

Incubation increases oxidative imbalance compared to chick rearing in a seabird, the Magellanic penguin (*Spheniscus magellanicus*)

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Abstract It is expected that activities which require a high use of energy could generate higher oxidative stress. In the present study, we have compared two breeding periods (incubation and chick rearing) with different energetic demands in the Magellanic penguin, predicting a higher oxidative unbalance during chick rearing since involves higher demanding activities such as chick feeding and greater nest protection than during incubation. Specifically, we predicted higher oxidative damage and lower antioxidant defences during chick rearing than during incubation. Fieldwork was conducted in a Magellanic penguin colony located in Estancia San Lorenzo (42°05'S, 63°49'W), Peninsula Valdes, Argentina, during the breeding season of 2014–2015. Surprisingly, our results did not support our initial prediction. Incubating adults had their oxidative status unbalanced showing significantly lower antioxidant levels than those rearing chicks. Moreover, oxidative damage did not show any significant variation between both breeding periods. Further, we did not find differences in oxidative status between sexes. Our results suggest that incubation is a highly demanding activity compared to chick rearing in terms of oxidative balance since the lower presence of antioxidants can be explained as they have probably depleted to limit oxidative damage by ROS. Differential foraging

effort could explain such results as Magellanic penguins adjust their foraging location to prey availability performing longer foraging trips during incubation than during chick rearing which increases the energy costs and therefore imbalance penguins oxidative status. Our results show the importance of examining physiological markers such as oxidative stress to assess differences during the breeding cycle and how the behaviour at sea could explain such differences in seabirds.

Introduction

Reproduction is one of the most energy-demanding life-history stages, nest construction and defences, egg incubation or chick feeding are energetically costly behaviours (Green et al. 2009); however, life-history demands might limit energy allocation to the different activities (Stearns 1992). To maximize fitness during reproduction, animals are assumed to specifically allocate energy to reproduction, growth, and metabolism (Roff 1992; Davies et al. 2012). Therefore, the relationship between individual costs and reproductive behaviour is a major topic in the study of life-history strategies.

Birds are iteroparous, that is, adults reproduce repeatedly during life, and each reproductive attempt includes distinct stages, such as egg laying, incubation and chick rearing. Energy demand in each stage is different due to the specific requirements, for example, thermal conditions during incubation or increased foraging when rearing chicks. Previous studies in some penguin species like the Little penguin (*Eudyptula minor*) have shown chick rearing to be the most energetically expensive period of the annual energetics cycle (Gales and Green 1990). On the other hand, it has been demonstrated that egg formation and incubation

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also generate costs (see Astheimer and Grau 1985 for energetic consequences of egg formation in Adélie penguin, *Pygoscelis adeliae*), and trade-offs between incubation and chick rearing have also been reported in seabirds (Heaney and Monaghan 1996). In contrast, another study found similar energy expenditure between incubation and chick rearing in females of Macaroni penguins (*Eudyptes chrysolophus*; Green et al. 2009).

Oxidative stress is one of the multiple physiological consequences of reproduction (Harshman and Zera 2007; Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009). Normal metabolic activities, such as aerobic cell respiration, produce a variety of free radicals as reactive oxygen species (ROS), which according to the free radical theory (Harman 1956), damage biomolecules (e.g., lipids, proteins and DNA) unless quenched by antioxidants (Balaban et al. 2005; Metcalfe and Alonso-Alvarez 2010). The accumulation of oxidative damage over time has been suggested to be one of the causal agents of ageing, because full biological mitigation efficiency (protection and repair systems) is not achieved (Beckman and Ames 1998; Finkel and Holbrook 2000). Therefore, oxidative stress is defined as disturbance in the pro-oxidant-antioxidant balance in favour of the former, leading to biomolecular damage caused by reactive species attacking the constituent parts of living organisms (Halliwell and Gutteridge 2007).

