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## Research paper

## An integrative appraisal of the hormonal and metabolic changes induced by acute stress using king penguins as a model



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## ABSTRACT

A large number of studies have focused on the reactivity of the hypothalamic–pituitary–adrenal (HPA) axis and the consequences of glucocorticoids (GC) in mediating life-history trade-offs. Although short-term increases in GCs are viewed as adaptive, mobilizing energy substrates allowing animals to deal with impending threats (e.g. stimulating hepatic gluconeogenesis, stimulating lipolysis, mobilizing amino acids), few studies have actually measured the exact time-course of substrate mobilisation in response to acute stress in natural conditions. We evaluated the hormonal and metabolic components of the stress response to acute stress in 32 free-living king penguins (*Aptenodytes patagonicus*). We monitored changes in blood GCs (corticosterone, CORT), glucose, lactate, ketone bodies ( $\beta$ -hydroxybutyrate), non-esterified fatty acids, and uric acid in response to a standardized capture-restraint protocol lasting for up to 90 min. Furthermore, we tested whether the vigilance status of the animal (alert or asleep) affected its perception of the capture, thereby modulating the hormonal and metabolic stress responses. The time course of energy mobilisation followed the characteristic pattern expected from laboratory and theoretical models, with a rapid depletion of those energy stores linked to rapid adrenergic responses (i.e. glucose and ketone bodies), followed by a mobilisation of energy stores associated with the sustained longer-term GC response (i.e. fats and protein stores). HPA reactivity was generally slower than reported in other birds, and there was high inter-individual variability. Sleeping birds had higher GC and glucose responses to acute stress, suggesting a more rapid mobilization of energy stores. Our results highlight the importance of considering HPA and metabolic responses to acute stress against species-specific life history and ecological relevant backgrounds.

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## 1. Introduction

The stress response may be defined as an ensemble of physiological and behavioural adaptations enabling the organism to cope with acute perturbations (both internal and external) superimposed to the normal diel and seasonal fluctuations of homeostasis (Landys et al., 2006; McEwen and Wingfield, 2003; Romero et al., 2009). Faced with an impending threat, physiological systems allowing animals to cope are mobilised, and those not needed are concurrently inhibited (Wingfield et al., 1998). For instance,

stress responses may redirect behaviour and energy resources from reproductive to somatic functions, especially in species where adult survival contributes more to lifetime reproductive success than annual fecundity (Elliott et al., 2014; Goutte et al., 2011).

The physiological response to an acute perturbation (a stressor) involves two main pathways. First, a rapid response is activated within seconds of exposure to a stressor by the release of catecholamine (namely adrenaline and noradrenaline) from the adrenal medulla under sympathetic activation of the autonomic nervous system (Sapolsky et al., 2000; De Boer et al., 1990). This first rapid wave of the stress response and the adrenaline rush that occurs triggers the typical “fight-or-flight” response: heart rate increases, air passages dilate, vigilance is enhanced, and energy substrates are mobilized via glycogenolysis in the liver and muscle, glycolysis in the muscle, and lipolysis in the adipose tissue. The

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second response occurs with the progressive activation of the hypothalamic-pituitary-adrenal (HPA) axis (within minutes rather than seconds) resulting in the release of glucocorticoids (GCs) by the adrenal cortex (Sapolsky et al., 2000; De Boer et al., 1990). The main GC in birds is corticosterone (CORT) whereas it is cortisol in most mammalian species (Palme et al., 2005). GCs (and catecholamine) act in a number of ways to deal with stressors by mobilizing energy substrates (MbangKollo and DeRoos, 1983; Peckett et al., 2011; Sapolsky et al., 2000), facilitating the movement of immune cells to fight an infection (Dhabhar, 2006; Dhabhar and Mcewen, 1997), or acting in the brain to promote the formation of memories of events which should be considered as dangerous in the future (Roozendaal, 2000; McEwen and Wingfield, 2003).

Whereas a number of studies have considered the adaptive nature of GC responses to acute stress and their modulation by an extensive range of factors (individual age, experience, life history, sociality, reproductive strategy) (Angelier et al., 2015, 2007; Bókonyi et al., 2009; Boonstra et al., 2001; Breuner et al., 1999; Goutte et al., 2011; Wingfield and Romero, 2011), fewer have concurrently examined the metabolic response to acute stress in terms of both GCs and the sequential mobilisation of energy stores (e.g. Jentoft et al., 2005), especially in natural settings (Corbel et al., 2010; Delehanty and Boonstra, 2011, 2009; Viblanc et al., 2016). Thus, the precise kinetic of metabolite regulation and sequence of mobilisation remains less well defined. In addition, a precise understanding of the benefits of high GC responses in promoting energy mobilisation is lacking. For instance, one might ask whether higher GC increases in response to an acute stressor actually promote a more efficient mobilisation of energy stores, and whether some threshold is met beyond which any additional increase in GCs has no substantial, or even deleterious, consequences.

In this study, we evaluate the hormonal and metabolic components of the stress response to an acute stressor in king penguins (*Aptenodytes patagonicus*). King penguins are colonially breeding seabirds that provide a good model for studies on stress responses related to energy mobilisation. Those animals are confronted to a variety of stressful stimuli in their natural environment. They are highly sensitive to their aggressive conspecifics (Côté, 2000), as shown by both acute heart rate stress responses to observed conflicts between neighbouring birds (Viblanc et al., 2012b) and elevated baseline GCs when breeding in dense social neighbourhoods (Viblanc et al., 2014a). They are exposed to land-based predators (giant petrels *Macronectes* sp. and brown skuas, *Stercorarius lonnbergi*; Descamps et al., 2005), and inclement weather conditions (Stonehouse, 1960; Viera et al., 2006), all of which are susceptible to elicit stress responses (Bize et al., 2010; Clinchy et al., 2013). We have recently shown that acute stress responses may reflect energy mobilisation in terms of non-esterified fatty acids in a context-dependent manner in those breeding seabirds. Specifically, energy-mobilisation is high for birds not yet engaged into reproduction, but rapidly attenuated later in the breeding season (Viblanc et al., 2016).

