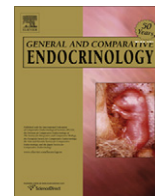


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Ambient temperature and pregnancy influence cortisol levels in female guinea pigs and entail long-term effects on the stress response of their offspring

C.L. Michel*, O. Chastel, X. Bonnet

CEBC CNRS, UPR 1934, Villiers en Bois, 79360 Beauvoir sur Niort, France

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ABSTRACT

Mammals generally respond to the important metabolic requirements imposed by thermoregulation and pregnancy by increasing plasma concentrations of glucocorticoid that promote the mobilization of body reserves and enhance energy use by tissues. This study examined the impact of distinct ambient temperatures and reproductive status on cortisol plasma levels in female guinea pigs (*Cavia aperea f. porcellus*). We also examined cortisol profiles of their offspring. Forty adult females were placed in individual boxes, 20 were exposed to a neutral thermal regime (mean ambient temperature 22.1 ± 1.5 °C) and 20 were maintained under a cool thermal regime (15.1 ± 1.5 °C). Within each treatment, 12 females were pregnant and 8 were non-pregnant. Pregnancy generated a marked elevation of baseline cortisol. Ambient temperature also affected cortisol concentrations. Compared to the pregnant females from the neutral thermal regime, pregnant females maintained under cool conditions exhibited lower baseline levels of cortisol, were less active, but they displayed a greater stress response (i.e. rapid increase of plasma cortisol) following handling. Thermal treatment did not influence reproductive output, reproductive effort, or offspring characteristics. This suggests that pregnant female guinea pigs cope with cool (but not extreme) thermal conditions by reducing activity and baseline cortisol levels, possibly to save energy via an adaptive response. Interestingly, the greater amplitude of the stress response of the cool regime females was also observed in their offspring 2 months after parturition, suggesting that hormonal ambience experienced by the individuals *in utero* shaped their stress response long after birth.

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1. Introduction

Glucocorticoid hormones are notably involved in the mobilization and use of energy in various physiological processes, including cardiovascular, metabolic, immunologic and homeostatic processes. These hormones are therefore central in the energy budgets of vertebrates [2,8,11,14,16,80]. Hormonal regulations are complex however, and different factors such as stress, age, photoperiod, and social context influence plasma glucocorticoid levels [6–8,62]. In female mammals, thermoregulation and reproduction involve major energy expenditure [52]. As both of these processes are under hormonal regulation, including glucocorticoid hormones, interactions between ambient temperature, gestation, and glucocorticoid levels are thus expected [61,62]. This issue remains poorly explored however. Glucocorticoids also play important roles in the stress response. The concept of stress is complex and subjected to various definitions (e.g. water balance stress, energetic stress, and emotional stress are all investigated in different ways and they involve different effectors). In a broad

context however, individuals are considered as stressed when their neuro-physiological demands exceed their regulatory capacities [5]. Stress is thus generally perceived as a temporary state existing between homeostasis and pathology, and in most cases stressors provoke consistent physiological responses with a rapid elevation of the plasma levels of several circulating hormone such as catecholamine, glucocorticoid, and vasopressin notably [5,13]. In the current study we limit the investigations of stress to marked elevations of glucocorticoid plasma levels under different ambient temperatures (an environmental stressor), considering reproductive status (a physiological stressor), and following handling (thereby mimicking a predator attack).

The influence of ambient temperature on reproduction has been documented in mammals, mostly in livestock, perhaps owing to their economic value [18,25,50,70]. Heat-stress, for example, can perturb reproduction in mammals [26,35]. However, very few studies have been performed on the effect of cold ambient temperatures on pregnant females and their offspring [85]. Embryos are sensitive to intra-uterine environmental conditions; their development and thus the resulting neonate phenotypes are optimized under stable conditions [22]. Pregnant females are under strong selection to buffer the perturbations that may reach their embryos [20] and hormones play a central role in these buffering processes

* Corresponding author. Fax: +33 549096526.

E-mail address: catherine.louise.michel@gmail.com (C.L. Michel).

[84]. When pregnant females are exposed to low temperatures, strong hormonal responses are expected. The embryos may consequently be exposed to hormonal fluctuations that can in turn influence their phenotype. Steroids are especially active in this process as they cross biological membranes easily and thus rapidly reach the embryo [86]. A considerable proportion of maternal effects on offspring phenotypes are indeed mediated via hormones [55]. The possible cascade of effects of low ambient temperatures during gestation on offspring phenotypes mediated by hormones has not been examined in mammals.