The relationship between the cost of reproduction and oxidative stress has been addressed in several animal species (see Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Beaulieu et al. 2011; Ołdakowski et al. 2012; Costantini et al. 2014a; Xu et al. 2014), but the taxonomic variation in reproductive strategies results in oxidative stress costs to reproduction having various roles (Costantini 2014). Reproductive costs compromise future reproduction, self-maintenance, and/or growth (Stearns 1989). It has been hypothesized that a heavy investment in reproduction would generate an oxidative cost resulting in faster somatic deterioration, reducing life expectancy unless antioxidant defences increase, because resources allocated to reproduction are not invested in oxidative stress defense (Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Beaulieu et al. 2011; Ołdakowski et al. 2012). However, experimental support for this hypothesis has not been clearly demonstrated (Metcalfe and Monaghan 2013; Speakman and Garratt 2014) due to inadequate experimental designs which reflect individual quality or access to resources (see Beaulieu et al. 2011; Ołdakowski et al. 2012) and its focused on antioxidants rather than oxidative damage or repair (see Alonso-Alvarez et al. 2004; Wiersma et al. 2004; Christie et al. 2012). Lately, a few experimental demonstrations have fulfilled previous biases, although evidence in favour of an oxidative cost of reproduction is still vague (Costantini et al. 2014b; Wegmann et al. 2015). Moreover, environmental conditions or individual quality may stimulate self-maintenance,

decreasing oxidative damage (Costantini et al. 2014a). Several studies relating oxidative stress and reproduction have focused on the chick rearing period (for examples, see Costantini 2014), and little attention has been paid concerning the oxidative costs of incubation. However, some studies have recently included this period in their studies showing, for instance, that oxidative status of incubating Iberian Pied Flycatchers (*Ficedula hypoleuca iberiae*) females did relate to egg-attendance behaviour (López-Arrabé et al. 2014), and diet quality could affect more the oxidative status of pigeons (*Columba livia*) during chick feeding than while incubation (Costantini 2010). In this study, we tested whether two different breeding periods, incubation and chick rearing, which demand different energy requirements due to differential reproductive behaviour result in an oxidative imbalance in a penguin species.

Penguins offer an excellent opportunity for such study due to dramatic differences in effort between those two reproductive stages. In penguins, chick-rearing is the most energetically expensive period and is particularly extreme towards the end of this stage due to chick growth demands (Gales and Green 1990). Magellanic penguins (*Spheniscus magellanicus*) breed in large colonies, placing their nests on the surface or underground (digging burrows), and frequently under bushes (Bertellotti 2013). After mating, females lay two eggs and then, share incubation with the males for around 40 days in which both adults go to the sea alternatively (Williams 1995; Bertellotti 2013). Chicks usually hatch in mid-to-late November remaining in the nest around 29 days in which parents alternate feeding visits and guard duties, and followed by an unattended period of 40–70 days until chicks become independent (Williams 1995; Bertellotti 2013).

Since more activity should increase ROS production unless antioxidant defences rise, the stronger investment during chick rearing should trigger susceptibility to oxidative stress (Alonso-Alvarez et al. 2004; Costantini et al. 2008; but see Salin et al. 2015 and Speakman and Garratt 2014 for recent contrasting results showing how complicate the link might be). Therefore, we hypothesized that during the chick rearing period, which involves a high parental investment in activities as chick feeding and greater nest protection, oxidative stress should be higher than during incubation. Specifically, we predicted higher oxidative damage and lower antioxidant defences during chick rearing than during incubation.

Methods

Fieldwork

Fieldwork was conducted in the Magellanic penguin colony located in Estancia San Lorenzo (42°05'S,

63°51'W), Peninsula Valdes, Argentina, during the breeding season of 2014–2015 (November and December of 2014; Fig. 1). The total population size of the colony is estimated at about 58,000 breeding pairs (Bertellotti 2013). Sixty-five adults were captured at the nest, 37 incubating and 28 rearing chicks (26 for non-protein thiols due to the lack of two samples). All the sampled penguins were nesting under shrubs incubating two eggs or rearing two chicks, in order to avoid uneven parental efforts. Eggs and chicks were kept safe while adults were being handled. After manipulation, adults were replaced in the nest, and their resumption of care of eggs or chicks was confirmed. Immediately after capture, a blood sample was taken with a heparinized capillary tube after pricking a peripheral foot vein with a sterilized needle. Then, they were kept in a cooler and later centrifuged at 12,000 rpm for 15 min to separate plasma from red blood cells, both of which were frozen at $-20\text{ }^{\circ}\text{C}$ until lab processing. In addition, adults were weighted (to the nearest 50 g with a spring balance) and bill length and depth were measured (to the nearest 0.1 mm) with a digital calliper. Finally, sex was obtained by means of discriminant functions based on morphometry (Bertellotti et al. 2002).