Here, we quantify changes in plasma glucocorticoids (corticosterone, CORT) and metabolites reflecting the usage of specific energy store compartments in the body. Besides blood glucose (GLUC), we focused on lactate (LACT) stemming mostly from the glycolysis pathway,  $\beta$ -hydroxybutyrate ( $\beta$ -OHB) and non-esterified fatty acids (NEFA) stemming from the lipolysis pathway, and uric acid (UA) stemming from the proteolysis pathway, in up to 32 free-living king penguins, in response to a capture-restraint test lasting up to 90 min. The joint examination of glucocorticoid and metabolic responses should provide a comprehensive appraisal of the kinetics and sequence of hormonal and nutrient-mobilisation changes the organism experiences when facing an acute external perturbation.

## 2. Methods

This study was conducted over 3 breeding seasons from January to March 1996, 1997 and 1998, on adult king penguins during courtship in the breeding colony of “La Baie du Marin” located at Possession Island, Crozet Archipelago (46°25'S–51°45'E). Authorisation to enter the breeding colony and perform the experiments described below were obtained from the Terres Australes and Antarctiques Françaises after approval from an independent Ethics Committee of the Institut Français pour la Recherche et la Technologie Polaire (present name Institut Polaire Paul-Emile Victor). They follow the ‘Agreed Measures for the Conservation of Antarctic and Subantarctic Fauna’, and comply with current French laws.

### 2.1. Hormonal and metabolic response to acute stress

First, we studied the acute stress response (CORT and plasma metabolites) of 18 alert males (sexed by song) that had been fasting for a few days in the breeding colony. Birds were caught at the onset of courtship, while displaying to tentative partners, when the CORT and NEFA responses to acute stress are maximal (Viblanc et al., 2016). Hidden experimenters first randomly selected a bird without attracting its attention. A stopwatch was started, the bird was rapidly caught, its head was covered with a linen mask, and a first blood sample (2 mL) was obtained from a flipper vein usually under 3 min (mean  $\pm$  sd =  $2.1 \pm 0.7$ , range = 0.3–3.2 min) using a heparinised syringe. The bird was rapidly transferred to a nearby shelter (within 5–10 m of their capture location), restrained by an experimenter, and subsequent samples were taken at  $5.1 \pm$  (sd) 0.2 (range = 5.0–6.0) min,  $10.0 \pm 0.0$  (10.0–10.1) min,  $15.1 \pm 0.2$  (15.0–16.0) min and  $30.0 \pm 0.0$  (30.0–30.0) min after the stopwatch was started. During all stress-restraint sessions, the bird was lying on its side and maintained under the experimenter's legs, its eyes covered by a hood. Second, in 7 of the 18 courting males, we prolonged the duration of the restraint stress session up to 90 min aiming to reach a well-defined plateau in metabolic responses. For those individuals, additional blood samples were taken at  $22.5 \pm$  (sd) 0.2 (range = 22.0–23.0) min,  $45.0 \pm 0.0$  (45.0–45.0) min,  $60.0 \pm 0.0$  (60.0–60.0) min,  $75.0 \pm 0.0$  (75.0–75.0) min and  $90.0 \pm 0.0$  (90.0–90.0) min. After the last blood sample was obtained, all 18 birds were weighed ( $\pm 20$  g) and released in the same area they were caught.

### 2.2. Arousal state effects on the stress response

We studied whether individual's state of arousal affected its hormonal and metabolic response during the early phase of acute stress. Following the same procedure as described above, but in different birds, hidden experimenters selected 7 males that were resting but alert (head tucked in shoulders, eyes open) and 7 males that were sleeping (bill tucked under flipper, eyes closed; see Telliez and Dewasmes, 2000) for at least 2 min. Males were selected within breeding pairs during the courtship period, by direct size comparison with their partner (males are typically larger than females; Stonehouse, 1960). A first blood sample (2 mL) was taken at  $43.5 \pm$  (sd) 14.3 (range = 24.0–60.0) s and  $33.6 \pm 11.2$  (19.0–53.0) s following the start of the stopwatch, for alert and sleeping birds, respectively. Following samples were taken as fast as possible below 3 min and then at  $2.0 \pm$  (sd) 0.2 (range = 1.7–2.3) min,  $3.2 \pm 0.3$  (2.6–3.5) min,  $5.1 \pm 0.3$  (4.6–5.5) min,  $7.1 \pm 0.1$  (6.9–7.2) min,  $9.1 \pm 0.2$  (8.8–9.4) min and  $12.3 \pm 0.2$  (9.6–10.4) min. Birds were weighed ( $\pm 20$  g) after the final blood draw, and released in the same area they were caught.

