

Breeding status affects the hormonal and metabolic response to acute stress in a long-lived seabird, the king penguin



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ABSTRACT

Stress responses are suggested to physiologically underlie parental decisions promoting the redirection of behaviour away from offspring care when survival is jeopardized (e.g., when facing a predator). Besides this classical view, the “brood-value hypothesis” suggests that parents’ stress responses may be adaptively attenuated to increase fitness, ensuring continued breeding when the relative value of the brood is high. Here, we test the brood-value hypothesis in breeding king penguins (*Aptenodytes patagonicus*), long-lived seabirds for which the energy commitment to reproduction is high. We subjected birds at different breeding stages (courtship, incubation and chick brooding) to an acute 30-min capture stress and measured their hormonal (corticosterone, CORT) and metabolic (non-esterified fatty acid, NEFA) responses to stress. We found that CORT responses were markedly attenuated in chick-brooding birds when compared to earlier stages of breeding (courtship and incubation). In addition, NEFA responses appeared to be rapidly attenuated in incubating and brooding birds, but a progressive increase in NEFA plasma levels in courting birds suggested energy mobilization to deal with the threat. Our results support the idea that stress responses may constitute an important life-history mechanism mediating parental reproductive decisions in relation to their expected fitness outcome.

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1. Introduction

Because parents are limited both in the time and energy to invest into offspring, decision rules about breeding and parental care should be selected considering a trade-off between the relative value of current reproduction vs. that of expected future breeding opportunities (Williams, 1966; Trivers, 1972; Maynard-Smith, 1977). According to the reproductive value hypothesis, parents may invest more into parental care as the clutch or offspring age, because of the higher probability of progeny surviving until sexual maturity (Andersson et al., 1980; Clutton-Brock, 1991). Consistently, several studies have shown that parental investment may change with offspring age, i.e. with the relative reproductive value of the young (Andersson et al., 1980; Redondo and Carranza, 1989; Cézyly et al., 1994; Albrecht and Klvana, 2004). For instance, birds are known to increase the amount of parental nest defence as offspring age, both throughout the incubation (Sjöberg, 1994;

Albrecht and Klvana, 2004; Osiejuk and Kuczynski, 2007) and chick-brooding (Redondo and Carranza, 1989) periods.

Mechanistically, stress responses have been suggested to physiologically underpin parental decisions by promoting the redirection of behaviour away from offspring care when survival is jeopardized. Stress responses allow animals to cope with unpredictable environmental challenges (i.e. labile perturbations, reviewed in Wingfield, 2003) by rapidly mounting a series of physiological and behavioural modifications. For instance, increases in glucocorticoid hormones (mainly corticosterone CORT, in birds) may promote the mobilization of energy substrates required to deal with impending threats (e.g. approaching predator, inclement weather). Breeding parents may then redirect their energy investment towards survival, thereby reducing or giving-up altogether on parental duties (Wingfield et al., 1998). The latter should especially apply to long-lived species for which lifetime reproductive success depends more on adult survival than seasonal fecundity, individuals behaving as ‘prudent parents’ (Drent and Daan, 1980; Stearns, 1992). However, when the value of the current breeding attempt is high relative to that of future reproductions, stress responses may be attenuated to ensure reproduction is not abandoned (Wingfield and Sapolsky, 2003).

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The ‘brood value hypothesis’ suggests that the magnitude of stress responses to acute challenges should match the level of parental commitment to the brood (Wingfield et al., 1995; Lendvai et al., 2007; Lendvai and Chastel, 2008; Goutte et al., 2011a). When brood value is high, parental stress responses should be attenuated to ensure continued breeding. Over the past couple of decades, this idea has been tested in numerous studies, yet conclusions remain equivocal. Whereas some studies have found corticosterone stress responses to be attenuated at advanced stages of breeding (Holberton and Wingfield, 2003; Adams et al., 2005; Williams et al., 2008; Krause et al., 2015), when brood value is high (Goutte et al., 2011a; Schmid et al., 2013), when breeding opportunities are limited (Wingfield et al., 1995; Romero et al., 1997; Heidinger et al., 2006), when parental care towards the offspring is increased (Meddle et al., 2003), or in the sex investing more into parental care (O’Reilly and Wingfield, 2001); this does not always appear to be the case (Chastel et al., 2005; Angelier et al., 2009, 2013; Riou et al., 2010). Other studies have found stress responses to be modulated according to life-history stage (e.g., in breeding vs. non-breeding individuals: Romero, 2002; Romero et al., 2006), or shown that circulating glucocorticoid levels may play a role in determining the onset of breeding (Goutte et al., 2010, 2011b).

