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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63 ## 1. Introduction

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

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

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

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

Table 1: **Summary table of avian studies testing the impact of acute stress exposure on changes in body surface temperatures measured with thermal imaging** (studies related to heat and food stress are not included). *Area* corresponds to the body region being measured, response to the direction of the temperature response (=: no significant change, ↘: significant decrease and ↗: significant increase, *Δ T°C* to the amplitude of the temperature response, *stressor* the nature and duration of the experimental stressor. Some studies measured temperature responses at different time points, and when the effects of stress exposure change through time, the responses are shown with consecutive symbols (*e.g.* =, ↗: initial absence of change followed later on by an increase in surface temperature).

Species	Condition	State	Stressor	Area	Response	Δ T°C	Ref.
Blue tit	Wild	Adult	Trap closure + handling	T_{eye}	↘, ↗,=	-1.0, +1.0°C, =	Jerem et al. 2019
Cyaniste caerulescens			(3min)				
Budgerigars	Captive	Adult	Handling	T_{eye}	↗, =	+0.7°C, =	Ikkatai and Watanabe 2015
Melopsittacus undulatus			(30 min)	T_{legs}	=	=	
Chicken	Captive	Adult	Air puff	T_{eye}	↘	-1.0°C	Edgar et al. 2011
Gallus gallus domesticus			(10 min)				
Chicken	Captive	Adult	Handling	T_{eye}	↘, =	-0.5°C, =	Edgar et al. 2013
Gallus gallus domesticus			(20 min)	T_{head}	=, ↗	=, +1.0°C	
				T_{comb}	↘, =	-1.5°C, =	
Chicken	Captive	Chick	Air puff	T_{eye}	↘	-1.0°C	Edgar et al. 2018
Gallus gallus domesticus		(9 weeks old)	(10 min)				
Chicken	Captive	Adult	Handling: mild/severe	T_{eye}	↘	-0.4°C	Herborn et al. 2015
Gallus gallus domesticus				T_{face}	=	=	
			(20 min)	T_{wattle}	↘	-0.7 / -1.3°C	
				T_{comb}	↘	-0.5 / -2.2°C	
Chicken	Captive	Adult	Enrichment removal	T_{eye}	↘	-0.8°C	Herborn et al. 2018
Gallus gallus domesticus			(within 2h)	T_{face}	↘	-0.7°C	
				T_{comb}	↘	-3.5°C	
Chicken	Captive	Chick	Handling	T_{head}	↗	+0.76°C	Moe et al. 2017
Gallus gallus domesticus		(30 days old)	(10 min)	T_{feet}	↘	-0.45°C	
Chicken	Captive	Chick	Visual	T_{eye}	=	=	Pijpers et al. 2022
Gallus gallus domesticus		(9 days old)	(45 min)	T_{beak}	=, ↗	+0.5°C	
Domestic pigeon	Captive	Adult	Handling	T_{eye}	=	-0.4°C	Tabh et al. 2021
Columbia livia domestica			(3.5 min)	T_{beak}	↘	-2.1°C	
King penguin	Wild	Adult	Handling	T_{eye}	↗	+0.8°C	This study
Aptenodytes patagonicus			(5-10 min)	T_{beak}	↘	-1.5°C	
	Wild	Chick	Handling	T_{eye}	↘	-0.9°C	
		(20 days old*)	(5-10 min)	T_{beak}	↘	-3.3°C	
Little Auk *Alle alle*	Wild	Adult	Handling (30 min)	T_{eye}	↗	+1,7°C	Jakubas et al. 2022
Pheasant	Captive	Juvenile	Agression	T_{head}	↗	+0.6°C	Knoch et al. 2022
Phasianus colchicus		(42 to 49 days old)	(20 s)				
Song sparrow	Captive	Adult	Handling	T_{eye}	↘, =	-1.5°C, =	Zuluaga et al. 2023
Melospiza melodia			(13 min)	T_{beak}	↘, ↗,=	-2.6°C, +2.6°C, =	
* king penguins chicks are not thermally emancipated at this age							

