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## **► To cite this version:**

Agnès Lewden, Chelsea Ward, Aude Noiret, Sandra Avril, Lucie Abolivier, et al.. Surface temperatures are influenced by handling stress independently of glucocorticoid levels in wild king penguins. 2023. <hal-04235532>

**HAL Id: hal-04235532**

**<https://hal.science/hal-04235532v1>**

Preprint submitted on 10 Oct 2023

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# Surface temperatures are influenced by handling stress independently of glucocorticoid levels in wild king penguins

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## Highlights:

- Experimental increase in CORT does not affect king penguin's surface temperature
- Acute handling stress increases eye but decreases beak surface temperature in adults
- Acute handling decreases both eye and beak surface temperatures in young chicks
- Parental CORT treatment does not affect egg surface temperature during incubation
- Chicks brooded by non-implanted partners of CORT parents are warmer

## Abstract

Assessing the physiological stress responses of wild animals opens a window for understanding how organisms cope with environmental challenges. Since stress response is associated with changes in body temperature, the use of body surface temperature through thermal imaging could help to measure acute and chronic stress responses non-invasively. We used thermal imaging, acute handling-stress protocol and an experimental manipulation of glucocorticoid hormone levels in breeding king penguins (*Aptenodytes patagonicus*), to assess: 1. the potential contribution of the Hypothalamo-Pituitary-Adrenal (HPA) axis in mediating chronic and acute stress-induced changes in adult surface temperature, 2. the influence of HPA axis manipulation on parental investment through thermal imaging of eggs and brooded chicks, and 3. the impact of parental treatment on offspring thermal's response to acute handling.

Eye temperature ( $T_{eye}$ ) increased and beak temperature ( $T_{beak}$ ) decreased in response to handling stress in adults, but neither basal nor stress-induced surface temperatures were significantly affected by glucocorticoid implants. While egg temperature was not significantly influenced by parental treatment, we found a surprising pattern for chicks: chicks brooded by the (non-implanted) partner of glucocorticoid-implanted individuals exhibited higher surface temperature (both  $T_{eye}$  and  $T_{beak}$ ) than those brooded by glucocorticoid-implanted or control parents. Chick's response to handling in terms of surface temperature was characterized by a drop in both  $T_{eye}$  and  $T_{beak}$  independently of parental treatment.

We conclude that the HPA seems unlikely to play a major role in determining chronic or acute changes in surface temperature in king penguins. Changes in surface temperature may primarily be mediated by the Sympathetic-Adrenal-Medullary axis (SAM) in response to stressful situations. Our experiment did not reveal a direct impact of parental HPA manipulation on parental investment (egg or chick temperature), but a potential influence on the partner's brooding behaviour (*i.e. family-transmitted stress hypothesis*).

**Key words:** Thermal imaging, Stress, Corticosterone, Heterothermy, Bird, Beak, Eye

## 53    **Abbreviations:**

54    Ambient air temperature:  $T_a$

55    Glucocorticoid hormones (corticosterone or cortisol): CORT

56    Egg surface temperature:  $T_{egg}$

57    Hypothalamic-pituitary-adrenal axis: HPA

58    Maximum eye region surface temperature:  $T_{eye}$

59    Minimum beak surface temperature:  $T_{beak}$

60    Sympathetic-adrenal-medullary axis: SAM

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# 1. Introduction

Measuring physiological stress is of central interest in wild animals to understand how they cope with environmental change (Ellenberg et al., 2007; Romero, 2004). Traditionally, ecologists have evaluated individual stress by assessing the activation of the Sympathetic-Adrenal-Medullary (SAM) or Hypothalamic-Pituitary-Adrenal (HPA) axes, either at baseline levels or in response to acute disturbances in the environment, by metrics such as increased heart rate (Cabanac and Guillemette, 2001; Viblanc et al., 2012), ventilation rate (Carere and Oers, 2004) or glucocorticoid hormone (GC) secretion (Sapolsky et al., 2000). Stress exposure is also known to trigger core body and surface temperature changes (Oka, 2018). As the latter can be measured using thermal imaging (McCafferty, 2013), it led to a recent burgeoning of thermal imaging studies to measure the stress response of captive and wild endotherms (McCafferty et al., 2021). Both the SAM and HPA can potentially influence changes in body surface temperature, but the importance of these two pathways in mediating the stress response(s) measured through thermal imaging remains largely unknown. This information is however essential for the proper interpretation of the physiological stress response measured by thermal imaging.

SAM responds within seconds to a stressor by releasing catecholamines hormones (adrenaline and noradrenaline) into the blood stream, which induces an immediate increase in heart rate (tachycardia) and vasoconstriction of peripheral blood vessels (Wingfield and Romero, 2015). This redistribution of peripheral blood flow to essential internal organs generally leads to a response called stress-induced hyperthermia along with a decrease of surface temperature (Cabanac and Guillemette, 2001; Herborn et al., 2015; Oka, 2018). The SAM response is followed by the slower (within a few minutes) activation of HPA that releases

GC into the blood stream (Wingfield and Romero, 2015). GC are metabolic hormones that helps responding to a stressor by mobilizing energy resources and by triggering an increase in behavioural activity and metabolic rate (Wingfield and Romero, 2015). Circulating GC levels are usually considered at baseline (*i.e.* without an inducing stressor) or at stress-induced levels, which are often interpreted as mirroring chronic stress/metabolic demand and acute stress response respectively. Yet, the actual interpretation of circulating GC levels is likely more complex (MacDougall-Shackleton et al., 2019; Lemmonier et al. 2022). GC-mediated changes in metabolism may cause an increase in internal heat production that, in turn, may lead to changes in body surface temperature (Oka, 2018). Additionally, previous evidence suggest that glucocorticoids might be necessary for catecholamine-induced thermogenic effects such as shivering, free fatty acid mobilization or vasoconstriction (Deavers and Musacchia, 1979).

Therefore, both the SAM and HPA axes could trigger, independently from each other, or together, a change in body core and/or surface temperatures (Ouyang et al., 2021). The immediate activation of SAM could lead to a decrease of peripheral temperature independently of CORT release. On the other hand, the short to long-term elevation of baseline CORT might lead to increased metabolic rate, heat production and, ultimately, higher peripheral temperature (to facilitate heat dissipation and maintain homeothermy) independently of SAM. To date, the use of thermal imaging as a non-invasive tool to measure stress in unmanipulated animals, as well as the roles of SAM and HPA axes in mediating changes in surface temperature, remains debated. For instance, data in captive and wild birds show that acute stress exposure is often leading to a decrease in body surface temperature (reviewed in Table 1, but note some discrepancies between species and/or body parts), as predicted if these changes are driven by the SAM axis.

