Surface temperatures are influenced by handling stress independently of corticosterone levels in wild king penguins (*Aptenodytes patagonicus*)

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Highlights:

- Experimental increase in corticosterone 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 corticosterone treatment does not affect egg surface temperature during incubation
- Chicks brooded by non-implanted partners of corticosterone parents are warmer than chicks brooded by others adults.

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 corticosterone (the main glucocorticoid hormone in birds) 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.

Maximum eye temperature (T_{eye}) increased and minimum beak temperature (T_{beak}) decreased in response to handling stress in adults, but neither basal nor stress-induced surface temperatures were significantly affected by corticosterone implant. 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 corticosterone-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 axis 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 (SAM) axis in response to stressful situations. Our experiment did not reveal a direct impact of parental HPA axis manipulation on parental investment (egg or chick temperature), but a potential influence on the partner's brooding behaviour.

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

62 **Abbreviations:**

63 Adrenocorticotropic hormone: ACTH

64 Ambient air temperature: T_a

65 Corticosterone: CORT

66 Glucocorticoid hormones: GC

67 Egg surface temperature: T_{egg}

68 Hypothalamic-pituitary-adrenal: HPA

69 Linear mixed models: LMMs

70 Maximum eye region surface temperature: T_{eye}

71 Minimum beak surface temperature: T_{beak}

72 Sympathetic-adrenal-medullary: SAM

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74

75 ## 1. Introduction

76 Measuring stress response is of central interest in wild animals to understand how they cope
77 with environmental change (Ellenberg et al., 2007; Romero, 2004). Traditionally, ecologists
78 have evaluated acute stress by assessing the activation of the Sympathetic-Adrenal-Medullary
79 (SAM) or Hypothalamic-Pituitary-Adrenal (HPA) axes, either at baseline levels or in response
80 to acute disturbances in the environment, by metrics such as increased heart rate (Cabanac
81 and Guillemette, 2001; Viblanc et al., 2012), ventilation rate (Carere and Oers, 2004) or
82 glucocorticoid hormone (GC) secretion (Sapolsky et al., 2000). Stress exposure is also known
83 to trigger core body and surface temperature changes (Oka, 2018). As the latter can be
84 measured using thermal imaging (McCafferty, 2013), it led to a recent burgeoning of thermal
85 imaging studies to measure the stress response of captive and wild endotherms (McCafferty
86 et al., 2021). Both the SAM and HPA axes can potentially influence changes in body surface
87 temperature, but the importance of these two pathways in mediating the stress response(s)
88 measured through thermal imaging remains largely unknown (but see Jerem and Romero
89 (2023)). Considering that both HPA and SAM axes provide strong and very reactive responses
90 to stress, teasing apart their contribution to changes in peripheral temperature is challenging.

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

99 that helps responding to a stressor by mobilizing energy resources and by triggering an
100 increase in behavioural activity and metabolic rate (Wingfield and Romero, 2015). Circulating
101 GC levels are usually considered at baseline (*i.e.* without an inducing stressor) or at stress-
102 induced levels, which are often interpreted as mirroring chronic stress/metabolic demand and
103 acute stress response respectively. Yet, the actual interpretation of circulating GC levels is
104 likely more complex (MacDougall-Shackleton et al., 2019). GC-mediated changes in
105 metabolism may cause an increase in internal heat production that, in turn, may lead to
106 changes in body surface temperature (Oka, 2018). Additionally, previous evidence suggests
107 that GC might be necessary for enabling the SAM axis to induce shivering, free fatty acid
108 mobilization or vasoconstriction (Deavers and Musacchia, 1979).
109 Therefore, both the SAM and HPA axes could trigger, independently from each other, or
110 together, a change in body core and/or surface temperatures (Ouyang et al., 2021). The
111 immediate activation of SAM axis could lead to a decrease of peripheral temperature
112 independently of GC release by the HPA axis. On the other hand, the short to long-term
113 elevation of baseline GC might lead to increased metabolic rate, heat production and,
114 ultimately, higher peripheral temperature (to facilitate heat dissipation and maintain
115 homeothermy) independently of the SAM axis. The use of thermal imaging as a non-invasive
116 tool to measure stress in unmanipulated animals is actively developing, but the implication of
117 the SAM and HPA axes in mediating changes in surface temperature remains uncertain (Jerem
118 and Romero, 2023; Ouyang et al., 2021). For instance, data in captive and wild birds show that
119 acute stress exposure often leads to a decrease in body surface temperature (reviewed in
120 Table 1, but note some discrepancies between species and/or body parts), as predicted if
121 these changes are driven by the SAM axis. However, evidence for an impact of chronic
122 stressors on body surface temperature (Herborn et al., 2018; Robertson et al., 2020a, 2020b;

123 Winder et al., 2020) or for a correlation between circulating GC levels and body surface
124 temperature (Giloh et al., 2012; Jerem et al., 2018; Weimer et al., 2020) remains unclear.

125 In this study, we took up the challenge of distinguishing the role of HPA and SAM axes
126 on changes in body surface temperatures using an experimental approach in adult king
127 penguins (*Aptenodytes patagonicus*). Breeding king penguins were subcutaneously implanted
128 with either a corticosterone (CORT, the main GC hormone in avian species) implant or a
129 placebo implant. The use of CORT implants in king penguin (see methods and Fig. 1), as in
130 many other bird species (see Torres-Medina et al., 2018), leads to higher baseline CORT levels,
131 while inhibiting the acute CORT response in the days following implantation. Indeed, CORT-
132 implanted birds no longer exhibit high blood CORT release in response to acute stress induced
133 by a handling stress protocol because of the HPA negative feedback loop (Torres-Medina et
134 al., 2018). Specifically, high circulating CORT levels are inhibiting the activity of the
135 paraventricular nucleus in the hypothalamus through binding with glucocorticoid receptors.
136 This inhibits the release of corticotrophin-releasing hormone and the afferent HPA cascade
137 through the pituitary (secreting the adrenocorticotropic hormone, ACTH) and adrenal
138 (secreting CORT) glands (Smulders, 2021). Hence, the use of CORT implants in conjunction
139 with handling stress allows for the separation of the contributions of the HPA and SAM axes
140 to acute changes in body surface temperatures. Here, we therefore measured the effects of
141 our CORT treatment, together with a handling stress protocol, on surface temperatures in the
142 king penguin. If changes in surface temperatures are mainly driven by activating the HPA axis,
143 we expect to find higher initial body surface temperatures in CORT-implanted individuals (with
144 elevated baseline CORT) prior to capture and handling. However, because CORT implants shut-
145 down the release of CORT in response to handling, we expect to find no change in body surface
146 temperature in response to handling stress in CORT individuals if such changes are mediated