Laboratory analyses

Oxidative balance in blood plasma was measured with both oxidant and antioxidant components using d-reactive oxygen metabolites (d-ROM) and oxy-adsorbent tests (Diacron International, Grosseto, Italy), respectively. The d-ROM test measures plasmatic hydroperoxydes, a type of reactive oxygen metabolite (ROM) resulting from oxidative damage (attack of ROS on organic substrates such as amino acids, proteins, nucleotides, etc.). The oxy-adsorbent test measures the total plasma antioxidant capacity (OXY), quantifying the contribution of a large section of exogenously and endogenously synthesised antioxidants by adding a highly potent oxidant, hypochlorous acid (HClO). Then the unreacted HClO radicals can be measured photometrically, because they react with a chromogenic substrate, see Beaulieu et al. (2010) for a similar approach. Both d-ROM and oxy-adsorbent test procedures were carried out following Beaulieu et al. (2010) with minor modifications, such as using a vortex machine (15 s) and short spins for thawed plasma and mixtures (plasma-reagent, calibrator-reagent and blank-reagent), and incubation with shaking (speed 6 of 10) in an Amersham Bioscience Hybridization Oven/Shaker for better homogenization. Absorbance was read with a spectrophotometer at $37\text{ }^{\circ}\text{C}$ and shaking

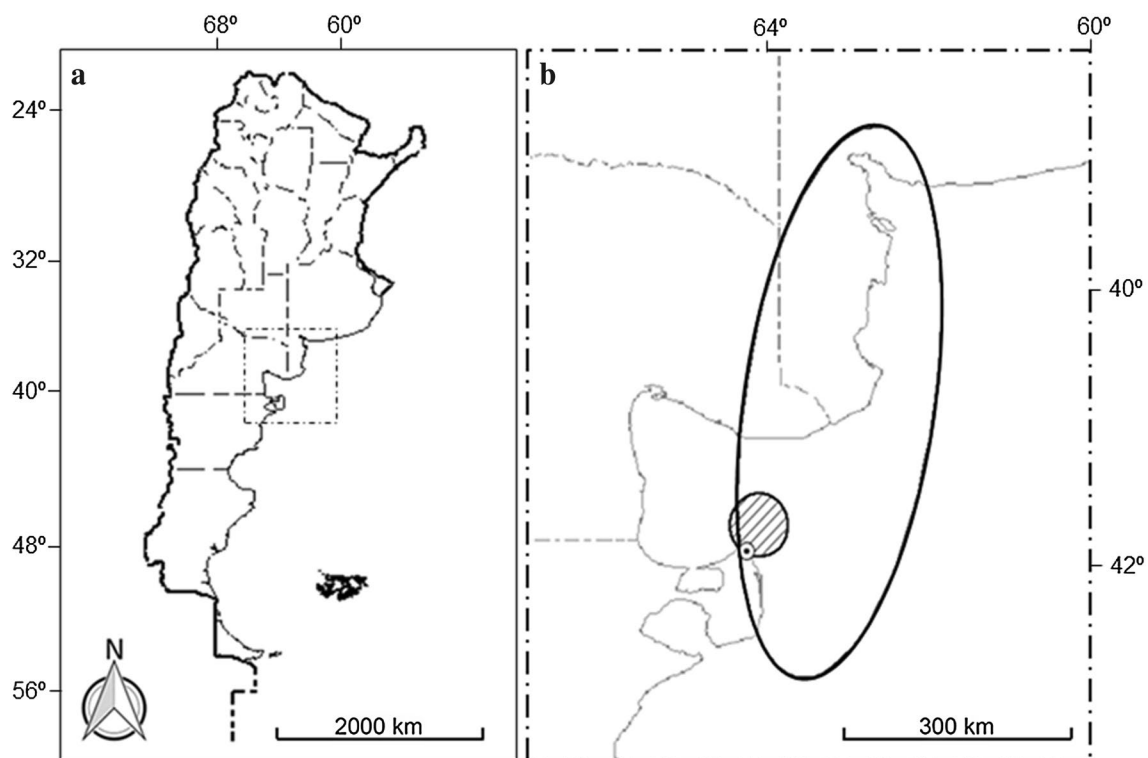


Fig. 1 Map of the study area: **a** Argentina. **b** The dot marks the location of the San Lorenzo penguin rookery. The foraging distribution of Magellanic penguins (*Spheniscus magellanicus*) during incubation

is circled (Wilson et al. 2005). The striped circle shows the foraging area during chick rearing (Sala et al. 2012a)

(medium speed) the microplate for a minute before reading at a wavelength of 490 nm (Multi-Mode Microplate Reader, Synergy™ HT, BioTek). Measurements are expressed as mg H₂O₂ dL⁻¹ and μmol HClO mL⁻¹ sample for the d-ROM and oxy-adsorbent tests, respectively. Both d-ROMs and oxy-adsorbent tests were performed using microplates where all treatments were evenly distributed together with duplicates per each sample, blanks and calibrators. Repeatability was calculated for both tests and per each plate, d-ROMs and OXY repeatabilities were 98.6% (plate I), 97.7% (plate II), 89.2% (plate I) and 85.2% (plate II), respectively. Intra-assays coefficients of variation for OXY and d-ROMs for plate I and II were 6.41, 10.44, 12.28 and 8.20%, respectively; and inter-assay coefficients of variation for OXY and d-ROMs were 23% and 5.08%.