### 2.3. Blood processing, hormone and metabolites assays

Once collected, blood was immediately transferred in polyethylene tubes, and kept 15–30 min in crushed ice until being centrifuged. Aliquots of plasma were then frozen ( $-20^{\circ}\text{C}$ ) until analysis. Total corticosterone (ng/mL) was measured from plasma aliquots using a commercial  $^{125}\text{I}$  RIA kit as previously described and validated for king penguins (Bernard et al., 2002). Uric acid (UA) and non-esterified fatty acids (NEFA) (both mmol/L) were assayed from plasma using enzymatic-colorimetric commercial kits (Boehringer Mannheim and WAKO chemicals, respectively). For glucose (GLUC),  $\beta$ -hydroxybutyrate ( $\beta$ -OHB) and lactate (LACT) assays (all mmol/L), plasma was deproteinized in 7% perchloric acid. The supernatant solution, neutralized with 10% KOH, was used for enzymatic determination using commercially available kits (Boehringer Mannheim). UA was not measured in birds studied for arousal state for lack of plasma.

### 2.4. Statistical analyses

All statistical analyses were conducted in the R statistical computing environment (R Core Team, 2016). Results are presented as means  $\pm$  s.e.

#### 2.4.1. CORT and metabolite kinetics in response to acute stress

To characterise changes over time of the different metabolites and CORT levels in plasma, we regressed plasma parameter concentrations on time since capture of the individuals. Because data were clustered within individuals, we fitted mixed effects models to include individuals as a random effect, allowing for an intercept effect. As we expected non-linear relationships, we fitted GAMMs using a full tensor product smooth for the main effect (“mgcv” package; Wood, 2006). The analysis was performed separately on the 18 alert individuals, and on the 14 asleep or alert individuals. For those 14 birds, we further tested for an effect of arousal status on the intercept.

From model predictions from the first analysis, we determined the time point at which CORT and metabolites exceeded or fell below baseline levels. This was done by comparing the confidence interval of the baseline levels to the levels predicted by the model at later points in time. Post-baseline levels were considered to deviate from baseline levels when the post-baseline levels no longer overlapped with the confidence interval (68%) given by one standard error.

#### 2.4.2. Magnitude of CORT responses and efficiency of metabolite mobilisation

To explore the relationship between CORT responses and the magnitude of metabolic responses, we examined the correlations

(Spearman rank correlations) between all combinations of relative maximum increases and areas under the curves (AUC) for all responses. For each individual and each metabolite, the AUC was calculated starting from where individual baseline metabolite concentrations were, thus correcting for inter-individual differences in baseline metabolite levels. The AUC was calculated using the R package “pracma” (Borchers, 2015). The relative maximum increase was calculated as the (maximum – baseline levels)/baseline levels within the first 30 min of the stress response (see ESM1 for absolute changes).

From the values predicted by the GAMM in the first analysis, we calculated the first order derivatives of each curve and located the relative extrema in the different kinetics of CORT and the various metabolites (i.e. when the first order derivative changes sign).

## 3. Results

### 3.1. Effect of body mass on basal levels

A linear model was fitted to assess the potential effect of body mass on hormonal and metabolite levels. No effect of body mass on basal CORT, GLUC, LACT, BOH, NEFA and AU level was detected (CORT: estimate =  $0.36 \pm 0.26$ ,  $P = 0.220$ ; GLUC:  $0.11 \pm 0.15$ ,  $P = 0.510$ ; LACT:  $-0.29 \pm 0.71$ ,  $P = 0.703$ ;  $\beta$ -OH:  $0.01 \pm 0.08$ ,  $P = 0.957$ , NEFA:  $0.02 \pm 0.04$ ,  $P = 0.558$ , and AU:  $0.02 \pm 0.02$ ,  $P = 0.276$ ).

### 3.2. Hormonal response to stress

Measured CORT levels ranged from 1.26 to 71.41 ng/mL. The corresponding GAMM (Table 1) predicted that CORT levels initially increased, peaking at  $40.12 \pm 3.68$  ng/mL at the 84th minute and then reached a plateau (Fig. 1A). CORT levels only differed significantly from basal levels after 5 min of capture, increasing from  $1.49 \pm 2.21$  to  $3.83 \pm 1.84$  ng/mL. On average, sleeping birds tended to have higher CORT levels during capture than birds fully awake ( $P = 0.056$ ) (Table 2, Fig. 1B).

### 3.3. Metabolic response to stress

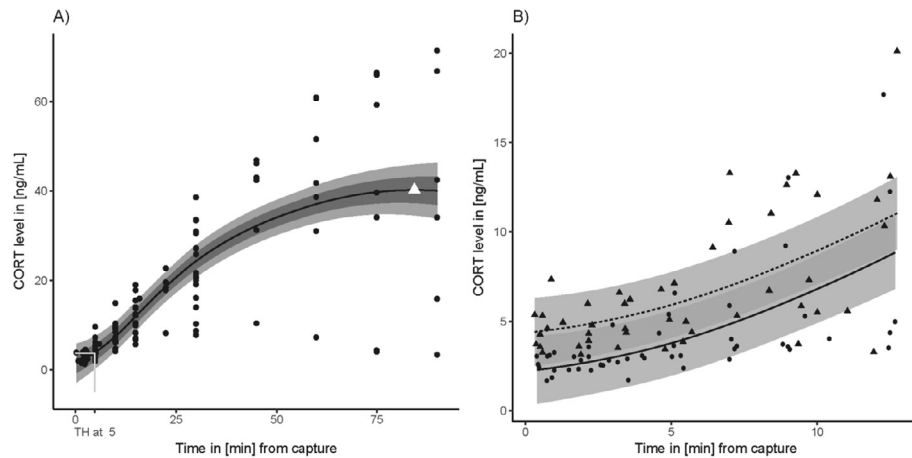
#### 3.3.1. Glucose response

Measured glucose levels ranged from 9.39 to 16.35 ng/mL. Within the first minutes after capture, plasma glucose levels significantly increased. After only 2.5 min, levels had already differed from basal levels, increasing from  $10.88 \pm 0.28$  to  $11.35 \pm 0.28$  mmol/L as predicted by the corresponding GAMM (Table 1). The response initially increased at a decelerating rate in the first hour to a maximum of  $14.21 \pm 0.19$  mmol/L (Fig. 2A). Arousal status affected birds' glucose levels: whereas the response

**Table 1**

Results of the GAMMs for the different metabolites including only individuals that were awake. Var. stands for variable, expl. for explanatory, edf for estimated degree of freedom, and dev. for deviance. We reported the fixed effect (s(time)) and the random effect (s(ID)).