147 by the HPA axis only. On the contrary, if changes in surface temperatures are mainly driven by
148 the activity of the SAM axis, we expect our CORT treatment to have no effect on body surface
149 temperatures (both before or after capture and handling).

150 In addition to direct effects on adults, parental stress exposure or increased CORT
151 levels may also affect the following generation, for instance by affecting parental care (Thierry
152 et al., 2013a) or by conveying information about a stressful environment (Brandl et al., 2022).
153 The latter idea has been framed as the concept of family-transmitted stress by Noguera et al.
154 (2017). Therefore, we also aimed to investigate whether thermal imaging could be used to
155 quantify indirect effects of stress on king penguin's progeny. To this end, we measured egg
156 surface temperature on the same day as the body surface temperatures of implanted parents,
157 and the surface temperatures of their chicks. Based on experimental data in Adélie penguin
158 (*Pygoscelis adeliae*) showing that elevated baseline CORT levels in adults led to lower parental
159 care (Thierry et al., 2013a), we hypothesized that increasing CORT levels may lower the surface
160 temperatures of eggs and/or chicks during incubation and brooding respectively, as parents
161 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 king penguin is a heterothermic bird whose body temperature largely varies between core and peripheral tissues (Lewden et al., 2017a; Lewden et al., 2017b). Its thick body plumage provides a very efficient insulation, while counter-current vascular heat exchangers in the appendages allows for a decrease in 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).

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 early 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 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 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). Twenty days after hatching, king penguin chicks are able to maintain their core body temperature under mild environmental conditions (> 10°C) but still rely on their parent's brooding behaviour (Duchamp et al., 2002), and are able to express a HPA-dependent stress response (X et al. unpublished).

188

189 *2.2. Use of sub-cutaneous CORT and SHAM implants*

190 We divided the 49 breeding pairs into two groups of either female-treated pairs (N =
191 23) or male-treated pairs (N = 26). In the female-treated pairs, we implanted females with
192 either a CORT (NG-111, 50 mg corticosterone, 90-days release, 8 mm diameter and 3mm
193 thickness; N = 12 females) or a Placebo (NC-111, same vehicle but no CORT; N = 11 females)
194 implant. Sub-cutaneous implants were purchased from Innovative Research of America
195 (Sarasota, FL, USA). Females were implanted during their first incubation shift, 3 days after
196 returning from sea. Similarly, in the male-treated pairs, we implanted 13 males with a CORT
197 and 13 males with a Placebo implant, 3 days after returning from sea. In males, the first
198 incubation shift is immediately after egg laying, and males have already been fasting for ~15
199 days during courtship. Therefore, we chose to implant the males during their second
200 incubation shift so that they would have comparable fasting times compared to females, and
201 therefore both sexes would be in a comparable physiological state during measurements.
202 Allocation of birds to specific groups was designed to avoid spatial or temporal biases within
203 and between groups. Male and female partners in, respectively, the female-treated and male-
204 treated pairs were not implanted. Hereafter, they are referred to as "Partner-CORT" or
205 "Partner-Placebo" regardless of their sex. Partner penguins were only considered in the chick
206 analysis (see below).

207 On the day of the implantation, adults were captured by hand while incubating their
208 egg and restrained by one experimenter with a hood covering the head to keep them calm.
209 While maintaining individuals on their territory within the colony to avoid risking reproductive
210 abandonment, sub-cutaneous CORT or placebo implants were inserted in the upper part of
211 the back by a veterinary surgeon under local anaesthesia (*ca.* 0.5 mg/kg xylocaine combined

212 with 0.0001 mg/kg adrenalin, Aspen Pharma). Immediately after implantation, the small
213 incision (*ca.* 15 mm) was closed using 3 sterile surgical staples (SurgiClose™ Skin Stapler), and
214 a prophylactic dose of antibiotics (cephalexin *ca.* 50 mg/kg, Rilexine®, Virbac) was injected to
215 prevent any risk of infection. The incision was checked 3 days later for implant rejection; no
216 sign of infection or implant rejection were noticed and staples were removed 6 days after
217 implantation.

218 To verify the efficacy of our CORT treatment, 6 days after implantation, 1 mL of blood
219 was sampled from the flipper vein using a heparinized syringe in less than 4 min after being
220 detected by the focal individual (baseline CORT) and after 30 min of standardized handling
221 (stress-induced, see Stier et al. (2019) and Viblanc et al. (2018)). Blood samples were taken
222 between 09:00 and 13:00 to limit variation linked to circadian variation in plasma CORT levels.
223 Blood samples were centrifugated within one hour of sampling at 3000 *g* for 10 minutes. The
224 plasma fraction was then removed and plasma aliquots were stored at −20°C until the end of
225 the day, before being transferred to −80°C until laboratory analyses in 2023. Plasma total
226 CORT levels were measured from 25 μL of plasma by immunoassay according to guidelines
227 provided by the manufacturer (Corticosterone Enzyme Immunoassay Kit, Arbor Assay, USA).
228 Intra-plate coefficient of variation based on duplicates was 8.17 ± 0.90% (mean ± SE), and
229 inter-plate coefficient of variation based on one repeated sample was 8.08%. We tested the
230 effect of implant on log-transformed plasma CORT using a linear mixed model (LMM) including
231 as fixed factors: Handling stress (Baseline *vs.* Handling stress), Treatment (CORT *vs.* Placebo)
232 and their interaction, as well as penguin ID as random effect. Implants were successful in
233 raising circulating baseline CORT levels and in inhibiting stress-induced CORT release (Fig. 1).
234
235 *2.3. Thermal image collection and analysis*