Species	Condition	State	Stressor	Area	Response	Δ T°C	Ref.
Blue tit	Wild	Adult	Trap closure + handling	T <sub>eye</sub>	↘, ↗, =	-1.0, +1.0°C, =	Jerem et al. 2019
<i>Cyaniste caeruleus</i>			(3min)				
Budgerigars	Captive	Adult	Handling	T <sub>eye</sub>	↗, =	+0.7°C, =	Ikkatai and Watanabe 2015
<i>Melopsittacus undulatus</i>			(30 min)	T <sub>legs</sub>	=	=	
Chicken	Captive	Adult	Air puff	T <sub>eye</sub>	↘	-1.0°C	Edgar et al. 2011
<i>Gallus gallus domesticus</i>			(10 min)				
Chicken	Captive	Adult	Handling	T <sub>eye</sub>	↘, =	-0.5°C, =	Edgar et al. 2013
<i>Gallus gallus domesticus</i>			(20 min)	T <sub>head</sub>	=, ↗	=, +1.0°C	
				T <sub>comb</sub>	↘, =	-1.5°C, =	
Chicken	Captive	Chick	Air puff	T <sub>eye</sub>	↘	-1.0°C	Edgar et al. 2018
<i>Gallus gallus domesticus</i>		(9 weeks old)	(10 min)				
Chicken	Captive	Adult	Handling: mild/severe	T <sub>eye</sub>	↘	-0.4°C	Herborn et al. 2015
<i>Gallus gallus domesticus</i>				T <sub>face</sub>	=	=	
				(20 min)	T <sub>wattle</sub>	↘	
				T <sub>comb</sub>	↘	-0.5 / -2.2°C	
Chicken	Captive	Adult	Enrichment removal	T <sub>eye</sub>	↘	-0.8°C	Herborn et al. 2018
<i>Gallus gallus domesticus</i>			(within 2h)	T <sub>face</sub>	↘	-0.7°C	
				T <sub>comb</sub>	↘	-3.5°C	
Chicken	Captive	Chick	Handling	T <sub>head</sub>	↗	+0.76°C	Moe et al. 2017
<i>Gallus gallus domesticus</i>		(30 days old)	(10 min)	T <sub>feet</sub>	↘	-0.45°C	
Chicken	Captive	Chick	Visual	T <sub>eye</sub>	=	=	Pijpers et al. 2022
<i>Gallus gallus domesticus</i>		(9 days old)	(45 min)	T <sub>beak</sub>	=, ↗	+0.5°C	
Domestic pigeon	Captive	Adult	Handling	T <sub>eye</sub>	=	-0.4°C	Tabh et al. 2021
<i>Columbia livia domestica</i>			(3.5 min)	T <sub>beak</sub>	↘	-2.1°C	
King penguin	Wild	Adult	Handling	T <sub>eye</sub>	↗	+0.8°C	This study
<i>Aptenodytes patagonicus</i>			(5-10 min)	T <sub>beak</sub>	↘	-1.5°C	
	Wild	Chick	Handling	T <sub>eye</sub>	↘	-0.9°C	
		(20 days old*)	(5-10 min)	T <sub>beak</sub>	↘	-3.3°C	
Little Auk <i>Alle alle</i>	Wild	Adult	Handling (30 min)	T <sub>eye</sub>	↗	+1,7°C	Jakubas et al. 2022
Pheasant	Captive	Juvenile	Agression	T <sub>head</sub>	↗	+0.6°C	Knoch et al. 2022
<i>Phasianus colchicus</i>		(42 to 49 days old)	(20 s)				
Song sparrow	Captive	Adult	Handling	T <sub>eye</sub>	↘, =	-1.5°C, =	Zuluaga et al. 2023
<i>Melospiza melodia</i>			(13 min)	T <sub>beak</sub>	↘, ↗, =	-2.6°C, +2.6°C, =	
* king penguins chicks are not thermally emancipated at this age							

Evidence for an impact of chronic stressors on body surface temperature (Herborn et al., 2018; Robertson et al., 2020a, 2020b; Winder et al., 2020) or for a correlation between circulating GC levels and body surface temperature (Giloh et al., 2012; Jerem et al., 2018; Weimer et al., 2020), however, remains mixed. Therefore, the question of whether acute or chronic elevation of GC causally induces changes in surface temperature remains largely untested (but see Ouyang et al., 2021). Since both HPA and SAM axes provide strong and very reactive responses to stress, teasing apart their contribution to changes in peripheral temperature is challenging.

In this study, we took up the challenge of distinguishing the role of HPA and SAM on changes in body surface temperatures using an experimental approach in adult king penguins (*Aptenodytes patagonicus*). The king penguin is a heterothermic bird whose body temperature largely varies between core and peripheral tissues (Lewden et al., 2017a, 2017b) . Its thick body plumage provides a very efficient insulation, while counter-current vascular heat exchangers in the appendages allow to decrease peripheral temperature loss (Thomas et al., 2011). These adaptations allow birds to conserve heat when foraging in cold Antarctic waters (Handrich et al., 1997), and to rapidly recover to normothermia when exiting the water (Lewden et al., 2020). Thermal imaging has been frequently used in the last years to investigate acute (see an overview in Table 1) and chronic (Herborn et al., 2018; Robertson et al., 2020a, 2020b; Tabh et al., 2022; Winder et al., 2020) stress-response in birds. Because of their dense and watertight plumage, the use of thermal imaging in penguins is restricted to measuring bare parts of the body, such as the eye (periorbital region) and beak (Lewden et al., 2020). These regions are highly vascularised and provide valuable information on body surface temperatures.