A better understanding of the mechanisms mediating parental decisions related to breeding would benefit from the joint study of glucocorticoid stress responses and associated metabolic responses. Specifically, studying metabolic responses such as the release of free fatty acids (NEFA) in response to acute stressors during different breeding stages should inform us on the modulation of energy substrate mobilization aimed at promoting adult survival, especially in animals relying on fat energy reserves because fasting during reproduction. Thus, in this study, we considered the hormonal and metabolic (energetic) responses to acute stress in relation to breeding status in the king penguin (*Aptenodytes patagonicus*).

King penguins provide an interesting model for testing this question. In this species, the energy commitment to reproduction is particularly high (Cherel et al., 1994; Groscolas and Robin, 2001). The reproductive cycle from egg-laying to chick fledging lasts for over a year, during which breeding parents alternate between periods of long-term fasting on land and periods foraging at sea (Stonehouse, 1960). Parents are only able to raise a single chick within a season and clutch replacement almost never occurs (Weimerskirch et al., 1992). Parental effort increases throughout the breeding season (Weimerskirch et al., 1992), as likely reflected by an increase in both baseline glucocorticoid levels (Viblanc et al., 2014; Bonier et al., 2009) and territorial defence (Côté, 2000). Due to the length of the breeding season, successful birds are constrained to breed late in the subsequent season, with virtually no chance of success (Weimerskirch et al., 1992). Given the above, one might expect the value of reproduction to increase with the advancing breeding season as the clutch or offspring age (Montgomerie and Weatherhead, 1988; Côté, 2000), and parents to be more committed to reproduction during later stages of breeding. Consistent with this hypothesis, we recently found that the “fight-or-flight” response (as measured by heart rate increase) of breeding birds to acute stressors was attenuated with advancing breeding (Viblanc et al., 2015). However, whether those short-term adrenergic-based changes reflect an overall modulation of bird stress to advancing breeding, especially in terms of hormonal responses and energy substrate mobilization, is unknown.

We thus examined the modulation of total corticosterone (CORT) and circulating non-esterified free fatty acid (NEFA) responses to acute 30-min captures on breeding birds at the stages of courtship, incubation and chick brooding, of expected increasing reproductive value. In fasting penguins, NEFA plasma levels are a

good index of fat store mobilization (Groscolas, 1986; Cherel et al., 1988; Groscolas et al., 2008; Corbel et al., 2010). Baseline NEFA levels remain steady during phase II of fasting, increase at the transition from phase II to phase III, and decrease again when fat stores are close to exhaustion (Groscolas, 1986; Cherel et al., 1988). If stress responses are attenuated to prevent brood desertion when reproductive value is high, we expect CORT responses and energy substrate mobilization (as reflected in blood NEFA) to be highest during courtship, intermediate during incubation and lowest during chick brooding. The joint consideration of hormonal and metabolic responses to stress is expected to provide a relatively complete overview of the potential fine-tuning of stress responses to changes in breeding status.

2. Methods

2.1. Study animals

King penguins were studied on Possession Island, Crozet Archipelago (46°25’S, 51°45’E). During the 2010–2011 breeding season, we measured the adrenocortical and metabolic response of breeding birds to an acute and standardized capture-immobilization stress. Birds were sampled at three stages of the breeding cycle that differed in the amount of time and effort invested into the current breeding attempt: (1) at the very onset of reproduction during courtship when birds arrived ashore to breed, (2) during the first incubation shift (beginning or end), and (3) when birds were brooding a young non-thermally emancipated chick.