Total non-protein thiols concentration was determined by assessing the thiols group capacity (-SH) for reducing 5-5'-dithiobis-nitrobenzoic acid (DTNB) in plasma. Plasma (3 μL) was homogenized with 1 ml HClO₄, then centrifuged for 20 min at 3000 rpm and neutralized with Na₃PO₄ (0.44 M) to pH7 before adding 50 μL of DTNB 6 mmol in NaHCO₃ 0.5% w/v. Absorbance was read at 412 nm after 30 s (ϵ 13.6 mmol⁻¹ cm⁻¹). In addition, kidney protein extracts were used as positive controls and the reaction buffer as blanks. Results are expressed as mmol L⁻¹ of total non-protein thiols.

Statistical analyses

All variables were normally distributed (Kolmogorov–Smirnov test >0.05). Statistical analyses included general linear mixed models (GLMMs). GLMMs were performed on OXY, ROM, non-protein thiol groups and weight (gr) with breeding periods (incubation and guard phase) and sex as fixed factors as well as its interaction, and individual, pair and the plate used in the laboratory analyses as random. The mean square (MS) and the degrees of freedom (*df*) of the error terms were estimated following the Kenward & Roger method (Kenward and Roger 1997). All *P*-values below 0.05 were considered significant. We used the R packages *lme4* and *lmerTest* (Kuznetsova et al.

2014; Bates et al. 2015) run under version 3.3.1 to perform the GLMMs (R Core Team 2014).

Results

Our results showed that adults during incubation had lower antioxidant defences than while rearing chicks. Antioxidant defences, total antioxidant capacity (OXY) and non-protein thiols, were significantly lower during incubation (Table 1; Figs. 2, 3). Furthermore, neither sex nor the interaction between sex and breeding period showed any significant effect in the level of antioxidant defences (Table 1). Oxidative damage (ROM) did not show significant differences in any factor analysed between periods (Table 1). Finally, adults during incubation were significantly heavier than while rearing chicks ($F_{1,48.714} = 18.429$, $P < 0.001$; incubation and chick rearing mean weight \pm SD = 4248.65 \pm 774.46 g, $n = 37$ and 3691.67 \pm 532.64 g, $n = 27$, respectively). Moreover, male weighted significantly more than

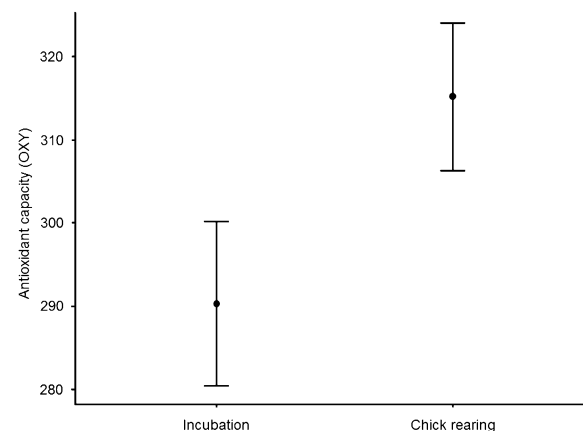


Fig. 2 Total antioxidant capacity (OXY, μmol HClO mL⁻¹) differences between reproductive periods in Magellanic penguins (*Spheniscus magellanicus*). Squares show model-predicted means \pm standard errors

Table 1 Results of a general mixed ANOVA GLMM, examining the effects of Magellanic penguin breeding periods and sex on oxidative status (total antioxidant capacity, OXY; and oxidative damage, ROM) and non-protein thiols

	OXY				ROM				Non-protein thiols			
	Estimate	DF	F	P	Estimate	DF	F	P	Estimate	DF	F	P
Period	13.935	1,48.35	4.907	0.032	-0.163	1,49.002	0.045	0.833	12.273	1,44.0	13.134	<0.001
Sex	-6.944	1,43.077	1.134	0.293	-0.335	1,42.414	0.163	0.689	4.808	1,43.366	2.042	0.16
Period \times sex	4.423	1,49.223	0.496	0.485	-0.852	1,49.196	1.242	0.271	0.619	1,43.829	0.035	0.853