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

143 In this study, we took up the challenge of distinguishing the role of HPA and SAM on
144 changes in body surface temperatures using an experimental approach in adult king penguins
145 (*Aptenodytes patagonicus*). The king penguin is a heterothermic bird whose body temperature
146 largely varies between core and peripheral tissues (Lewden et al., 2017a, 2017b) . Its thick
147 body plumage provides a very efficient insulation, while counter-current vascular heat
148 exchangers in the appendages allow to decrease peripheral temperature loss (Thomas et al.,
149 2011). These adaptations allow birds to conserve heat when foraging in cold Antarctic waters
150 (Handrich et al., 1997), and to rapidly recover to normothermia when exiting the water
151 (Lewden et al., 2020). Thermal imaging has been frequently used in the last years to
152 investigate acute (see an overview in Table 1) and chronic (Herborn et al., 2018; Robertson et
153 al., 2020a, 2020b; Tabh et al., 2022; Winder et al., 2020) stress-response in birds. Because of
154 their dense and watertight plumage, the use of thermal imaging in penguins is restricted to
155 measuring bare parts of the body, such as the eye (periorbital region) and beak (Lewden et
156 al., 2020). These regions are highly vascularised and provide valuable information on body
157 surface temperatures.

158 Breeding king penguins were subcutaneously implanted with either a corticosterone
159 (CORT) implant or a placebo implant. The use of CORT implants in king penguin (see methods
160 and Fig. 1), as in many other bird species (see Torres-Medina et al., 2018), leads to higher
161 baseline GC (or CORT) levels, while inhibiting the acute CORT responses in the days following
162 implantation. Indeed, CORT-implanted birds no longer exhibit high blood CORT release in
163 response to acute stress induced by a handling stress protocol. Hence, the use of CORT
164 implants in conjunction with handling stress allows for the separation of the contributions of
165 the HPA and SAM axes to acute changes in body surface temperatures. Here, we therefore
166 measured the effects of our CORT treatment, together with a handling stress protocol, on
167 surface temperatures in the king penguin. If changes in surface temperatures are mainly
168 driven by activating the HPA axis, we expect to find higher initial body surface temperatures
169 in CORT-implanted individuals (with elevated baseline CORT) prior to capture and handling.
170 However, because CORT implants shut-down the release of CORT in response to handling, we
171 expect to find no change in body surface temperature in response to handling stress in CORT
172 individuals if such changes are mediated by the HPA only. On the contrary, if changes in
173 surface temperatures are mainly driven by the activity of the SAM axis, we expect our CORT
174 treatment to have no additional effect on body surface temperatures (both before or after
175 capture and handling). We also measured egg surface temperature on the same day as the
176 body surface temperatures of implanted parents and the surface temperatures of chicks at
177 day 20 after hatching, before they were fully thermally emancipated. Based on experimental
178 data in Adélie penguin showing that elevated baseline CORT led to lower parental care (Thierry
179 et al., 2013a), we hypothesized that increasing CORT levels may lower the surface
180 temperatures of eggs and/or chicks during incubation and brooding respectively, as parents
181 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

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

213 On the day of the implantation, adults were captured while incubating their egg and
214 restrained by one experimenter with a hood covering the head to keep them calm. Sub-
215 cutaneous CORT or placebo implants were inserted in the upper part of the back by a
216 veterinary surgeon (SA) under local anaesthesia (*ca.* 0.5mg/kg xylocaine and 0.0001mg/kg
217 adrenalin, Aspen Pharma). Immediately after implantation, the small incision was closed using
218 3 sterile surgical staples, and a prophylactic dose of antibiotics (cephalexin *ca.* 50mg/kg,
219 Rilexine®, Virbac) was injected to prevent any risk of infection. The wound was checked 3 days
220 later, when taking the thermal images without any sign of infection on individuals, nor implant
221 rejection, and staples were removed 6 days after implantation.

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

228 sample was 8.08%. Implants were successful in raising circulating baseline CORT levels and in

229 inhibiting stress-induced CORT release (Fig. 1).