The energetic impacts of gestation and cool ambient temperatures should be particularly severe in relatively small and precocial mammals: the production of large and well-developed neonates requires a considerable reproductive investment during gestation and this is cumulated with an unfavorable body mass/surface ratio for endothermy in small mammals [31]. Guinea pigs (*Cavia aperea f. porcellus*, Caviomorph rodents) provide an excellent opportunity to examine these issues. In this species pregnancy lasts 68 days on average, entails a heavy maternal investment and females give birth to very large and well-developed offspring relative to their own size [44]. On average, relative litter mass represents 30% of the post-parturient maternal mass. Maternal mass can increase by more than 50% during gestation; impeding maternal mobility and sustained by an intensive foraging activity. Neonates are almost independent and the lactation period is very brief [43,44]. Because neonates are physiologically mature for many functions (e.g. central nervous system, endocrine and cardiovascular systems), their phenotypes, including endocrine characteristics, should largely reflect the impact of the prolonged intra-uterine period. Adult females are relatively small (approximately 800 g), and thus are subject to the difficult physiological challenge of maintaining their body temperature under low thermal conditions. Adding to the suitability of this species for this study is the existing background data: the reproductive physiology and ecology of these animals have been intensively studied [40,44,71,72].

We experimentally examined the influence of distinct (neutral versus cool) ambient temperatures on baseline and stress cortisol levels in pregnant and non-pregnant female guinea pigs. We used in this study the cortisol level as a proxy of the stress response. Although we thus assessed only one aspect of an otherwise complex stress response, the use of changes of cortisol levels (routinely measured in vertebrates) has been validated and is now widely employed [13]. We also assessed the impact of experimental treatment on reproductive output and the offspring. We notably assessed baseline and stress cortisol levels of the offspring 2 months after birth. This issue is important to determine if environmental conditions can influence offspring stress physiology, a prerequisite to explore the adaptive value of maternal effects [9]. We addressed two main questions:

- (1) Do gestational status and ambient temperature influence maternal cortisol levels?
- (2) If so, does this translate into different stress responses in the offspring?

2. Materials and methods

2.1. Study species

The domestic guinea pig reproduces throughout the year and, in contrast to rats and mice, irrespective of photoperiod, assuming sufficient food and thermally neutral ambient conditions [71]. Caviomorphs are distinct from other rodents as they produce extremely precocial offspring, guinea pigs in particular. Compared to altricial species, neonates are physiologically mature (except for reproductive functions), agile and relatively independent at birth,

resembling miniature adults: they have open eyes, exhibit fully developed fur, feeding apparatus, and start to forage almost immediately after birth. In guinea pigs, most of the offspring development is achieved before birth [44]. Neonates survive weaning at 5 days old [77], and in experimental conditions it is possible to separate them from their mother 6 days after parturition. Pregnancy entails a massive maternal investment and enhances feeding rate [44]. Maternal body mass increases markedly (50% elevation) during gestation, and impedes mobility due to the overload-handicap. Overall the potential effects of post-natal maternal care are very limited compared to the long gestation period during which most of the morphological characteristics are set.

Previous studies provided an important background on guinea pig life history traits, and reproductive physiology and ecology [4,36,38,47,59,65]. Notably, the role of the HPA (hypothalamic-pituitary-adrenal axis) in relation with social status and stress responses has been investigated [29,30,39,53]. Importantly, these studies shown that in guinea pigs plasma variations of glucocorticoids can be used as a proxy of the stress response. But they also revealed that such response is relatively independent of several confounding factors. For instance, social environment affects behavior and androgen levels, but not cortisol concentrations in pregnant females [37] and their young [41]. These results limited possible confounding social influence impacts during pregnancy on plasma cortisol levels, and thus enabled us to focus on the impact of ambient temperatures in interaction with reproductive status.

2.2. Experimental design

Forty adult female guinea pigs were involved in the experiments (February 2008 to April 2008). Individuals originated from a colony maintained at the Centre d'Etude Biologique de Chizé (France). Using natural color markings, each individual was easily identified (e.g. variations of the main color morphs: "yellow", "black", "brown", and "gray"). All the adult females involved had previously successfully produced 1 or 2 litters, but none of them had recently reproduced at the onset of the experiment. At least 100 days had elapsed between the last parturition and the beginning of the experiment.

We placed each female with an adult male (randomly selected from twenty males) in an individual cage for 10 days, and then with a different male for the same amount of time. We expected that most (i.e. >70%) of the females would become pregnant and that the rest of the females would not reproduce for various uncontrolled factors as observed in closely related experimental designs [71]. We then randomly allocated the females to two thermal treatments. Maternal body mass, body size, color morph and age did not differ significantly between the two experimental groups (Wilcoxon–Mann–Whitney-*U* test, all $p > 0.1$).

Most endotherms better tolerate cool rather than hot ambient temperatures; therefore we used a neutral regime versus cool regime design [28]. In guinea pigs, thermal conductance is relatively low and stable when individuals are placed under ambient temperatures (T_a) close to 20 °C; central body (colonic) temperature is easily maintained at 38 °C when T_a is between 22 °C and 30 °C [24]. For the current experiment, we set up a "cool" mean temperature regime at 15 °C, and a "neutral" mean temperature regime at 22 °C. Such experimental ambient temperatures, although distinct, were not particularly harsh for the animals (the 15 °C cool regime was well tolerated and no sign of disorder was observed), thereby limiting a potential overstress impact on reproduction. The experiment was performed in winter in a large shed that protected the animals from bad weather (extreme cold temperatures and precipitations) and buffered external temperature fluctuations. Each of the 40 females was kept in an individual box (60 cm × 50 cm × 35 cm) with a wood shelter (20 cm × 15 cm × 15 cm) and hay as substratum, a

