Role of huddling on the energetic of growth in a newborn altricial mammal

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Huddling is considered as a social strategy to reduce thermal stress and promote growth in newborn altricial mammals. So far, the role of huddling on the allocation of saved energy has not been quantified nor have the related impacts on body temperature rhythms. To determine the energy partitioning of rabbit pups either raised alone or in groups of eight, four, or two individuals, when thermoregulatory inefficient (TI) and efficient (TE), we first investigated their total energy expenditure and body composition. We then monitored body temperature and activity rhythms to test whether huddling may impact these rhythms, centered on the suckling event. Pups in a group of eight utilized 40% less energy for thermogenesis when TI than did pups alone and 32% less energy when TE. Pups in groups of eight and four had significantly lower thermoregulatory costs in the TI period, whereas pups in groups of two, four, and eight had lower costs during the TE period. Huddling pups could therefore channel the energy saved into processes of growth and accrued more fat mass (on average 4.5 ± 1.4 g) than isolated pups, which lost 0.7 g of fat. Pups in groups of four and eight had a body temperature significantly higher by 0.8°C than pups in groups of two and one when TI, whereas no more differences were noted when the TE period was reached. Moreover, pups alone showed an endogenous circadian body temperature rhythm that differed when compared with that of huddling pups, with no rise before suckling. Thus huddling enables pups to invest the saved energy into growth and to regulate their body temperature to be more competitive during nursing, particularly at the early time when they are TI.

Huddling is used by many adult birds and mammals to survive during harsh climatic conditions. When energy is essentially needed for maintenance, this thermoregulatory behavior was shown to decrease energy expenditure, thus promoting energy saving and hence survival (e.g., see Refs. 2–4, 6, 10, 11, 17, 18, 20, 21, 34, 38, 39, 46, 47, 49). It is not quite clear, however, how huddling promotes allocation of energy when animals are faced with conflicting energetic demands such as for survival, reproduction, and/or growth.

In the context of reproduction, we have shown that huddling induces metabolic depression without hypothermia in breeding emperor penguins (19). Huddling thus avoids the high cost of rewarming that would be required in such large birds but more importantly allows the maintenance of the high body temperature needed for egg incubation. Male emperor penguins, fasting for 4 mo to assume their breeding task during the Antarctic winter, represent an extreme and unique example of how huddling promotes fine physiological adjustments to save energy in the context of a trade-off for energy allocation.

Another extreme physiological situation where social thermoregulation should optimize energy allocation is growth. Altricial mammal pups are indeed born with relatively little insulation and are not thermoregulatory efficient. Early studies have shown that huddling altricial pups invest more energy into growth and maintain a higher body temperature than pups alone (1, 5, 47). However, the thermal benefits of huddling and its physiological mechanisms in mammal pups have been poorly investigated. One explanation is the difficulty to dissociate the effects of huddling per se from the environmental effect provided by the mother. Indeed, many mammalian mothers spend long periods within the nest, feeding, cleaning, or rewarming their offspring. The European rabbit (Oryctolagus cuniculus) doe is an exception, as it leaves the pups after birth, and only nurses them for 3–5 min once a day (25, 30, 31, 35, 53). Nursing events are under strict circadian control, and pups anticipate nursing by increasing their body temperature before suckling (33). Pups are, however, able to fast for 1 day in the case that they miss a suckling event, which may be common (16, 25). It has been shown that rabbit pups thermally benefit from their littermates’ presence, especially during the first 5 days of life when they are thermoregulatory inefficient, i.e., when they have no fur and a large surface area relative to body mass but are capable of heat production through activation of brown adipose tissue (5). At ~10 days of age, their body mass is increased by 150% and fur is present. Pups are considered thermoregulatory efficient, as they are able to maintain constant their body temperature when thermally stressed (5, 23). Although previous studies evaluated the benefits of huddling in altricial pups (1, 5, 47), none of them focused on the role huddling plays in their acquisition of efficient thermoregulatory capacities, notably through the regulation of body temperature, linked to the energetic trade-off altricial pups face along their growth.

The overall objective of this study was therefore to extend the knowledge we gained from the emperor penguins model to understand the mechanisms by which social thermoregulation optimizes energy allocation when newborn mammals have to face conflicting energetic demands. We therefore investigated how huddling influences energy partitioning in thermoregulatory situations.
tory inefficient vs. efficient rabbit pups, and to what extent energy is invested in the thermal challenge in place of growth. We hypothesize first that huddling promotes growth via the accretion of fat mass, providing a better insulation that enhances the acquisition of an effective thermoregulatory capacity. We moreover suggest that energy partitioning strategies depend on both the number of pups involved in huddling and their thermoregulatory capacities.