Breeding king penguins were subcutaneously implanted with either a corticosterone (CORT) implant or a placebo implant. The use of CORT implants in king penguin (see methods and Fig. 1), as in many other bird species (see Torres-Medina et al., 2018), leads to higher baseline GC (or CORT) levels, while inhibiting the acute CORT responses in the days following implantation. Indeed, CORT-implanted birds no longer exhibit high blood CORT release in response to acute stress induced by a handling stress protocol. Hence, the use of CORT implants in conjunction with handling stress allows for the separation of the contributions of the HPA and SAM axes to acute changes in body surface temperatures. Here, we therefore measured the effects of our CORT treatment, together with a handling stress protocol, on surface temperatures in the king penguin. If changes in surface temperatures are mainly driven by activating the HPA axis, we expect to find higher initial body surface temperatures in CORT-implanted individuals (with elevated baseline CORT) prior to capture and handling. However, because CORT implants shut-down the release of CORT in response to handling, we expect to find no change in body surface temperature in response to handling stress in CORT individuals if such changes are mediated by the HPA only. On the contrary, if changes in surface temperatures are mainly driven by the activity of the SAM axis, we expect our CORT treatment to have no additional effect on body surface temperatures (both before or after capture and handling). We also measured egg surface temperature on the same day as the body surface temperatures of implanted parents and the surface temperatures of chicks at day 20 after hatching, before they were fully thermally emancipated. Based on experimental data in Adélie penguin showing that elevated baseline CORT led to lower parental care (Thierry et al., 2013a), we hypothesized that increasing CORT levels may lower the surface temperatures of eggs and/or chicks during incubation and brooding respectively, as parents may pay less attention in keeping the egg/chick covered and warm under their brood pouch.

## 2. Material and Methods

### 2.1. Study site and species

The study was conducted in 2018-2019 in a king penguin colony of ca 22,000 breeding birds at 'La Baie du Marin' on Possession Island (Barbraud et al. 2020), Crozet Archipelago, in the Southern Indian Ocean (46°26' S, 51°52' E). We followed 49 breeding pairs from courtship (early November) until the onset of the Austral winter (April). All adults were identified by a hair dye mark on the breast feathers. During the breeding season, male and female alternate between periods on-land caring for their single-egg or chick and periods foraging at sea (Weimerskirch et al., 1992). The male takes care of the first incubation shift while the female forages at sea. The female returns ~15 days later to relieve her partner, and the parents continue to alternate shifts throughout the incubation (~53 days) and early chick brooding. Chicks become thermally emancipated from the parents around one month of age, allowing both parents to go back at sea simultaneously to forage (Weimerskirch et al., 1992).

### 2.2. Use of sub-cutaneous CORT and SHAM implants

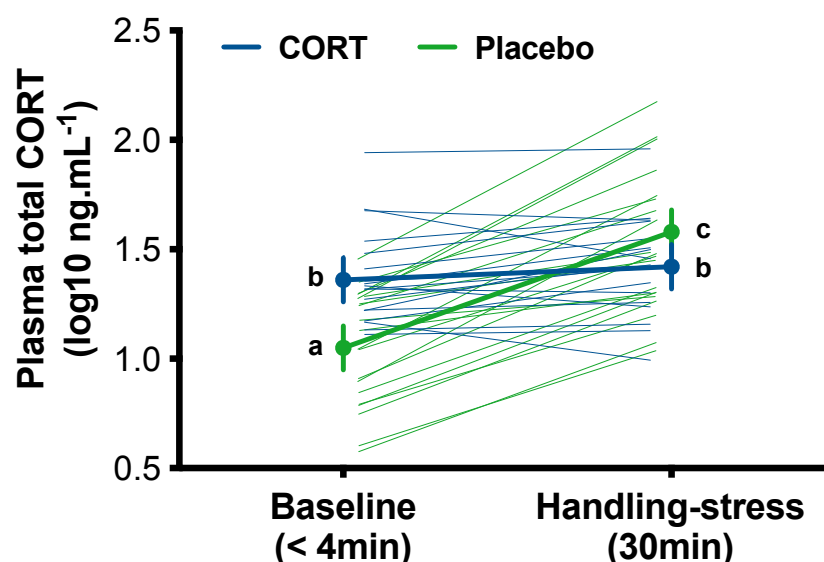
We randomly divided the 49 breeding pairs into two groups of either female-treated pairs (N = 23) or male-treated pairs (N = 26). In the female-treated pairs, we randomly implanted females with either a CORT (NG-111, 50mg corticosterone, 90-days release; N = 12 females) or a Placebo (NC-111, same vehicle but no CORT; N = 11 females) implant. Sub-cutaneous implants were purchased from Innovative Research of America (Sarasota, FL, USA). Females were implanted during their first incubation shift, 3 days after returning from sea. Similarly, in the male-treated pairs, we randomly implanted 13 males with a CORT and 13 males with a Placebo implant, 3 days after returning from sea. In males, the first incubation

shift is immediately after egg laying, and males have already been fasting for ~15 days during courtship. Therefore, we chose to implant the males during their second incubation shift so that they would have comparable fasting times to the females, and therefore both sexes would be in a comparable physiological state during measurements. Male and female partners in, respectively, the female-treated and male-treated pairs were not implanted. Hereafter, they are referred to as “Partner-CORT” or “Partner-Placebo” regardless of their sex. Partner penguins were only considered in the chick analysis (see below).

On the day of the implantation, adults were captured while incubating their egg and restrained by one experimenter with a hood covering the head to keep them calm. Subcutaneous CORT or placebo implants were inserted in the upper part of the back by a veterinary surgeon (SA) under local anaesthesia (*ca.* 0.5mg/kg xylocaine and 0.0001mg/kg adrenalin, Aspen Pharma). Immediately after implantation, the small incision was closed using 3 sterile surgical staples, and a prophylactic dose of antibiotics (cephalexin *ca.* 50mg/kg, Rilexine®, Virbac) was injected to prevent any risk of infection. The wound was checked 3 days later, when taking the thermal images without any sign of infection on individuals, nor implant rejection, and staples were removed 6 days after implantation.

To verify the efficacy of our CORT treatment, blood samples were taken 6 days after implantation in less than 4 min (baseline CORT) and after 30 min of standardized handling (stress-induced, see Stier et al., 2019; Viblanc et al., 2018). Plasma total CORT levels were determined by immunoassay according to guidelines provided by the manufacturer (Corticosterone EIA Kit, Arbor Assay, USA). Intra-plate coefficient of variation based on duplicates was  $8.17 \pm 0.90\%$ , and inter-plate coefficient of variation based on one repeated

sample was 8.08%. Implants were successful in raising circulating baseline CORT levels and in inhibiting stress-induced CORT release (Fig. 1).

