Stress hormones in relation to breeding status and territory location in colonial king penguin: a role for social density?

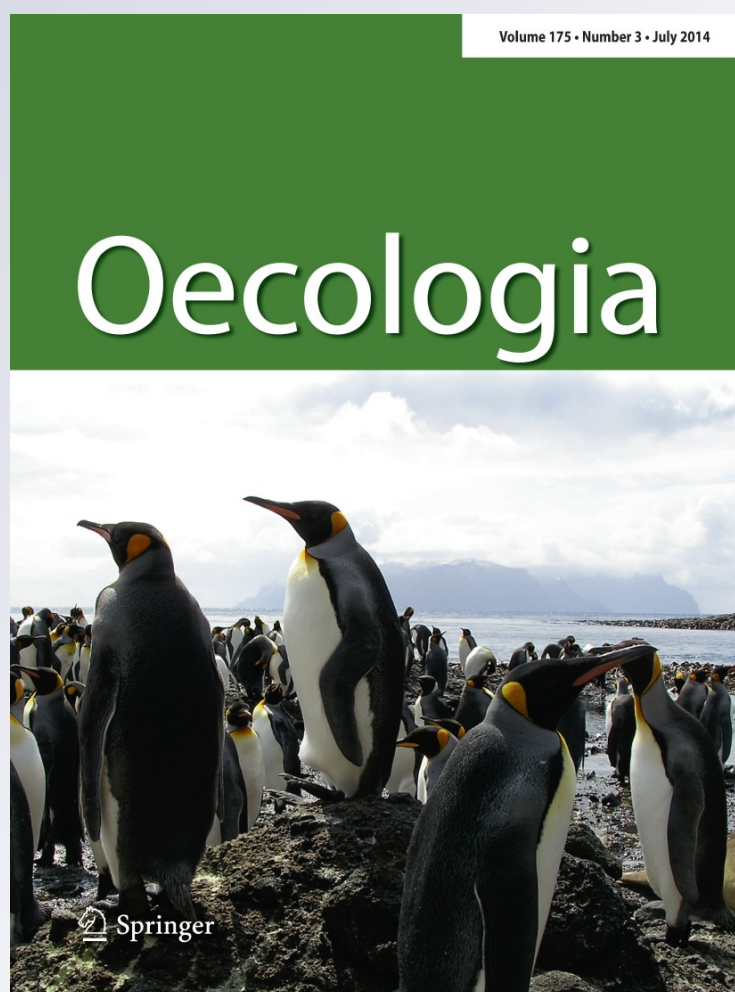## Vincent A. Viblanc, Benoit Gineste, Antoine Stier, Jean-Patrice Robin & René Groscolas

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Stress hormones in relation to breeding status and territory location in colonial king penguin: a role for social density?

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Abstract Because glucocorticoid (stress) hormones fundamentally affect various aspects of the behaviour, life history and fitness of free-living vertebrates, there is a need to understand the environmental factors shaping their variation in natural populations. Here, we examined whether spatial heterogeneity in breeding territory quality affected the stress of colonial king penguin (*Aptenodytes patagonicus*). We assessed the effects of local climate (wind, sun and ambient temperature) and social conditions (number of neighbours, distance to neighbours) on the baseline levels of plasma total corticosterone (CORT) in 77 incubating and 42 chick-brooding birds, breeding on territories of central or peripheral colony location. We also assessed the oxidative stress status of a sub-sample of central vs. peripheral chick-brooders to determine whether chronic stress arose from breeding on specific territories. On average, we found that brooders had 55 % higher CORT levels than incubators. Regardless of breeding status, central birds experienced greater social density (higher number of neighbours, shorter distance between territories) and had higher CORT levels than peripheral birds. Increasing social density positively explained 40 % of the variation in CORT levels of both incubators and brooders, but the effect was more pronounced in brooders. In contrast, climate was similar among breeding territories and did not significantly affect the CORT levels of breeding birds. In brooders, oxidative stress status was not affected by local density or weather conditions. These results highlight that local heterogeneity in breeding (including social) conditions may strongly affect the stress levels of breeding seabirds. The fitness consequences of such variation remain to be investigated.

Keywords Coloniality · Corticosterone · Crowding · Population density · Glucocorticoids · Oxidative stress · Seabird · Stress · Territory location

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Introduction

Glucocorticoid (GC) hormones, corticosterone (CORT) in birds, are products of the physiological stress response enabling vertebrates to cope adaptively with predictable and unpredictable changes in the environment (Wingfield and Romero 2001; Sapolsky 2002; Boonstra 2004). At baseline levels, they regulate the energy balance to meet the different energy demands associated with specific life history stages (Landys et al. 2006; Romero et al. 2009). In response to acute stressors, transient increases in GCs trigger physiological and behavioural changes aimed at increasing individual fitness (Wingfield et al. 1998). Given these critical functional roles, there is a need to understand the relationships between inter- and intra-individual GC

variation, the intrinsic and extrinsic factors that stimulate their release, and animal fitness (Bonier et al. 2009). For instance, GC levels are affected by various environmental factors including weather conditions (Wingfield et al. 1983; Bize et al. 2010), predation pressure (Boonstra et al. 1998; see Boonstra 2013; Clinchy et al. 2013), or social stimuli such as conspecific aggressiveness (McCormick 2006) and density (Raouf et al. 2006; Dantzer et al. 2013, reviewed in Creel et al. 2013).

