.22%, 10.65%, and 5.34%, respectively. Inter-assay coefficients of variation for OXY and d-ROMs were 10.82% and 13.88%, respectively. Finally, birds were sexed using molecular markers following Fridolfsson and Ellegren (1999).

Statistical analysis. All variables were normally distributed (Kolmogorov-Smirnov test ≥ 0.2) except for the H/L ratio and ROM that were $\log_{10}$ transformed to achieve normality prior to analysis. Three dependent variables, including the H/L ratio, OXY, and ROM, were analyzed with general linear models (GLMs), with sex and life-history stage (breeding vs. molting) as fixed factors; H/L ratio, OXY, and ROM were considered covariates when they were not the dependent variable. In addition, simple regressions were also performed. Statistica 9.0 (Statsoft Inc., Tulsa, OK) was used for all analyses. P values < 0.05 were considered significant. Values are presented as $\text{mean}_{\log_{10} \text{ ROM}} \pm \text{SE}$.

RESULTS

The H/L ratio of Chinstrap Penguins was significantly higher during breeding ($\text{mean}_{\log_{10} \text{ H/L ratio}} = 0.03 \pm 0.04$) than molting ($\text{mean}_{\log_{10} \text{ H/L ratio}} = -0.54 \pm 0.05$; Tables 1 and 2), but differences between the sexes and the interaction between factors and covariates were not significant (Tables 1 and 2). ROM values were also higher, but not significantly higher (although the difference was significant without the outlier marked with an arrow in Fig. 1, also see Table 2), during breeding ($\text{mean}_{\log_{10} \text{ ROM}} = 0.90 \pm 0.04$) than molt ($\text{mean}_{\log_{10} \text{ ROM}} = 0.85 \pm 0.02$; Tables 1 and 2), and were also higher in males ($\text{mean}_{\log_{10} \text{ ROM}} = 0.91 \pm 0.03$) than females ($\text{mean}_{\log_{10} \text{ ROM}} = 0.85 \pm 0.03$; Tables 1 and

2). In contrast, we found no differences in the interaction between the factors and ROMs, and between OXY and all fixed factors analyzed (Tables 1 and 2). However, our analysis revealed significant negative correlations between ROM and OXY during breeding ($N = 27$, $r = -0.42$, $P = 0.029$) and molt ($N = 29$, $r = -0.45$, $P = 0.014$; Fig. 1, Table 2). We found no relationship between the H/L ratio and any of the covariates analyzed (Table 2).

DISCUSSION

We found that the H/L ratio was lower during molting than breeding, suggesting that breeding is more stressful for Chinstrap Penguins, even though molting in this species is one of the most intense in birds and requires high energy expenditure (Brown 1985, Gales et al. 1988). This result is consistent with those of previous studies where metabolic rates of Chinstrap Penguins increased 4–4.7 times during breeding (Moreno and Sanz 1996). In addition, Green et al. (2009) reported that metabolic rates of Macaroni Penguins increased 3.6 - 4 times during breeding, and 2.9 - 3.1 times during molt (Green et al. 2009). Studies of Little (*Eudyptula minor*) and Chinstrap penguins have also revealed increasing energetic demands during breeding (Mortimer and Lill 2007, D’Amico et al. 2016a, b, Palacios et al. 2018).

Differences in H/L ratio between breeding and molting could also been explained by agonistic interactions that occur to protect nests and offspring during breeding (Viñuela et al. 1995) that also increase glucocorticoid levels (Davis et al. 2008) and contribute to a higher H/L ratio during breeding than during the socially inactive period of molting (van der Meer and van Oers 2015). Osmoregulatory demands resulting from foraging at sea during the breeding period could have also increased the H/L ratio because more time at sea would increase both salt intake and blood plasma