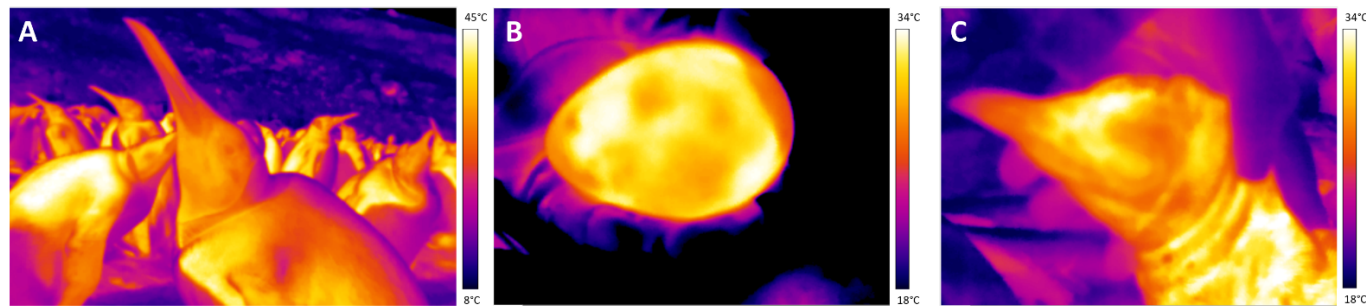


**Fig. 1:** Baseline and stress-induced plasma corticosterone levels of breeding king penguins (N = 20 individuals per group) implanted either with a Placebo, or a CORT subcutaneous implant (50 mg, 90 days release) during incubation. Birds were blood sampled 6 days after implantation. CORT levels were influenced by the interaction between handling stress (baseline: < 4 min vs. stress-induced: 30 min of handling stress) and CORT treatment (LMM on log-transformed plasma CORT:  $F_{1,37.2} = 62.4$ ,  $p < 0.001$ ). Letters indicate significant differences according to post-hoc tests with Tukey adjustments ( $p < 0.030$ ).

### 2.3. Thermal image collection and analysis

Thermal pictures of implanted adults (Fig. 2A) and their egg (Fig. 2B) were taken 3 days after implantation and thermal pictures of chicks (Fig. 2C) were taken at day 20 after hatching (40 to 60 days after CORT or Placebo implant of the focal parent, *i.e.* within the 90-days release given by the CORT pellet manufacturer). Thermal pictures were taken using FLIR E8 thermal imaging camera, Lewden et al., 2020). When approaching the adults and/or chicks, we started a stopwatch when the focal individual showed the first signs of alarm behaviour to measure the duration of our disturbance at the time the different pictures were taken. In adults, we

took a first set of pictures (1 to 2 pictures per individual) before handling, on average (mean  $\pm$  SE)  $38 \pm 4$  sec after the first sign of alarm behaviour at a distance of *ca.* 2 meters from the focal individuals. Adults were then captured-restrained, wound healing was checked, and we took a second set of pictures (1 to 2 pictures per individual) just after terminating handling stress (mean  $\pm$  SE handling stress duration was  $8.3 \pm 0.3$  min; a timeframe within which CORT is known to increase by *ca.* 100% above baseline levels in king penguins (Viblanco et al., 2018). Just after capturing the adult, we removed the single-egg from the brood pouch and immediately took a thermal picture at a distance of *ca.* 0.5 m. We then measured egg length and breadth using callipers and estimated egg volume ( $\text{cm}^3$ ) following Narushin (2005). When chicks were 20 days old, we took a first set of pictures (1 to 2 pictures per individual) at a distance of *ca.* 0.5 m immediately after they were removed from the brood pouch (*i.e.* before handling:  $44 \pm 5$  sec after the first sign of alarm behavior from the parent) and a second set of pictures (1-2 pictures per individual) was taken after handling,  $5.4 \pm 0.3$  min later. Thermal images of the chicks were only taken in non-rainy days, which reduced the sample size from 38 chicks that survived until day 20, to 16 chicks with thermal images available. Chick sex was unknown and body mass was not significantly influenced by the treatment (CORT:  $1.14 \pm 0.11$  vs. Placebo  $1.32 \pm 0.09$ ,  $N = 8/\text{group}$ ,  $t = -1.28$ ,  $p = 0.22$ ). Chicks were kept at ambient temperature ( $14.9 \pm 0.9^\circ\text{C}$ ) between the first and second set of pictures, a temperature at which they are known to be able to maintain their internal body temperature constant (Duchamp et al., 2002). In adults and chicks, we defined the first set of measures taken  $< 1$  minute as ‘before handling’ surface temperatures and the second set of measures taken after  $> 5$  minutes of handling stress as ‘after handling’ surface temperatures.



**Fig. 2: Infrared images of (A) breeding adult, (B) incubated egg and (C) 20 days-old chick**

Thermal images were analysed using the ThermoCAM TM Researcher Pro 2.10 software (Flir systems, Wilsonville, Oregon, USA). Only profile pictures were analysed to avoid surface temperature errors related to head's orientation (Playà-Montmany and Tattersall, 2021; Tabh et al., 2021). For each image we set the emissivity at 0.98 and controlled in the analyses for daily variation in air temperature ( $T_a$ ) and relative humidity using daily climatic measures from a permanent weather station located 2 km inland from the colony ([https://rp5.ru/Archives\\_m%C3%A9t%C3%A9o\\_sur\\_la\\_base\\_Alfred-Faure](https://rp5.ru/Archives_m%C3%A9t%C3%A9o_sur_la_base_Alfred-Faure)). We extracted measures of surface temperatures on maximum eye temperature ( $T_{eye}$ ) and minimum beak temperature ( $T_{beak}$ ) in adults and chicks. We used maximum  $T_{eye}$  as recommended by (Jerem et al., 2015, 2019) and the minimum  $T_{beak}$  to gain insight on the maximum state of vasoconstriction for this body surface. Finally, we estimated egg surface temperature ( $T_{egg}$ ) as the mean of average length and width surface temperature.

#### 2.4. Ethical note

All the procedures were approved by the French Ethical Committee (APAFIS#16465-2018080111195526 v4) and the Terres Australes et Antarctiques Françaises (Arrêté TAAF A-2018-118).