For colonial seabirds, environmental heterogeneity associated with the selection of a breeding territory is likely to have strong effects on GC levels (Kitaysky et al. 1999; Shultz and Kitaysky 2008). For instance, in black-legged kittiwakes (*Rissa tridactyla*), the dynamics of circulating CORT levels differ substantially between birds breeding at food-rich vs. food-poor colonies, with higher baseline CORT levels and lower acute adrenocortical responses found in birds breeding at a food-poor colony (Kitaysky et al. 1999). Such studies confirm that widescale differences in environmental conditions related to breeding site location affect GC levels. However, how local (within-colony) variation in breeding territory characteristics affects GC levels, and which specific factors best explain these levels, remain to be investigated.

In this study, we considered the effects of breeding status and local territory characteristics on the GC levels of a colonially breeding seabird, the king penguin (*Aptenodytes patagonicus*). King penguins breed on remote subantarctic islands in large groups of several thousands of pairs [up to 500,000 pairs (Guinet et al. 1995)]. After courtship, reproductive pairs walk through the colony and select a small breeding territory (either in central or peripheral colony locations) on which they incubate and raise their single offspring on their feet, throughout the breeding season (Stonehouse 1960). Incubation and chick-rearing until thermal emancipation occur on this fixed territory, which both males and females aggressively and relentlessly defend using threat displays and physical blows (Côté 2000). During this period, breeding territories shift by only a few centimetres (e.g. as penguins turn their body according to wind direction), but birds avoid longer displacements which instantaneously induce agonistic responses of neighbours, with substantial risks of injury and egg or chick loss. The surprisingly high rate of aggressiveness in this species (up to 112 interactions/bird per hour; Côté 2000) suggests high benefits to territorial defence.

Breeding territories vary in a number of key aspects. First, avian predation is at least twice as great on the outskirts of the colony, where subantarctic skuas (*Catharacta loonbergi*) and giant petrels (*Macronectes giganteus* and *Macronectes halli*) predate on eggs and young chicks (Côté 2000; Descamps et al. 2005); and breeding success has thus been suggested to be higher in central territories (Côté

2000; Bried and Jouventin 2001; but see Descamps et al. 2009). Second, birds on the periphery are less buffered from detrimental weather events such as storms and floods (Bried and Jouventin 2001; Viera et al. 2006). Third, birds in the centre have to contend with more numerous (aggressive) conspecifics (Côté 2000). For instance, the rate of aggression with body contact (pecking and flipper blows) elicited by brooding (though not incubating) birds has been shown to increase towards the centre of the colony (Côté 2000). Finally, to reach their breeding territory, central birds typically have to navigate through more aggressive conspecifics with greater risk of fights and injuries. Indeed, the proportion of birds being attacked by neighbours while walking through the colony increases with distance to the edge (Côté 2000).

Thus, specific predictions on how GC levels should vary with territory characteristics in king penguins can be made. If predation risk and weather conditions are crucial factors determining individual stress, birds breeding on central territories should have lower total baseline CORT levels than birds breeding on peripheral territories. Conversely, if social density and conspecific aggressiveness are more important, peripheral birds should have lower CORT levels than central birds. To distinguish between these hypotheses, we simultaneously considered the effects of local climate (wind, sun and ambient temperature) and social (number of neighbours, distance between neighbours, bird density) conditions on the total baseline CORT levels of 119 penguins either incubating an egg or brooding a young chick in various locations of the colony (defined by their distance from the edge). In addition, we examined the oxidative stress status of a sub-sample of brooding birds to assess whether specific territory characteristics were associated with a state of chronic stress (Breuner et al. 2013). High levels of GCs have indeed been suggested to disrupt the balance between pro-oxidants and antioxidant defences, leading to oxidative damage of important biomolecules such as lipids, proteins or DNA under stressful situations (Halliwell and Gutteridge 2007; Zafir and Banu 2009; Haussmann et al. 2012).