Twelve birds were stressed at each breeding stage, each bird being stressed only once. Courting birds were stressed while searching for a partner (songs, displays) on the beach adjacent to the breeding colony shortly after having arrived ashore for breeding. When capturing courting birds, we always caught the largest member of pair, and thus all courting birds were most likely males (as indeed confirmed by their morphometric measurements; average mass (mean \pm s.d.) = 13.4 \pm 0.8 kg; average wing length = 335.6 \pm 7.6 mm; average beak length = 135.9 \pm 4.5 mm; see Fahlman et al., 2006). In order to obtain birds at the onset and end of the first incubation shift, we marked 24 non-incubating pairs on the belly from a 1-m distance with spray animal dye (Procimark®, Kruuse, Langeskov, Denmark), once settled on their breeding territory. We then performed daily visual observations at a distance using binoculars. The first time one member of the pair was observed alone incubating an egg, it was identified as a male at day 1 of incubation (Stonehouse, 1960; Weimerskirch et al., 1992; Descamps et al., 2002). Twelve males were stressed the next day (day 2 of incubation), while the other twelve were monitored until close to the end of the incubation shift, when they were stressed. Since the average duration of the first incubation shift is 16 days (Weimerskirch et al., 1992), we stressed those birds on day 15 of the shift to avoid their premature departure to sea. Brooding birds were stressed when caring for a small chick of approximately 10 days of age or less (as could be visually inferred from its size) and kept in their brood-pouch. This time period corresponds to the first brooding stage after hatching, during which the male is typically the one tending the chick (Stonehouse, 1960; Weimerskirch et al., 1992; Descamps et al., 2002). To avoid further displacing and disturbing incubating and brooding birds and risking breeding abandonment, we did not obtain any mass or size measurements for those groups.

The adrenocortical response was estimated from changes in the plasma concentration of total corticosterone and the metabolic response was determined from changes in the plasma concentration of non-esterified fatty acids, a good index of metabolic response to stress in penguins (Corbel et al., 2010) (see below).

2.2. Stress protocol

During stress protocols, birds were approached from the back and we timed the moment of their reaction (alertness) to the experimenter, usually at a few meters distance. Then, the bird was rapidly caught and hooded to keep it calm. A first blood sample (c.a. 1 mL) was taken from the marginal flipper vein, as quickly as possible. In this study, initial samples were all obtained in less than 3.5 min following alertness, and were considered as providing baseline values for the measured plasma parameters. This is slightly higher than the recommended 3 min for most avian species (see Romero and Reed, 2005), but considerably less than the 5 min threshold at which a significant stress-induced increase in CORT over baseline levels is observed in king penguins (Ménard, 1998). Following successful initial blood sampling, the bird was kept immobilized for 30 min and a second and third blood sample were taken after 10 and 30 min of immobilization, respectively. Blood was kept on ice in the field, and centrifuged within an hour of sampling using a laboratory set-up in the vicinity of the colony. Blood was centrifuged for 10 min at 3500 rpm and the plasma was kept frozen at -80°C until analyses. Plasma concentrations of CORT were determined by radio-immuno-assay (RIA) using a commercial kit (MP Biomedicals, cat. no 07-120103), as previously described and validated for king penguins (Bernard et al., 2002; Groscolas et al., 2008; Corbel et al., 2010). The sensitivity of the assay was $1\text{ ng}\cdot\text{mL}^{-1}$ and the intra- and inter-assay coefficients of variation were 7.6% and 9.5%, respectively. Plasma concentration of NEFA, the product of lipolysis in adipose tissue and the major fuel in fasting penguins (Groscolas, 1990), was measured by an enzyme-colorimetric method using a commercial kit (Randox, Ref. FA 115).

2.3. Data analyses

The adrenocortical response to stress was calculated as the increase in plasma CORT concentration from baseline levels to 10 min after the stress (rapid response), or from baseline levels to 30 min after the stress (longer-term response). Because baseline CORT levels differed significantly between breeding stages (see Results), we calculated the relative change in CORT levels as $(\text{CORT}_{10\text{ or }30} - \text{CORT}_{\text{baseline}})/\text{CORT}_{\text{baseline}}$ (expressed in %).

The metabolic response to stress was calculated as changes in plasma NEFA levels. First, because baseline NEFA values also differed between breeding stages, the short term NEFA response to stress was calculated as the relative change in plasma NEFA after 10 min $(\text{NEFA}_{10} - \text{NEFA}_{\text{baseline}})/\text{NEFA}_{\text{baseline}}$ (expressed in %). However, for the longer-term NEFA response, we calculated the area under the curve relating NEFA concentrations to time (expressed in $\text{mmol}\cdot\text{min}\cdot\text{L}^{-1}$). This different calculation method was chosen to better capture the nature of NEFA kinetic after 30 min (CORT levels increased continuously over the 30-min period, whereas NEFA levels generally increased before decreasing; see Fig. 1).