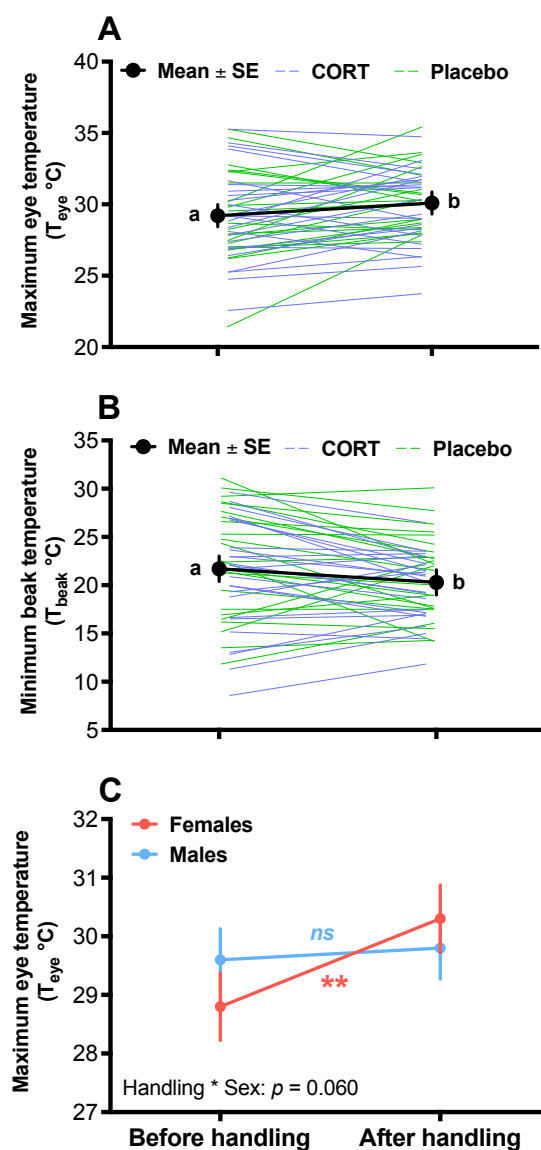
294

## 295 2.5. Statistical analysis

296 We ran separate analyses for adults, eggs and chicks. In adults, we investigated  
 297 variations in  $T_{\text{eye}}$  and  $T_{\text{beak}}$  using two separated linear mixed models (LMMs) where treatment  
 298 (CORT vs. Placebo), handling stress (before vs. after), Sex and  $T_a$  were specified as fixed effects.  
 299 Relative humidity was initially tested but removed from final models since it was never  
 300 significant. Two-ways interactions were also initially included but removed from final model if  
 301  $p > 0.10$  using a backward stepwise procedure. The p-values (just before removal in the  
 302 backward stepwise procedure) for the focal interaction between treatment and handling  
 303 stress are reported in tables. Bird identity was included as a random intercept to control for  
 304 repeated measures. We investigated variation in  $T_{\text{egg}}$  by entering parental CORT treatment  
 305 (CORT vs. Placebo), the sex of the parent,  $T_a$  and egg volume as fixed effects. We investigated  
 306 variation chick  $T_{\text{eye}}$  and  $T_{\text{beak}}$  by entering parental treatment (CORT vs. Placebo), the category  
 307 of the brooding parent at the time of measurement (implanted individual vs. non-implanted  
 308 partner), handling stress (before vs. after), and  $T_a$  as fixed effects, and chick identity as a  
 309 random intercept. Interactions were treated as described above for adults. All statistical  
 310 analyses were conducted using R Studio (version 3.6.2) and *lme4*, *emmeans* and *pbkrtest*  
 311 packages. Results are reported as least-square means  $\pm$  SE.

### 3. Results

#### 3.1. Adult surface temperatures



**Fig. 3: Adult king penguin surface temperature responses to an experimental manipulation of glucocorticoid levels (CORT vs. Placebo subcutaneous implants) and handling stress: (A) Maximum eye temperature response to handling stress, (B) Minimum beak temperature response to handling stress, and (C) Sex-specific response of eye temperature to handling stress.** In panels A and B, CORT (blue) vs. Placebo (green) individual responses (raw data) are presented. Since CORT treatment had no significant effect either alone or in interaction with handling stress (see Table 2 for details), the overall mean  $\pm$  SE is shown in black. Least-square means  $\pm$  SE from final statistical models (Table 2) are presented. Different letters indicate significant differences in panels A and B, and \*\* in panel C represent the significant increase of  $T_{eye}$  observed only in females.



Treatment (CORT vs. Placebo), either alone or in interaction with handling stress, had no significant effect on eye ( $T_{eye}$ ) or beak ( $T_{beak}$ ) surface temperature in incubating adults (Table 2, Fig. 3A and 3B). Handling stress significantly affected  $T_{eye}$  and  $T_{beak}$ , in opposite directions (Table 2): over an 8-min handling stress,  $T_{eye}$  increased by  $0.8 \pm 0.3^{\circ}\text{C}$ , on average (Fig. 3A), while  $T_{beak}$  decreased by  $-1.5 \pm 0.4^{\circ}\text{C}$ , on average (Fig. 3B). The increase in  $T_{eye}$  appeared to be sex-specific (marginally significant interaction Handling \* Sex:  $p = 0.060$ ), with only females showing a significant increase in  $T_{eye}$  in response to handling stress (Fig. 3C; males:  $t = 0.63$ ,  $p = 0.53$ ; females:  $t = 3.08$ ,  $p = 0.003$ ). As expected,  $T_{eye}$  and  $T_{beak}$  were significantly and positively associated with  $T_a$  (Table 2).

**Table 2: Summary of linear mixed models (LMMs) investigating the determinants of (A) maximum eye temperature ( $T_{eye}$ ) and (B) minimum beak temperature ( $T_{beak}$ ) in adult king penguins.**

A. $T_{eye}$ (n = 138 observations; N = 49 individuals)						
Random effects:		Variance				
Bird ID	Intercept	5.04				
Residual		3.21				
Fixed effects:		Estimate	Std. Error	t	df	p
Intercept		24.94	1.71	14.54	47.9	<0.001
Treatment (CORT)		-0.10	0.72	-0.15	45.8	0.89
Handling (after)		<b>1.45</b>	<b>0.47</b>	<b>3.04</b>	<b>89.3</b>	<b>0.003</b>
Sex (Male)		0.76	0.78	0.97	63.7	0.34
$T_a$		<b>0.29</b>	<b>0.13</b>	<b>2.33</b>	<b>45.0</b>	<b>0.024</b>
Handling*Sex		-1.19	0.63	-1.90	89.7	0.060
(Treatment x Handling)						(0.44)