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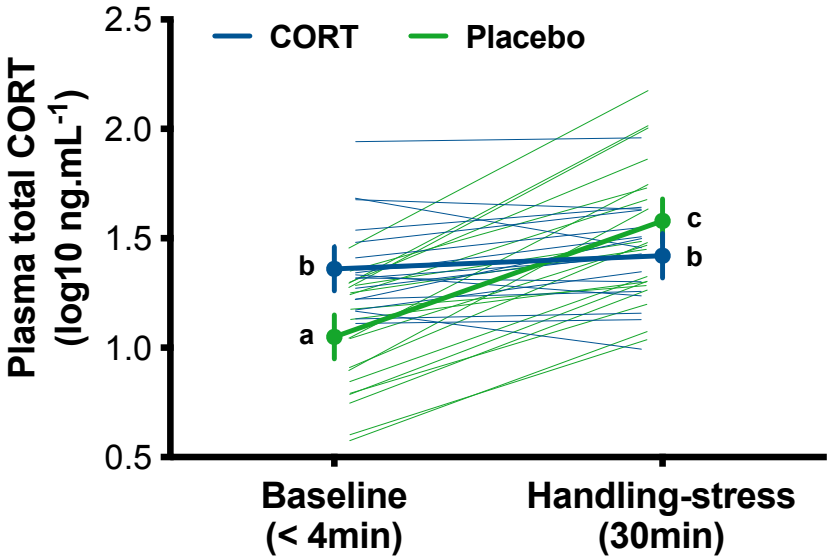

237 **Fig. 1**: **Baseline and stress-induced plasma corticosterone levels of breeding king penguins** (N = 20
238 individuals per group) implanted either with a Placebo, or a CORT subcutaneous implant (50 mg, 90
239 days release) during incubation. Birds were blood sampled 6 days after implantation.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

243

244 *2.3. Thermal image collection and analysis*

245 Thermal pictures of implanted adults (Fig. 2A) and their egg (Fig. 2B) were taken 3 days

246 after implantation and thermal pictures of chicks (Fig. 2C) were taken at day 20 after hatching

247 (40 to 60 days after CORT or Placebo implant of the focal parent, *i.e.* within the 90-days release

248 given by the CORT pellet manufacturer). Thermal pictures were taken using FLIR E8 thermal

249 imaging camera, Lewden et al., 2020). When approaching the adults and/or chicks, we started

250 a stopwatch when the focal individual showed the first signs of alarm behaviour to measure

251 the duration of our disturbance at the time the different pictures were taken. In adults, we

252 took a first set of pictures (1 to 2 pictures per individual) before handling, on average (mean ±
253 SE) 38 ± 4 sec after the first sign of alarm behaviour at a distance of *ca*. 2 meters from the
254 focal individuals. Adults were then captured-restrained, wound healing was checked, and we
255 took a second set of pictures (1 to 2 pictures per individual) just after terminating handling
256 stress (mean ± SE handling stress duration was 8.3 ± 0.3 min; a timeframe within which CORT
257 is known to increase by *ca.* 100% above baseline levels in king penguins (Viblanc et al., 2018).
258 Just after capturing the adult, we removed the single-egg from the brood pouch and
259 immediately took a thermal picture at a distance of *ca.* 0.5 m. We then measured egg length
260 and breadth using callipers and estimated egg volume (cm^3) following Narushin (2005). When
261 chicks were 20 days old, we took a first set of pictures (1 to 2 pictures per individual) at a
262 distance of *ca.* 0.5 m immediately after they were removed from the brood pouch (*i.e.* before
263 handling: 44 ± 5 sec after the first sign of alarm behavior from the parent) and a second set of
264 pictures (1-2 pictures per individual) was taken after handling, 5.4 ± 0.3 min later. Thermal
265 images of the chicks were only taken in non-rainy days, which reduced the sample size from
266 38 chicks that survived until day 20, to 16 chicks with thermal images available. Chick sex was
267 unknown and body mass was not significantly influenced by the treatment (CORT: 1.14 ± 0.11
268 vs. Placebo 1.32 ± 0.09, N = 8/group, t = -1.28, p = 0.22). Chicks were kept at ambient
269 temperature (14.9 ± 0.9°C) between the first and second set of pictures, a temperature at
270 which they are known to be able to maintain their internal body temperature constant
271 (Duchamp et al., 2002). In adults and chicks, we defined the first set of measures taken < 1
272 minute as 'before handling' surface temperatures and the second set of measures taken after
273 > 5 minutes of handling stress as 'after handling' surface temperatures.