In addition, we studied the physiological mechanisms involved in huddling and isolated rabbit pups and investigated how this behavior influences body temperature and activity rhythms that would impair their competitiveness with littermates during suckling. We hypothesize that the heat-buffering effect of huddling reduces the consequences of an acute energy shortage and is thus critical for the survival of thermoregulatory inefficient pups, and that huddling favors an optimized body temperature rhythm for suckling.

To test these hypotheses, we used the doubly labeled water technique along with measurements of deep body temperature, two methods that have never been used in such a context.

MATERIALS AND METHODS

Animals and Housing Conditions

The experiments were carried out on rabbits of a crossed strain, "Hyplus" from Grimaud (New Zealand White × Californian rabbits; Grimaud La Corbière, Roussay, France; http://www.grimaud.fr/). Four does and two males were housed individually in cages 50 × 100 cm and 50-cm high. Parturition occurred spontaneously after 30–31 days of gestation. The sliding door of a litter box (30 × 50 cm, 40-cm high) filled with fresh straw and hooked to the doe’s cage was opened 3 days before parturition. Room temperature varied from 18 to 23°C, and a 16:8-h light-dark cycle was maintained. The animals were kept and treated in accordance with the European Guidelines for Animal Care with full approvals from the French Government, the Centre National de la Recherche Scientifique and the Direction of the Veterinary Services (no. 67–188).

Data were obtained from a total of 12 litters. Mean number of pups at birth per litter was 8.5 ± 0.6. We reduced the number of pups to eight for the oversized litters. The number of pups per litters thus varied from six to eight, to reduce any effect of the number of pups when suckling (16): we used two litters constituted of six pups, one litter of seven pups, and nine litters of eight pups. The huddling reference group was of eight pups, corresponding to the average number of pups per litter for rabbits. Once a litter was born, pups were left a few hours with the doe in the nesting box to allow them to suckle once without disturbance. Then pups were separated from the doe, color-marked on their back with an animal marking stick (Raidex, Dettingen/Erms, Germany) for identification, and weighed.

Pups were placed in plastic boxes with an open top (28 × 42 cm, 16-cm high) and randomly allocated to groups of eight (G8), four (G4), two (G2), or one (G1). The total number of pups studied was 91, allocated to 4 G8, 5 G4, 10 G2, and 19 G1 groups. All pups were placed with the doe until weaning at 28–30 days of age. Parturition occurred spontaneously after 30 –31 days of gestation. The sliding door of a litter box (30 × 50 cm, 40-cm high) filled with fresh straw and hooked to the doe’s cage was opened 3 days before parturition. Room temperature varied from 18 to 23°C, and a 16:8-h light-dark cycle was maintained. The animals were kept and treated in accordance with the European Guidelines for Animal Care with full approvals from the French Government, the Centre National de la Recherche Scientifique and the Direction of the Veterinary Services (no. 67–188).

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Depending on the time of day when pups were born (early morning or evening) and to homogenize the age of pups for their first nursing, suckling was allowed once a day, around 9:30 AM (8 litters) or around 5:00 PM (4 litters). Suckling lasted for 3–4 min on average. Pups from different groups were gathered in their original litter for nursing. One-half of the pups of each litter were killed at 12 days of age, according to the recommendations of French animal care committees, to sample blood and analyze body composition. The remaining pups were placed with the doe until weaning at 28–30 days of age. All pups were sexed by visual inspection or after death.

Total Energy Expenditure and Body Composition

Total energy expenditure (TEE) in the thermoregulatory inefficient (TI; 3–5 days old) and thermoregulatory efficient periods (TE; 8–10 days old) was determined during a 2-day period by the two-point doubly labeled water (DLW) methodology described by Schoeller et al. (43). About 6 h after the last suckling event, a baseline urine sample was collected (by stimulating urination manually), and a premaxed 1.50 mg/g dose of DLW was injected intraperitoneally. The dose was composed of 1.7 mg/g 94.4% 2H218O (Rotem Industries, Israel) and 0.55 g/kg estimated total body water) 99.9% 18O2O (Cambridge Isotope Laboratories, Andover, MA) and was diluted with 0.9% NaCl. The doses were calculated to ensure an in vivo enrichment of −500 and 150 ppm for 18-oxygen and deuterium, respectively. The isotopic equilibration in body water was determined through a blood sample collected in the jugular vein at 1 h postdose. Urine samples were collected on day 2 after dosing to complete DLW calculations. Immediately after collection, blood was transferred into glass capillaries rapidly flame-sealed and stored at 5°C until further analysis by mass spectrometry. Urine was stored at −28°C in cryogenically stable tubes.