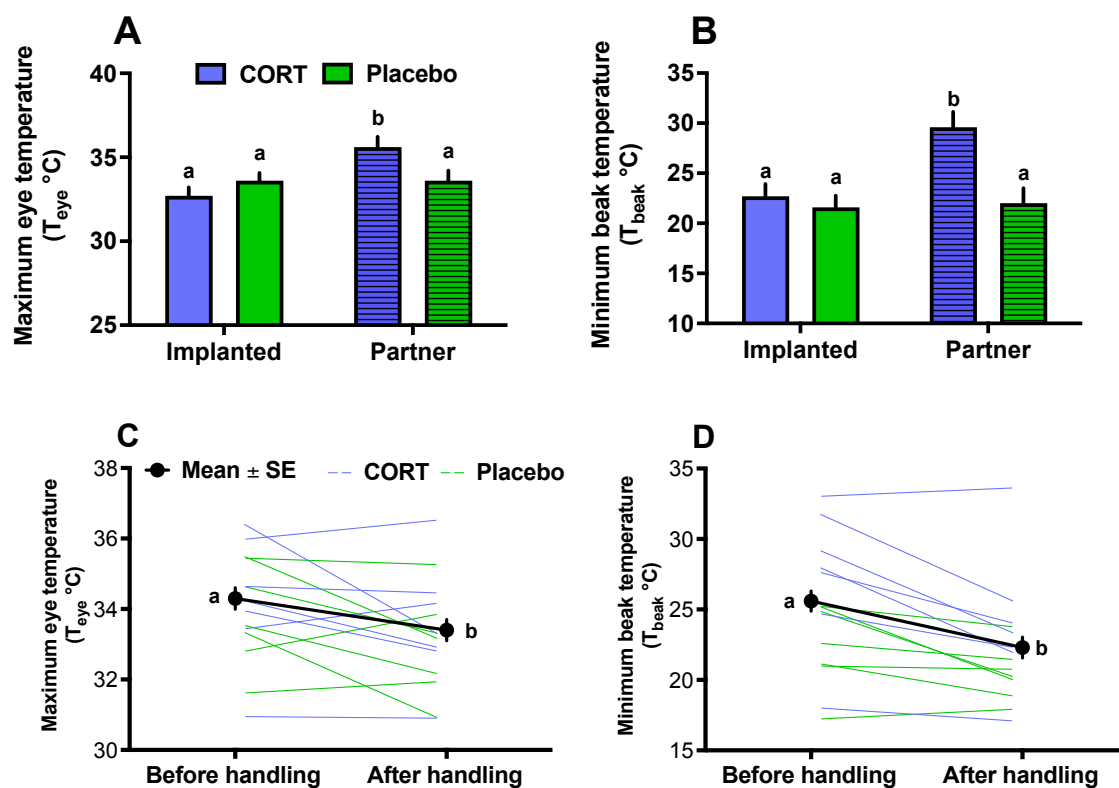
  

B. $T_{beak}$ (n = 138 observations; N = 49 individuals)						
Random effects:		Variance				
Bird ID	Intercept	15.91				
Residual		6.40				
Fixed effects:		Estimate	Std. Error	t	df	p
Intercept		13.84	2.92	4.74	45.0	<0.001
Treatment (CORT)		-1.13	1.24	-0.91	44.5	0.37
Handling (after)		<b>-1.47</b>	<b>0.45</b>	<b>-3.29</b>	<b>84.6</b>	<b>0.001</b>
Sex (Male)		0.90	1.23	0.73	44.6	0.47
$T_a$		<b>0.54</b>	<b>0.22</b>	<b>2.45</b>	<b>43.8</b>	<b>0.018</b>
(Treatment x Handling)						(0.61)

### 3.2. Egg surface temperature

Neither parental treatment ( $F_{1,45} = 1.38$ ,  $p = 0.25$ ; CORT:  $34.5 \pm 0.2$  vs. Placebo:  $34.9 \pm 0.3^\circ\text{C}$ ;  $N = 46$ ), sex ( $F_{1,45} = 0.47$ ,  $p = 0.49$ ), ambient temperature ( $F_{1,45} = 0.45$ ,  $p = 0.50$ ), nor egg volume ( $F_{1,45} = 0.01$ ,  $p = 0.93$ ) significantly affected  $T_{\text{egg}}$ .

### 3.3. Chick surface temperatures



**Fig. 4:** King penguin chick surface temperature in response to parental glucocorticoid manipulation and handling: (A) Maximum eye temperature according to parental treatment and category of the parent brooding the chick (*i.e.* partners are not implanted), (B) Minimum beak temperature according to parental treatment and category of the parent brooding the chick, (C) Maximum eye temperature response to handling, and (D) Minimum eye temperature response to handling. In panels C and D, CORT (blue) vs. Placebo (green) chick's individual responses (raw data) are presented, but only the global mean  $\pm$  SE are presented since there was no significant interaction between parental CORT treatment and handling (see Table 3 for details). Least-square means  $\pm$  SE from final statistical models (Table 3) are presented. Different letters indicate significant differences.

The interaction between parental treatment (CORT vs. Placebo) and the category of the parent brooding the chick (implanted vs. non-implanted partner) at the time of measurement significantly affected chick  $T_{eye}$  ( $F_{1,11} = 6.37$ ,  $p = 0.028$ , Table 3) and chick  $T_{beak}$  ( $F_{1,11} = 5.49$ ,  $p = 0.039$ , Table 3). Chicks brooded by the partner of CORT-implanted individuals had higher overall  $T_{eye}$  and  $T_{beak}$  than chicks brooded by CORT parents, or by Placebo and associated partners (Fig 4A and 4B). While we found no significant interaction between parental treatment and Handling (Table 3), both chick  $T_{eye}$  (Fig 4C) and chick  $T_{beak}$  (Fig 4D) significantly decreased after handling (by  $-0.9 \pm 0.3^{\circ}\text{C}$  and  $-3.3 \pm 0.6^{\circ}\text{C}$  respectively, Table 3). As expected, chick  $T_{eye}$  and  $T_{beak}$  were significantly positively associated with  $T_a$  (Table 3).

**Table 3: Summary of linear mixed models (LMMs) investigating the determinants of (A) maximum eye temperature ( $T_{eye}$ ) and (B) minimum beak temperature ( $T_{beak}$ ) in king penguin chicks.** Only one parent was implanted with CORT or Placebo implant, thus we considered both parental treatment (CORT vs. Placebo), the category of the parent brooding the chick at the time of measurement (implanted parent vs. non-implanted partner) and their interaction in our analyses.