First, we analysed changes in CORT and NEFA concentrations during the stress for each breeding stage. Because birds were sampled for blood at discrete times T_0 , T_{10} , T_{30} , we used a mixed model approach to determine changes in CORT and NEFA over time, with individual ID specified as a random variable to account for repeated sampling. Second, we compared the overall CORT and NEFA responses among breeding stages using one-way ANOVA since birds were only stressed once per breeding stage. Multiple comparisons were performed using post hoc Tukey tests for honest significant differences (Tukey HSD). Results are given as means \pm standard error (s.e.) and differences considered significant for $P < 0.05$. Finally, we considered the correlation between CORT and NEFA levels using Pearson correlation tests. All statistics were run in R v. 3.0.2 (R Core Team, 2013).

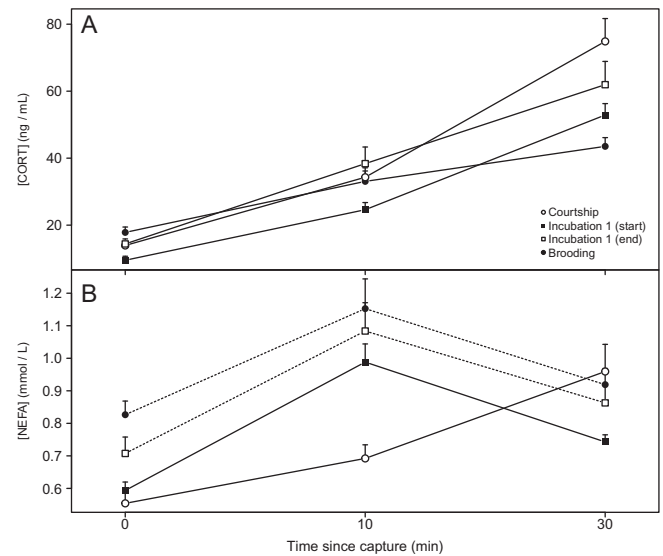


Fig. 1. Changes in plasma concentrations of (A) corticosterone [CORT] and (B) non-esterified fatty acids [NEFA] during a capture-immobilization stress in breeding-fasting king penguins. Incubation 1 (start): onset of the first incubation shift; Incubation 1 (end): end of the first incubation shift. Full lines: values significantly different from each other at times 0, 10 and 30 min for $P < 0.05$ (except for NEFA concentrations in courting birds between T_0 and T_{10} , see text). Dotted lines: values at 0 and 30 min not significantly different. Changes in plasma concentrations of corticosterone and non-esterified fatty acids were investigated using mixed models with bird ID specified as a random variable to account for repeated sampling on the same individual. Differences between times 0, 10 and 30 were asserted using post hoc Tukey HSD tests. Results are presented as means \pm s.e. $N = 12$ for all values.

3. Results

3.1. Changes in CORT and NEFA concentrations during the stress

Regardless of breeding status, the capture-immobilization stress induced a progressive increase in plasma CORT concentrations over baseline levels (Fig. 1A). For all birds, the increase was significant as of 10 min from capture (T_{10}) and continued to increase until 30-min post-capture (T_{30}). NEFA concentrations, however, did not exhibit a similar trend in all birds (Fig. 1B). In courting birds, NEFA concentrations increased gradually from T_0 to T_{30} , the T_{10} concentration however, not yet being significantly different from baseline levels (Tukey HSD; $P = 0.08$). On the other hand, both in incubating and brooding birds, NEFA concentrations significantly increased during the first 10 min of the capture-immobilization stress, but then subsequently decreased from T_{10} to T_{30} (Fig. 1B). Both in males which were stressed close to the end of their first incubation shift and in brooding birds, NEFA concentrations measured at T_{30} were not significantly different from basal NEFA concentrations measured at the beginning of the stress (T_0).