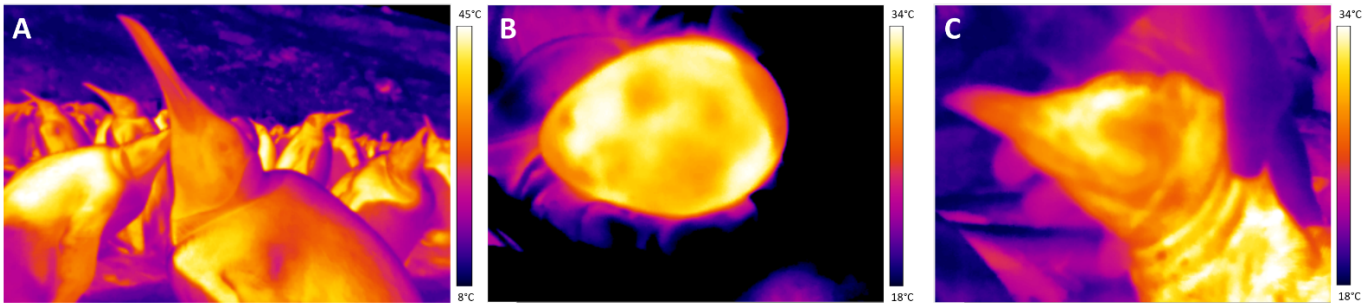

Fig. 2: **Infrared images of (A) breeding adult, (B) incubated egg and (C) 20 days-old chick**

Thermal images were analysed using the ThermaCAM 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). 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).

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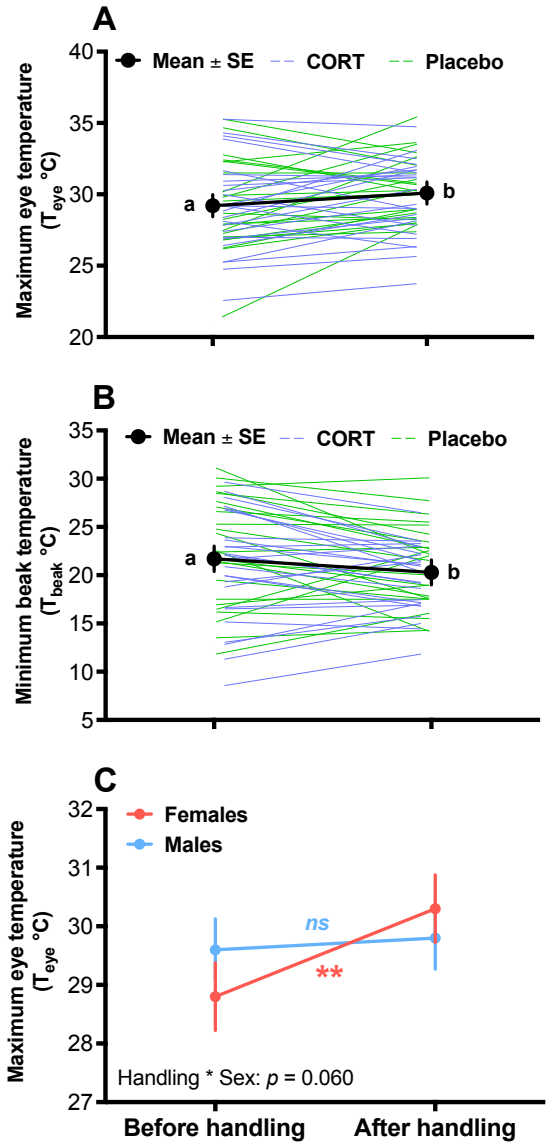
295 *2.5. Statistical analysis*

296 We ran separate analyses for adults, eggs and chicks. In adults, we investigated
297 variations in T_{eye} and T_{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_{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_{eye} and T_{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 ± SE.