Materials and methods

Territory location, breeding and nutritional status of sampled birds

This study was performed over the 2010–2011 breeding season (austral summer), in a king penguin colony of ca. 24,000 breeding pairs on Possession Island (Crozet Archipelago; 46°25′S, 51°45′E). Based on sampling dates (see below) and on the duration of incubation in king penguin [54 days (Weimerskirch et al. 1992)], all birds were early

breeders (late breeders start breeding by the end of January). Incubating ($n = 77$) and chick-brooding ($n = 42$) penguins were selected at random both in central and peripheral colony locations, and their sex was unknown. Incubating birds were sampled at an unknown point of incubation (potentially during any sex-specific incubation shift from days 1 to 53 of incubation). There are no data available suggesting that baseline plasma CORT levels change over the course of incubation in king penguins. Sampling incubating penguins of standardized incubation duration would have required catching and marking birds (and their egg) at the time of egg laying, and monitoring them daily. This was avoided to reduce colony disturbance. On the other hand, brooding birds were tightly synchronized at blood sampling. All were caring for a young chick of around 1 week of age (estimated from the size of the chick) kept in their brood pouch. The breeding stage (incubation or brooding) was first estimated from a distance (see below) and then confirmed at the end of blood sampling by checking the content (egg or chick) of the brood pouch.

Whereas all birds in this study were fasting while incubating/brooding their egg/chick ashore, the number of days they had been fasting was not known. Based on the duration of sex-specific incubation and brooding shifts in king penguin (Weimerskirch et al. 1992), it would have ranged from 1 to 15 days in incubating birds and from 1 to 12 days in birds brooding a young chick. To avoid a potential bias linked to nutritional status, we did not sample birds of critically low body girth [and thus body mass (Viblanc et al. 2012)] which might have been at an advanced stage of fasting (i.e. phase III). Indeed, during phase III, protein catabolism occurs and GC levels increase in fasting king penguins (Cherel et al. 1988). Our results thus only concern birds in phase II fasting, a nutritional status characterized by the maintenance of baseline CORT levels at low steady values (Cherel et al. 1988). This was indeed confirmed by the observation of bird behaviour prior to sampling, revealing that no bird had lost the drive to incubate or brood, characteristic of penguins entering fasting phase III (Groscolas et al. 2000). Actually, as only very few breeding birds reach such an advanced fasting stage in natural conditions [ca. 3 % (Gauthier-Clerc et al. 2001)], we are confident our findings are representative of the vast majority of king penguins naturally breeding in this colony. Further details on sampling protocol are provided in the Electronic Supplemental Materials (ESM) of this paper (see ESM 1).

Breeding territory characteristics

Prior to blood sampling and to avoid bird disturbance, the overall location (central or peripheral) and status (incubating or brooding) of the focal bird was determined at a distance of >25 m, using binoculars. This distance was chosen as preliminary tests in the study colony indicate that the physiological detection distance of king penguins when approached by humans, i.e. the distance at which their heart rate starts increasing, is around 20–25 m (V. A. Viblanc and R. Groscolas, unpublished data). The centrality of a bird in the colony was also quantified by counting the number of bird ranks between the focal individual and the edge of the colony. For instance a bird of rank 2 would have two bird ranks separating it from the edge of the colony. The mean rank ($\pm$SE) of central birds was 9.8 ± 0.4, whereas peripheral birds were virtually always right on the outskirts of the colony (mean rank = 0.04 ± 0.03). Also, before sampling and from a distance, the number of immediate neighbours of the focal bird was counted and distances between the focal bird and each of its neighbours were estimated at $\pm$10 cm. Only neighbours with which the focal bird could have agonistic interactions (within less than 1.5 m as determined from preliminary observations) were considered. Estimates of distances between the focal bird and neighbours were refined at the time of capture, before birds started moving. For each sampled bird, a social density index was calculated as number of neighbours/average distance to neighbours. For example, a density index of 2 could correspond to a focal bird having two neighbours at an average 1-m distance, whereas a density index of 10 could correspond to a focal bird having six neighbours at an average distance of 0.6 m.

Local weather conditions at the time of blood sampling were recorded on the breeding territory of each sampled bird. Ambient temperature at a 0.5-m (penguin) height was recorded at $\pm$0.5 °C using a mercury thermometer. Wind speed was scored by a single experimenter as: 0, no wind; 1, moderate wind; 2, strong wind. Intermediate categories (e.g. 0/1) were also considered. Solar status was scored as: 0, no sun; 1, moderate sun, sky cloudy; 2, strong sun, no clouds, intermediate categories also being considered. Blood sampling was performed only on non-rainy days. In addition, no major unpredictable climatic event (e.g. storms, heavy rain falls, flooding) occurred during the study.