236 Because of their dense and watertight plumage, the use of thermal imaging in seabirds
237 is usually restricted to measuring the eye (periorbital region) and beak regions (Gauchet and
238 Grémillet, 2022). These regions are highly vascularised and provide valuable information on
239 body surface temperatures. Thermal pictures of implanted adults (Fig. 2A) and their egg (Fig.
240 2B) were taken 3 days after implantation and thermal pictures of chicks (Fig. 2C) were taken
241 at day 20 after hatching (mean ± SE: 49.8 ± 1.4 days after CORT or Placebo implant of the focal
242 parent, *i.e.* within the 90-days release given by the CORT pellet manufacturer). Thermal
243 pictures were taken at a distance between *ca.* 0.8 and 2 m using FLIR E8 thermal imaging
244 camera (320 x 240 pixels) resulting in a spotsize between 1.95 and 4.88 mm (Playa-Montmany
245 and Tatersall 2021). King penguins' beaks being approximately 120 mm long, and the eye
246 region being approximately 18 mm of diameter, our target areas exceeded the 3 times larger
247 spotsize recommended by Playa-Montmany and Tatersall (2021). When approaching the
248 adults and/or chicks, we started a stopwatch when the focal individual showed the first signs
249 of alarm behaviour (*e.g.* stopping activity, looking towards the experimenter) to measure the
250 duration of our disturbance at the time the different pictures were taken. In adults, we took
251 a first set of pictures (1 to 2 pictures per individual) before handling, on average (mean ± SE)
252 0.63 ± 0.07 min after the first sign of alarm behaviour at a distance of *ca*. 1.5 to 2 m from the
253 focal individuals. Adults were then captured-restrained, incision healing was checked, and we
254 took a second set of pictures (1 to 2 pictures per individual) after terminating handling stress
255 (mean ± SE handling stress duration was 8.3 ± 0.3 min; a timeframe within which CORT is
256 known to increase by *ca.* 100% above baseline levels in king penguins (Viblanc et al., 2018).
257 Just after capturing the adult, we removed the single-egg from the brood pouch and
258 immediately (*ca.* within 10 s) took a thermal picture at a distance of *ca.* 0.8 m. We then
259 measured egg length and breadth using a Vernier calliper (Mitutoyo; accuracy ± 0.02 mm) and

260 estimated egg volume (cm^3) following (Narushin, 2005). When chicks were 20 days old, we
261 took a first set of pictures (1 to 2 pictures per individual) at a distance of *ca.* 0.8 m immediately
262 (*ca.* within 10 s) after they were removed from the brood pouch (*i.e.* before handling: 0.73 ±
263 0.08 min after the first sign of alarm behavior from the parent) and a second set of pictures
264 (1-2 pictures per individual) was taken after handling, 5.40 ± 0.30 min later. Thermal images
265 of the chicks were only taken on non-rainy days, which reduced the sample size from 38 chicks
266 that survived until day 20, to 16 chicks with thermal images available. Chick sex was unknown
267 and body mass was not significantly influenced by the parental treatment (CORT: 1.14 ± 0.11
268 kg *vs.* Placebo 1.32 ± 0.09 kg, N = 8/group, t-test: t = -1.28, p = 0.22). Chicks were kept within
269 the colony at ambient temperature (14.9 ± 0.9°C) between the first and second set of pictures,
270 a temperature at which they are known to be able to maintain their internal body temperature
271 (Duchamp et al., 2002). In adults and chicks, we defined 'before handling' surface
272 temperatures, the temperatures extracted from the first set of pictures taken in < 1 min and
273 'after handling' surface temperatures, the temperatures extracted from the second set of
274 pictures taken after > 5 minutes of handling stress.

275 We analysed thermal images using the ThermaCAM TM Researcher Pro 2.10 software
276 (Flir systems, Wilsonville, Oregon, USA). Only profile pictures were analysed to avoid surface
277 temperature errors related to head orientation (Playà-Montmany and Tattersall, 2021; Tabh
278 et al., 2021). For each image we set the emissivity at 0.98, reflected temperature at 20°C and
279 controlled in the analyses for daily variation in air temperature (T_a) and relative humidity using
280 daily climatic measures from a permanent Météo France weather station located 2 km inland
281 from the colony (https://rp5.ru/Archives_m%C3%A9t%C3%A9o_sur_la_base_Alfred-Faure).
282 We extracted measures of surface temperatures on maximum periorbital temperature (T_{eye})
283 and minimum beak temperature (T_{beak}) in adults and chicks. We used maximum T_{eye} as

recommended by Jerem et al. (2015 & 2019) as well as recorded in recent studies (*e.g.* Robertson et al. 2020a; Zuluaga & Danner 2023, and the minimum T_{beak} to gain insight on the maximum state of vasoconstriction for this body surface (Tattersall et al., 2009). 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).