Response var.	Dev. explained	Expl. var.	edf	F	P-value
Corticosterone	76.9%	s(Time)	2.64	81.36	<0.001
		random effect ID	11.14	3.24	<0.001
Glucose	88.0%	s(Time)	2.90	123.43	<0.001
		random effect ID	16.22	22.91	<0.001
Lactate	89.9%	s(Time)	17.90	26.58	<0.001
		random effect ID	15.09	7.03	<0.001
$\beta$ -hydroxybutyrate	70.7%	s(Time)	3.74	24.02	<0.001
		random effect ID	15.39	17.00	<0.001
Non-esterified fatty acids	65.0%	s(Time)	6.56	17.97	<0.001
		random effect ID	12.85	2.58	<0.001
Uric Acid	85.0%	s(Time)	2.71	12.09	<0.001
		random effect ID	5.89	47.25	<0.001



**Fig. 1.** (A-B) CORT kinetics over the acute stress protocol. The curve is the trend over time predicted at population level by the corresponding GAMM with the 95% confidence interval (light grey area) and in (A) with the confidence interval given by one standard error (dark grey area). The large white triangle indicates the local maximum. The threshold (TH) indicates the time at which the post-basal levels are higher than the upper confidence interval given by one standard error of the basal levels, i.e. when the hormone levels are distinct from basal levels. (B) CORT kinetics as predicted by the model fitted on individuals awake (dots; full prediction line) and asleep (triangles; dashed prediction line).

**Table 2**

Results of the GAMMs including the vigilance state for the different metabolites. Var. stands for variable, expl. for explanatory, edf for estimated degree of freedom, and dev. for deviance. We reported the intercept, the fixed effects (s(time)), states (asleep) and the random effect (random effect ID).

Response var.	Dev. explained	Expl. var.	Estimate	edf	S.E.	F	P-value
Corticosterone	75.9%	Inter.	4.33		0.79		
		State (asleep)	2.14		1.12		0.059
		te(Time)		2.14		40.86	<0.001
		random effect ID		10.52		7.63	<0.001
Glucose	58.5%	Inter.	9.96		0.26		
		State (asleep)	0.93		0.38		0.016
		te(Time)		1.56		6.50	<0.001
		random effect ID		9.40		4.75	<0.001
Lactate	75.6%	Inter.	5.10		0.89		
		State (asleep)	-0.41		1.26		0.745
		te(Time)		2.85		24.84	<0.001
		random effect ID		11.06		12.88	<0.001
$\beta$ -hydroxybutyrate	94.7%	Inter.	1.00		0.17		
		State (asleep)	0.23		0.23		0.327
		s(Time)		3.02		132.51	<0.001
		random effect ID		11.86		61.78	<0.001
Non-esterified fatty acids	64.8%	Inter.	0.49		0.06		<0.001
		State (asleep)	0.06		0.09		0.514
		s(Time)		3.34		10.17	<0.001
		random effect ID		10.72		8.14	<0.001

to acute stress was generally similar, sleeping birds had overall higher glucose levels than alert individuals (Table 2; Fig. 3A).

### 3.3.2. Lactate response

Measured lactate levels ranged from 0.37 to 14.60 ng/mL. Plasma lactate levels increased rapidly following the onset of the stress. A GAMM (Table 1) detected that plasma lactate levels followed a rapid increase peaking at a predicted  $7.59 \pm 0.84$  min after the onset of the stress, and subsequently decreased almost as rapidly until the 30th minute where the curve reached a local minimum (Fig. 2B). Basal levels ( $6.86 \pm 1.17$  mmol/L) were exceeded after 5 min ( $8.14 \pm 0.72$  mmol/L), just before reaching the local maximum. Arousal status did not affect the lactate response to acute stress (Table 2, Fig. 3B).

### 3.3.3. $\beta$ -OHB response

Measured  $\beta$ -OHB levels ranged from 0.38 to 2.28 ng/mL. The levels of  $\beta$ -OHB were predicted by the corresponding GAMM (Table 1) to initially decrease for the first 12 min from  $1.17 \pm 0.08$  mmol/L to a minimum of  $0.61 \pm 0.08$  mmol/L, before increasing again in a linear fashion (Fig. 2C).  $\beta$ -OHB levels rapidly

differed from basal levels. After only 1.45 min of stress onset,  $\beta$ -OHB levels were significantly different from basal levels, falling from  $1.45 \pm 0.09$  to  $1.30 \pm 0.08$  mmol/L. Arousal status did not affect the  $\beta$ -OHB response to acute stress (Table 2, Fig. 3C).