Blood sampling protocol

Baseline GC levels are affected by various parameters such as season or daytime (Romero 2002), approach or handling stress (Romero and Reed 2005), and activity levels (Jessop et al. 2002). In order to obtain baseline levels, we standardized the sampling protocol as follows. First, we ensured that for each of the two breeding statuses considered, blood sampling of peripheral and central birds was performed at the same time of year, within a time frame as narrow as possible. For incubating birds, blood samples were taken on average on 2 January (range 20

December–25 January) for peripheral birds and on 7 January (range 16 December–26 January) for central birds. For brooding birds, blood samples were taken on average on 7 February (range 25 January–12 February) for peripheral birds and on 9 February (range 6–12 February) for central birds. Sampling dates between central and peripheral birds did not differ significantly for brooders (median test, $Z = -1.30$, $P = 0.30$). However, there was a slight difference in sampling dates for central and peripheral incubators, though it did not reach statistical significance ($Z = -1.79$, $P = 0.07$). Second, all blood samples were taken in the afternoon, between 1400 and 1600 hours to avoid a bias linked to a possible daily rhythm in CORT secretion. Third, to ensure that birds had not been recently approached and were thus potentially stressed, we did not sample birds for a period of at least 2 h in areas which had previously been visited by experimenters. This was possible because the colony on which we work is large (24,000 breeding pairs) and extends over a wide area (30,000 m²), allowing the sampling of birds located at least 100 m away from the zone of the colony where the preceding blood sampling and disturbance occurred. This distance is greater than the maximum radius of the zone around a sampled bird to where disturbance can extend (personal observation based on birds' behaviour), and also higher than the ca. 25-m individual detection distance described above. In addition, it has been previously shown that in king penguins, plasma CORT level recovers to baseline values within 1 h following a major disturbance, such as several minutes of handling (Ménard 1998). Fourth, for blood sampling, birds were approached from the back and we timed the moment at which they reacted to our approach by becoming vigilant, usually at a 5- to 8-m distance. This time was considered as time 0 of blood sampling and only blood samples obtained less than 3 min after time 0 were considered for analysis. In a previous study in king penguins (Ménard 1998) we determined that plasma CORT levels do not increase significantly due to capture-handling stress within the first 3 min. Fifth, before sampling we observed the behaviour of the focal bird and of its neighbours from a distance of >25 m. Sampling was only performed if the bird had been in a resting state for at least 5 consecutive min, continuously sitting either on its egg or chick. Immediately upon capture (and throughout handling and sampling) a hood was placed over the bird's head to keep it calm. Thus, we are confident that the total CORT levels measured in this study were indeed baseline resting levels. Blood (1 mL) was taken from a flipper vein using a G22-1 1/2 needle fitted to a 2.5-mL heparinized syringe. It was centrifuged at 4,000 r.p.m. for 5 min, within 10 min of sampling. Plasma was frozen at −80 °C and analysed within 4 months.

Total plasma CORT measurements

Total plasma CORT was measured in duplicate using a commercial double-antibody ^{125}I radioimmunoassay (RIA) kit (catalogue no. 07-120103; MP Biomedicals, Orangeburg, NY), without preliminary plasma extraction. The use of this RIA kit to measure total plasma CORT levels has been previously described and validated in birds (Washburn et al. 2002), including in king penguins (Bernard et al. 2002). Samples from central and peripheral birds at the same breeding status were analysed in the same run. Assay sensitivity was 1.0 ng/mL. Intra- and inter-assay coefficients of variation were 6 and 9 %, respectively. Recovery of exogenous CORT is 100.1 %, the minimum detectable dose is 0.008 ng/L and % of cross-reaction is 100.00 % for CORT, 0.34 % for desoxycorticosterone, 0.10 % for testosterone, <0.05 % for cortisol and other hormones (data from MP Biochemicals).

Oxidative stress measurements

To assess the degree of oxidative stress, we measured birds' plasma concentrations of reactive oxygen metabolites (ROMs) and 8-hydroxy-2′-deoxyguanosine (8-OHdG), as well as plasma's antioxidant capacity (antioxidant defences; OXY) [see Stier et al. (2012) for a review of these measures]. OXY was evaluated using the OXY adsorbent test (Diacron International, Italy) that quantifies the ability of plasma to buffer a massive oxidation reaction in response to hypochlorous acid. Results are expressed as micromoles of HClO neutralised per millilitre. ROMs were measured using the derivative (d)-ROM test (Diacron International, Italy), which is based on the Fenton reaction and measures primarily hydroperoxides (in mg H_2O_2 equivalent/dL) as an indicator of potential exposure to oxidative stress. 8-OHdG (ng/mL) was quantified using a competitive immunoassay (plasma diluted 1/10e; Assay Designs DNA damage enzyme-linked immunosorbent assay kit, Enzo Life Sciences, USA). 8-OHdG is one of the predominant forms of free radical-induced oxidative lesions on DNA, and has been widely used as a marker of oxidative stress (Halliwell and Gutteridge 2007). Plasmatic levels of 8-OHdG integrate whole-body oxidative damage to DNA, since 8-OHdG stems from damaged cellular DNA of any cell type, passes through the blood and is excreted into urine. Due to limitations on plasma quantity, we were only able to run OXY/ROM measurements for ten central and 18 peripheral brooders and 8-OHdG measurements for nine central and 18 peripheral brooders. All samples were run in duplicates in the same run. Coefficients of variation were 2.0 % for OXY, 4.1 % for ROM, and 2.7 % for 8-OHdG measures.