drinking bowl connected to an automatic system provided clean fresh water. Dry food (Commercial pellets, ©Moissons du Clos, France) was provided ad libitum and the diet was supplemented with a standard number of pieces of carrot, endive, and apple. Dry food (pellets) provided most of the energetic resources, with fresh food providing vitamin and other oligo-element supplements. We used two types of individual box. Twenty boxes were fitted with a heater (150 W Elstein© Ceramic Infrared Heater, such device does not produce light) the distance and the power of the heaters were set in order to generate mean ambient temperatures close to thermal neutrality (see Section 3). The two types of boxes (cool versus neutral) were placed in alternation in an overall grid design (producing an experimental checkerboard made of cool and neutral boxes) to limit the influence of other environmental factors. The boxes were inspected daily. Each female was allocated to a box (cool or neutral) and was examined for reproductive and general status, and was weighed once a week.

The random mating design generated an equilibrated distribution of the gravid versus non-pregnant females: in each group, 12 females became pregnant (66%) and 8 were non-pregnant (34%). The date of parturition was recorded; as the boxes were examined every day and because births occurred in early morning, the maximum deviation from the precise timing of parturition was of 12 h. Number, sex and morphological characteristics (mass, size, color pattern) of the pups were recorded as soon as parturition was observed. We separated the pups from their mother 7 days later [77]. Following separation, the pups were maintained under stable thermal conditions close the thermal neutrality (mean ambient temperature = 19.1 ± 1.7 °C). The female offspring were kept alone in a box (the same type of box than those used for the mother), and the male offspring were placed together in a larger enclosure (4 m*2 m, note that social environment does not influence cortisol concentrations in young guinea pigs; [40]); both types of boxes were placed in the large shed, and hence all individuals were exposed to similar climatic conditions. All offspring were fed ad libitum.

2.3. Temperature monitoring

Ambient temperatures in individual boxes were monitored using data loggers (iButtons Thermochron®). In order to estimate the range of thermal gradients available in the boxes (especially in those fitted with the ceramic heater), two data loggers were placed in each box: one on the floor and one on the roof of the shelter. The data loggers recorded ambient temperatures every 10 min during the entire experiment. We assessed the external temperature of the fur of the guinea pigs with a laser thermometer (Raytek MX2, Fotronic Corporation, USA, calibrated for the targeted substrate using a digital thermometer fitted with a probe) and targeted three different points to calculate a mean value.

2.4. Behaviors and activity level

During 1 week we scanned daily the boxes at regular time intervals (8:00 h, 11:00 h, 14:00 h, 17:00 h, and 20:00 h) and recorded the position and behavior of each female. For that we carefully approached individuals because although the guinea pigs involved in the experiment were accustomed human observers, a rapid movement could alarm them. In a step by step approach we firstly determined the daily activity rhythm of the guinea pigs. In each box, we notably noted if the female was sheltered or not, her exact position in the box (back, middle, front), or basking under the ceramic heater. We noted precisely such attitude in order to examine if for instance females placed under the lower thermal regime remained more often under the shelter, for example curled up in the rear of the box. For simplicity, we used two types of attitudes: inactive

versus active. The term inactive referred to behaviors such as lying motionless, sleeping in the box for example; whilst the term active was associated to behaviors such as walking, eating, grooming, or drinking.

2.5. Food consumption

Daily food consumption was calculated as the net difference between the food ration supplied and the remaining food after 24 h (grams of dry food, number of pieces of fresh food). In practice this calculation was mostly relevant to the dry food because guinea pigs tended to rapidly eat all the fresh food (and to not totally consume the dry food (on average the females consumed $31.6 \pm 4\%$ of the pellets available). Pregnant females especially tended to eat most (or all) the fresh food. For clarity, dry and fresh foods were considered separately.

2.6. Blood sampling and hormonal assays

We blood sampled all the females around mid-gestation (4 weeks before the first parturition occurred). Pups were blood sampled 2 months after birth. To assess acute stress response, we used a standardized capture/handling stress protocol [79]. All blood samples were taken without anesthesia, between 13h30 and 15h00 to limit daily variations. Samples were taken from the main marginal ear vein with a sterile needle (0.9 × 40 mm) and blood was collected in heparinized capillaries [64]. The samples were immediately centrifuged ($3' \times 10000g$) and the plasma was collected and stored at -25 °C until assays. For baseline cortisol levels, all guinea pigs were sampled within 3 min after removal from their box. Each guinea pig was subsequently kept in a calico bag to generate handling stress. After 1.5 h in the bag, we took a second blood sample for stress-induced cortisol levels following the same method. The guinea pigs were then returned to their box.