312 # 3. Results

313 *3.1. Adult surface temperatures*



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315 **Fig. 3**: **Adult king penguin surface temperature responses to an experimental manipulation of**
316 **glucocorticoid levels (CORT *vs.* Placebo subcutaneous implants) and handling stress: (A) Maximum**
317 **eye temperature response to handling stress, (B) Minimum beak temperature response to handling**
318 **stress, and (C) Sex-specific response of eye temperature to handling stress.** In panels A and B, CORT
319 (blue) *vs.* Placebo (green) individual responses (raw data) are presented. Since CORT treatment had no
320 significant effect either alone or in interaction with handling stress (see Table 2 for details), the overall
321 mean ± SE is shown in black. Least-square means ± SE from final statistical models (Table 2) are
322 presented. Different letters indicate significant differences in panels A and B, and ** in panel C
323 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 ± 0.3°C, on average (Fig. 3A), while T_{beak} decreased by -1.5 ± 0.4°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)	**1.45**	**0.47**	**3.04**	**89.3**	**0.003**
Sex (Male)	0.76	0.78	0.97	63.7	0.34
T_a	**0.29**	**0.13**	**2.33**	**45.0**	**0.024**
*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)

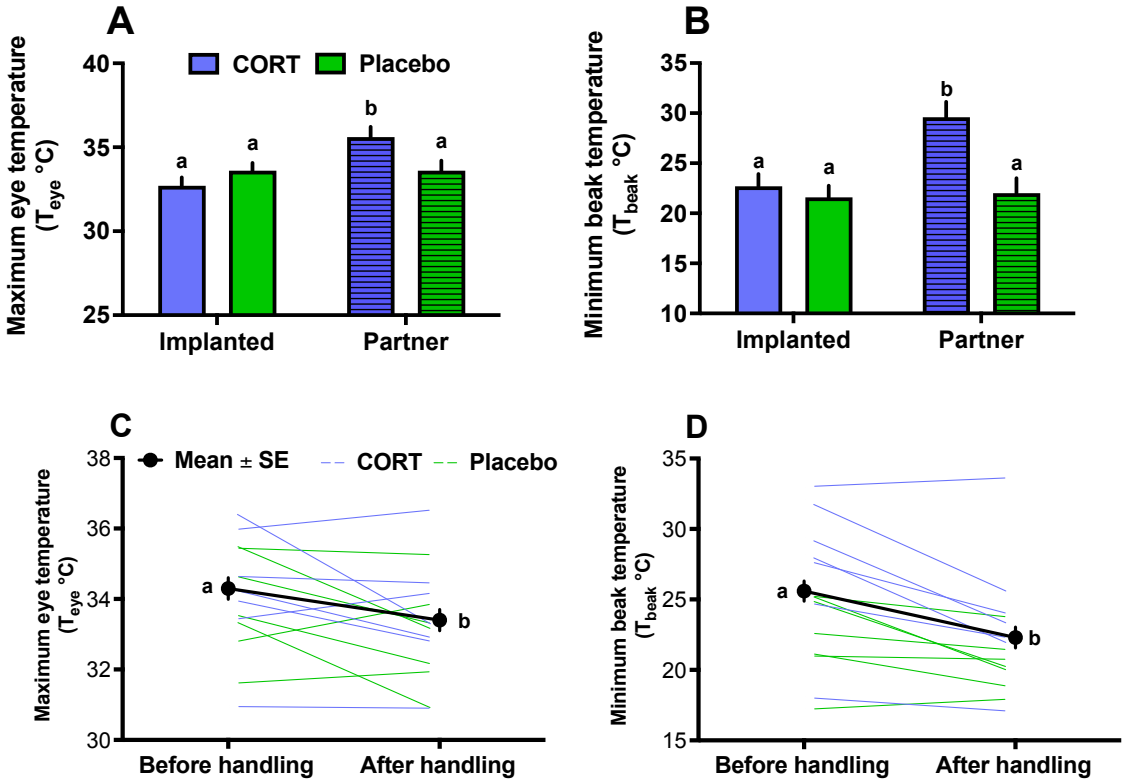
Random effects:		**Variance**			
Bird ID	Intercept	15.91			
Residual		6.40			
Fixed effects:	**Estimate**	**Std. Error**	**t**	**df**	**p**
Intercept	13.84	2.92	4.74	45.0	<0.001
Treatment (CORT)	-1.13	1.24	-0.91	44.5	0.37
Handling (after)	**-1.47**	**0.45**	**-3.29**	**84.6**	**0.001**
Sex (Male)	0.90	1.23	0.73	44.6	0.47
T_a	**0.54**	**0.22**	**2.45**	**43.8**	**0.018**
(Treatment x Handling)					(0.61)

336 *3.2. Egg surface temperature*

337 Neither parental treatment ($F_{1,45}$ = 1.38, p = 0.25; CORT: 34.5 ± 0.2 *vs.* Placebo: 34.9 ±
338 0.3°C; N = 46), sex ($F_{1,45}$ = 0.47, p = 0.49), ambient temperature ($F_{1,45}$ = 0.45, p = 0.50), nor egg
339 volume ($F_{1,45}$ = 0.01, p = 0.93) significantly affected T_{egg}.