Statistics

Analyses were performed using JMP 9.0.0 (SAS Institute 2010) statistical software. The effects of local environmental conditions on CORT and oxidative stress levels in incubating and brooding penguins were investigated in a two-step analyses. First, we used principal components analyses (PCA) to identify major axis of covariation amongst local environmental parameters characterizing breeding territories. Those included bird rank in the colony as a measure of centrality, weather conditions (sun score, wind score, ambient temperature) and social conditions (number of neighbours, distance between neighbours, social density index). PCA was based on a correlation matrix [Bartlett's sphericity test, $\chi^2 = 509.7$, $P < 0.001$; KMO factor adequacy $= 0.67$ (Budaev 2010)] which was appropriate given that variables were measured on different scales. We thus produced independent orthogonal vectors (no factor rotation) describing local conditions of breeding territories. We only retained principal components (PCs) with eigenvalues >1 which together explained over 80 % of cumulative percent variance (Valle et al. 1999). Second, we used general linear models (LMs) to investigate the effects of PCs and breeding status (incubation vs. brooding) on CORT and oxidative stress levels. PCs, breeding status and their interactions were entered as independent variables in the models. Sampling date was added as a covariate to account for its potential effects on CORT levels. We ran all possible models, i.e. all combinations between possible variables including PCs, breeding status, sampling date, and all second-order interactions between PCs and breeding status (see ESM2). We retained models with the lowest Akaike information criteria corrected for small sample size (AICc) as the best fits (Burnham and Anderson 2002). If the ΔAICc between a model and the best fit was <2, we selected the most parsimonious model. Model selection using Bayesian information criterion (BIC) yielded similar results. Results are presented as mean ± SE. For model estimates, JMP does not use a specific level as a reference. Rather, it compares the mean of the dependent variable for a given level to the overall mean of the dependent variable. Consequently, the parameter for a nominal level is interpreted as the differences in the predicted response for that level from the average predicted response over all levels (given by the intercept). Significant results are given for $P < 0.05$ (two-tailed tests).

Ethical note

Birds were kept in their natural incubation or brooding stance while blood sampling took place. Overall handling took less than 5 min. This procedure never resulted in egg or chick abandonment. Protocols were approved by the

Table 1 Major independent axes obtained from a principal component (*PC*) analysis describing the local breeding environment of king penguins holding central or peripheral territories in the Baie du Marin colony (Ile de la Possession, Crozet Archipelago)

	PC1	PC2	PC3
Rank	*0.49*	−0.11	−0.07
Number of neighbours	*0.54*	−0.08	−0.01
Distance to neighbours	*−0.36*	0.19	−0.20
Density index	*0.52*	−0.14	0.06
Ambient temperature (°C)	0.23	*0.60*	−0.38
Sun score	0.13	*0.73*	0.11
Wind score	0.02	0.21	*0.89*
% Variance	45.7	20.4	15.5
Cumulated % variance	45.7	66.1	81.6

The strongest loadings for each axis are given in *italic*. Variables included bird rank in the colony as a measure of centrality, social conditions (number of neighbours, distance between neighbours, social density index) and weather conditions (ambient temperature, sun score, and wind score)

Ethical Committee of the Institut Polaire Français Paul-Emile Victor. Authorization to enter the colony and to manipulate birds was obtained from the Terres Australes et Antarctiques Françaises. The experiments comply with the current laws of France.

Results

Breeding territory characteristics

Of the various environmental parameters we recorded for penguin territories, over 81 % of the total variance in local breeding conditions could be explained by three PC axes (Table 1). Bird's rank vs. the edge the colony, its number of neighbours and local density index especially loaded on PC1, and were all inversely correlated to the distance between breeding neighbours (Table 1). In other words, as breeding territories increased in rank in the colony, so did number of neighbours and bird density, whereas the average distance between breeding territories decreased. PC2 and PC3 mainly described weather conditions. Sun score and ambient temperature strongly loaded on PC2 (Table 1), whereas wind score especially loaded on PC3 (Table 1) (see also ESM3).

When tested in a LM including breeding status (incubator vs. brooder), territory location (central vs. peripheral), and the interaction breeding status × territory location, PC1 significantly differed between peripheral and central territories (LM, $t = 28.56$, $P < 0.0001$), but not between incubating and brooding birds ($t = 0.78$, $P = 0.44$), nor was the interaction term significant ($t = -0.39$, $P = 0.70$).