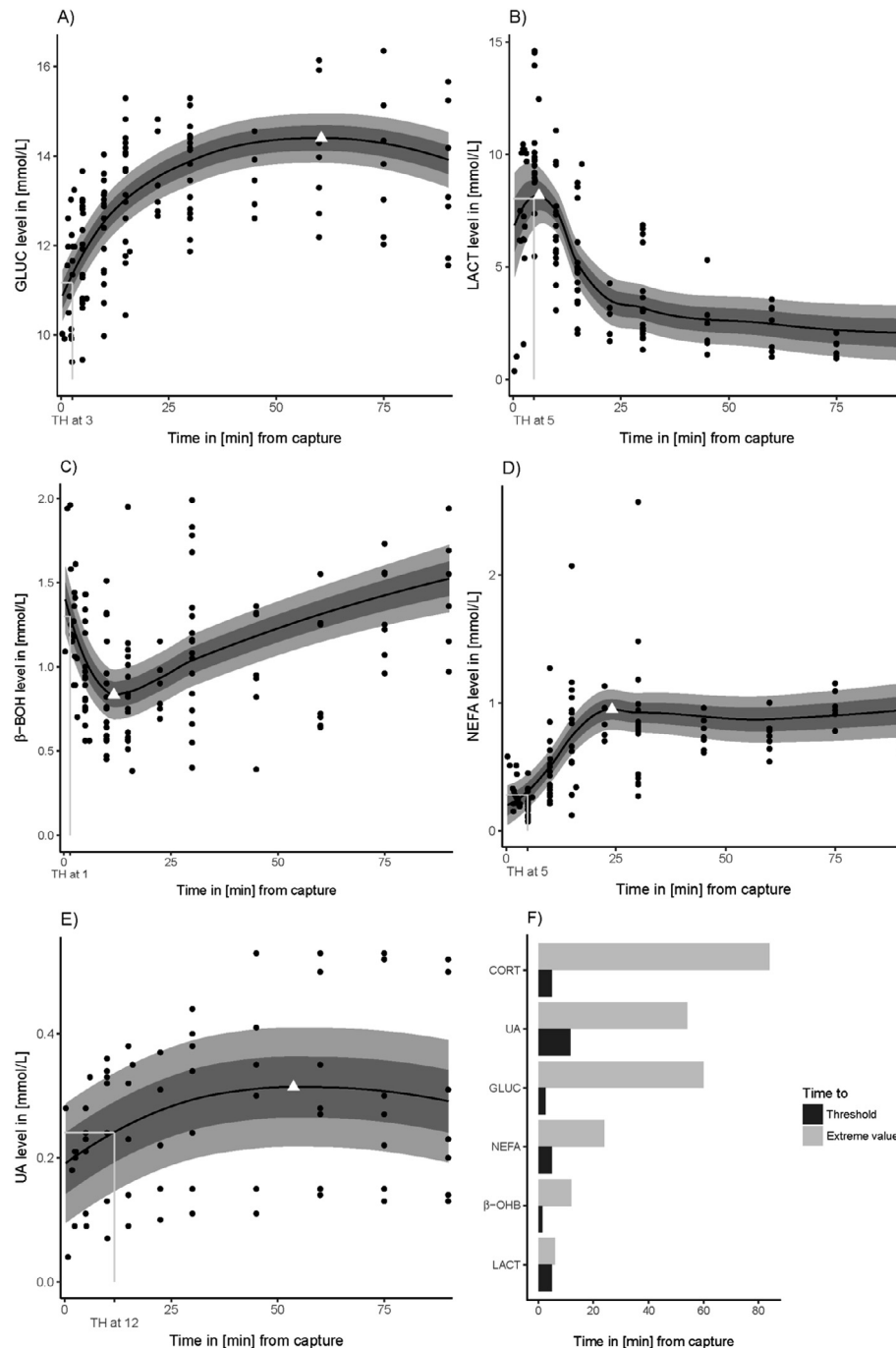
### 3.3.4. NEFA response

Measured NEFA levels ranged from 0.07 to 2.57 ng/mL. According to the corresponding GAMM (Table 1), NEFA plasma levels initially increased from  $0.20 \pm 0.08$  mmol/L up to a maximum of  $0.95 \pm 0.11$  mmol/L within the first 24 min of the stress, before reaching a plateau (Fig. 2D). After 5 min, post-basal levels ( $0.30 \pm 0.08$ ) significantly exceeded basal levels ( $0.20 \pm 0.07$ ). Arousal status did not affect the NEFA response to acute stress (Table 2, Fig. 3D).

### 3.3.5. Uric acid response

Measured UA levels ranged from 0.04 to 0.53 ng/mL. Plasma UA levels were predicted by a GAMM (Table 1) to initially increase before reaching a maximum value of  $0.31 \pm 0.02$ , within 54 min of stress initiation (Fig. 3E). UA was the metabolite which plasma levels changed the slowest. Post-basal levels ( $0.24 \pm 0.05$ ) were sig-