Denominator degrees of freedom follow the Kenward–Roger approach to GLMM

Significant differences ($P < 0.05$) marked in bold. For more details see the “Methods” section

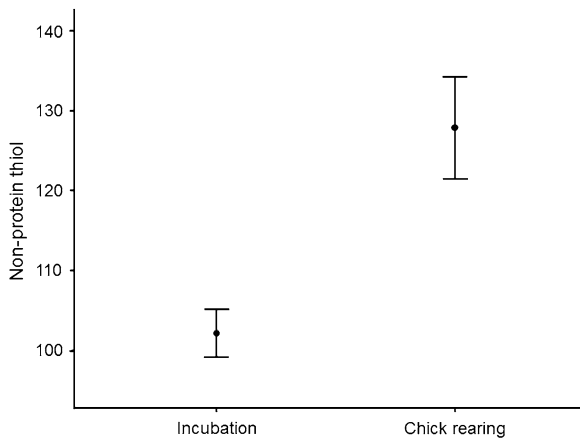


Fig. 3 Total differences in non-protein thiol groups (mmol L⁻¹) between reproductive periods in Magellanic penguins (*Spheniscus magellanicus*). Squares show model-predicted means \pm standard errors

females ($F_{1,43,270} = 50.249$, $P < 0.001$; male and female mean weight \pm SD = 4440.71 ± 680.67 g, $n = 35$ and 3498.28 ± 376.66 g, $n = 29$, respectively). Nevertheless, the interaction of sex and breeding period did not show significant differences for the weight of the penguins ($F_{1,48,714} = 2.567$, $P = 0.116$).

Discussion

Incubation and chick rearing require different energy demands due to the particular behaviour in each, such as maintaining thermal conditions during incubation or chick feeding after hatching. In penguins, energy expenditure during chick rearing seems to be high due to chick growth demands which oblige adults to go to sea and back to the colony very frequently to feed them (Davis et al. 1989; Gales and Green 1990). On the contrary, incubation is considered a period of lower energy expenditure, although costs of egg formation and thermal demands of eggs during incubation have been demonstrated (Astheimer and Grau 1985; Moreno and Carlson 1989; Monaghan and Nager 1997 and references therein; Reid et al. 2000; Visser and Lessells 2001). Indeed, energy use during incubation and chick care from different penguin species showed that the former might be only 1–2 times the basal metabolic rate whereas the latest might be even four times (Croxall 1982; Moreno and Sanz 1996). Based on these differences in activity, a higher oxidative cost would be expected during the chick rearing period. Surprisingly, our results did not support this prediction. We found that both antioxidant defences analysed (OXY and non-protein thiols) were significantly lower during incubation, although no difference was found for oxidative damage. Antioxidant defences

contribute to the prevention of pro-oxidants and to their blockage once formed to avoid oxidative damage (Sies 2007). Even though defences are essential, complete efficiency is never achieved, and therefore, ROS might damage biomolecules such as lipids, proteins and DNA (Beckman and Ames 1998; Sies 2007; Selman et al. 2012). The metabolic dynamics of oxygen radicals generates oxidants, decreases antioxidant defences and results in damage to the organism (Beckman and Ames 1998). Hence, our results suggest that incubation would be a more demanding situation in terms of oxidative balance, as we have found a reduction in the antioxidant defences probably as a consequence of a limitation of oxidative damage by ROS. In contrast, a study on Macaroni penguins (*E. chrysolophus*) found similar energy expenditure throughout all the breeding period including incubation and chick rearing (Green et al. 2009). Furthermore, our results showed no significant differences for sex or its interaction with breeding period, which suggests there is no sex-bias in oxidative status in either of the studied periods in contrast with energy expenditure findings (Davis et al. 1989).