340

341 *3.3. Chick surface temperatures*
342



343

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

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

362 **Table 3: Summary of linear mixed models (LMMs) investigating the determinants of (A) maximum**
363 **eye temperature (T_{eye}) and (B) minimum beak temperature (T_{beak}) in king penguin chicks.** Only one
364 parent was implanted with CORT or Placebo implant, thus we considered both parental treatment
365 (CORT *vs.* Placebo), the category of the parent brooding the chick at the time of measurement
366 (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_a	**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)

367

368 ## 4. Discussion

369 By experimentally manipulating CORT levels in adult breeding king penguins, we show
370 that glucocorticoids are unlikely to play a major role in determining changes in surface
371 temperatures (both before or in response to handling stress) in this large seabird species.
372 Indeed, we found no significant difference in baseline surface temperature between CORT
373 and Placebo implanted individuals. However, whatever the parental CORT treatment, our
374 handling stress protocol led to an increase in T_{eye} and a decrease in T_{beak} in response to capture
375 and handling. The contrasting results between parental treatment and handling stress suggest
376 that changes in surface temperature are probably driven primarily by the activity of the SAM
377 axis in king penguins (but see Ouyang et al. (2021), for a potential causal involvement of CORT
378 in house sparrows *Passer domesticus*). Contrary to our prediction, parental treatment had no
379 significant effect on egg temperature. Yet, we found an unexpected pattern on chick surface
380 temperatures: chicks brooded by the non-implanted partner of CORT individuals had higher
381 surface temperatures (both T_{eye} and T_{beak}) than chicks brooded by Placebo (or associated non-
382 implanted partner) or CORT individuals. Finally, in the thermally non-emancipated chicks, both
383 T_{eye} and T_{beak} decreased with handling, but irrespectively of the parental treatment.
384

385 *4.1. CORT and adult surface temperatures*

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

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

399 that our handling stress protocol for thermal imaging was too short to detect a potential role
400 of stress-induced CORT in influencing changes in surface temperatures, since in 8 minutes of
401 handling, CORT levels are expected to double in king penguin, while the maximum CORT levels
402 are only attained after *ca.* 80 minutes of handling in our study species (Viblanc et al., 2018).
403 Overall, our results suggest that changes in surface temperature are probably more likely
404 mediated by the SAM rather than the HPA in adult king penguins. Future studies testing for a
405 direct acute stimulation of the HPA axis (using ACTH) or SAM axis (using catecholamines) in
406 the king penguin and other bird species could be useful to gain greater insight on the
407 contribution of the HPA and SAM axes in mediating changes in body surface temperature
408 (Ouyang et al., 2021).
409
410 *4.2. Stress-induced changes in surface temperatures*
411 In breeding adults, we observed a mild (sex-specific) significant increase of T_{eye} and a
412 significant decrease of T_{beak} in response to acute capture-handling stress. Differences in
413 temperature change between body regions in response to acute stress are not rare (see Table
414 1). For instance, Moe et al. (2017) measured a decrease of T_{eye} contrasting with an increase of
415 T_{feet} in chicken, whereas in captive budgerigars (*Melopsittacus undulatus*) T_{eye} increased
416 transiently after exposure to a stressor while T_{feet} did not change (Ikkatai and Watanabe,
417 2015); Table 1). Anatomical and functional reasons can likely explain the difference of stress-
418 induced changes in surface temperature between body regions in penguins, with for instance
419 a potential interest in maintaining or increasing eye blood flow to maintain or enhance visual
420 acuity when acutely threatened by an environmental challenge.
421 T_{eye} has also been shown to positively correlate with internal (cloacal) temperature in
422 various avian species (budgerigars: Ikkatai and Watanabe (2015); chicken: Cândido et al.
423 (2020)). Consequently, it is possible that the increase in T_{eye} we observe in adults could reflect
424 an increase in internal body temperature (Oka, 2018). Consistently, in closely related Emperor
425 penguins (*Aptenodytes forsteri*), acute stress leads to an approximate 1.5°C increase in
426 internal (stomach) temperature (Regel and Pütz, 1997). Such an increase in T_{eye} in response to
427 handling stress has been reported two times in avian species to the best of our knowledge
428 (Ikkatai and Watanabe, 2015; Jakubas et al., 2022), while most previous studies have shown
429 either a significant decrease (6 studies) or no significant change (2 studies; Table 1). Those
430 contrasted findings might be explained by at least three factors: 1. variation in body size, since