2.5. Statistical analysis

We ran separate analyses for adults, eggs and chicks. In adults, we investigated variations in T_{eye} and T_{beak} using two separated linear mixed models (LMMs) where Treatment (CORT *vs.* Placebo), Handling stress (before *vs.* after), Sex and T_a were specified as fixed effects. Relative humidity was initially tested but removed from final models since it was never significant. All two-ways interactions were also initially included but removed from the final model if $p > 0.10$ using a backward stepwise procedure. The p-values (just before removal in the backward stepwise procedure) for the focal interaction between Treatment and Handling stress are reported in tables. Bird identity was included as a random intercept to control for repeated measures. We investigated variation in T_{egg} by entering parental CORT Treatment (CORT *vs.* Placebo), the Sex of the parent, T_a and egg volume as fixed effects. Eggs were always measured when incubated by the implanted parents (*i.e.* 3 days after implantation of CORT or Placebo implants), while chicks at day 20 could be brooded by the implanted parent or its non-

308 implanted partner. Thus, we investigated variation chick T_{eye} and T_{beak} by entering parental
309 Treatment (CORT *vs.* Placebo), the category of the brooding parent at the time of
310 measurement (implanted individual *vs.* non-implanted partner), Handling stress (before *vs.*
311 after), and T_a as fixed effects, and chick identity as a random intercept. Parental sex was not
312 included in the models for chick T_{eye} and T_{beak} due to limited sample size available for chicks.
313 Interactions were treated as described above for adults. All statistical analyses were
314 conducted using *R* (version 3.5.3) and the packages *lme4* (Bates et al., 2015), *emmeans* (Lenth
315 et al., 2018) and *pbkrtest* (Halekoh and Højsgaard, 2014). Results are reported as least-square
316 means ± SE.

317 ## 3. Results

318

319 *3.1. Adult surface temperatures*

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

329

330 *3.2. Egg surface temperature*

331 Neither parental Treatment ($F_{1,45} = 1.38$, $p = 0.25$; CORT: 34.5 ± 0.2°C *vs.* Placebo: 34.9
332 ± 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
333 egg volume ($F_{1,45} = 0.01$, $p = 0.93$) significantly affected T_{egg}.

334

335 *3.3. Chick surface temperatures*

336

337 The interaction between parental Treatment (CORT *vs.* Placebo) and the category of
338 the parent brooding the chick (implanted *vs.* non-implanted partner) at the time of
339 measurement significantly affected chick T_{eye} ($F_{1,11} = 6.37$, $p = 0.028$, Table 3) and chick T_{beak}
340 ($F_{1,11} = 5.49$, $p = 0.039$, Table 3). Chicks brooded by the partner of CORT-implanted individuals
341 had higher overall T_{eye} and T_{beak} than chicks brooded by CORT parents, or by Placebo and
342 associated partners (Fig 4A and 4B). While we found no significant interaction between

343 parental Treatment and Handling stress (Table 3), both chick T_{eye} (Fig 4C) and chick T_{beak} (Fig

344 4D) significantly decreased after handling (by -0.9 ± 0.3°C and -3.3 ± 0.6°C respectively, Table

345 3). As expected, chick T_{eye} and T_{beak} were significantly positively associated with T_a (Table 3).

346 ## 4. Discussion

347 By experimentally manipulating CORT levels in adult breeding king penguins, we show
348 that corticosterone is unlikely to play a major role in determining changes in surface
349 temperatures (both before or in response to handling stress) in king penguins. Indeed, we
350 found no significant difference in baseline surface temperature between CORT and Placebo
351 implanted individuals. However, whatever the parental CORT treatment, our handling stress
352 protocol led to an increase in T_{eye} and a decrease in T_{beak} in response to capture and handling.
353 The absence of effect of the CORT treatment on baseline surface temperature and its
354 dynamics during acute handling stress suggest that changes in surface temperature are
355 probably driven primarily by the activity of the SAM axis in king penguins (but see below for a
356 potential causal involvement of CORT in house sparrow *Passer domesticus* (Ouyang et al.,
357 2021)). Contrary to our prediction, parental treatment had no significant effect on egg
358 temperature. Yet, we found an unexpected pattern for chick surface temperatures: chicks
359 brooded by the non-implanted partner of CORT individuals had higher surface temperatures
360 (both T_{eye} and T_{beak}) than chicks brooded by Placebo (or associated non-implanted partner) or
361 CORT individuals. Finally, in the thermally non-emancipated chicks, both T_{eye} and T_{beak}
362 decreased with handling, but irrespectively of the parental treatment.
363
364 *4.1. CORT and adult surface temperatures*
365 Previous correlative studies have considered baseline and stress-induced CORT levels
366 to investigate the relationship between GC signalling and changes in body surface
367 temperature in birds (blue tit, *Cyanistes caerulescens*, (Jerem et al., 2018); domestic chicken,
368 *Gallus gallus domesticus,* (Giloh et al., 2012; Weimer et al., 2020)). To go deeper in the
369 involved mechanisms, we experimentally manipulated CORT in the king penguin to help to
370 causally distinguish the contribution of the HPA and SAM axes to changes in body surface
371 temperature. Since we did not detect any significant effect of CORT manipulation on baseline
372 or stress-induced surface temperatures, our results provide little support for a causal
373 involvement of the HPA axis in mediating stress-related changes in surface temperature.
374 Our results contrast with a recent study showing that an acute stimulation of the HPA
375 axis with adrenocorticotropic hormone (ACTH) induced a decrease in T_{eye} within 5 min in
376 captive-held house sparrows (Ouyang et al., 2021), which suggests a causal involvement of