Cortisol is the main glucocorticoids in guinea pigs [30]. All hormonal assays were performed at the Centre d'Etudes Biologiques de Chizé. Plasma concentrations of glucocorticoids were determined using radioimmunoassay (RIA; [83]) a technique routinely employed in our laboratory. Baseline and stress-induced plasma cortisol was extracted with ether (100 µL extracted from 15 µL of plasma) and then measured by RIA using a polyclonal rabbit antiserum (Sigma, USA) (immunogen was cortisol-21-hemisuccinyl-thyroglobulin). For each extracted sample, duplicate aliquots were incubated overnight at 4 °C with antiserum and 8000 cpm of 3H -cortisol (Amersham GE Healthcare, UK). The bound cortisol was separated from the free cortisol by adding dextran-coated charcoal and after centrifugation, the radioactivity of the bound fraction was counted on a liquid scintillation analyzer. Sensitivity of the assay was 300 pg/ml. All samples were run in three assays, the coefficients of intra and inter-assay variation being 6.2% and 8.5%, respectively. The antiserum cross-reacted with relevant steroids as follows: androstenedione (<0.1%), Compound S (7%), corticosterone (0.1%), 11deoxycorticosterone (0.1%), progesterone (7%), and testosterone (<0.1%).

The relatively high plasma levels of cortisol we recorded in guinea pigs (often >100 ng ml⁻¹, see Section 3) are in the range of the values observed in other rodents of comparable body size (e.g. 100–400 ng ml⁻¹ in the ground squirrel, *Spermophilus saturatus*, 200–800 ng ml⁻¹ in the chipmunk, *Tamias amoenus*; [63]). Such results are important for comparative purposes because plasma levels of glucocorticoids vary greatly among amniotes depending upon taxonomy and reproductive strategy [63]. For example, average baseline levels are low in certain species (e.g. 5 ng ml⁻¹ for the non-breeding Texas horned lizard, *Phrynosoma cornutum*; [75]; 2 ng ml⁻¹ for house sparrows, *Passer domesticus*, [48]), but they are extremely elevated in others (e.g. 1270 ng ml⁻¹ in the fruit

bat, *Pteropus hypomelanus*, [78]; and 3000–8000 ng ml⁻¹ in the Lemming, *Lemmus trimucronatus*, [63]).

2.7. Analyses

Statistical analyses were performed using R7.1. (R-Development Core Team 2008). Potential deviations from the assumptions of the models were checked using graphical diagnostic tools [19]. Mean values are presented with standard error unless otherwise stated. To limit problems associated with autocorrelation of temperature data, we calculated mean temperature for each box in order to compare ambient temperature between groups. To analyze effect of ambient temperature on changes of maternal body mass and food consumption we used ANOVA for repeated measures. Female body mass and litter mass had no effect on the cortisol levels and were therefore not included in the models. We analyzed offspring characteristics using linear mixed effects (LME) models; maternal identity was used as a random factor in a nested design. In preliminary models, litter size had an effect on offspring body mass, litter size was therefore included in the models as covariate. Maximal cortisol levels after handling were positively correlated with baseline levels ($F_{1,38} = 47.30$, $r = 0.55$, $p < 0.001$). Consequently, we calculated the stress response, the elevation of cortisol levels following handling, as the residual from the regression between stress levels following handling against baseline levels of cortisol. Model selection was performed using stepwise backward procedure, removing non-significant terms beginning with the interactions with the largest p -value in each step. For conciseness, in Section 3, we provide only final models. Interaction terms were all non-significant unless otherwise reported.

3. Results

3.1. Temperature monitoring of the boxes

Mean ambient temperatures were significantly different between the two treatments (Table 1), and were respectively 22.11 ± 1.55 °C for the neutral regime and 15.13 ± 1.55 °C for the cool regime. Circadian variations caused by the imperfect buffering of the shed with respect to external natural fluctuations remained relatively low, on average 0.70 ± 0.74 °C versus 0.78 ± 0.74 °C for the neutral and cool regimes, respectively. The mean fur temperatures were significantly different between the two treatments for the neutral (31.45 ± 0.62 °C) and the cool (23.35 ± 0.60 °C) groups (ANOVA; $F_{1,38} = 28.134$, $p < 0.0001$).

Table 1

Summary of the main maternal traits measured, and of reproductive output characteristics, in guinea pigs placed under two thermal regimes. Means are expressed with standard deviation and range in brackets. Significant, or marginally significant, effects are indicated in italics.

Trait	Thermal regime		Covariate	df	F	p-Value
	Cool	Neutral				
Ambient temperature (°C)	15.3 ± 1.55	22.11 ± 1.55		1.38	67.76	<0.001
Maternal mass increase (g)	251.4 ± 32.8	249.6 ± 21.3	Litter mass	1.22	0.352	0.559
Food consumption per day (g)	28.2 ± 5.7	32.3 ± 7.3		1.22	2.55	0.114
Activity at 8:00 h (% of time active)	0.25 ± 0.11	0.67 ± 0.12		5.34	4.405	0.010
Activity at 17:00 h (% of time active)	0.31 ± 0.12	0.60 ± 0.13		5.34	2.78	0.075
Activity at 20:00 h (% of time active)	0.12 ± 0.08	0.40 ± 0.13		5.34	2.732	0.058
Maternal basal cortisol level (ng/ml)	231.14 ± 18.12	283.91 ± 32.93		1.22	3.972	0.058
Maternal stress response (residuals)	0.41 ± 0.16	0.17 ± 0.12		1.22	5.028	0.035
Pup basal cortisol level (ng/ml)	56.21 ± 6.98	65.14 ± 7.16		1.54	2.767	0.012
Pup stress response (residuals)	2.25 ± 0.37	0.9 ± 0.13		1.54	2.07	0.052
Parturition date (date)	13 June ± 8 days	10 June ± 6 days		1.22	1.114	0.303
Litter size	3.9 ± 1.3 (2 to 6)	3.4 ± 0.9 (1 to 4)	Maternal mass	1.61	1.186	0.288
Litter mass (g)	339.1 ± 82.5	306.3 ± 75.5	Maternal mass	1.61	1.034	0.320
Pup mass (g)	86.56 ± 12.97	90.39 ± 12.65		1.61	0.114	0.737
Pup size (mm)	134.05 ± 6.59	132.32 ± 8.63		1.61	0.135	0.714
Pup body condition (residuals)	0.89 ± 7.9	0.78 ± 8.91		1.61	0.009	0.921