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3 levels of adrenocorticoid hormones because salt gland secretion is dependent on the
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5 presence of those hormones (Harvey et al. 1984a, see Harvey et al. 1984b for different
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7 corticosterone increments depending on the amount of NaCl and the exposure time of
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9 birds to NaCl). Finally, hormonal stress (i.e., corticosterone response as the primary
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11 glucocorticoid in birds) may be down-regulated during molting in some species of birds
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13 (e.g., Zebra Finches, *Taeniopygia guttata*), and this could potentially contribute to the
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15 lower H/L ratio during this stage (Cornelius et al. 2011).
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19 Excluding an outlier (Fig. 1; Table 2), penguins in our study had higher ROM
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21 values during breeding than during molt. This outlier, a breeding female, seems to have
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23 a biological base as we can exclude errors during the laboratory analysis because
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25 duplicates were very similar. Biological explanations include those producing a
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27 decrease of antioxidant in the plasma such as the present of infections, although cellular
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29 immunity values did not differ from other birds; differences in diet, differences in
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31 parental care or differences in foraging effort, among others. Energetically demanding
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33 activities that increase metabolic rate have been found to contribute to an oxidative
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35 imbalance (Costantini et al. 2008). Nevertheless, the relationship between increased
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37 metabolic rate and oxidative stress is likely more complex, also depending in some
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39 cases on mechanisms at the level of mitochondrial inner membranes. Reduction of the
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41 inner-membrane potential (by increasing the flow of protons back through the inner
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43 membrane to the matrix) would reduce free-radical production without generating ATP
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45 (Speakman and Garratt 2014, Salin et al. 2015). For Chinstrap Penguins, increased
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47 metabolic rates during breeding (i.e., 1.5-1.8 and 4.0-4.7 times higher when guarding
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49 and caring for chicks, respectively, compared to basal metabolic rate; Moreno and Sanz
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51 1996) likely trigger greater production of ROS, resulting in greater levels of oxidative
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53 damage. The negative correlation between OXY and ROM in our study suggests that
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antioxidant defenses can counteract oxidation to some extent (see Beaulieu et al. 2010 for similar results with Adelie Penguins). However, complete antioxidant efficiency may not always be achieved, leading to ROS-mediated damage of organic substrates (Beckman and Ames 1998, Sies 2007, Selman et al. 2012). Although some investigators have reported high levels of oxidative stress during molt in birds (Raja-aho et al. 2012, Costantini 2014), the greater metabolic increment experienced during breeding than during molt (Croxall 1982, Green et al. 2009) suggests that the oxidative status of Chinstrap Penguins is worse (greater oxidative damage and lower antioxidant defenses) during breeding and better (less oxidative damage) during molt (but see Salin et al. 2015 and Speakman and Garratt 2014 for results about the complexity behind this potential relationship).

Total antioxidant capacity did not differ between molting and breeding Chinstrap Penguins, suggesting that antioxidants are used at the same level during both periods. Indeed, both periods unbalance homeostasis in the same way because there was no significant difference in the slopes of the regression analysis (with outlier: $t_{1,52} = -0.3$, $P = 0.23$; without outlier: $t_{1,51} = -0.9$, $P = 0.61$). Although antioxidant defenses may similarly limit oxidation during both stages, as mentioned above, the oxidative status of Chinstrap Penguins is likely worse during breeding than molt due to a greater increase in metabolic rate (Croxall 1982, Moreno and Sanz 1996, Green et al. 2009, Speakman and Garratt 2014, Salin et al. 2015).

Although ROM was higher in males than females in our study, as predicted, neither the H/L ratio nor OXY differed between the sexes. However, in a previous study of the same penguin rookery, males had higher H/L ratios than females during breeding and molting (Palacios et al. 2018). Different environmental conditions (e.g., sea-ice distribution; Clarke et al. 2006, Widmann et al. 2015) in the same study area in different

years could lead to ecophysiological and/or immunological sex differences, e.g., in terms of foraging effort and diet, energy consumption, or body condition (Ismar et al. 2017, Pereira et al. 2018). Similarly, sex differences in oxidative status have been found in other species of non-breeding birds (Schmidt et al. 2007) and other species of *Pygoscelis* penguins during the breeding cycle (Colominas-Ciuró et al. 2017b, but see Beaulieu et al. 2010, 2011). Therefore, sex differences in oxidative status in our study could have been due to differences in nest defense, with male Chinstrap Penguins engaging in more agonistic behaviors than females both during incubation (Moreno et al. 1995) and the guard phase (Viñuela et al. 1995). More agonistic interactions might lead to a metabolic increment that could trigger more ROS production and, as a consequence, greater oxidative damage (Alonso-Alvarez et al. 2004) and higher glucocorticoid levels (Davis et al. 2008, but see Thierry et al. 2014), although this level of stress seems not to affect H/L ratios. Moreover, antioxidant defenses may play a similar role in both sexes. Also, and as mentioned for the H/L ratio, other ecological factors such as differences in foraging effort, diet, energy consumption, or body condition (Widmann et al. 2015, Ismar et al. 2017, Pereira et al. 2018) could also explain differences between the sexes in oxidative damage.