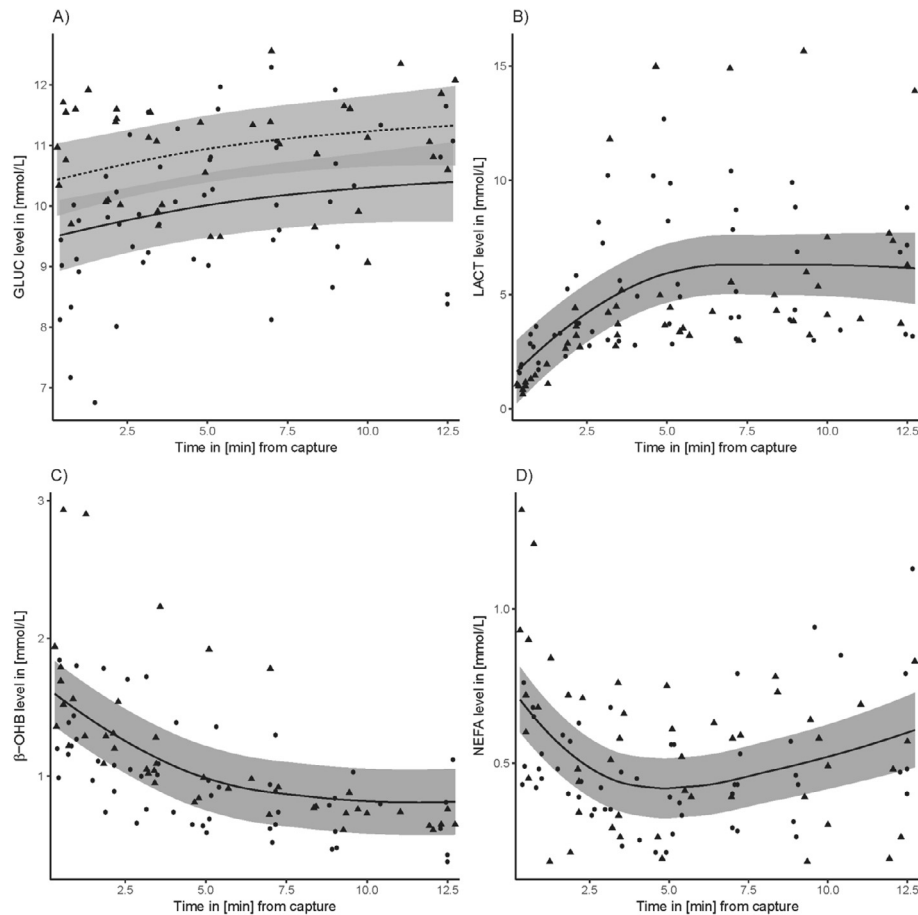
**Fig. 2.** Metabolite kinetics over the acute stress protocol for (A) blood glucose (GLUC, mmol/L), (B) lactate (LACT, mmol/L), (C)  $\beta$ -hydroxybutyrate ( $\beta$ -OHB, mmol/L), (D) non-esterified fatty acids (NEFA, mmol/L), (E) and uric acid (UA mmol/L). The curve is the trend over time predicted at population level by the corresponding GAMM with the 95% confidence interval (light grey area) and with the confidence interval given by one standard error (dark grey area). The large white triangle indicates the local extreme point (either maximum or minimum). The threshold (TH) indicates the time at which the post-basal levels are higher (lower) than the upper (lower) confidence interval given by one standard error of the basal levels i.e. when the metabolite levels are distinct from basal level. (F) Timeframe describing when metabolites reached the threshold (black bars) and first relative extrema (maximum or minimum; grey bars) in minutes elapsed since capture.

nificantly different from basal levels ( $0.19 \pm 0.05$ ) only after 11 min.

#### 3.4. Local extremes in hormonal and metabolic profiles and their interrelationships

The first metabolite predicted to reach its first local extreme was LACT after 6 min of stress onset, followed by  $\beta$ -OHB after 12 min, and NEFA after 24 min from the start. UA, GLUC and CORT peaked

later, after 54, 60 and 84 min, respectively (Fig. 2 F). The relative maximum increases in all metabolites were strongly positively correlated to their corresponding areas under the curves (AUC) ( $0.64 < r < 0.92$ ) (see ESM 2). There was a strong positive correlation between the AUCs for the CORT and glucose responses ( $r = 0.63$ ), and a positive but weaker correlation for the CORT and LACT responses ( $r = 0.44$ ). In contrast, the AUC of the CORT response was negatively correlated with the AUCs of  $\beta$ -OHB ( $r = -0.43$ ) and NEFA ( $r = -0.31$ ) responses to acute stress (see ESM 1 and 2).



**Fig. 3.** Metabolite responses to stress in king penguins that were either sleeping or awake when caught for (A) blood glucose (GLUC, mmol/L), (B) lactate (LACT, mmol/L), (C)  $\beta$ -hydroxybutyrate ( $\beta$ -OHB, mmol/L), and (D) non-esterified fatty acids (NEFA, mmol/L). The curve is the trend over time by the corresponding GAMM with the 95% confidence interval. When the effect of status was not significant (see Table 2), only one curve is drawn. Individuals awake are represented by dots (full prediction line) and asleep by triangles (dashed prediction line).

## 4. Discussion

### 4.1. Hormonal and metabolic responses to acute stress

It is generally admitted that GCs play a major role in the animal's stress response by mobilizing energy substrates through preparatory, permissive and stimulatory effects on glycolysis, lipolysis, proteolysis and neoglucogenesis (Campbell et al., 2011; Hasselgren, 1999; Peckett et al., 2011; Pilgis and Granner, 1992; Xu et al., 2009). In captive fish for instance, acute handling stress typically mobilizes energy substrates from carbohydrate, fat and protein catabolism both through HPA and sympathetic pathways (Barton and Iwama, 1991; Vijayan and Moon, 1992; Wendelaar Bonga, 1997). Similarly, in captive birds and mammals, acute GC increases typically enhance the mobilisation of energy stores through preparatory, permissive and stimulating effects (Dallman et al., 1993; Eigler et al., 1979; Remage-Healey and Romero, 2001; Tomas et al., 1979; Sapolsky et al., 2000). In contrast, fewer studies have actually considered an integrative view of the metabolic response to stress and time course of substrate mobilisation during acute stress in the wild (Corbel et al., 2010; Davies et al., 2013; Deleahanty and Boonstra, 2011, 2009; Viblanc et al., 2016). The present results in wild king penguins highlight important points on the mobilisation of energy substrates in response to acute stress. As could be expected, CORT levels in penguins increased substantially from baseline levels following stress onset (Corbel et al., 2010; Viblanc et al., 2016). The predicted increase