3.2. Body mass and food intake variations in relation to pregnancy and thermal regime

As expected the body mass of pregnant females increased over time while the body mass of non-pregnant females remained stable (ANOVA with successive female body mass as repeated measures and reproductive status as a factor; Wilk $\lambda = 0.092$, $F_{15,23} = 15.16$, $p < 0.001$; specific of time, $F_{14,532} = 23.700$, $p < 0.0001$). Changes in maternal mass over time were positively correlated with litter size ($F_{1,22} = 14.367$, $r^2 = 0.346$, $p = 0.002$) and litter mass ($F_{1,22} = 11.68$, $r^2 = 0.395$, $p = 0.001$) indicating that a significant proportion of maternal mass variation was caused by developing embryos. Pregnant females consumed greater quantities of dry food compared to non-pregnant females: $34.67 \text{ g} \pm 4.58 \text{ g}$ versus $28.50 \text{ g} \pm 6.88 \text{ g}$ (ANOVA with mean mass of dry food eaten per day as the dependent variable and reproductive status as the factor; $F_{1,38} = 2.781$, $p = 0.033$) and of fresh food (same design ANOVA with the mean number of pieces eaten per day as the dependent variable; $F_{1,38} = 3.718$, $p < 0.01$).

Unexpectedly however, females placed under neutral thermal regime did not increase more in mass compared to the females from the cool regime; and such absence of effect held true both in pregnant (Table 1), and non-pregnant females (ANOVA; $F_{1,14} = 0.097$, $p = 0.761$). We found no difference between the females from the two thermal regimes in terms of dry food consumption, both in pregnant (Table 1) and non-pregnant females (ANOVA with mean food mass eaten per day as the dependent variable and ambient temperature as a factor; $F_{1,14} = 2.175$, $p = 0.202$). We also found no difference between the females from the two thermal regimes in terms of fresh food consumption, both in pregnant (ANOVA with mean number of pieces fresh food eaten per day as the dependent variable and ambient temperature as a factor; $F_{1,22} = 1.890$, $p = 0.183$) and non-pregnant females (same design ANOVA; $F_{1,14} = 2.218$, $p = 0.160$).

3.3. Activity in relation to pregnancy and thermal regime

Female guinea pigs were more active (eating, walking, grooming) in the early morning (08:00 h) and to a lesser extent in late afternoon (17:00 h) (repeated measures ANOVA, specific effect of time; $F_{5,34} = 27.813$, $p < 0.0001$). Irrespective of the experimental ambient temperature and the time of the day, the positions adopted by guinea pigs inside their box did not produce any clear pattern (repeated measures ANOVA, with time and temperature as the factors; $F_{1,38} = 2.213$; $p = 0.120$), and females under cool

temperature regimes did not spend larger amounts of time under their shelter compared to the females from the neutral regime (repeated measures ANOVA with time and temperature as the factors; $F_{7,31} = 0.519$, $p = 0.813$). However, guinea pigs placed under neutral regime were more active compared to the individuals placed under the cool regime during the peaks of activity (one way ANOVA with ambient temperature as a factor and activity level as the dependent variable measured at 8:00 h: $F_{5,34} = 4.405$; $p = 0.01$; at 17:00 h: $F_{5,34} = 2.78$; $p = 0.075$; and at 20:00 h: $F_{5,34} = 2.732$; $p = 0.058$).

3.4. Maternal baseline and stress cortisol levels in relation to pregnancy and thermal regime

Both reproductive status and thermal treatment significantly influenced baseline and stress-induced cortisol plasma levels in adult female guinea pigs (Fig. 1).

Baseline levels of cortisol were higher in pregnant females compared to non-pregnant females (one way ANOVA, reproductive status as the factor; $F_{1,38} = 20.15$, $p < 0.01$). Similarly, stress-induced cortisol levels were higher in pregnant females (same design ANOVA; $F_{1,38} = 10.57$, $p < 0.01$). Thus, based on cortisol plasma concentrations, pregnancy was associated with a more intense activity of the HPA-axis. In both pregnant and non-pregnant females, thermal treatment did not influence baseline cortisol levels (Table 1, and non-pregnant females $F_{1,14} = 0.004$, $p = 0.900$). However, stress response following handling was more marked in the pregnant females maintained under cool regime compared to the pregnant females from the neutral regime (Table 1). Fig. 1 suggests that pregnant females from the neutral regime group exhibited an almost suppressed acute stress response with no significant difference between baseline and stress cortisol levels; plasma cortisol levels were high before and after handling (paired Student *t*-test; $p > 0.1$). In contrast, pregnant females from the cool treatment displayed a marked stress response with baseline levels situated in an intermediate position between pregnant females from the neutral thermal treatment (high values) and non-pregnant females (low values) (paired Student *t*-test; $p < 0.01$), and an elevated stress level.