In conclusion, our results, based on both the H/L ratio and oxidative status, suggest that breeding is more stressful and more demanding for Chinstrap Penguins than molting. Our results provide further support for the relationship between effort, in terms of increased metabolic rate, and oxidative balance. Notably, however, our study was based on measurements taken only during the chick-rearing phase so stress levels during other breeding phases, such as incubation, should also be studied to determine if effort (metabolic rate) and, consequently, H/L ratios and oxidative stress, vary throughout the breeding season.

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LITERATURE CITED

ADAMS, N. J., AND R. BROWN. 1990. Energetics of molt in penguins. In: Penguin biology (L. S. Davis and J. T. Darby, eds.), pp. 297-316. Academic Press, San Diego, CA.

ALONSO-ALVAREZ, C., S. BERTRAND, G. DEVEVEY, J. PROST, B. FAIVRE, AND G. SORCI. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. Ecology Letters 7: 363-368.

BARBOSA, A., E. DE MAS, J. BENZAL, J. I. DIAZ, M. MOTAS, S. JEREZ, L. PERTIERRA, J. BENAYAS, A. JUSTEL, P. LAUZURICA, F. J. GARCIA-PEÑA, AND T. SERRANO. 2013. Pollution and physiological variability in Gentoo Penguins at two

- rookeries with different levels of human visitation. *Antarctic Science* 25: 329-338.
- BARBOSA, A., J. MORENO, J. POTTI, AND S. MERINO. 1997. Breeding group size, nest position and breeding success in the Chinstrap Penguin. *Polar Biology* 18: 410-414.
- BEAULIEU, M., S. REICHERT, Y. LE MAHO, A. ANCEL, AND F. CRISCUOLO. 2011. Oxidative status and telomere length in a long-lived bird facing a costly reproductive event. *Functional Ecology* 25: 577-585.
- BEAULIEU, M., Y. ROPERT-COUDERT, Y. LE MAHO, A. ANCEL, AND F. CRISCUOLO. 2010. Foraging in an oxidative environment: relationship between $\delta^{13}\text{C}$ values and oxidative status in Adelie Penguins. *Proceedings of the Royal Society B* 277: 1087-1092.
- BECKMAN, K. B., AND B. N. AMES. 1998. The free radical theory of aging matures. *Physiological Reviews* 78: 547-581.
- BLOUNT, J. D., E. I. VITIKAINEN, I. STOTT, AND M. A. CANT. 2016. Oxidative shielding and the cost of reproduction. *Biological Reviews* 91: 483-497.
- BRIDGE, E. S. 2009. How does imping affect wing performance. *Journal of Wildlife Rehabilitation* 29: 4-9.
- BROWN, C. R. 1985. Energetic cost of moult in Macaroni Penguins (*Eudyptes chrysolophus*) and Rockhopper Penguins (*E. chrysocome*). *Journal of Comparative Physiology B* 155: 515-520.
- CLARKE, J., L. M. EMMERSON, AND P. OTAHAL. 2006. Environmental conditions and life history constraints determine foraging range in breeding Adélie Penguins. *Marine Ecology Progress Series* 310: 247-261.