reached a maximum 14.5 times baseline levels within 84 min of stress initiation before levelling-off. Thus, maximum (zenith) levels were reached a substantial amount of time *after* stress initiation. Further, the increase in CORT levels was relatively slow, plasma titres differing significantly from baseline measures only after 5 min. This suggests that the reactivity of the HPA axis in penguins is slower than what is often reported in other studies both in terms of the onset of the CORT response and the time needed to reach a physiological maximum. In comparison, over 5 different avian and one reptile species, Romero and Reed (2005) found that GC generally increased within 1–3 min depending on species. In rats (Dallman and Bhatnagar, 2001) and sandpipers (Mizrahi et al., 2001) increases in plasma GCs were not detectable before some 3–5 min. Early studies on HPA reactivity to acute stress have suggested that peak GC levels may be reached as early as 10 min following stressor initiation (Wingfield et al., 1994, 1992), a clear contrast with the present results (peak CORT levels reached after >1 h of capture).

Taken together, those results highlight the context-dependency of the stress response, which should be evaluated against the life history and ecological background of the species under consideration (Crespi et al., 2013). Penguins are long-term fasting seabirds that breed in an environment of moderate-risk but frequent harassment by on-land predators, generally poor weather, and of constant aggression by neighbours (Côté, 2000; Descamps et al., 2005; Groscolas et al., 2008; Groscolas and Robin, 2001; Stonehouse, 1960; Viera et al., 2006). Such factors likely shaped

acute stress responses (both HPA and sympathetic) and reactivity to external threats (Viblanc et al., 2016, 2015, 2014a,b, 2012a,b), possibly explaining the high inter-individual variability observed in our study. CORT plays major roles in the breeding cycle of penguins. During long-term fasts on land, baseline CORT levels are maintained at minimal levels but markedly increase when the bird's energy stores are critically depleted (Cherel et al., 1988; Groscolas and Robin, 2001): this increase is involved in the re-feeding signal triggering adults to abandon reproduction in favour of self-maintenance and survival (Groscolas et al., 2008). Baseline CORT levels also increase throughout the breeding season (Viblanc et al., 2016, 2014a), possibly to meet the high energy demands of chick rearing (Bonier et al., 2009). In contrast, CORT increases in response to acute stress appear to be modulated by breeding status, and are attenuated at advanced stages of breeding (Viblanc et al., 2016). In the present study, all males were of similar breeding and fasting status (caught shortly after courtship and of similar mass), and it is unlikely those factors had an effect on baseline CORT levels. However, larger samples sizes including birds of known life history may prove useful in identifying the environmental and individual characteristics shaping both baseline CORT levels and stress-induced CORT responses. For instance, recent studies highlight the importance of early environmental effects in shaping the HPA axis of both birds and mammals (Love et al., 2013; Love and Williams, 2008; Matthews, 2002), including epigenetic modulation of the expression of GC receptor gene promoters (Weaver et al., 2004). Whether early environmental stimuli may also have long-lasting consequences on HPA reactivity in penguins is yet to consider, given that (1) marked differences in rearing conditions occur early and late in the season both in terms of food resources (Gauthier-Clerc et al., 2002) and colonial conditions (i.e. social density; Viblanc et al., 2014b), and (2) chicks raised early or late in the breeding season exhibit markedly different baseline GCs profiles shortly after hatching (Stier et al., 2014). In addition, whereas our focus was on males in the present study, it would be interesting to test if the metabolic mobilisation of energy reserves in response to acute stress differs between the sexes. In this species, males endure a longer overall fasting period than females during the early stages of incubation (Stonehouse, 1960; Weimerskirch et al., 1992), and the necessity of energy savings at that time may constrain the strength of the metabolic response to acute stress or favour a more rapid down-regulation of nutrient mobilisation when the stressor has subsided (Viblanc et al., 2016). Further, it should be noted that the CORT responses reported here are to one specific type of stressor, i.e. capture and restraint. Sympathetic heart rate responses to stress are stressor-specific in this species, and captures are known to elicit maximal adrenergic responses when compared to milder stressors such as loud sounds or distant human approaches (Viblanc et al., 2015, 2012a). Whether HPA responses are also stressor-specific (e.g. predators, human disturbance, inclement weather, aggressive conspecifics) remains to be investigated. Finally, it would be interesting to study the possible influence of proactive vs. reactive bird personalities in shaping the CORT response to acute stress (Bousquet et al., 2015; Carere et al., 2003; Cockrem, 2007). In birds, proactive/reactive individuals typically exhibit relatively low/high CORT and active/passive behavioural responses to acute stressors (Cockrem, 2007). Such variation along a proactive-reactive continuum may also explain the large inter-individual variation observed in terms of CORT responses in the present study.