Our results could be explained by a differential foraging effort at sea, and thereby different energy expenditure, which might result in an oxidative imbalance (Costantini et al. 2008; Green et al. 2009; Beaulieu et al. 2010). Different foraging efforts for incubation and chick rearing have been recorded in Magellanic penguin throughout its distribution (Wilson et al. 2005; Sala et al. 2012a). At Isla Martillo, Beagle Channel, penguins made longer foraging trips during incubation than while brooding (Rey et al. 2012). Similarly, incubating adults at Punta Tombo, Argentina, also showed longer foraging trips while brooding than during chick rearing, suggesting increased probability of encountering their preferred prey (Walker and Boersma 2003). In our study area, this seems to be the case, as Magellanic penguins perform longer foraging excursions during incubation than during chick rearing (Wilson et al. 2005; Sala et al. 2012a; Fig. 1). These excursions fit to the stocks of their main prey, anchovies. During the incubation period (October–November), the main anchovy stock, the Bonaerense, is found from Southern Brazil to 41°S, while during chick rearing (December), the major anchovy stock is located in the central Patagonian maritime front area (41°S–47°S; Pastous Madureira et al. 2009; Pájaro et al. 2011). Therefore, our results suggest that a greater foraging effort was made to feed on the Bonaerense stock during incubation, which would increase the metabolic rate and likely trigger higher production of ROS resulting in oxidative damage. However, we have not detected such situation probably due to the use of antioxidants resulting in a depletion of their own levels as our results show. Thus, feeding plasticity of Magellanic penguins as a consequence of the

availability of their prey (see Sala et al. 2012a, 2014; Scioscia et al. 2014 for examples) could explain the oxidative imbalance found between breeding periods. Previous studies have related energetically demanding activities which increase metabolic rate, with an oxidative imbalance (Butler et al. 2016; Ziomkiewicz et al. 2016). For instance, Costantini et al. (2008) found a close relationship between the flight effort and the oxidative status in homing pigeons (*C. livia*). However, the precise mechanism linking metabolic rate to increase oxidative damage is far to be simple and therefore more research is needed (Speakman and Garratt 2014; Salin et al. 2015).

Food deprivation during incubation shifts and its consequences may also explain our results. Recent studies have showed the link between prolonged fasting and oxidative stress in birds. Indeed, a progressive body mass reduction and antioxidants depletion has been found in different species during long-term fasting (Sylvie et al. 2012; Barbosa et al. 2013; Schull et al. 2016). However, this seems not be the case in our study as we did not found a lower body mass for incubating individuals as expected if they were in a prolonged fasting situation. Further, the minimum Magellanic penguin weight occurs at the end of incubation (male 3060 g and females 2300 g; P. D. Boersma, unpublished data in Williams 1995) which is considerably lower than the weight in our sampled birds during incubation. Therefore, based on the weights of our sampled individuals, we assume that we sampled the incubating penguins at the beginning of fasting.

Our results might also be explained based on likely different diet composition between both study periods. Nutrition influences the physiology of organisms in several ways. Diet is a source of antioxidants mitigating ROS damage (Surai 2002; Hulbert 2005; Pamplona and Barja 2007). Dietary antioxidants (e.g., vitamins and carotenoids) have a major role in the entire antioxidant network, and because birds cannot synthesise carotenoids, its efficiency may be affected by its diet (Goodwin 1984; Catoni et al. 2008 and references therein; Cohen et al. 2009). For instance, diet quality impacts on the oxidative status of birds (Costantini 2010). However, we can rule out such explanation as in our study area, Magellanic penguins fed exclusively on anchovies (*Engraulis anchoita*) in both study periods, accounting for 90 or more percent of the diet (Wilson et al. 2005; Sala et al. 2012b).

An alternative interpretation to the antioxidant findings is that these compounds are upregulated during the chick rearing period to cope with the higher free radical production due to foraging effort or remobilization of antioxidants among tissues over the transition from incubation to the chick rearing phase. However, the likely differences in foraging during both periods as shown above seem to exclude this explanation.

Finally, our results show that no oxidative damage differences were found between breeding periods. We suggest that the antioxidant barrier could be the responsible of this situation as antioxidants have been acting by limiting oxidative damage producers and showing no differences between periods (Gaál et al. 2006). In addition, since the detection of an oxidative cost of parental care might be affected by the sampling time (see Losdat et al. 2011), oxidative damage differences could not be found due to a lack of time to become detectable or because damage have been cleaned up after its occurrence (Costantini et al. 2014b).

In conclusion, our results suggest that antioxidant defences (OXY and non-protein thiols) decrease during incubation for Magellanic penguins. Hence, plasticity in foraging behaviour of Magellanic penguins in response to the availability of prey around the breeding colony could be the most likely explanation triggering oxidative susceptibility between breeding periods due to a probable difference in the foraging effort. Further research integrating oxidative physiology and foraging ecology during breeding periods would be necessary to confirm our suggestion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal handling and experimental procedures were approved by the Office of Tourism and Protected Areas of Chubut Province and Fauna and Flora Department, Argentina.

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