Water was extracted from ~100-μl blood and urine samples by vacuum distillation (52). Using a gas isotope ratio mass spectrometer (Optima Fisons), as previously described (50), 18-oxygen and deuterium isotopic enrichments were determined by CO2 equilibration and zinc reduction in microliter urine samples. The 18O/16O and 2H/1H isotope ratios were measured in triplicate and repeated if the SD exceeded 0.5 ppm for deuterium and 0.2 ppm for 18-oxygen. CO2 production was calculated according to the single-pool equation of Speakman (45): rCO2 = N(0.48123ko – 0.48743ko). N represents the average isotope dilution space of 18-oxygen over the DLW measurement period. Initial N was calculated according to Coward et al. (12) by the plateau method, using the 1-h postdose sample. The values for initial pool size as percentage of initial body mass were applied to the final body mass to estimate the final pool size. During growth, change in N is presumably exponential rather than linear. The error in the calculated average N is, however, small, as an unrealistic 50% increase in N over the 2 DLW measurement days will not introduce a difference >1.5% between the two estimates (12). k0 and k1 represent the isotope constant elimination rates calculated by linear regression of the natural logarithm of isotope enrichment as a function of elapsed time from day 1 samples. TEE was calculated using Weir’s equation (51) with a food quotient calculated from rabbit milk composition (36) and corrected for the changes in body composition observed during the study. As expected from the literature (8), the effect on TEE was minimal (4.0 ± 3.6%). In our hand, the DLW method has a precision of 5–8% and an accuracy of 1% (9).

Total body water (TBW) was measured from the dilution space of 18-oxygen corrected for isotope exchange by the factor 1.007 (40). Fat-free mass (FFM) was calculated from TBW by assuming hydration coefficients of 0.814 and 0.790 at, respectively, 3 and 8 days of age. Fat mass (FM) was calculated by the difference of FFM from body mass. The hydration coefficients of body mass were measured by lyophilization of the carcasses of 31 pups, aged between 1 and 14 days old, as routinely done in our laboratory (37). Then FFM was calculated by subtracting FM content from body mass, FM content being determined through lipid extraction (37). Hypothesizing that FM had a null hydration coefficient, we calculated a hydration coefficient of FFM at 3 and 8 days of age, according to the

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following regression line: $y = -0.4775 \times x + 83.091$, with $r^2 = 0.936$ (SE for constant, 0.244; SE for variable, 0.023).

Metabolized energy intake (MEI) was calculated as the sum of TEE and energy stored in the form of FM and FFM. Energy stored was calculated from the changes in body composition (FM and FFM) between the two DLW doses and expressed on a per day basis, multiplied by the energy equivalent of 38.7 kJ/kg for fat stored (41) and 4.84 kJ/kg for FFM stored, with a 23.5 kJ/kg energy equivalent of protein stored (41), protein representing 20.6% of FFM (44).

**Milk Intake and Index of Milk Conversion Efficiency**

Daily milk intake was obtained by subtracting the prenursing weight from the postnursing weight ($\pm 0.1$ g). We calculated milk intake as a percentage of the pups’ weight at day 1. Body mass accretion as a function of milk ingested was estimated using an index of milk conversion efficiency (IC) for milk ingested over $t$ days: 

$$IC_t = \frac{\text{incress in prenursing weight over } t + 1 \text{ days (g) }}{\text{weight of milk ingested over } t \text{ days (g)}}$$

(previously described in Bautista et al. (5) and Drummond et al. (16). This conversion index was calculated from day 1 to day 5 and from day 6 to day 10. We also tested slope differences in the accretion of body mass as a function of milk intake, which gave similar results to the ratio. Four pups (because of technical problems) and pups implanted with a transmitter (see Body Temperature and Physical Activity Monitoring, below) were excluded from this analysis.