Overall, pregnancy caused a strong elevation of plasma cortisol concentrations. Cool ambient temperatures were associated with a down regulation of baseline levels, but not of the maximal levels

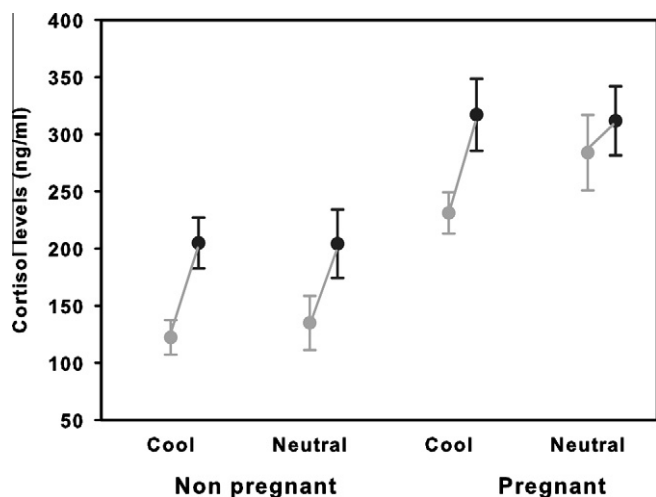


Fig. 1. Comparison of the cortisol plasma levels of pregnant females ($N = 24$) and non reproductive female ($N = 16$) guinea pigs placed under two thermal treatments: neutral ($22\text{ }^{\circ}\text{C}$) versus cool ($15\text{ }^{\circ}\text{C}$) ambient conditions. Both baseline (gray symbols) and handling-induced stress levels (dark gray symbols) are represented. Mean values are expressed with their error type. Arrows indicate the elevation of cortisol following handling stress.

following handling (i.e. stress response) in pregnant females. Non-pregnant females presented low baseline levels and moderate levels during the stress response irrespective of the thermal regime.

3.5. Reproductive output in relation with thermal regime

Thermal treatment did not influence parturition date (Table 1), the proportion of stillborns (3 stillborns versus 41 healthy offspring, and 1 stillborn versus 47 healthy offspring in the neutral and cool regime respectively; $\chi^2 = 1.24$, $p = 0.27$), mean litter size (Table 1), mean litter mass (Table 1), or sex ratio (neutral regime: 18 females and 23 males $\chi^2 = 0.610$, $p = 0.435$ versus cool regime: 23 females and 24 males $\chi^2 = 0.0213$, $p = 0.884$).

Post-parturition maternal mass did not differ between the two groups (repeated measures ANOVA with ambient temperature as a factor; $F_{1,22} = 0.106$, $p = 0.748$; interaction between temperature and time; $F_{7,154} = 0.789$, $p = 0.597$). Relative litter mass, an index of reproductive effort, was not influenced by ambient temperatures (ANCOVA with thermal treatment as a factor, litter mass as the dependent variable and post-parturition maternal mass as a covariate; $F_{1,37} = 0.254$, $p = 0.617$).

Thermal treatment did not influence the characteristics of the offspring: body mass (Table 1), body size (Table 1), body condition (Table 1), or post-natal survival (only two pups died, both from the same litter).

3.6. Plasma cortisol levels of the offspring in relation to maternal thermal regime

We collected blood samples from a total of 56 offspring, 24 from the neutral regime and 32 from the cool regime. We found no effect of sex of the offspring on cortisol levels (LME models, baseline levels: $F_{1,54} = 0.177$, $p = 0.676$; stress-induced levels: $F_{1,54} = 0.166$, $p = 0.203$). In addition, this result suggests that the two social conditions experienced by the young females (alone) and young males (in group) respectively did not influence cortisol levels (as already observed by Kemme et al. [41]). There was no significant relationship between offspring body mass and plasma cortisol levels (LME models, baseline levels: $F_{1,54} = 1.101$, $p = 0.299$; stress levels: $F_{1,54} = 0.018$, $p = 0.895$).

Although we found no significant influence of the thermal treatment on the cortisol plasma levels of the offspring (LME models, baseline levels: $t_{1,54} = 1.265$, $p = 0.141$; stress-induced levels: $t_{1,54} = 1.533$, $p = 0.140$; Table 1), the amplitude of the stress response was greater in the pups born from the cool regime females (LME models, using two different measures of stress response: S-TB/TB [where TB = baseline corticosterone level and S = stress-induced corticosterone level]: $t_{1,54} = 2.767$, $p = 0.012$, or the residuals from the regression between stress-induced levels against baseline plasma cortisol levels: $t_{1,54} = 2.070$, $p = 0.052$; Fig. 2; Table 1). This suggests that the greater stress response observed in the mothers subjected to cool ambient temperatures was somehow transferred to their offspring. Because the tests were performed 2 months after parturition, this maternal influence persisted long (at least 2 months) after the environmental conditions necessary for the induction of this effect were removed.