3.2. Effect of breeding status on CORT and NEFA responses to stress

3.2.1. Baseline CORT and NEFA values

CORT baseline values differed significantly between breeding stages (ANOVA; $F_{3,44} = 6.60$, $P < 0.001$), being highest for brooding birds (Fig. 2A). On average, birds at the start of their incubation shift had baseline CORT levels 47% lower than brooding birds. Other breeding stages did not differ significantly (Tukey HSD; all $P \geq 0.06$). Similarly, NEFA baseline values differed significantly between breeding stages ($F_{3,44} = 9.72$, $P < 0.001$), increasing progressively from incubation to brooding (Fig. 2B). In courting birds for which we had mass measurements, neither baseline CORT

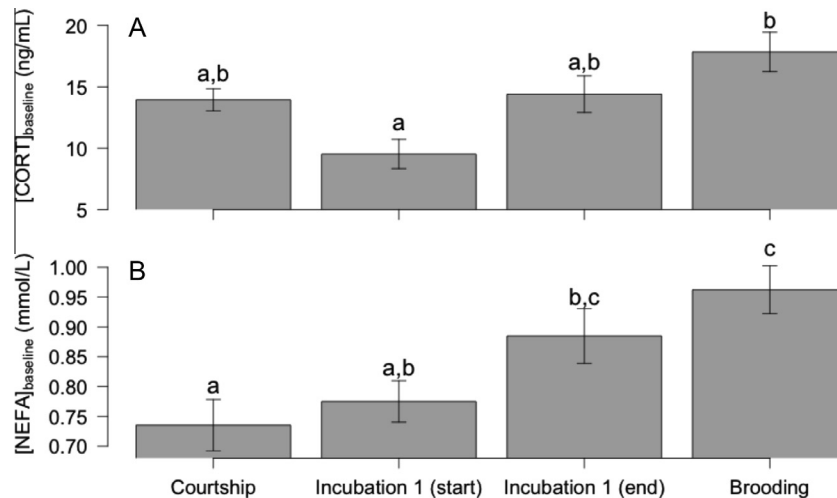


Fig. 2. Baseline concentrations of plasma corticosterone [CORT] and non-esterified fatty acids [NEFA] in relation to breeding status in fasting king penguins. Values (N = 12 in all cases) are means \pm SE. Values not sharing a common superscript letter are significantly different, $P < 0.05$.

($r = 0.52$, $P = 0.08$) or NEFA ($r = 0.19$, $P = 0.55$) values were significantly related to body mass.

3.2.2. CORT response to stress

Within 10 min of capture, bird's relative CORT increase differed significantly according to breeding stage (ANOVA; $F_{3,44} = 3.30$, $P = 0.03$; Fig. 3A). Overall, the CORT response to stress decreased with advancing breeding stage, but was only significantly different between early incubation and brooding (52% lower in brooding birds; Tukey HSD; $P = 0.002$). After 30 min of capture, the change in relative CORT increase with advancing breeding stage was more pronounced (Fig. 3B), and differed significantly according to breeding status (ANOVA; $F_{3,44} = 10.60$, $P < 0.001$). Brooding birds had the lowest relative CORT increase (54–71% lower than all other breeding stages; Tukey HSD; all $P < 0.05$), and incubating birds had somewhat lower relative CORT increases than courting birds, though not significantly (Tukey HSD; all $P > 0.42$). In courting birds for which we had mass measurements, neither bird's relative CORT increase after 10 min ($r = 0.30$, $P = 0.34$) or after 30 min ($r = -0.05$, $P = 0.87$) of capture was significantly related to body mass.

3.2.3. NEFA response to stress

Within 10 min of capture, bird's relative NEFA increase differed only slightly between breeding stages (ANOVA; $F_{3,44} = 0.30$, $P = 0.05$) (Fig. 4A). It tended to be highest in birds during early incubation, though not significantly (Tukey HSD; all $P > 0.058$). The overall metabolic response to stress after 30 min of capture did not differ significantly between breeding stages (ANOVA; $F_{3,44} = 0.72$, $P = 0.55$; Tukey HSD; all $P > 0.57$) (Fig. 4B). In courting birds for which we had mass measurements, bird's relative NEFA increase after 10 min of capture was not significantly related to body mass ($r = -0.17$, $P = 0.60$). Similarly, the overall metabolic response to stress after 30 min was not significantly related to body mass ($r = -0.38$, $P = 0.22$).