- COLOMINAS-CIURÓ, R., M. BERTELOTTI, E. CARABAJAL, V. L. D'AMICO, AND A. BARBOSA. 2017a. Incubation increases oxidative imbalance compared to chick rearing in a seabird, the Magellanic Penguin (*Spheniscus magellanicus*). *Marine Biology* 164: 99.
- COLOMINAS-CIURÓ, R., M. SANTOS, N. CORIA, AND A. BARBOSA. 2017b. Reproductive effort affects oxidative status and stress in an Antarctic penguin species: an experimental study. *PLoS ONE* 12: e0177124.
- CORNELIUS, J. M., N. PERFITO, R. ZANN, C. W. BREUNER, AND T. P. HAHN. 2011. Physiological trade-offs in self-maintenance: plumage molt and stress physiology in birds. *Journal of Experimental Biology* 214: 2768-2777.
- COSTANTINI, D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. *Ecology Letters* 11: 1238-1251.
- COSTANTINI, D. 2014. Oxidative stress and hormesis in evolutionary ecology and physiology: a marriage between mechanistic and evolutionary approaches. Springer-Verlag, Berlin and Heidelberg, Germany.
- COSTANTINI, D., G. CASASOLE, AND M. EENS. 2014. Does reproduction protect against oxidative stress? *Journal of Experimental Biology* 217: 4237-4243.
- COSTANTINI, D., G. DELL'ARICCIA, AND H. P. LIPP. 2008. Long flights and age affect oxidative status of homing pigeons (*Columba livia*). *Journal of Experimental Biology* 211: 377-381.
- CROXALL, J. P. 1982. Energy costs of incubation and moult in petrels and penguins. *Journal of Animal Ecology* 51: 177-194.
- CHRISTE, P., O. GLAIZOT, N. STREPPARAVA, G. DEVEVEY, AND L. FUMAGALLI. 2012. Twofold cost of reproduction: an increase in parental effort leads to higher

- malarial parasitaemia and to a decrease in resistance to oxidative stress.
- Proceedings of the Royal Society B 279: 1142-1149.
- D'AMICO, V. L., N. CORIA, M. G. PALACIOS, A. BARBOSA, AND M. BERTELLOTTI. 2016a. Physiological differences between two overlapped breeding Antarctic penguins in a global change perspective. *Polar Biology* 39: 57-64.
- D'AMICO, V. L., B. MARCELO, J. BENZAL, N. CORIA, V. VIDAL, J. I. DIAZ, AND A. BARBOSA. 2016b. Leukocyte counts in different populations of Antarctic *Pygoscelid* penguins along the Antarctic Peninsula. *Polar Biology* 39: 199-206.
- DAVIS, A. K., D. L. MANEY, AND J. C. MAERZ. 2008. The use of leukocyte profiles to measure stress in vertebrates: a review for ecologists. *Functional Ecology* 22: 760-772.
- FINKEL, T., AND N. J. HOLBROOK. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* 408: 239-247.
- FRIDOLFSSON, A.-K., AND H. ELLEGREN. 1999. A simple and universal method for molecular sexing of non-ratite birds. *Journal of Avian Biology* 20: 116-121.
- GALES, R., AND B. GREEN. 1990. The annual energetics cycle of Little Penguins (*Eudyptula minor*). *Ecology* 71: 2297-2312.
- GALES, R., B. GREEN, AND C. STAHEL. 1988. The energetics of free-living Little Penguins *Eudyptula minor* (Spheniscidae), during molt. *Australian Journal of Zoology* 36: 159-167.
- GARRATT, M., A. VASILAKI, P. STOCKLEY, F. MCARDLE, M. JACKSON, AND J. L. HURST. 2011. Is oxidative stress a physiological cost of reproduction? An experimental test in house mice. *Proceedings of the Royal Society B* 278: 1098-106.
- GEIGER, S., S. KAUFFMAN, Y. LE MAHO, J. P. ROBIN, AND F. CRISCUOLO. 2012. Of the importance of metabolic phases in the understanding of oxidative stress in

- prolonged fasting and refeeding. *Physiological and Biochemical Zoology* 85: 415-420.
- GREEN, J. A., I. L. BOYD, A. J. WOAKES, N. L. WARREN, AND P. J. BUTLER. 2009. Evaluating the prudence of parents: daily energy expenditure throughout the annual cycle of a free-ranging bird, the Macaroni Penguin *Eudyptes chrysolophus*. *Journal of Avian Biology* 40: 529-538.
- HARMAN, D. 1956. Aging: a theory based on free radical and radiation chemistry. *Journal of Gerontology* 11: 298-300.
- HARVEY, S., J. G. PHILLIPS, A. REES, AND T. R. HALL. 1984a. Stress and adrenal function. *Journal of Experimental Zoology* 232: 633-645.
- HARVEY, S., H. KLANDORF, W. J. RADKE, AND J. D. FEW. 1984b. Thyroid and adrenal responses of ducks (*Anas platyrhynchos*) during saline adaptation. *General and Comparative Endocrinology* 55: 46-53.
- ILMONEN, P., D. HASSELQUIST, Å. LANGEFORS, AND J. WIEHN. 2003. Stress, immunocompetence and leukocyte profiles of Pied Flycatchers in relation to brood size manipulation. *Oecologia* 136: 148-154.
- ISMAR, S. M. H., D. RAUBENHEIMER, S. J. BURY, C. D. MILLAR, AND M. E. HAUBER. 2017. Sex-specific foraging during parental care in a size-monomorphic seabird, the Australasian Gannet (*Morus serrator*). *Wilson Journal of Ornithology* 129: 139-147.
- JENNI, L., AND R. WINKLER. 1994. Molt and ageing of European passerines. Academic Press, London, UK.
- JONES, D. P. 2006. Redefining oxidative stress. *Antioxidants & Redox Signaling* 8: 1865-1879.