4. Discussion

Our results show that pregnancy entailed a chronic elevation of plasma levels of cortisol, and that this effect was associated with behavioral and morphological variations (increase in food consumption and body mass). Such co-variations between changes in glucocorticoid hormone levels, behaviors, and body mass were

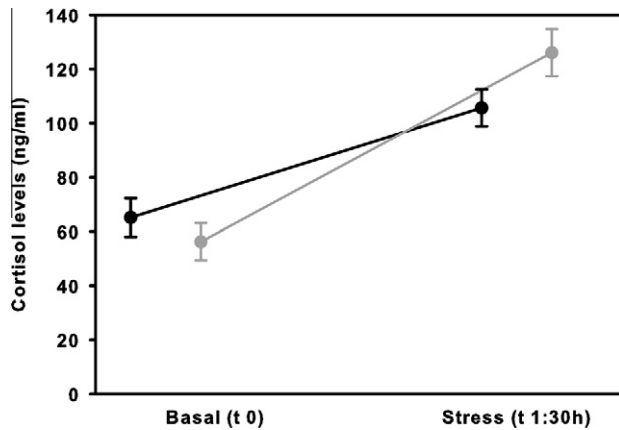


Fig. 2. Comparison of the stress response – difference between baseline level (t0: blood sampling immediately after capture) and post-handling stress-induced levels of cortisol (t1h30: second blood sample taken 1h30 later) – of the pups from mothers maintained in neutral (black symbols) versus cool (gray symbols) conditions. Mean values are expressed with their error type.

probably associated with the increasing energy demands of the developing fetuses [42,51,81]. Food was not limited in our experimental design. When facing challenging metabolic conditions (i.e. reproduction), female guinea pigs responded positively and ingested larger food rations than non-pregnant females [67].

Surprisingly however, ambient temperatures did not affect these same two parameters of the maternal energy budget. When placed under cool thermal regime, the females, pregnant or not, did not increase food intake. Similarly, thermal treatment did not influence reproductive output (offspring number, offspring mass) and reproductive effort (relative litter mass). Experimental subjects were not exposed to extreme environmental conditions, and thus they did not reach the physiological limits of the species (e.g. prolonged negative temperatures). It is possible that a seven-degree deviation from thermal neutrality was insufficient to trigger a significant metabolic response. However this is unlikely as guinea pigs are relatively small endotherms, not particularly well adapted to cold climates, and even small variations in ambient temperatures impact the thermoregulatory metabolism in small or medium sized mammals ([31]; our unpublished results on guinea pigs). In the current experimental design, females were submitted to distinct thermal regimes (15 °C versus 22 °C) over prolonged time periods, and such difference necessarily influenced their respective metabolism. Consequently, the absence of a significant effect of ambient temperature suggests that compensatory mechanisms enabled the guinea pigs to buffer environmental constraints. More precisely, our data revealed that when confronted with cool ambient temperatures, physiological and behavioral regulations mediated by plasma cortisol levels were involved without influencing reproductive output: compared to females placed under neutral thermal conditions, pregnant females under cool temperatures reduced their activity level and baseline levels of cortisol, possibly to save energy; but they maintained unchanged their reproductive output. Cooler temperatures modulated stress regulation in guinea pigs, possibly adaptively in pregnant female (e.g. via energy saving); further investigation are required to test such assumption however.

In mammals, the elevation of baseline cortisol and specific transport protein (CBG) levels during pregnancy positively influence myometrial activity, uterine blood flow, and glucose release from body reserves; probably in relation with increasing metabolic requirements [12,34,46,57]. The elevation of baseline plasma cortisol in pregnant female guinea pigs related to the mobilization of resources in response to increasing metabolism was therefore

expected. Such generalized increase in cortisol levels induced by gestation was therefore taken into account to compare all experimental groups that involved thermal and reproductive factors. Clearly, pregnant females from the cool thermal regimes exhibited intermediate baseline cortisol concentrations between the high values recorded in the pregnant females from the neutral regime and the relatively low values of the non-pregnant females. This result firstly demonstrates that ambient temperatures actually influenced maternal physiology, and thus that our thermal experimental design impacted the animals. Secondly, this provides a physiological clue for the absence of effect of cool thermal regime on maternal food intake and reproductive output: lower plasma cortisol levels were possibly associated with a lower maternal metabolism. Apparently, to buffer moderately cool environmental conditions, female guinea pigs firstly slightly reduced their overall behavioral and physiological activity without consequences on reproductive output. Our results suggest that this compensatory mechanism was notably mediated by a decrease of locomotor activity: females placed under cool regime spend more time immobile than the females from the neutral regime. Measurements of oxygen consumption on pregnant females under different experimental thermal conditions, and following cortisol (and antagonist) injections are essential to accurately test this hypothesis however. The characteristics of the litter were unaffected by the thermal treatments, hence the perceived fitness of mother; the hormonal regulations we observed were thus apparently contained within the range of normal adaptive responses, likely below maximal physiological limits (i.e. non-pathological).