377 the HPA axis in mediating stress-related changes in surface temperature. Yet, a recent study
378 in the same species found a significant correlation between the acute change in surface
379 temperature and heart rate variability, a measure of SAM axis activation (Jerem and Romero,
380 2023). This suggests that both the HPA and SAM axes could be involved in mediating stress-
381 related changes in surface temperature in house sparrow. While we used an experimental
382 approach and the lack of significant effect of baseline CORT on body surface temperatures
383 appears to be robust, we cannot fully exclude that our handling stress protocol for thermal
384 imaging was too short to detect a potential role of stress-induced CORT in influencing changes
385 in surface temperatures. Indeed, in 8 minutes of handling, CORT levels are expected to double
386 in king penguin, while maximum CORT levels are only attained after *ca.* 80 minutes of handling
387 in our study species (Viblanc et al., 2018). Additionally, we may have missed some early
388 changes in surface temperatures occurring in response to the stress of our approach prior to
389 bird capture and handling. Indeed, changes in T_{eye} and T_{beak} within 25 seconds before capture
390 have been observed in house sparrow (*Passer domesticus*) (Jerem and Romero, 2023) and our
391 pre-handling thermal pictures were taken on average 38 seconds after the first sign of alarm
392 behaviour. Yet, our results suggest that changes in surface temperature are probably more
393 likely mediated by the SAM axis rather than the HPA axis in adult king penguins. Future studies
394 testing for a direct acute stimulation of the HPA axis (using ACTH) or SAM axis (using
395 catecholamines) in the king penguin and other bird species could be useful to gain greater
396 insight on the contribution of the HPA and SAM axes in mediating changes in body surface
397 temperature (Jerem and Romero, 2023; Ouyang et al., 2021).
398
399 *4.2. Stress-induced changes in surface temperatures*
400 In breeding adults, we observed a mild sex-specific significant increase of T_{eye} and a
401 significant decrease of T_{beak} in response to acute capture-handling stress. Differences in
402 temperature change between body regions in response to acute stress are not rare (see Table
403 1). For instance, (Moe et al., 2017) measured a decrease of T_{eye} contrasting with an increase
404 of T_{feet} in chicken, whereas in captive budgerigars (*Melopsittacus undulatus*) T_{eye} increased
405 transiently after exposure to a stressor while T_{feet} did not change (Ikkatai and Watanabe, 2015;
406 Table 1). Anatomical and functional reasons can likely explain the difference of stress-induced
407 changes in surface temperature between body regions in penguins, with for instance a

408 potential interest in maintaining or increasing eye blood flow to maintain or enhance visual
409 acuity when acutely threatened by an environmental challenge.

410 T_{eye} has also been shown to positively correlate with internal (cloacal) temperature in
411 various avian species (budgerigars: Ikkatai and Watanabe, 2015; chicken: Cândido et al.,
412 2020). Consequently, it is possible that the increase in T_{eye} we observe in adults could reflect
413 an increase in internal body temperature (Oka, 2018). Consistently, in closely related Emperor
414 penguins (*Aptenodytes forsteri*), acute stress leads to an approximate 1.5°C increase in
415 internal (stomach) temperature (Regel and Pütz, 1997). Such an increase in T_{eye} in response to
416 handling stress has been reported two times in avian species to the best of our knowledge
417 (Ikkatai and Watanabe, 2015; Jakubas et al., 2022), while most previous studies have shown
418 either a significant decrease (4 studies) or no significant change (4 studies; Table 1). Those
419 contrasted findings might be explained by at least three factors: 1. variation in body size, since
420 smaller individuals/species are expected to favour internal heat conservation by reducing
421 more markedly peripheral blood flow and surface temperature compared to large ones
422 (*thermoprotective hypothesis*; Robertson et al., 2020a); 2. differences in the thermal
423 environment, since it has been shown that during acute stress response, heat conservation is
424 favoured below the thermoneutral zone while heat dissipation is favoured above its upper
425 limit (Robertson et al., 2020a); 3. the various delays at which T_{eye} was measured in response
426 to acute stress, since for instance the increase in T_{eye} found by Ikkatai and Watanabe (2015)
427 was short-lived (visible 5 min after the stressor, but not later on during the 30 min
428 measurement period). It is important to note that T_{eye} and T_{beak} can show very rapid and non-
429 linear changes in response to acute stress, that are likely to be missed by most studies not
430 using continuous thermal image recording (Jerem et al., 2019; Jerem and Romero, 2023;
431 Zuluaga and Danner, 2023). For instance, Zuluaga et al. (2023) found an initial drop in T_{beak}
432 following exposure to stressor, with a recovery to baseline levels after only 2 minutes, while
433 Jerem et al. 2019 found an initial drop in T_{eye} lasting less than 15s, followed by an increase
434 above baseline from 15 to 75 seconds.

435 Although the interaction between handling stress and sex was only marginally
436 significant (p = 0.060), it appeared that only females exhibited a noticeable increase in T_{eye} in
437 response to handling stress in our study. Differences in body size or mass between sexes are
438 very modest (*ca.* 5%) in king penguin (Kriesell et al., 2018), which thus seems unlikely to fully
439 explain the sex-difference observed here. There are known sex-differences in stress

440 physiology (Handa and McGivern, 2017), but sex differences in surface temperature changes
441 induced by acute stress have been rarely investigated (but see Robertson et al. (2020a) for a
442 result opposite to ours between sexes). We previously observed no sex effect in HPA axis
443 responsiveness between males and females king penguins (Viblanc et al., 2016), but as
444 mentioned above, the SAM axis is the likely driver of acute changes in surface temperatures.
445 Consequently, this suggests that sexes might differ in the stress-sensitivity of their SAM axis,
446 although this would need to be confirmed by direct measurements of SAM axis activity.
447 The decrease we observe in T_{beak} in response to acute stress in adults appears like the
448 typical peripheral vasoconstriction response previously reported for instance by Tabh et al.
449 (2021). Such peripheral vasoconstriction enables the redistribution of the peripheral blood
450 circulation to internal organs and tissues, such as the brain or muscles, favouring their
451 oxygenation and nutrition to sustain the fight-or-flight response. King penguins breed in a
452 highly dense and aggressive colonial environment (up to 500 aggressive interactions per hour;
453 (CôTé, 2000). Aggressive interactions, frequently leading to injuries, are known to result in
454 increased heart rates (Viblanc et al., 2012). A decrease of T_{beak} during acute stress response
455 likely reflects peripheral vasoconstriction, that is likely widespread to other peripheral body
456 parts, which according to the *haemoprotective hypothesis* could help to reduce blood loss in
457 case of injury (Robertson et al., 2020a).
458 The changes observed in surface temperatures in chicks differ from those found in
459 adults. Indeed, we observed a significant decrease in chick's T_{eye}, whereas T_{eye} increased in
460 adult females and stayed stable in adult males. While T_{beak} significantly decreased as observed
461 in adults, the decrease was more pronounced in chicks (-3.3 ± 0.6°C *vs.* -1.5 ± 0.4°C, in chicks
462 and adults, respectively). This is likely explained by greater peripheral thermal losses in chicks
463 suddenly exposed to an ambient temperature drop (*ca.* 35°C under the brood pouch of the
464 adults *vs.* 15°C when taken out for measurement and handling), despite their ability to
465 maintain their internal temperature at this stage at ambient temperature (Duchamp et al.,
466 2002). The more pronounced drop in surface temperature we observed in chicks during acute
467 handling could reflect their greater need to conserve heat at a relatively mild T_a compared to
468 adults.
469
470 *4.3. Impact of parental CORT on egg and chick surface temperature*