**A.  $T_{eye}$  (n = 63 observations; N = 16 chicks)**

Random effects:		Variance				
Bird ID	Intercept	0.81				
Residual		1.18				
Fixed effects:		Estimate	Std. Error	t	df	p
Intercept		29.47	1.39	21.26	11.1	<0.001
Treatment (CORT)		-0.88	0.68	-1.29	11.3	0.22
Parent (implanted)		-2.84	0.82	3.48	11.1	0.005
Handling (after)		-0.85	0.28	-3.08	47.2	0.003
$T_a$		0.21	0.09	2.41	10.9	0.035
Treatment*Parent		2.78	1.10	2.53	11.0	0.028
(Treatment x Handling)						(0.79)

**B.  $T_{beak}$  (n = 63 observations; N = 16 chicks)**

Random effects:		Variance				
Bird ID	Intercept	5.53				
Residual		4.89				
Fixed effects:		Estimate	Std. Error	t	df	p
Intercept		13.55	3.42	3.96	11.1	0.002
Treatment (CORT)		1.15	1.68	0.69	11.3	0.51
Parent (implanted)		-6.82	2.02	-3.38	11.1	0.006
Handling (after)		-3.26	0.56	-5.77	46.9	<0.001
T <sub>a</sub>		0.57	0.21	2.65	11.0	0.023
Treatment*Parent		6.37	2.72	2.34	11.0	0.039
(Treatment x Handling)						(0.94)

## 4. Discussion

By experimentally manipulating CORT levels in adult breeding king penguins, we show that glucocorticoids are unlikely to play a major role in determining changes in surface temperatures (both before or in response to handling stress) in this large seabird species. Indeed, we found no significant difference in baseline surface temperature between CORT and Placebo implanted individuals. However, whatever the parental CORT treatment, our handling stress protocol led to an increase in  $T_{eye}$  and a decrease in  $T_{beak}$  in response to capture and handling. The contrasting results between parental treatment and handling stress suggest that changes in surface temperature are probably driven primarily by the activity of the SAM axis in king penguins (but see Ouyang et al. (2021), for a potential causal involvement of CORT in house sparrows *Passer domesticus*). Contrary to our prediction, parental treatment had no significant effect on egg temperature. Yet, we found an unexpected pattern on chick surface temperatures: chicks brooded by the non-implanted partner of CORT individuals had higher surface temperatures (both  $T_{eye}$  and  $T_{beak}$ ) than chicks brooded by Placebo (or associated non-implanted partner) or CORT individuals. Finally, in the thermally non-emancipated chicks, both  $T_{eye}$  and  $T_{beak}$  decreased with handling, but irrespectively of the parental treatment.

### 4.1. CORT and adult surface temperatures

Previous correlative studies have considered baseline and stress-induced CORT levels to investigate the relationship between GC signalling and changes in body surface temperature in birds (blue tit, *Cyanistes caeruleus*, Jerem et al. (2018); domestic chicken, *Gallus gallus domesticus*, Giloh et al. (2012); Weimer et al. (2020)). To go deeper in the involved mechanisms, we experimentally manipulated CORT in king penguin to help to causally distinguish the contribution of the HPA and SAM axes to changes in body surface temperature. Since we did not detect any significant effect of CORT manipulation on baseline or stress-induced surface temperatures, our results provide little support for a causal involvement of the HPA in mediating stress-related changes in surface temperature.

Our results contrast with a recent study showing that an acute stimulation of the HPA axis with ACTH induced a decrease in  $T_{eye}$  within 5 min in captive-held house sparrows (Ouyang et al., 2021). While we used an experimental approach and the lack of significant effect of baseline CORT on body surface temperatures appears to be robust, we cannot fully exclude

that our handling stress protocol for thermal imaging was too short to detect a potential role of stress-induced CORT in influencing changes in surface temperatures, since in 8 minutes of handling, CORT levels are expected to double in king penguin, while the maximum CORT levels are only attained after *ca.* 80 minutes of handling in our study species (Viblanco et al., 2018). Overall, our results suggest that changes in surface temperature are probably more likely mediated by the SAM rather than the HPA in adult king penguins. Future studies testing for a direct acute stimulation of the HPA axis (using ACTH) or SAM axis (using catecholamines) in the king penguin and other bird species could be useful to gain greater insight on the contribution of the HPA and SAM axes in mediating changes in body surface temperature (Ouyang et al., 2021).

#### 4.2. Stress-induced changes in surface temperatures

In breeding adults, we observed a mild (sex-specific) significant increase of  $T_{eye}$  and a significant decrease of  $T_{beak}$  in response to acute capture-handling stress. Differences in temperature change between body regions in response to acute stress are not rare (see Table 1). For instance, Moe et al. (2017) measured a decrease of  $T_{eye}$  contrasting with an increase of  $T_{feet}$  in chicken, whereas in captive budgerigars (*Melopsittacus undulatus*)  $T_{eye}$  increased transiently after exposure to a stressor while  $T_{feet}$  did not change (Ikkatai and Watanabe, 2015); Table 1). Anatomical and functional reasons can likely explain the difference of stress-induced changes in surface temperature between body regions in penguins, with for instance a potential interest in maintaining or increasing eye blood flow to maintain or enhance visual acuity when acutely threatened by an environmental challenge.

$T_{eye}$  has also been shown to positively correlate with internal (cloacal) temperature in various avian species (budgerigars: Ikkatai and Watanabe (2015); chicken: Cândido et al. (2020)). Consequently, it is possible that the increase in  $T_{eye}$  we observe in adults could reflect an increase in internal body temperature (Oka, 2018). Consistently, in closely related Emperor penguins (*Aptenodytes forsteri*), acute stress leads to an approximate 1.5°C increase in internal (stomach) temperature (Regel and Pütz, 1997). Such an increase in  $T_{eye}$  in response to handling stress has been reported two times in avian species to the best of our knowledge (Ikkatai and Watanabe, 2015; Jakubas et al., 2022), while most previous studies have shown either a significant decrease (6 studies) or no significant change (2 studies; Table 1). Those contrasted findings might be explained by at least three factors: 1. variation in body size, since

smaller individuals/species are expected to favour internal heat conservation by reducing more markedly peripheral blood flow and surface temperature compared to large ones (*thermoprotective hypothesis*; Robertson et al., 2020a); 2. differences in the thermal environment, since it has been shown that during acute stress response, heat conservation is favoured below the thermoneutral zone while heat dissipation is favoured above its upper limit (Robertson et al., 2020a); 3. the various delays at which  $T_{eye}$  was measured in response to acute stress, since for instance the increase in  $T_{eye}$  found by Ikkatai and Watanabe (2015) was short-lived (visible 5 min after the stressor, but not later on). Although the interaction between handling stress and sex was only marginally significant ( $p = 0.060$ ), it appeared that only females exhibited a noticeable increase in  $T_{eye}$  in response to handling stress in our study. There are known sex-differences in stress physiology (Handa and McGivern, 2017), but sex differences in surface temperature changes induced by acute stress have been rarely investigated (but see Robertson et al. (2020a), for a result opposite to ours between sexes). We previously observed no sex effect in HPA responsiveness between males and females king penguins (Vibanc et al., 2016), but as mentioned above SAM is the likely driver of acute changes in surface temperatures. Consequently, this suggests that sexes might differ in the stress-sensitivity of their SAM, although this would need to be confirmed by direct measurements of the SAM activity.