Table 2 Model estimates for factors explaining the variation observed in total baseline corticosterone (CORT) levels (ng/mL) of king penguins holding central or peripheral breeding territories in the Baie du Marin colony, Possession Island (Crozet Archipelago)

Term	Estimate ± SE	t-ratio	P
Intercept	10.37 ± 0.31	33.05	<0.0001***
PC1	0.86 ± 0.18	4.93	<0.0001***
Breeding status (brooding)	2.33 ± 0.31	7.43	<0.0001***
PC1 × Breeding status	0.41 ± 0.18	2.36	0.02*

According to model selection information criteria (Akaike information criterion corrected for small sample size and Bayesian information criterion), CORT was best explained by breeding status (incubating vs. brooding), PC1 describing local social density conditions (see Table 1), and the interaction between both factors. The estimated parameter for the breeding status is interpreted as the difference in the predicted response for brooders from the average predicted response over both incubator and brooder levels (given by the intercept). The t-ratio tests whether the true value of the parameter is zero. It is the ratio of the estimate to its SE and has a Student's t-distribution under the null hypothesis

Similarly, neither PC2 nor PC3 differed between central and peripheral territories (PC2, $t = -0.70$, $P = 0.48$; PC3, $t = -0.60$, $P = 0.55$), or between incubators and brooders (PC2, $t = -0.85$, $P = 0.39$; PC3, $t = 0.55$, $P = 0.58$); both their interaction terms were also non-significant (all $P > 0.35$).

Effects of local breeding environment on baseline total CORT

The best and most parsimonious model explained 40 % of the variance in baseline total CORT and only retained PC1, breeding status and their interaction as explanatory variables ($F_{3,117} = 25.9$, $P < 0.0001$; Table 2). Thus, neither PC2, PC3, nor sampling date were important variables affecting CORT levels in this study. Overall, brooders had higher baseline total CORT than incubators (12.6 ± 0.8 vs. 8.1 ± 0.3, respectively; Table 2; Fig. 1). Whereas PC1 impacted plasma CORT levels both in incubators and brooders, it had a greater effect on brooding birds (Table 2; Fig. 1).

Effects of local breeding environment on oxidative stress

In brooding birds for which we had oxidative stress measurements, the best models identified by both AIC and BIC did not retain any of the PCs as explanatory variables (i.e. only the intercept remained) (see ESM3). Thus, neither OXY nor concentration of ROMs, or levels of 8-OHdG were affected by breeding status, sampling date or PC1–3. Pairwise (Pearson's) correlation tests showed that baseline total CORT was not significantly related to OXY ($r = 0.20$, $t = 1.02$, $P = 0.32$), ROM ($r = -0.14$, $t = -0.71$,
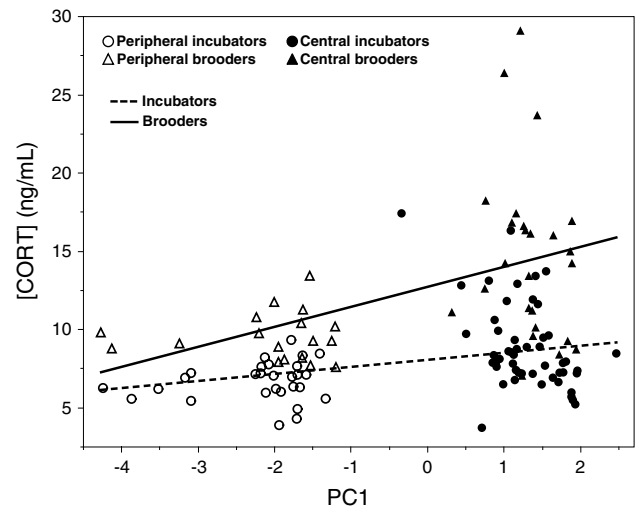
Fig. 1 Influence of a principal component (*PC1*) describing local social density conditions on total baseline corticosterone (*CORT*) levels (ng/mL) in king penguins holding central or peripheral breeding territories in the Baie du Marin colony, Possession Island (Crozet Archipelago). *Regression lines* based on the best model are given for incubating and brooding birds

$P = 0.48$), or 8-OHdG measurements ($r = 0.04$, $t = 0.21$, $P = 0.84$). OXY and ROMs ($r = -0.30$, $t = -0.71$, $P = 0.12$), and ROMs and 8-OHdG ($r = -0.02$, $t = -0.11$, $P = 0.91$) were not correlated either. However, we did find a negative relationship between OXY and 8-OHdG ($r = -0.49$, $t = -2.85$, $P = 0.009$) (see Fig. 2).

Discussion

In this study, central and peripheral breeding territories mainly differed in terms of their social environment. Birds on the outskirts of the colony had fewer neighbours, a greater overall distance to their nearest neighbours, and as a consequence, experienced lower breeding density. Peripheral birds might have been expected to suffer from chronic stress due to adverse weather conditions (e.g. stronger winds) elevating GC levels (Wingfield et al. 1983; Bize et al. 2010). However, our analyses indicated that local climate conditions (PC2 and PC3) did not differ substantially between breeding sites, nor did they ultimately affect baseline total CORT levels. Because CORT may be affected by persistently (rather than punctually) poor weather (Romero et al. 2000), it is possible that our single measures of climate conditions at the time of sampling were not enough to detect relevant effects on baseline CORT. Nonetheless, the fact that peripheral birds actually had lower CORT levels than central birds (contrary to our initial hypothesis) suggests that they did not suffer from more detrimental weather conditions. Similarly, CORT was not affected by