471 Contrary to our prediction, we observed no significant impact of parental CORT
472 treatment on egg temperature, suggesting little alteration of incubation behaviour by
473 increased CORT levels, contrary to a previous study in Adelie penguin reporting a reduction of
474 1.3 ± 0.2°C in T_{egg} (Thierry et al., 2013a). It is possible that incubating two eggs in the colder
475 environment of Adélie penguins is more challenging (and thus more likely to be impacted by
476 high CORT levels) than incubating a single egg under the milder climate experienced by king
477 penguins. Alternatively, it is also possible that king penguins are more resilient to increased
478 CORT levels than Adelie penguins, which is supported by the good reproductive success of
479 king penguins implanted with CORT (X et *al.* unpublished), contrary to what has been observed
480 in Adelie penguins (Thierry et al., 2013b). Measurements of incubation quality using a dummy
481 egg with temperature and rotation sensors (Thierry et al., 2013a) may provide more accurate
482 data on this question, but it is important to note that the T_{egg} measured in this study (Placebo:
483 34.9 ± 0.3 °C) was close to the T_{egg} measured in the same penguin colony using internal sensors
484 in dummy eggs (35.7 ± 0.4°C; (Groscolas et al., 2000)). Our results therefore suggest that
485 further studies may benefit from the use of minimally invasive thermal imaging to measure
486 incubation quality, for instance in the context of parental behaviour and climate change (Cook
487 et al., 2020).
488 The measure of chick surface temperatures revealed some surprising patterns. While
489 we expected CORT chicks to have lower surface temperatures due to poorer parental care
490 (less efficient brooding), we found some opposite result: chicks had higher surface
491 temperatures when brooded by the non-implanted partner of the CORT parent. This result
492 should be considered with caution considering the limited sample size available for chick
493 surface temperatures (N = 16). One possibility is that partners from CORT-implanted
494 individuals somehow perceived the 'stress' levels of their partner (as shown between siblings
495 in yellow legged gulls (*Larus michahellis*); Noguera et al., 2017) and somehow compensated
496 for parental care through more efficient brooding. This hypothesis requires further study to
497 determine whether the concept of 'family-transmitted stress' (Noguera et al., 2017) applies
498 to the king penguin. In this species, both parents must rely on each other over more than one
499 year to successfully fledge their single chick, which itself is entirely dependent on its parents
500 for its food supply throughout its growth. Hence, the king penguin could be promising system
501 to investigate the 'family-transmitted stress' hypothesis.
502

503 *4.4. Conclusion*

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

513

514 ## 5. Declarations

515 *5.1. Author contribution*

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

520

521 *5.2 Data availability*

522 The datasets used in this manuscript are available on *FigShare*
523 (10.6084/m9.figshare.20134181).

524

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5.4. Competing interests

None

6. References

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719 **Figures**

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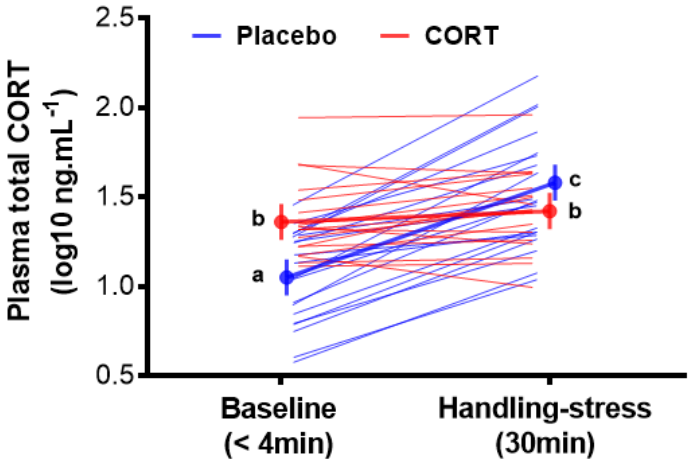