3.2.4. Relationships between CORT and NEFA values

Overall, CORT and NEFA baseline values were positively and significantly related at T_0 ($r = 0.51$, $P < 0.001$). Similarly, CORT and NEFA values were positively and significantly related at T_{30} ($r = 0.30$, $P = 0.037$), but not at T_{10} ($r = 0.03$, $P = 0.862$). Overall, NEFA (area under the curve) and CORT (relative increase)

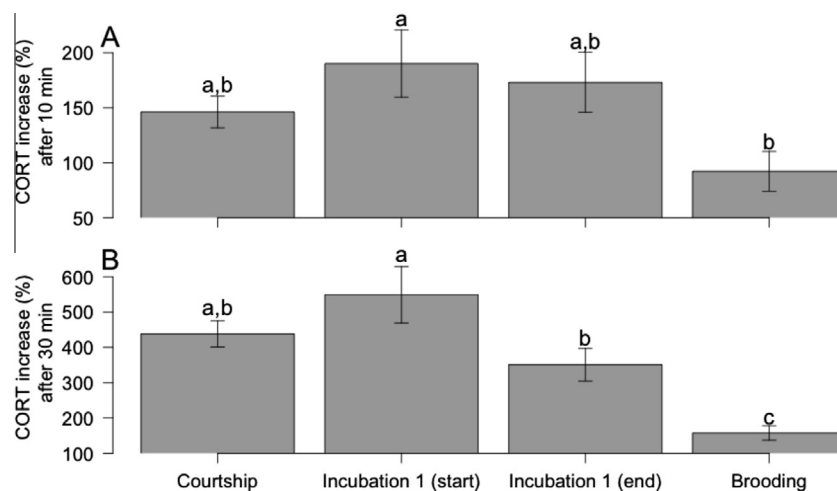


Fig. 3. Hormonal response to a capture-immobilization stress in fasting king penguins of different breeding status. (A) Relative increase in plasma corticosterone concentration [CORT] from the initial baseline level to the level measured at 10 min. (B) Relative increase in plasma [CORT] from the initial baseline level to the level measured at 30 min. Values (N = 12 in all cases) are means \pm SE. Values not sharing a common superscript letter are significantly different, $P < 0.05$.

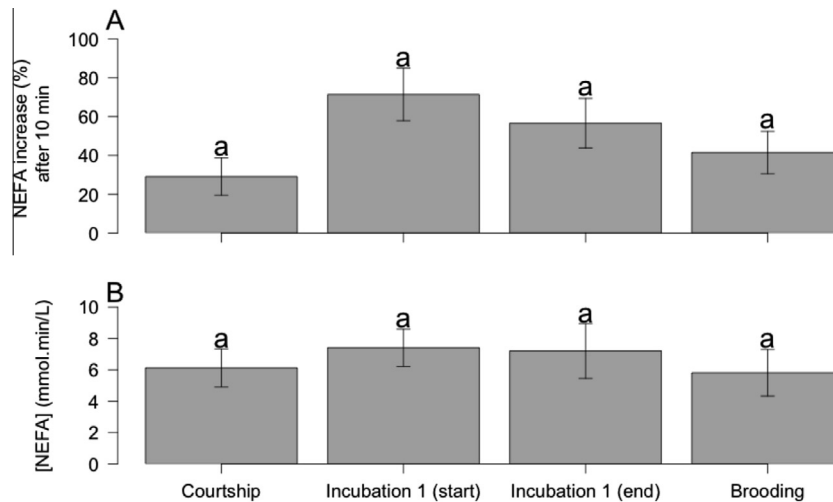


Fig. 4. Metabolic response to a capture-immobilization stress in fasting king penguins of different breeding status. (A) Relative increase in plasma non-esterified fatty acids concentration [NEFA] from the initial baseline level to the level measured at 10 min. (B) Area under the curve relating [NEFA] to time. Values ($N = 12$ in all cases) are means \pm SE. Values not sharing a common superscript letter are significantly different, $P < 0.05$.

responses to the 30-min acute captures were positively related ($r = 0.31$, $P = 0.037$).