- KULASZEWICZ, I., K. WOJCZULANIS-JAKUBAS, AND D. JAKUBAS. 2018. Breeding phased dependent oxidative balance in a small High Arctic seabird, the Little Auk. *Journal of Avian Biology* 49: e01702
- LINDSTRÖM, Å., G. H. VISSER, AND S. DAAN. 1993. The energetic cost of feather synthesis is proportional to basal metabolic rate. *Physiological Zoology* 66: 490-510.
- METCALFE, N. B., AND C. ALONSO-ALVAREZ. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology* 24: 984-996.
- METCALFE, N. B., AND P. MONAGHAN. 2013. Does reproduction cause oxidative stress? An open question. *Trends in Ecology & Evolution* 28: 347-350.
- MORENO, J., J. BUSTAMANTE, AND J. VIÑUELA. 1995. Nest maintenance and stone theft in the Chinstrap Penguin (*Pygoscelis antarctica*). 1. Sex-roles and effects on fitness. *Polar Biology* 15: 533-540.
- MORENO, J., L. M. CARRASCAL, J. J. SANZ, J. A. AMAT, AND J. J. CUERVO. 1994. Hatching asynchrony, sibling hierarchies and brood reduction in the Chinstrap Penguin *Pygoscelis antarctica*. *Polar Biology* 14: 21-30.
- MORENO, J., A. DE LEÓN, J. A. FARGALLO, AND E. MORENO. 1998. Breeding time, health and immune response in the Chinstrap Penguin *Pygoscelis antarctica*. *Oecologia* 115: 312-319.
- MORENO, J., S. MERINO, J. MARTINEZ, J. J. SANZ, AND E. ARRIERO. 2002. Heterophil/lymphocyte ratios and heat-shock protein levels are related to growth in nestling birds. *Écoscience* 9: 434-439.

- 1
2
3 MORENO, J., AND J. J. SANZ. 1996. Field metabolic rates of breeding Chinstrap Penguins
4
5 (*Pygoscelis antarctica*) in the South Shetlands. *Physiological Zoology* 69: 586-
6
7 598.
8
9
- 10 MORTIMER, L., AND A. LILL. 2007. Activity-related variation in blood parameters
11
12 associated with oxygen transport and chronic stress in Little Penguins.
13
14 *Australian Journal of Zoology* 55: 249-256.
15
16
- 17 OCHS, C. L., AND R. D. DAWSON. 2008. Patterns of variation in leucocyte counts of
18
19 female Tree Swallows, *Tachycineta bicolor*: repeatability over time and
20
21 relationships with condition and costs of reproduction. *Comparative*
22
23 *Biochemistry and Physiology A* 150: 326-331.
24
25
- 26 OŁDAKOWSKI, Ł., Ż. PIOTROWSKA, K. M. CHRZAŚCIK, E. T. SADOWSKA, P. KOTEJA, AND
27
28 J. R. E. TAYLOR. 2012. Is reproduction costly? No increase of oxidative damage
29
30 in breeding bank voles. *Journal of Experimental Biology* 215: 1799-1805.
31
32
- 33 PALACIOS, M. J., F. VALERA, R. COLOMINAS-CIURÓ, AND A. BARBOSA. 2018. Cellular
34
35 and humoral immunity in two highly demanding energetic life stages:
36
37 reproduction and moulting in the Chinstrap Penguin. *Journal of Ornithology*
38
39 159: 283-290.
40
41
- 42 PAYNE, R. B. 1972. Mechanisms and control of molt. In: *Avian biology* (D. S. Farmer
43
44 and J. R. King, eds.), pp. 103-155. Academic Press, New York, NY.
45
46
- 47 PEREIRA, J. M., V. H. PAIVA, R. A. PHILLIPS, AND J. C. XAVIER. 2018. The devil is in the
48
49 detail: small-scale sexual segregation despite large-scale spatial overlap in the
50
51 Wandering Albatross. *Marine Biology* 165: 55.
52
53
- 54 RAJA-AHO, S., M. KANERVA, T. EEVA, E. LEHIKONEN, P. SUORSA, K. GAO, D. VOSLOO,
55
56 AND M. NIKINMAA. 2012. Seasonal variation in the regulation of redox state and
57
58
59
60