**Microclimate Within the Groups: Cutaneous Temperature**

The microclimate within the nest was continuously monitored by a thermistor (Pt-RTD 100, Jumo, Jumo-Regulation, Metz, France) emerging from the drawsheet at the center of the box, directly below the pups. Since the temperature probes were in tight contact with the pups at the center of the group, they recorded their skin temperatures. The probes were connected to a Smart A/D computer card (model no. 619), and data were recorded every 30 s using Sensoredays Quicksense software (version 3.3; Smart A/D and Quicksense, Sensoray, Tigard, OR, http://www.sensoray.com). Before and after the experiments, the thermistors were calibrated against a reference thermometer in a thermostatic bath, from +20 to +40°C, with 5°C increments.

**Body Temperature and Physical Activity Monitoring**

Four randomly selected pups in G8, G2, and G1 and five pups in G4 were implanted with transmitters at 2.5 days of age (groups of 8, 4, and 2) and 3.5 days of age (groups of 1). TA10TA-F20 transmitters (Data Sciences International, St Paul, MN; 3.5 g, 1.75 cm$^3$) were placed intraperitoneally, under gaseous anesthesia (isoflurane, Forene) and strictly aseptic surgical conditions. The surgery took place at least 5 h after suckling, and a heating pad was used to prevent hypothermia. Antibiotics (oxytetacycline, Terramycine LA) and anti-inflammatory molecules (ketoprofen, Ketofen) were injected at the end of the surgery. The pup was brought back to its littermates ~1–2 h after. At weaning, the body mass of implanted animals did not significantly differ from that of control litters. Moreover, the quantity of milk ingested along the study period and the corticosterone plasma levels of implanted pups were not significantly different from those of animals not implanted. Transmitters recorded body temperature and physical activity (arbitrary units) at 15-s intervals, from 2.5 days of age to 11–12 days of age. Because of radio frequency interference, only one pup per group was implanted.

**Corticosterone, T3, and T4 Assays**

At 12 days of age, the pups were anesthetized, blood was sampled within a few minutes, and the pups were killed. Blood was sampled at approximately the same time of day for all pups. Blood was transferred to an EDTA-coated tube and centrifuged at 3,500 rpm at 4°C for 10 min. Plasma was stored at –20°C, and corticosterone was measured without extraction procedure, using an enzyme immunoassay kit (Correlate-EIA, Assay Designs, Ann Arbor, MI; http://www.assaydesigns.com). The intra- and interassay coefficients of variation were under 8.4 and 13.1%, respectively.

Plasma concentrations of free triiodothyronine (T3) and free thyroxine (T4) were measured without extraction procedure using a commercially available RIA kit (Immunootech SA, Marseille, France). The intra- and interassay coefficients of variation were under 6.4 and 5.5%, respectively.

**Data Analysis**

Since no gender differences were detected, males and females (34 males and 36 females) were pooled to compare differences between groups. When normality was respected (Kolmogorov-Smirnov test), the data were analyzed by a parametric ANOVA with repeated measures. When normality was not respected or when the sample size was too low to detect difference with a power of at least 80%, we performed nonparametric analyses. Normality and power-related sample size were only respected for body composition and energy expenditure data.

**Body composition, TEE, and hormones.** Two-way ANOVAs on repeated measures with huddling (group size) and growth (between the TI and TE periods) as factors were used to compare body composition, calculated MEI, and TEE. Values were weighted for the initial between-subjects variability in body composition. To evaluate the differences in maintenance costs between groups, we calculated an estimated minimum maintenance TEE (TEE$\text{min}$) by multiplying the mean TEE (kJ·g$^{-1}$·day$^{-1}$) of pups in groups of eight by each individual body mass of pups. We then individually subtracted actual TEE by the estimated TEE$\text{min}$ and expressed the results per gram of body mass. Differences in maintenance costs associated with huddling (kJ·g$^{-1}$·day$^{-1}$), constituted by a large part of extra thermoregulatory costs, were tested for TI and TE pups using an ANOVA with Tukey’s post hoc tests.

Between-groups differences in corticosterone, T3, and T4 plasma levels, milk intake as a percentage of body mass at day 1, and the index of conversion efficiency (along days 1–5 and days 6–10) were tested using Kruskal-Wallis ANOVAs with Dunn’s post hoc tests.