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

449 The decrease we observe in T_{beak} in response to acute stress in adults appears like the
450 typical peripheral vasoconstriction response previously reported for instance by Tabh et al.
451 (2021). Such peripheral vasoconstriction enables the redistribution of the peripheral blood
452 circulation to internal organs and tissues, such as the brain or muscles, favouring their
453 oxygenation and nutrition to sustain the fight-or-flight response. King penguins breed in a
454 highly dense and aggressive colonial environment (up to 100 aggressive interactions per hour;
455 Côté, 2000). Aggressive interactions, frequently leading to injuries, are known to result in
456 increased heart rates (Viblanc et al., 2012). A decrease of T_{beak} during acute stress response
457 likely reflects peripheral vasoconstriction, that is likely widespread to other peripheral body
458 parts, which according to the *haemoprotective hypothesis* could help to reduce blood loss in
459 case of injury (Robertson et al., 2020a).

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

463 in adults, the decrease was even more pronounced in chicks (-3.3 ± 0.6°C *vs.* -1.5 ± 0.4°C, in
464 chicks and adults, respectively). This is likely explained by greater peripheral thermal losses in
465 chicks suddenly exposed to an ambient temperature drop (*ca.* 35°C under the brood pouch of
466 the adults *vs.* 15°C when taken out for measurement and handling), despite their ability to
467 maintain their internal temperature at this stage and ambient temperature (Duchamp et al.,
468 2002). The more pronounced drop in surface temperature we observed in chicks during acute
469 handling could reflect their greater need to conserve heat at a relatively mild T_a compared to
470 adults.
471
472 *4.3. Impact of parental CORT on egg and chick surface temperature*
473 Contrary to our prediction, we observed no significant impact of parental CORT
474 treatment on egg temperature, suggesting little alteration of incubation behaviour by
475 increased CORT levels, contrary to a previous study in Adelie penguin reporting a reduction of
476 1.3 ± 0.2°C in T_{egg} (Thierry et al., 2013a). It is possible that incubating two eggs in the colder
477 environment of Adélie penguins is more challenging (and thus more likely to be impacted by
478 high CORT levels) than incubating a single egg under the milder climate experienced by king
479 penguins. Alternatively, it is also possible that king penguins are more resilient to increased
480 CORT levels than Adelie penguins, which is supported by the good reproductive success of
481 king penguins implanted with CORT (Stier et *al.* unpublished), contrary to what has been
482 observed in Adelie penguins (Thierry et al., 2013b). Measurements of incubation quality using
483 a dummy egg with temperature and rotation sensors (Thierry et al., 2013a) may provide more
484 accurate data on this question, but it is important to note that the T_{egg} measured in this study
485 (Placebo: 34.9 ± 0.3°C) was close to the T_{egg} measured in the same penguin colony using
486 internal sensors in dummy eggs (35.7 ± 0.4°C; Groscolas et al., 2000). Our results therefore
487 suggest that further studies may benefit from the use of minimally invasive thermal imaging
488 to measure incubation quality, for instance in the context of parental behaviour and climate
489 change (Cook et al., 2020).
490 The measure of chick surface temperatures revealed some surprising patterns. While
491 we expected CORT chicks to have lower surface temperatures due to poorer parental care
492 (less efficient brooding), we found the opposite result, but only when chicks were brooded by
493 the non-implanted partner of the CORT-parent. This result should be considered with caution
494 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.

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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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