The stress response – rapid elevation of cortisol plasma concentrations recorded following handling – provides complementary results. The stress-induced elevation of cortisol in non-pregnant females remained relatively moderate (Fig. 1), at least compared to the baseline levels of pregnant females. Pregnant females from the two thermal regimes responded differently: the magnitude of the stress response was greater under cool conditions and the acute cortisol stress response was somehow suppressed in pregnant females under neutral regime. Two alternative explanations can be proposed. Firstly, the absence of stress response in the pregnant females from the neutral regime reflects the saturation in the functioning of a specific segment of the HPA-axis; very high quantities of cortisol are chronically released: baseline and post-handling plasma values were indeed all at the maximal values recorded, possibly indicating a saturation of the releasing capacities. We note that this does not mean that other segments of the overall stress response (e.g. release of catecholamine, vasopressin, etc.) were suppressed. Alternatively, the magnitude of the stress response is sometimes considered to reflect the parental motivation in a stressful context [48]; the suppression of the stress response in the females under neutral thermal conditions may mirror their greater commitment to the current reproduction compared to the mothers under cold regime. This second hypothesis is poorly supported however due to the absence of difference in terms of reproductive effort or reproductive output between the females from the two thermal regimes; in addition viviparity imposes far more rigid physiological constraints compared to egg brooding or chick-rearing.

The greater amplitude of the stress response of the cool regime females was also observed in their offspring 2 months after parturition. Alternatively, such difference between the two groups may arise from physiological adaptation to post-natal conditions if we consider that more energy is required to adapt from 15 °C to 19 °C than from 22 °C to 19 °C. However, this cannot explain the fact that the sole significant effect was associated to the treatment experienced by the mothers whilst all the offspring were raised under similar thermal conditions. Furthermore, the possibility that maternal hormonal ambience experienced by the embryos during

their development shaped their post-natal stress response is supported by results from other studies. Notably, glucocorticoid hormones can cross the placenta barriers, and maternal cortisol can thus easily reach the fetus [15,86]. Experimental modifications of offspring exposure to glucocorticoids (injections, food supplementation, and subcutaneous implants) influence the HPA axis activity [58,73] and the number of adrenocorticoid receptors in the hippocampus area of the brain [21]. Several studies demonstrate the heritability of circulating steroid levels in primates and livestock [3,33,54,60,66]. Our results thus revealed a previously un-described maternal effect; the epigenetic transmission of differential amplitude of the stress response under distinct thermal environments during gestation. Epigenetic programming of the stress response has been documented in mammals [17]. We emphasize, however that previous studies used intensive stressors during the prenatal period (e.g. footshocks, strong alcohol challenge...) whilst the stressors we employed belong to a natural stressors commonly encountered by free ranging animals. Consequently, our study extends the range of factors capable to induce epigenetic effects from intensive and artificial stressors (e.g. strong drugs, previous studies) toward more natural and widespread factors (e.g. moderate fluctuations of ambient temperature, current study). Our results consequently provide a more natural basis to examine the possible adaptive value of epigenetic transfer such as the regulations reported in the current study.

In mammals, frequent exposure to strong stressors provokes chronic elevation of circulating glucocorticoids that in turn negatively impact the offspring: low offspring mass, permanent hypertension and hyperglycemia in adults, and degraded learning capacities [1,45,49,74]. On the other hand, when maternal cortisol levels fall to very low plasma concentrations (e.g. due to hypocorticism), negative effects on the offspring are also observed [12,82]. In the current experiment, the offspring from the two treatments were indistinguishable, most of them survived and none of them showed any sign of disorder. The different hormonal responses caused by the thermal treatments, recorded both in the mothers and in their offspring, were thus contained within non pathologic physiological limits. In the absence of a detectable pathological effect our result are more relevant to the notion that an epigenetic transfer can be adaptive. Perhaps that under cool conditions, higher stress response provides an adaptive advantage as it is related with greater capacity to mobilize resources? Currently we do not know if the offspring obtained an advantage by setting the amplitude of their stress response in relation with the environment experienced by their mothers, and hence that they were the most likely to encounter after birth; and a lack of similar study precludes further comparison. Therefore we cannot speculate on the possible adaptive value of the maternal effect we found [32,55,68,76].

In their recent review, Breuner et al. [8] suggested to further measure the heritability of glucocorticoid plasma levels in relation to environmental factors, and to assess fitness consequences to examine possible selection acting on those traits influenced by glucocorticoids. Many life history characteristics are concerned: locomotor activity, dispersal behavior, immunity, metabolism, growth rates and spatial memory [10,23,27,56,69]. The multiple effects of steroid hormones underlie trade-offs and provide a bridge between proximate and ultimate factors. Our results revealed several new effects which suggest that guinea pigs offer interesting possibilities to better understand the various levels of implication of glucocorticoids in the regulation of coping mechanisms associated with environmental fluctuations.

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