**Body temperature and physical activity monitoring.** Average body temperatures on days 2–3 and days 11–12 were compared using a Mann-Whitney test. Daily averaged body temperatures and physical activity indexes of rabbits in G8, G4, G2, and G1 were compared by a Kruskal-Wallis ANOVA with Dunn’s post hoc test.

The effect of huddling on daily body temperature rhythms was studied on hourly means using a Kruskal-Wallis ANOVA with groups as main factor and Dunn’s test for post hoc comparison. To study the variations in physical activity and body temperature associated to suckling, we compared the hourly mean body temperature and physical activity 3 h before suckling, just before suckling (taking into account the hourly mean just before suckling), just after sucking (taking into account the hourly mean just after suckling), 2 h after suckling, and 6 h after suckling to the mean body temperature and activity calculated over 1 day, centered on the suckling event, from day 3 to day 11. This time schedule was chosen according to previous studies on rabbit pup body temperature rhythm (32, 33). Variations of physical activity and temperature around suckling were analyzed by Kruskal-Wallis ANOVAs with Dunn’s post hoc tests. The same tests were used to compare between-groups differences for the six time classes described above.

All values reported are means ± SE. Analyses were conducted using SigmaStat, version 2.03 (SYSTAT Software, Point Richmond, CA) and JMP software, version 5.1.1 (SAS Campus Drive, Cary, NC). Differences were considered significant at $P < 0.05.$
RESULTS

Body Mass and Composition

Changes in body mass and body composition (weighted with the initial values of BM, FFM, and FM) are summarized in Fig. 1. Body mass increased with time (F1,61 = 297.5, P < 0.0001), but no huddling effects were observed (F3,61 = 0.5, P = 0.67; growth-by-huddling interaction F3,61 = 1.2, P = 0.31). Although significance was not reached, G4 had the highest increase in body mass (79 g) and G1 and G8 the lowest (61 g). The body mass change in G2 ranged between those extremes (72 g). Similar to body mass, FFM increased significantly along growth (F1,61 = 300.9, P < 0.0001), but no between-group differences were observed (F3,61 = 0.4, P = 0.79; growth-by-huddling F3,61 = 0.8, P = 0.50). Although not significant, the change in FFM followed the trends observed for body mass, with G4 having the highest FFM accretion (+72 g). G8 FFM growth was the lowest (+58 g), and G1 and G2 FFM growth was between those extreme changes (60 and 69 g, respectively). On the other hand, we observed a significant huddling effect for FM (F3,61 = 5.6, P = 0.005). The presence of a growth-by-huddling interaction (F3,61 = 5.4, P = 0.006) indicated that the evolution of FM across time significantly differed between groups (growth: F1,61 = 23.5, P < 0.0001). G1 lost 0.7 g of FM, whereas G8, G4, and G2 gained, respectively, 2.8, 7.4, and 3.2 g of fat tissue. The fact that those changes in FM did not translate into significant changes in body mass is likely explained by the small contribution of FM to body mass in pups and the observed variability between subjects.

Raw Milk Conversion Efficiency, Calculated MEI, TEE, and Extra Maintenance Cost

The mean index of milk conversion efficiency (IC; Fig. 2, top) over the TI period (from day 1 to 5) was significantly higher for pups in G8 than G1 by 25% (0.76 ± 0.01 and 0.61 ± 0.04, respectively; H = 14.0, df = 3, P = 0.003). Furthermore, pups raised in G8 had a mean IC over the TE period (from day 6 to 10) that was higher by 10% than G1, although not significantly with post hoc tests (0.67 ± 0.01 and 0.61 ± 0.02, respectively; H = 13.2, df = 3, P = 0.004). Calculated MEI (Fig. 2, middle) expressed on a per gram basis decreased significantly between the TI and TE periods (1.75 ± 0.07 and 1.16 ± 0.03 kJ·g⁻¹·day⁻¹, respectively; F1,61 = 159.6, P < 0.0001), but no group effect was noted (F3,61 = 2.4, P = 0.09).
The TEE (Fig. 2, bottom) of pups, expressed per gram of body weight, was significantly lower when TE than when TI (on average 0.66 ± 0.02 vs. 0.91 ± 0.04 kJ·g\(^{-1}\)·day\(^{-1}\), respectively). Furthermore, the change in TEE across time was significantly different between groups (group-by-time interaction \(F_{3,30} = 3.2, P = 0.04\)). Pups in G1 had the highest TEE in both the TI and TE periods (1.14 ± 0.09 and 0.83 ± 0.04 kJ·g\(^{-1}\)·day\(^{-1}\), respectively) when compared with pups in G2 (1.01 ± 0.05 and 0.64 ± 0.01 kJ·g\(^{-1}\)·day\(^{-1}\), respectively), G4 (0.80 ± 0.05 and 0.60 ± 0.03 kJ·g\(^{-1}\)·day\(^{-1}\), respectively), or G8 (0.68 ± 0.03 and 0.57 ± 0.01 kJ·g\(^{-1}\)·day\(^{-1}\), respectively). When expressed as percentage during the TI period, pups in G8 had a TEE reduced by 40% compared with pups in G1, by 32% compared with pups in G2, and by 15% compared with pups in G4. When the TE period was reached, pups in G8 had a TEE reduced by 32% compared with pups in G1, by 11% compared with pups in G2, and by 5% compared with pups in G4.