- some biotransformation enzyme activities in the Barn Swallow (*Hirundo rustica* L.). *Physiological and Biochemical Zoology* 85: 148-158.
- SAINO, N., S. CALZA, AND A. P. MØLLER. 1997. Immunocompetence of nestling Barn Swallows in relation to brood size and parental effort. *Journal of Animal Ecology* 66: 827-836.
- SALIN, K., S. K. AUER, A. M. RUDOLF, G. J. ANDERSON, A. G. CAIRNS, W. MULLEN, R. C. HARTLEY, C. SELMAN, AND N. B. METCALFE. 2015. Individuals with higher metabolic rates have lower levels of reactive oxygen species *in vivo*. *Biology Letters* 11:20150538.
- SCHMIDT, E. M. D. S., A. C. PAULILLO, E. SANTIN, R. L. DITTRICH, AND E. G. DE OLIVEIRA. 2007. Hematological and serum chemistry values for the Ring-necked Pheasant (*Phasianus colchicus*): variation with sex and age. *International Journal of Poultry Science* 6: 137-139.
- SCHULL, Q., V. A. VIBLANC, A. STIER, H. SAADAOU, E. LEFOL, F. CRISCUOLO, P. BIZE, AND J.-P. ROBIN. 2016. The oxidative debt of fasting: evidence for short to medium-term costs of advanced fasting in adult King Penguins. *Journal of Experimental Biology* 219: 3284-3293.
- SELMAN, C., J. D. BLOUNT, D. H. NUSSEY, AND J. R. SPEAKMAN. 2012. Oxidative damage, ageing, and life-history evolution: where now? *Trends in Ecology & Evolution* 27: 570-577.
- SIES, H. 2007. Total antioxidant capacity: appraisal of a concept. *Journal of Nutrition* 137: 1493-1495.
- SPEAKMAN, J. R., AND M. GARRATT. 2014. Oxidative stress as a cost of reproduction: beyond the simplistic trade-off model. *BioEssays* 36: 93-106.

- 1
2
3 THIERRY, A.-M., S. BRAJON, M. SPÉE, AND T. RACLOT. 2014. Differential effects of
4
5 increased corticosterone on behavior at the nest and reproductive output of
6
7 chick-rearing Adélie Penguins. *Behavioral Ecology and Sociobiology* 68: 721-
8
9 732.
10
11
12 VAN DE CROMMENACKER, J., D. S. RICHARDSON, A. M. KOLTZ, K. HUTCHINGS, AND J.
13
14 KOMDEUR. 2012. Parasitic infection and oxidative status are associated and vary
15
16 with breeding activity in the Seychelles Warbler. *Proceedings of the Royal*
17
18 *Society B* 279: 1466-1476.
19
20
21 VAN DER MEER, E., AND K. VAN OERS. 2015. Gender and personality differences in
22
23 response to social stressors in Great Tits (*Parus major*). *PLoS ONE* 10:
24
25 e0127984.
26
27
28 VIÑUELA, J., J. A. AMAT, AND M. FERRER. 1995. Nest defence of nesting Chinstrap
29
30 Penguins (*Pygoscelis antarctica*) against intruders. *Ethology* 99: 323-331.
31
32
33 VIÑUELA, J., J. MORENO, L. M. CARRASCAL, J. J. SANZ, J. A. AMAT, M. FERRER, J.
34
35 BELLIURE, AND J. CUERVO. 1996. The effect of hatching date on parental care,
36
37 chick growth, and chick mortality in the Chinstrap Penguin *Pygoscelis*
38
39 *antarctica*. *Journal of Zoology* 240: 51-58.
40
41
42 WEGMANN, M., B. VOEGELI, AND H. RICHNER. 2015. Physiological responses to
43
44 increased brood size and ectoparasite infestation: adult Great Tits favour self-
45
46 maintenance. *Physiology & Behavior* 141: 127-134.
47
48
49 WIDMANN, M., A. KATO, B. RAYMOND, F. ANGELIER, B. ARTHUR, O. CHASTEL, M.
50
51 PELLÉ, T. RACLOT, AND Y. ROPERT-COUDERT. 2015. Habitat use and sex-
52
53 specific foraging behaviour of Adélie Penguins throughout the breeding season
54
55 in Adélie Land, East Antarctica. *Movement Ecology* 3: 1-10.
56
57
58
59
60