4. Discussion

4.1. Kinetics of CORT and NEFA responses to acute stress

This study shows that the adrenocortical and metabolic responses to acute stress are modulated by breeding status in the king penguin. Over the 30-min capture, CORT and NEFA responses had markedly different dynamics. Whereas CORT responses increased continuously over the 30-min capture for all breeding groups, NEFA responses reached a maximum 10 min post-capture before subsequently decreasing both in incubating and brooding birds. In courting birds however, the increase in plasma NEFA concentrations was gradual and kept rising 30 min after capture. Unfortunately, we did not sample blood after T_{30} , so that we may not conclude on whether NEFA levels continued to rise (or not) in courting birds, nor can we make any inference on the recuperation of the CORT response. Thus, whereas the NEFA response (i.e. the area under the curve) measured in incubating and brooding birds likely integrated the entire stress response; the NEFA response of courting birds was likely underestimated.

The rapid increase in NEFA values at T_{10} was likely due to the lipolytic action of catecholamines (Das Neves et al., 2000) released by a sympathetic discharge of the nervous system, allowing birds to rapidly mobilize energy substrates during the early and short-lived phase of the stress response. This is consistent with the rapid changes in heart rate (sympathetic response to stress) we previously observed in penguins in response to captures (Viblanc et al., 2012, 2015). Consistently with the idea that increases in glucocorticoid hormones may promote the mobilization of energy substrates during acute stress, NEFA and CORT levels were also correlated both at baseline and stress-induced levels, though the correlation was less pronounced at stress-induced levels. This is not surprising given that lipolytic effects of glucocorticoids (whether genomic or not) are not expected to be immediate (Peckett et al., 2011; Campbell et al., 2010).

Whereas the overall NEFA response to the 30-min acute stress did not differ significantly between breeding stages (Fig. 4), the kinetics of the response was very different. NEFA concentrations were still progressively increasing after 30 min in courting birds (Fig. 1B), but had almost re-joined baseline levels in incubating

and brooding birds (having reached a maximum at T_{10} in this study). This suggests that in courting birds, energy is mobilized to flee/deal with the threat even after 30 min, whereas the response is rapidly attenuated for birds remaining on their breeding territory caring for their egg (incubators) or chick (brooders). Those results agree with predictions of the “brood value” hypothesis. Indeed, the parental investment required for replacing a chick is greater than that required for replacing an egg (Montgomerie and Weatherhead, 1988) and king penguins are only able to manage one chick within the same breeding season (Weimerskirch et al., 1992). Thus, brooding parents should be motivated to remain on their breeding territory (as illustrated by increased territorial defence against conspecifics; Côté, 2000), and costly stress responses may be attenuated (Lendvai et al., 2007; Bókonyi et al., 2009; Goutte et al., 2011a). Compared to courting birds that have not yet produced an egg, down-regulating the mobilization of energy stores would be especially relevant for penguins with an egg/chick as those are compelled to fast on land for several days/weeks while caring for their progeny (Weimerskirch et al., 1992). On the other hand, courting birds may flee and attempt reproduction later, and mobilizing energy reserves serve to deal with the potential threat favouring escape/fight behaviour and adult survival.

4.2. Attenuation of CORT responses with advancing breeding stage

On average, the CORT response to acute stress appeared to decrease with advancing breeding stage. This effect was already apparent 10 min after capture and clearly marked by T_{30} . Specifically, CORT responses were markedly lower in birds brooding a young chick than in courting birds and birds at the start of their incubation shift. Several alternative (non-mutually exclusive) hypotheses might explain those differences.

First, the lower CORT response observed in brooding birds (and generally with advancing breeding stage) may be due to differences in baseline CORT levels and physiological constraints on the maximum achievable CORT increase in response to acute stress. Indeed, baseline CORT levels increased slightly with breeding stage, being highest in brooding birds. This observation is consistent with our previous findings in king penguins (Viblanc et al., 2014), and with the notion that higher baseline CORT levels play a role in preparing parents to deal with the energetically demanding period of chick rearing (Bonier et al., 2009). This idea is also

consistent with the observed increase in baseline NEFA levels (energy substrates) from courtship to brooding. Given a maximum achievable CORT plasma level in response to stress, one possibility is that brooding birds with high baseline CORT levels may be constrained in the amplitude of a CORT response they mount compared to other breeding stages. However, the average maximum CORT plasma level reached in brooding birds at T₃₀ (43.5 ng/mL) was actually lower than all other stages (52.9–74.9 ng/mL), suggesting that those birds were not at a physiological maximum. Thus, although differences in baseline levels may have contributed to the observed differences in the overall CORT response to stress, this effect was likely small.