The calculated extra maintenance cost of being in G1, G2, or G4 compared with G8 mainly corresponds to extra thermoregulatory costs (Fig. 3). During the TI period, pups in G1 (0.45 ± 0.09 kJ·g\(^{-1}\)·day\(^{-1}\)) had thermoregulatory costs higher than for pups in G2 (0.33 ± 0.06 kJ·g\(^{-1}\)·day\(^{-1}\)), G4 (0.12 ± 0.05 kJ·g\(^{-1}\)·day\(^{-1}\)), and G8 (0.00 ± 0.03 kJ·g\(^{-1}\)·day\(^{-1}\); \(F_{3,30} = 10.7, P < 0.001\)). G2 pups also had extra thermoregulatory costs significantly higher than pups in G8 (\(P < 0.05\)). During the TE period, G1 pups had extra thermoregulatory costs (0.27 ± 0.04 kJ·g\(^{-1}\)·day\(^{-1}\)) significantly higher than pups in G2 (0.07 ± 0.01 kJ·g\(^{-1}\)·day\(^{-1}\)), G4 (0.03 ± 0.03 kJ·g\(^{-1}\)·day\(^{-1}\)), and G8 (0.00 ± 0.01 kJ·g\(^{-1}\)·day\(^{-1}\); \(F_{3,30} = 19.4, P < 0.0001\)). However, the G2 values were no longer significantly different from those of G8.

**Body Temperature and Physical Activity**

During the first 12 days of life, the mean body temperatures of pups in G8 (38.1 ± 0.1°C) were significantly higher by 0.4°C and 0.5°C than the body temperatures of pups in G2 (37.7 ± 0.1°C) and G1 (37.6 ± 0.2°C; \(H = 10.5, df = 3, P = 0.02\)), respectively. Body temperatures of all implanted pups increased significantly by 1.4°C from days 2–3 to days 11–12 (37.1 ± 0.2, \(n = 30\) and 38.5 ± 0.0°C, \(n = 28\), respectively; \(U = 1,244, P < 0.001\); Fig. 4, top).

To gain insights into the interpretation of the above data, we averaged physical activity indexes and body temperature over the period TEE was measured. Changes in body temperature (Fig. 4, middle) were also dependent on group allocation (huddling-by-growth interaction: \(F_{3,101} = 5.2, P = 0.003\)). For all groups, TE pups had a body temperature significantly higher than when TI (38.2 ± 0.1°C vs. 37.4 ± 0.1°C, respectively). Moreover, TI pups in G4 and G8 had a mean body temperature (37.8 ± 0.1°C) significantly higher by 0.8°C than pups in G2 and G1 (37.0 ± 0.2°C). No more differences were
noted when the TE period was reached. The changes in physical activity (Fig. 4, bottom) during growth were different across groups (huddling-by-growth interaction: F3,101 = 3.2, P = 0.03). G8 pups had a physical activity during the TI and TE periods (5.0 ± 0.3 vs. 4.4 ± 0.3, respectively) significantly higher than the physical activity of G1, G2, and G4 pups (on average 2.6 ± 0.1 when TI and 2.5 ± 0.2 when TE).

Microclimate Within the Groups: Cutaneous Temperature

Cutaneous temperatures were 31.9 ± 0.5°C in G8 (n = 4), 31.0 ± 0.5°C in G4 (n = 5), 31.3 ± 0.6°C in G2 (n = 8), and 29.8 ± 0.3°C in G1 (n = 10) along the 10 days of growth. Pups in G8 showed a cutaneous temperature significantly higher by 2.1°C than for pups alone (U = 48, P = 0.01).