The decrease we observe in  $T_{beak}$  in response to acute stress in adults appears like the typical peripheral vasoconstriction response previously reported for instance by Tabh et al. (2021). Such peripheral vasoconstriction enables the redistribution of the peripheral blood circulation to internal organs and tissues, such as the brain or muscles, favouring their oxygenation and nutrition to sustain the fight-or-flight response. King penguins breed in a highly dense and aggressive colonial environment (up to 100 aggressive interactions per hour; Côté, 2000). Aggressive interactions, frequently leading to injuries, are known to result in increased heart rates (Vibanc et al., 2012). A decrease of  $T_{beak}$  during acute stress response likely reflects peripheral vasoconstriction, that is likely widespread to other peripheral body parts, which according to the *haemoprotective hypothesis* could help to reduce blood loss in case of injury (Robertson et al., 2020a).

The changes observed in surface temperatures in chicks differ from those found in adults. Indeed, we observed a significant decrease in chick's  $T_{eye}$  whereas  $T_{eye}$  increased in adult females and stayed stable in adult males. While  $T_{beak}$  significantly decreased as observed

in adults, the decrease was even more pronounced in chicks ( $-3.3 \pm 0.6^{\circ}\text{C}$  vs.  $-1.5 \pm 0.4^{\circ}\text{C}$ , in chicks and adults, respectively). This is likely explained by greater peripheral thermal losses in chicks suddenly exposed to an ambient temperature drop (*ca.*  $35^{\circ}\text{C}$  under the brood pouch of the adults vs.  $15^{\circ}\text{C}$  when taken out for measurement and handling), despite their ability to maintain their internal temperature at this stage and ambient temperature (Duchamp et al., 2002). The more pronounced drop in surface temperature we observed in chicks during acute handling could reflect their greater need to conserve heat at a relatively mild  $T_a$  compared to adults.

#### 4.3. Impact of parental CORT on egg and chick surface temperature

Contrary to our prediction, we observed no significant impact of parental CORT treatment on egg temperature, suggesting little alteration of incubation behaviour by increased CORT levels, contrary to a previous study in Adelie penguin reporting a reduction of  $1.3 \pm 0.2^{\circ}\text{C}$  in  $T_{\text{egg}}$  (Thierry et al., 2013a). It is possible that incubating two eggs in the colder environment of Adélie penguins is more challenging (and thus more likely to be impacted by high CORT levels) than incubating a single egg under the milder climate experienced by king penguins. Alternatively, it is also possible that king penguins are more resilient to increased CORT levels than Adelie penguins, which is supported by the good reproductive success of king penguins implanted with CORT (Stier et al. unpublished), contrary to what has been observed in Adelie penguins (Thierry et al., 2013b). Measurements of incubation quality using a dummy egg with temperature and rotation sensors (Thierry et al., 2013a) may provide more accurate data on this question, but it is important to note that the  $T_{\text{egg}}$  measured in this study (Placebo:  $34.9 \pm 0.3^{\circ}\text{C}$ ) was close to the  $T_{\text{egg}}$  measured in the same penguin colony using internal sensors in dummy eggs ( $35.7 \pm 0.4^{\circ}\text{C}$ ; Groscolas et al., 2000). Our results therefore suggest that further studies may benefit from the use of minimally invasive thermal imaging to measure incubation quality, for instance in the context of parental behaviour and climate change (Cook et al., 2020).

The measure of chick surface temperatures revealed some surprising patterns. While we expected CORT chicks to have lower surface temperatures due to poorer parental care (less efficient brooding), we found the opposite result, but only when chicks were brooded by the non-implanted partner of the CORT-parent. This result should be considered with caution considering the limited sample size available for chick surface temperatures ( $N = 16$ ). One



possibility is that partners from CORT-implanted individuals somehow perceived the ‘stress’ levels of their partner (as shown between siblings; Noguera et al., 2017) and somehow compensated for parental care through more efficient brooding. This hypothesis requires further study to determine whether the concept of ‘family-transmitted stress’ (Noguera et al., 2017) applies to the king penguin. In this species, both parents must rely on each other over more than one year to successfully fledge their single chick, which itself is entirely dependent on its parents for its food supply throughout its growth. Hence, the king penguin could be promising system to investigate the ‘family-transmitted stress’ hypothesis.

#### 4.4. Conclusion

Evaluating stress levels and responses of free-living animals in a non-invasive manner using thermal imaging is a burgeoning field of research (Jerem et al., 2015; Tabh et al., 2022). However, little is still known about the underlying pathways of stress physiology that influence the changes in surface temperature. Our experimental study in king penguin points toward a likely preponderant role of the SAM. Additionally, by summarizing the current evidence for acute changes in surface temperatures during stress exposure (Table 1), we highlight the relative complexity and inconsistency between the effects observed by different studies. Further experimental studies related to SAM and HPA involvement are thus required to clarify what stress component(s) we are measuring through non-invasive thermal imaging.

## 5. Declarations

### 5.1. Author contribution

Study design: AS, VAV, PB and AL. Funding acquisition: JPR, PB, VAV, AS. Data collection in the field: AS, SA, TH, LA, CG, and JPR. Data collection in the lab: ER and AS. Data analysis: CW, AL, PB, AS. Writing original draft: AL, PB and AS. Writing review and editing: VAV, JPR, AN, SA, TH, LA, ER and CG.

### 5.2 Data availability



The datasets used in this manuscript are available on *FigShare* through a private link for review purposes (<https://figshare.com/s/73b39dff978b0dfd2545>), and public access with the following doi (10.6084/m9.figshare.20134181) will be enabled pending acceptance of the manuscript.

### 5.3. Acknowledgments

We are grateful to the French Polar Institute (IPEV) and the Terres Australes et Antarctiques Françaises for providing financial and logistical support for this study through the polar program #119 (ECONERGY). We wish to thank the Zone Atelier Antarctique et Terres Australes (ZATA) from the CNRS for financial support, and the members of the Alfred Faure field station for their help and support in the field. AL was supported by ISblue project, Interdisciplinary graduate school for the blue planet (ANR-17-EURE-0015) and co-funded by a grant from the French government under the program "Investissements d'Avenir" embedded in France 2030. AS was supported by a 'Turku Collegium for Science and Medicine' Fellowship and a Marie Skłodowska-Curie Postdoctoral Fellowships (#894963).

### 5.4. Competing interests

Antoine Stier is an Associate Editor of *J Thermal Biol* but took no part in the peer review and decision-making processes for this research article.

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