Fig. 2 Scatterplot matrix presenting the pairwise relationships between plasma values of oxidative stress and CORT (ng/mL) in chick-brooding king penguins holding central (*filled triangle*) or peripheral (*open triangle*) breeding territories in the Baie du Marin colony, Possession Island (Crozet Archipelago). Oxidative stress measures included plasma's antioxidant capacity ([*OXY*]; µM HClO/mL), levels of reactive oxygen metabolites ([*ROM*]; mg H_2O_2/dL), or levels of 8-hydroxy-2′-deoxyguanosine ([*8-OHdG*]; ng/mL). Bivariate normal density *ellipses* enclosing 95 % of the data points are in *grey*. The *width* and *orientation* of the ellipses show the strength and direction of the correlation between variables, respectively. Only the correlation between [OXY] and [8-OHdG] was significant for *$P < 0.05$
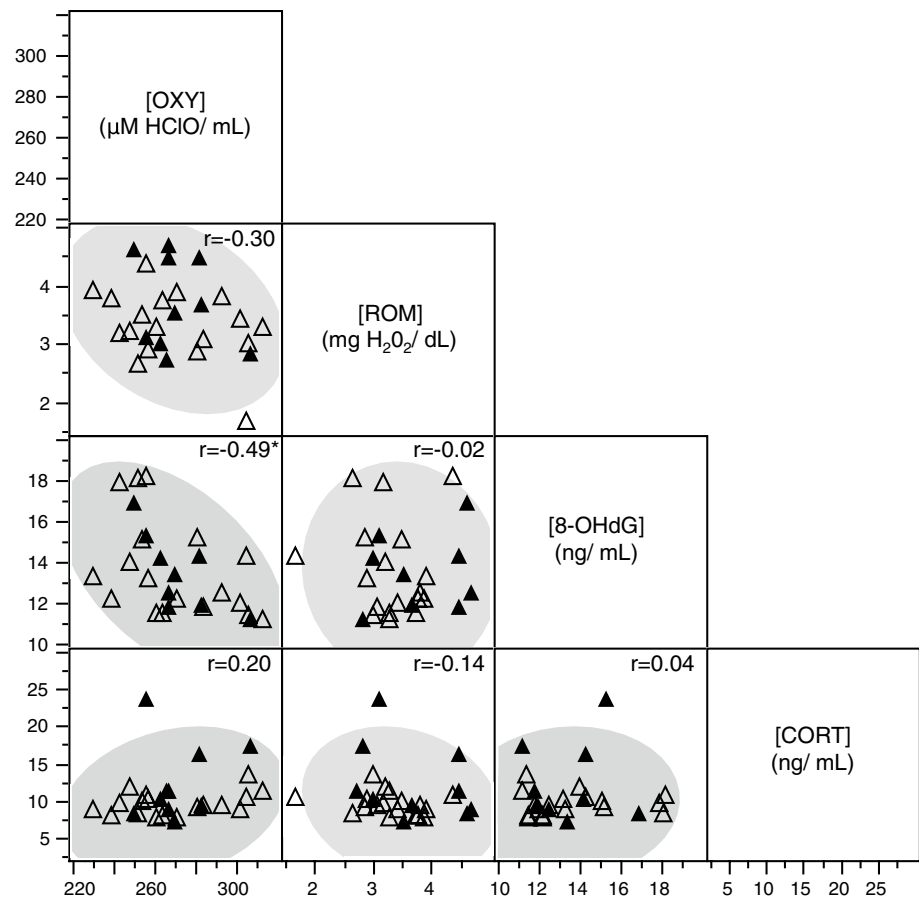
sampling date, nor likely predation risk. Again, CORT was actually lower in peripheral birds that might have experienced a higher risk of predation (Côté 2000; Descamps et al. 2005). Actually, only PC1 which described bird centrality and social conditions explained substantial (40 %) variation in CORT. Birds of more central rank, with a higher number of neighbours, and higher social density, also had higher CORT. Interestingly, this effect was more pronounced in brooders than in incubators. In addition, we found that regardless of their territory location, brooders had overall higher CORT than incubators.