732 **<u>Fig. 1</u>: Baseline and stress-induced plasma corticosterone levels of breeding king penguins**
733 implanted either with a Placebo, or a CORT subcutaneous implant (50 mg, 90 days release; n
734 = 80 observations, N = 40 individuals) during incubation. Birds were blood sampled 6 days
735 after implantation. CORT (red) *vs.* Placebo (blue) individual responses (raw data) are
736 presented along with group and time specific mean ± SE. CORT levels were influenced by the
737 interaction between handling stress (baseline: < 4 min vs. stress-induced: 30 min of handling
738 stress) and CORT treatment (LMM on log-transformed plasma CORT: $F_{1,37.2} = 62.4$, $p < 0.001$).
739 Letters indicate significant differences according to post-hoc tests with Tukey adjustments ($p <$
740 0.030).
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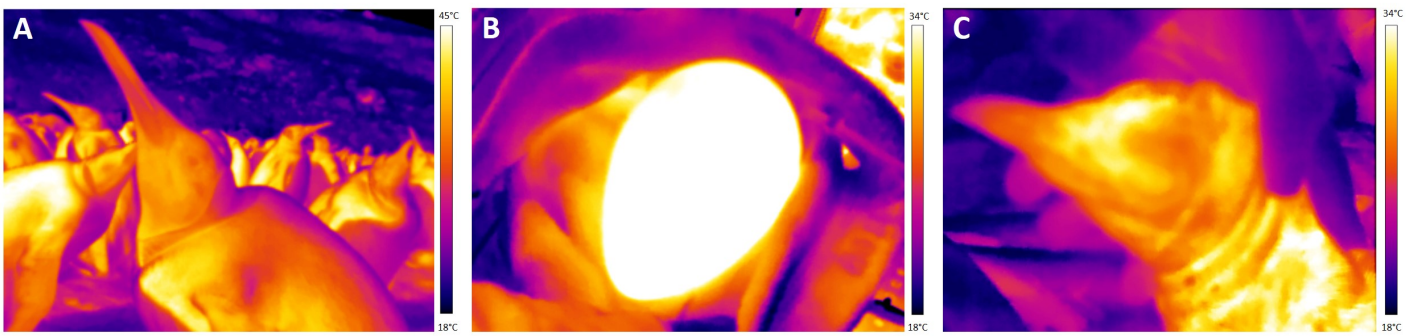

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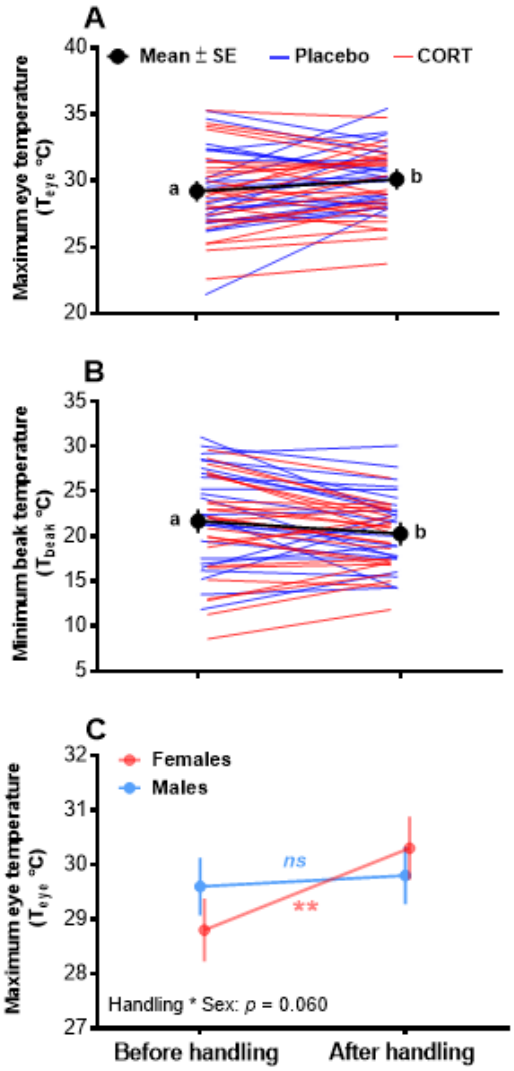
751 **<u>Fig. 2</u>: Infrared images of (A) breeding adult, (B) incubated egg and (C) 20 days-old chick**



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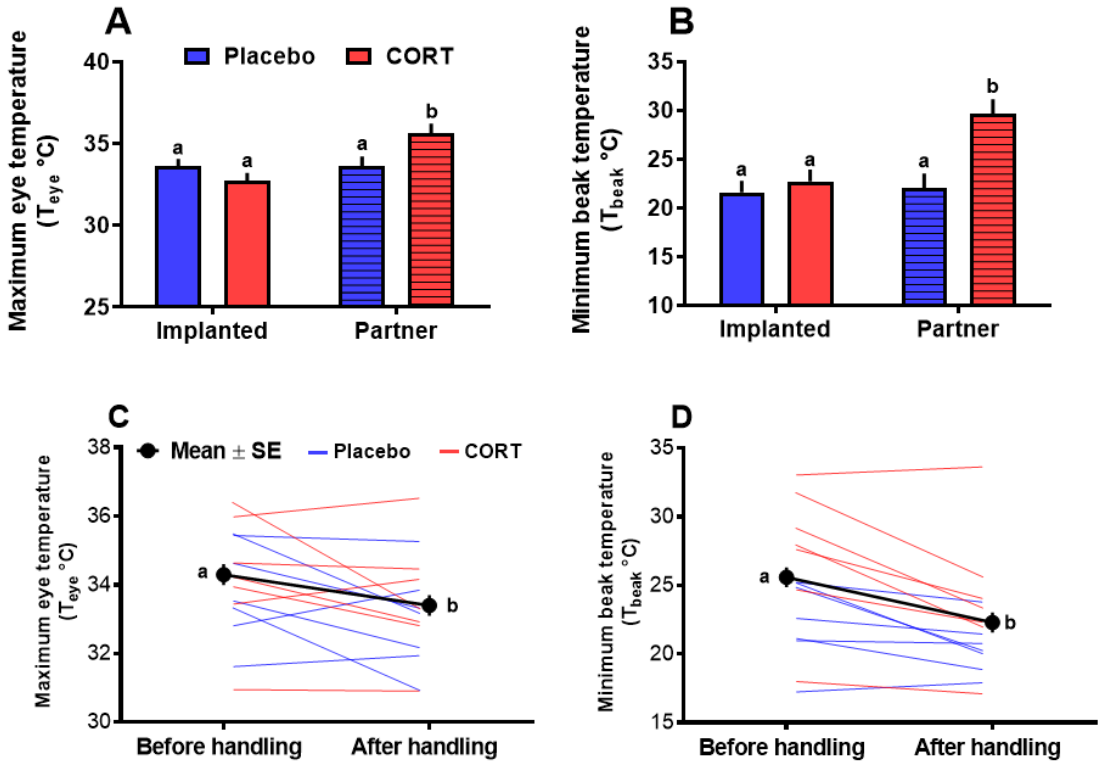
754 **Fig. 3**: **Adult king penguin surface temperature responses to an experimental manipulation**
755 **of glucocorticoid levels (CORT *vs.* Placebo subcutaneous implants) and handling stress: (A)**
756 **Maximum eye temperature response to handling stress, (B) Minimum beak temperature**
757 **response to handling stress, and (C) Sex-specific response of eye temperature to handling**
758 **stress.** In panels A and B, CORT (red) *vs.* Placebo (blue) individual responses (raw data) are
759 presented. Since CORT Treatment had no significant effect either alone or in interaction with
760 Handling stress (see Table 2 for details), the overall mean ± SE is shown in black. Least-square
761 means ± SE from final statistical models (Table 2; n = 138 observations; N = 49 individuals) are
762 presented. Different letters indicate significant differences in panels A and B, and ** in panel
763 C represent the significant increase of T_{eye} observed only in females.
764