In male king penguins, the CORT response (the area under the curve, AUC) was strongly correlated with the magnitude of glucose response ( $r = 0.63$ ), and to a lesser extent with the magnitude of the lactate response ( $r = 0.44$ ). However, its relation with other metabolites was actually negative ( $r = -0.43$  and  $-0.31$  for  $\beta$ -OHB and NEFA responses, respectively). In addition, CORT was

actually the last to reach peak levels in plasma (zenith, after 84 min), with lactate being the first (zenith, after 6 min at a time when CORT levels were still close to baseline values), followed by  $\beta$ -OHB (nadir, 12 min) and NEFA (zenith, 24 min). Importantly, the time-course of energy metabolite mobilisation should reflect different biochemical pathways, some of which are only partly influenced by the action of GCs. First, the rapid increase in blood lactate to maximum levels (a breakdown product of glucose) likely reflects the rapid usage of glucose stores through anaerobic glycolytic carbon flow, a process primarily stimulated by the release of catecholamine from the autonomic nervous system (viz. the first rapid wave of the stress response) independently of GCs (Fernandez et al., 1994; Le Maho et al., 1992; MbangKollo and DeRoos, 1983; Sapolsky et al., 2000). Similarly, the rapid decrease in plasma ketonemia ( $\beta$ -OHB levels) and re-increase after 12 min, likely reflects (1) their uptake as energy substrates in neoglucogenesis (Krebs, 1966; Exton and Park, 1967), and (2) the progressive oxidation of free fatty acids leading to the generation of acetyl-CoA, which excess is converted to ketone bodies. In contrast, the constant mobilisation of glucose paralleling the slow increase in CORT highlights the longer-term potentiating action of glucocorticoids (Sapolsky et al., 2000), also affecting lypolysis (Charmandari et al., 2005) and indirectly proteolysis, as reflected in the slower increase kinetics of NEFA and uric acid plasmatic concentrations, respectively. The lack of a positive relationship between the AUC for CORT and the AUC for NEFA or  $\beta$ -OHB is surprising. However, this is likely due to different factors. First, the kinetics of  $\beta$ -OHB that decreased before increasing again, probably precludes a useful meaning of AUC for this metabolite. Second, the AUC for CORT likely reflects a situation where both type I and type II GC receptors (GR) are saturated (Romero, 2004), and may not be a good index to test lipid mobilisation when compared to the actual maximal increase in CORT levels. For instance, in a separate study, we recently found that maximal increases in CORT levels were positively associated with the AUC of NEFA responses in breeding penguins (Viblanc et al., 2016). Finally, although direct non-genomic effects have been suggested (Campbell et al., 2011), it should be kept in mind that the action of GCs on substrate mobilisation is mostly indirect through binding to both cellular and nuclear GR altering gene transcription, e.g. lipase proteins (Fain and Saperstein, 1970; Slavin et al., 1994; Peckett et al., 2011). Thus the effects of GCs on lipid metabolism may be long-lasting, taking hours to days to occur (Fain et al., 1971; Peckett et al., 2011; Slavin et al., 1994; but see Viblanc et al., 2016). NEFA and  $\beta$ -OHB in plasma are terminal measures of lipid catabolism, so that they may be longer-term reflections of GC actions on fat mobilisation.

#### 4.2. Acute stress protocol in sleeping vs. awakened king penguins

Arousal state caused differences in the acute stress response of penguins, especially in terms of changes in plasma glucose, and marginally in CORT. Two main, non-mutually exclusive, hypotheses might explain those findings. First, differences in CORT and glucose levels may stem from circadian variations in the secretion of GCs (Landys et al., 2006). An increase in corticosteronemia during sleep is known to occur in several bird species (Breuner et al., 1999; Dufty and Belthoff, 1997; Romero and Ramage-Healey, 2000) and may allow promoting bird restfulness by decreasing responsiveness to external stimuli during sleeping periods (Buttemer et al., 1991). This would be especially adaptive for penguins that are subject to long-term fasting while on-land and are indeed known to increase deep-sleep time in fasting phase II, a phase of energy sparing (e.g. in emperor penguins; Dewasmes et al., 1989). Whether such effects differ during short-term day sleeping events (as often occurs in penguins) in contrast to night sleep, and associated circadian changes, remains to be determined.



Second, higher CORT responses and mobilisation of glucose in sleeping birds may allow to rapidly deal with an impending threat for which no prior conscious evaluation of the stimuli may have occurred. For instance, king penguins are known to modulate even rapid adrenergic heart rate responses depending on the intensity of a stressor (Viblanc et al., 2015, 2012a), which is likely confronted against a memory template of past traumatising experiences (Viblanc et al., 2012a; Sapolsky et al., 2000). In addition, current studies suggest that sleeping king penguins respond more often and more strongly in terms of behaviour to the playback of predator sounds than awakened individuals (van Walsum et al., unpublished data) suggesting a potentiation of stress responses in sleeping birds. Besides rapidly mobilizing energy stores, high GC responses at sleep arousal should also potentiate the action of catecholamines in forming long-lasting memories in relation to stressful experiences (McGaugh and Roozendaal, 2002). The present results highlight the importance of considering individual's arousal state in stress studies, to better understand inter-individual variation linked to HPA responses and mobilisation of energy stores during acute stress.

#### 4.3. Conclusion

We investigated the time course of GC and energy substrate mobilisation in response to acute stress in wild king penguins in their colonial environment, aiming to provide a comprehensive view of the metabolic response to stress in a natural setting. Taken together, our results confirm the cascade of energy mobilisation during acute stress highlighting both the short term rapid “fight-or-flight” response linked to sympathetic adrenergic activity, and the longer sustained response linked to HPA activity. Our study evidences substantial differences in the stress response depending on an individual's resting state and further highlights the importance of considering HPA-axis reactivity in light of the life history and ecological conditions of the considered species. High inter-individual variability in stress responses is possibly due to variation in early life conditions, a truly fascinating topic awaiting further research.

#### Author contribution

JPR and RG designed the study. JPR, RG and JJM did the field-work, the laboratory work, and pre-analysed the data. TC analysed the data and participated in writing the paper. AS provided critical comments on the manuscript. VAV and QS co-wrote the paper and discussed the data analyses with TC and JPR.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.ygcen.2017.08.024>.

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