WIERMSMA, P., C. SELMAN, J. R. SPEAKMAN, AND S. VERHULST. 2004. Birds sacrifice oxidative protection for reproduction. *Proceedings of the Royal Society B* 271: S360-S363.

WILLIAMS, T. D. 1995. *The penguins*. Oxford University Press, New York, NY.

WILLIAMS, T. D. 2018. Physiology, activity and costs of parental care in birds. *Journal of Experimental Biology* 221: jeb169433.

XU, Y.-C., D.-B. YANG, J. R. SPEAKMAN, AND D.-H. WANG. 2014. Oxidative stress in response to natural and experimentally elevated reproductive effort is tissue dependent. *Functional Ecology* 28: 402-410.

Table 1. Mean values ($\pm$ SE) of the response variables H/L ratio, oxidative damage (ROMs, mg H₂O₂ dl⁻¹), and total antioxidant capacity (OXY, μ mol HClO mL⁻¹) related to the fixed factors analyzed.

		<i>N</i>	H/L ratio	ROMs	OXY
Life-history stage	Breeding	27	1.18 $\pm$ 0.10	8.71 $\pm$ 0.80	252.37 $\pm$ 13.21
	Molting	29	0.34 $\pm$ 0.03	7.43 $\pm$ 0.38	243.88 $\pm$ 7.47
Sex	Male	26	0.81 $\pm$ 0.12	8.82 $\pm$ 0.73	249.25 $\pm$ 10.48
	Female	30	0.69 $\pm$ 0.10	7.38 $\pm$ 0.49	238.17 $\pm$ 10.67

Table 2. Results of general linear models examining the effects of life-history stage, sex, and two covariates (H/L ratio, OXY, and ROM when not the dependent variable) on the H/L ratio and oxidative status of Chinstrap Penguins. All df = 1,50. In addition, VIF (Variance Inflation Factors) have been included.

	Log ₁₀ H/L ratio			Log ₁₀ ROM			OXY		
	<i>F</i>	<i>P</i>	VIF	<i>F</i>	<i>P</i>	VIF	<i>F</i>	<i>P</i>	VIF
Life-history stage	77.3	<0.001	1.1	3.6	0.063 ^a	2.6	0.1	0.83	2.8
Sex	2.1	0.16	1.1	5.4	0.025	1.0	1.7	0.20	1.1
Life-history stage*Sex	0.2	0.68	1.0	0.7	0.40	1.0	1.3	0.25	1.0
Log ₁₀ H/L ratio	-	-	-	0.9	0.36	2.8	1.4	0.24	2.8
Log ₁₀ ROM	0.9	0.36	1.4	-	-	-	12.0	0.001	1.1
OXY	1.4	0.24	1.4	12.0	0.001	1.1	-	-	-

^a The three models were rerun without the outlier marked with an arrow in Fig. 1, and only the strength of the significance from this marginal *P* value was modified: $F_{1,49} = 7.5$, $P = 0.009^*$

FIGURE LEGENDS

Fig. 1. Correlations between oxidative damage (ROM) and antioxidant capacity (OXY) for Chinstrap Penguins during breeding and molting. The value indicated by the arrow is the outlier of the breeding distribution. Statistical analyses were performed both including and excluding this value for a better understanding of its impact (see Results section and Table 2).

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