Second, from a proximate perspective, a down-regulation of stress responses may also occur in energy-depleted birds. Several studies have indeed highlighted links between corticosterone baseline levels and energy status in different bird species (Kitaysky et al., 1999; Cockrem et al., 2006; Lynn et al., 2010; Angelier et al., 2015), including king penguins (Cherel et al., 1988). Based on plasma CORT and NEFA levels (Groscolas et al., 2008) measured a posteriori, all birds in our study were of comparable fasting status, i.e. the phase II fasting stage, where baseline CORT levels are maintained at relatively low levels (Cherel et al., 1988). Indeed, changes in circulating CORT levels in this species mainly concern the transition from phase II fasting (when birds rely essentially on fat stores) to phase III fasting (when protein catabolism increases) (Cherel et al., 1988). Thus, it seems unlikely that fasting status had a major effect on the observed differences between circulating CORT levels. On the other hand, CORT responses to acute stress have also been shown to decrease with decreasing body condition (e.g. Angelier et al., 2009, 2013), though not consistently (see Lynn et al., 2010; Angelier et al., 2015, for the opposite or no relationship). Again, it seems unlikely that such differences in nutritional status might explain the down-regulation of stress responses observed from courtship to brooding in our study, as in both cases birds had only been fasting for a few days in the colony upon capture and were all in PII fasting status. Further, the CORT response of birds at the end of their incubation shift (after fasting for 13-days) was not significantly different from that of birds during courtship, although the latter had only been fasting for a few days. Nonetheless, the possibility that the stress response of breeding king penguins might differ according to their nutritional status should not be discarded. Indeed, we found lower CORT responses in birds sampled at the end vs. birds sampled at the beginning of the first incubation shift, and reduced stress responses have also been observed in phase II fasting vs. fed (or phase I fasting when birds use up carbohydrates) king penguin chicks towards fledging (Corbel et al., 2010). Future studies should thus aim at comparing animals with strongly marked differences in their fasting status (e.g. fed vs. phase I, phase II or phase III fasting animals) at different breeding stages to clarify the importance of nutritional status in shaping individual stress responses in relation to breeding.

Third, from an ultimate perspective, a down-regulation of acute stress responses with advancing breeding is consistent with the idea that attenuated stress responses may prevent brood desertion when reproductive value is high (Wingfield et al., 1995; Lendvai et al., 2007; Lendvai and Chastel, 2008; Goutte et al., 2011a; Schmid et al., 2013). Interestingly, the present findings are in line with the observation that heart rate responses to acute stress are also attenuated with advancing reproduction in king penguins (Viblanc et al., 2015), and suggest an overall modulation of individual stress responses via the down-regulation of both sympatho-adrenal and hypothalamic-pituitary-adrenal (HPA) stress pathways.

Although we did not sex birds in our study, all individuals were most likely males (based on mass and size measurements for

courting birds, and the size of the chick and associated time-period of the reproductive cycle in brooding birds; Stonehouse, 1960; Weimerskirch et al., 1992; Descamps et al., 2002; Fahlman et al., 2006). Nonetheless, we cannot rule out the possibility that part of the differences observed between courting/incubating birds on the one hand and brooding birds on the other could be due to sex effects. Because CORT responses may be modulated by bird sex (e.g. Astheimer et al., 1994; Hood et al., 1998, but see Romero et al., 2006), it would be interesting to further consider the attenuation of hormonal and metabolic stress responses in brooding birds of known sex for this species, where specific predictions can be made. Indeed, for king penguins parental cooperation is a requirement for reproductive success, and sexual selection for high quality partners is mutual (Nolan et al., 2010; Viblanc et al., 2016). Given that the chances of re-nesting in the same season are extremely low (Weimerskirch et al., 1992), one would expect parental commitment to the brood to be similar in males and females, and stress responses to be shaped similarly in both sexes.

Taken together, the present results (and previous results on heart rate; Viblanc et al., 2015) suggest a down-regulation of acute stress responses with advancing breeding stage. Given that HPA and sympatho-adrenal stress pathways may be modulated independently in birds (Nephew et al., 2003), it would be interesting to know whether selection has generally favoured the coevolution of attenuated short and longer-term stress responses with increases in breeding value in long-lived species. We look forward to more data on the interactions between adrenergic and HPA responses, and on the interactions between bird condition, sex and breeding value in shaping parental decisions related to reproduction in the present and other species.

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