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767 **Fig. 4**: **King penguin chick surface temperature in response to parental glucocorticoid**
768 **manipulation and handling: (A) Maximum eye temperature according to parental treatment**
769 **and category of the parent brooding the chick (*i.e.* partners are not implanted), (B) Minimum**
770 **beak temperature according to parental treatment and category of the parent brooding the**
771 **chick, (C) Maximum eye temperature response to handling, and (D) Minimum eye**
772 **temperature response to handling.** In panels C and D, CORT (red) vs. Placebo (blue) chick's
773 individual responses (raw data) are presented, but only the global mean ± SE are presented
774 since there was no significant interaction between parental CORT Treatment and Handling
775 (see Table 3 for details). Least-square means ± SE from final statistical models (Table 3) are
776 presented (n = 63 observations, N = 16 chicks). Different letters indicate significant
777 differences.
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Tables

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). Please note that the area of interest for T_{eye} differs between studies (periorbital region *vs.* eyeball region) and that the surface temperature being measured can differ in nature between studies (*i.e.* maximum, average or minimum temperature of the area of interest).

Species	Condition	Age	Stressor	Area	Response	Δ T°C	Ref.
Blue tit *Cyaniste caerulescens*	Wild	Adult	Trap closure + handling (3min)	Eye	↓, ↑,=	-1.0, +1.0°C, =	(Jerem et al., 2019)
Budgerigars *Melopsittacus undulatus*	Captive	Adult	Handling (30 min)	Eye Legs	↑, = =	+0.7°C, = =	(Ikkatai and Watanabe, 2015)
Chicken *Gallus gallus domesticus*	Captive	Adult	Air puff (10 min)	Eye	↓	-1.0°C	(Edgar et al., 2011)
Chicken *Gallus gallus domesticus*	Captive	Adult	Handling (20 min)	Eye Head Comb	↓, = =, ↑ ↓, =	-0.5°C, = =, +1.0°C -1.5°C, =	(Edgar et al., 2013)
Chicken *Gallus gallus domesticus*	Captive	Chick (9 weeks)	Air puff (10 min)	Eye	↓	-1.0°C	(Edgar and Nicol, 2018)
Chicken *Gallus gallus domesticus*	Captive	Adult	Handling: mild/severe (20 min)	Eye Face Wattle Comb	↓ = ↓ ↓	-0.4°C = -0.7 / -1.3°C -0.5 / -2.2°C	(Herborn et al., 2015)
Chicken *Gallus gallus domesticus*	Captive	Adult	Enrichment removal (within 2 h)	Eye Face Comb	↓ ↓ ↓	-0.8°C -0.7°C -3.5°C	(Herborn et al., 2018)
Chicken *Gallus gallus domesticus*	Captive	Chick (30 days)	Handling (10 min)	Head Feet	↑ ↓	+0.76°C -0.45°C	(Moe et al., 2017)
Chicken *Gallus gallus domesticus*	Captive	Chick (9 days)	Visual (45 min)	Eye Beak	= =, ↑	= +0.5°C	(Pijpers et al., 2022)
Domestic pigeon *Columbia livia domestica*	Captive	Adult	Handling (3.5 min)	Eye Beak	= ↓	-0.4°C -2.1°C	(Tabh et al., 2021)
House sparrow *Passer domesticus*	Captive	Adult	Handling and injection (~1min)	Eye Beak	↓ ↓	-1.0°C -4.0°C	(Jerem and Romero, 2023)
King penguin *Aptenodytes patagonicus*	Wild	Adult	Handling (5-10 min)	Eye Beak	↑ ↓	+0.8°C -1.5°C	This study
	Wild	Chick (20 days*)	Handling (5-10 min)	Eye Beak	↓ ↓	-0.9°C -3.3°C	
Little Auk *Alle alle*	Wild	Adult	Handling (30 min)	Eye	↑	+1,7°C	(Jakubas et al., 2022)
Pheasant *Phasianus colchicus*	Captive	Juvenile (42 to 49 days)	Agression (20 s)	Head	↑	+0.6°C	(Knoch et al., 2022)
Song sparrow *Melospiza melodia*	Captive	Adult	Handling (13 min)	Eye Beak	↓, = ↓, ↑, =	-1.5°C, = -2.6°C, +2.6°C, =	(Zuluaga and Danner, 2023)

* king penguins chicks are not thermally emancipated at this age

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**	**SE**	**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**	**SE**	**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)

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

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