In line with other studies (e.g. Raouf et al. 2006; McCormick 2006; Dantzer et al. 2013), our results suggest that breeding density and conspecific crowding may have been important factors affecting CORT secretion in breeding penguins. In king penguins, Côté (2000) reported that breeding density increased with increasing distance to the edge of the colony, as did breeding bird aggressiveness towards transiting individuals. Thus, central penguins have to contend with more numerous and more aggressive conspecifics, and will indeed rather walk past sleeping individuals to avoid aggressive encounters when reaching their breeding site (Côté and Dewasmes 1999). Interestingly, Côté (2000) also found that the rate of aggressive

interactions (flipper blows and pecking) between neighbours was significantly higher for central vs. peripheral brooders, but not for central vs. peripheral incubators. This could explain why in our study, the effect of PC1 on CORT was stronger in brooding than in incubating birds. In addition to a more stressful environment, central brooders may have experienced greater metabolic activation (due to higher rates of aggression) than central incubators, in comparison to their peripheral counterparts. Higher flipper blow and pecking rates are indeed expected to increase physical activity with associated energetic costs (Viera et al. 2011). Thus, variations in CORT levels could result from a combination of differences in more or less stressful social environments and differences in metabolic rates (differential aggressiveness) associated with defending more or less crowded breeding territories. Differences in aggressiveness (Côté 2000) and higher metabolic activation may also explain the overall higher CORT levels observed in brooders. Non-exclusively, higher baseline total CORT during this stage may also reflect increased parental efforts in chick-rearing birds (Bonier et al. 2009). This is consistent with the idea that at baseline levels, CORT may play a preparative role allowing parents to deal with energetically demanding (reproductive) processes (Romero 2002;

Bókony et al. 2009; Hau et al. 2010). For instance, in addition to increasing territorial defence (Côté 2000), brooding king penguins also resume more frequent foraging trips at sea to provision their chick (Stonehouse 1960; Weimerskirch et al. 1992).

Could penguins breeding on more central territories be chronically stressed? Because chronic stress should affect energy mobilization and utilization, one downstream measure expected to increase in chronically stressed animals is oxidative stress (Breuner et al. 2013). Experimental evidence from laboratory mice suggests that crowding and social stress may increase oxidative stress (Miyashita et al. 2006; Nishio et al. 2007) and affect adult telomere length (Kotrschal et al. 2007), guanine-rich DNA sequences especially prone to oxidative damage (von Zglinicki 2002). Further, manipulations of GC levels have been shown to disrupt the oxidative balance both in laboratory rats (Zafir and Banu 2009) and free-living birds (Stier et al. 2009). However, using multiple markers of oxidative stress (as recommended by Selman et al. 2012), we found no difference in the oxidative stress status of chick-brooding penguins breeding in different locations, although birds with the highest plasma antioxidant capacity also presented the lowest cellular DNA damage. One caveat to our results is that we only performed plasmatic measures of oxidative markers [but see Geiger et al. (2012); Stier et al. (2014) for significant environment-related differences in these markers for king penguin chicks]. Yet, organisms' tissues widely differ in their exposure to reactive oxygen species, antioxidant defences and repair mechanisms (see Selman et al. 2012 for a review). Working on a wider range of tissues (e.g. muscle, liver) may provide a more accurate picture of the oxidative status of breeding birds, though this appears a major methodological limitation in wild penguins. In addition, multiplying measures of oxidative damage (e.g. protein carbonylation, lipid peroxidation) and defence (superoxide dismutase, glutathione peroxidase) and working on a larger sample of both incubating and brooding birds may yield further insight into this question (Selman et al. 2012). Alternately, in long-lived king penguins, the potential damaging effects of high CORT during a breeding season could be compensated by up-regulated antioxidant defences and repair mechanisms (e.g. DNA base excision repair or lipid peroxide repair through glutathione peroxidase 4 activity; Halliwell and Gutteridge 2007). This suggestion is somewhat supported by recent experimental evidence that a CORT increase does not affect the oxidative stress status of adult Adélie penguins (T. Raclot, personal communication).

Which alternative explanations may explain the territory-dependent CORT variations observed in our study colony? First, differences in CORT between peripheral and central birds may be related to differences in behavioural phenotypes [coping styles (Koolhaas et al. 1999)]. Whereas

this remains to be tested, higher CORT associated with higher individual aggressiveness or territoriality (e.g. Kitaysky et al. 2003) could provide a proximal mechanism by which more aggressive and competitive penguins secure high-quality territories at the centre of the colony. Second, variation in CORT levels could be related to individual attributes [such as age or breeding experience (Angelier et al. 2006; Heidinger et al. 2008)] determining breeding site selection in this species. Whether individuals of different age or breeding experience are preferentially distributed in central or peripheral colony locations remains to be thoroughly investigated. In this regard, long-term monitoring programs may provide valuable insights into the spatial distribution of breeding adults in relation to age and breeding experience (Descamps et al. 2009).

Finally, regardless of the factors explaining territory-dependent variation in CORT levels, our findings raise the puzzling question of what the consequences may be for king penguin chicks. Indeed, recent studies have revealed marked effects of maternal CORT on offspring phenotype (e.g. growth, HPA function, oxidative stress, telomere length) (Hayward and Wingfield 2004; Haussmann et al. 2012; Dantzer et al. 2013), including in response to changes in perceived conspecific density (Dantzer et al. 2013). Whether differences in social stimuli related to breeding territory location may also drive maternal effects affecting offspring phenotype in the colonial king penguin is a fascinating question opening exciting perspectives for future research.

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