Physical Activity and Body Temperature Rhythms

Pups showed an endogenous rhythm anticipating suckling, both in their physical activity and body temperature rhythms. Physical activity showed a significant rise on average by 47.8 ± 5.0% before suckling and by 45.5 ± 8.1% after suckling (H = 122.3, df = 4, P < 0.01). Similarly, body temperatures of pups rose significantly on average by 0.18 ± 0.04°C before suckling and by 0.22 ± 0.03°C after suckling (H = 66.0, df = 4, P < 0.001). However, failed suckling resulted in changes in body temperatures that differed with the number of pups per group. Indeed, pups huddling in G4 and G8 did not show any drop in their body temperature when they failed suckling. By contrast, G1 or G2 pups showed drops in body temperatures, even down to 34.3°C, when a pup failed suckling (Fig. 5). We then tested whether huddling had an impact on these endogenous rhythms. Huddling had no effect on the variation of physical activity around the mean 3 h before, just before suckling, and 2 and 6 h after suckling (Fig. 6, top). However, after suckling, G1 pups had a rise in physical activity significantly higher than for G8, G4, and G2 pups (90.5 ± 17.0, 10.9 ± 15.5, 48.3 ± 20.1, and 38.2 ± 8.3%, respectively). Considering body temperatures, pups in G4 showed an increase in body temperature 3 h before and just before suckling, and a decrease 2 and 6 h after suckling, which were both higher than for pups in other groups (Fig. 6, middle). Before suckling, G1 pups did not show any anticipatory rise in their body temperature (−0.05 ± 0.09°C), in contrast to G2, G4, and G8 pups (on average 0.24 ± 0.04°C). After suckling, G1 pups had a body temperature rise (0.45 ± 0.06°C) significantly higher than for G4 and G8 pups (on average 0.13 ± 0.04°C).

Fig. 5. Profiles of body temperature (hourly mean temperatures) and physical activity of 4 pups, either alone or in a group of 2, 4, and 8 pups. Milk intake (indicated with a black triangle) occurs once a day during the nursing event. Note the decrease in body temperature (indicated with a grey bar) after a failed suckling event (indicated with a circle) for pups in G1 and G2, whereas, in contrast, the body temperature of pups in G4 and G8 is maintained after the failed suckling.
Milk Intake

To relate the effect of body temperature and physical activity rhythms on the competitiveness of pups during suckling, we calculated milk intake as a percentage of body mass at day 1 (Fig. 6, bottom). This milk intake percentage from day 1 to 5 was significantly higher for pups in G8 (127.8 ± 3.8%) than for pups in G2 and G1 (104.4 ± 5.6 and 100.8 ± 8.9%, respectively; H = 12.1, df = 3, P = 0.007). In contrast, the milk intake percentages from day 6 to 10 and from day 1 to 10 did not reach significance between groups, although G1 pups had the lowest milk intakes (on average for all groups: 157.9 ± 4.5 and 273.6 ± 6.6%; H = 7.7, df = 3, P = 0.05, and H = 7.5, df = 3, P = 0.06, respectively).

Corticosterone, T3, and T4 Concentrations

Plasma corticosterone and T4 levels were not significantly affected by huddling (Fig. 7; on average for all groups: 9.31 ± 0.82 ng/ml and 25.50 ± 0.87 pmol/l, respectively). In contrast, G1 pups showed T3 concentrations significantly higher than for pups in G4 and G8 (3.10 ± 0.27, 2.05 ± 0.14, and 2.17 ± 0.17 pmol/l, respectively).

DISCUSSION

Rabbit pups provide a unique model to study the impact of huddling on energy allocation to growth vs. thermoregulation. Although they are able at birth to heat themselves up endogenously via a functional brown adipose tissue, they are considered thermoregulatory efficient only at ~10 days of age (5, 7, 13, 14, 22, 26–29). To study huddling energetic benefits, most of previous studies evaluated the saved energy induced by huddling through confinement of the whole huddling group a few hours in a calorimetry chamber (1, 3, 17, 18, 20, 21, 38, 39, 47, 49). The novelty of our study is that we used the doubly labeled water method to go further in the study of allocation of energy to growth in place of thermoregulation in huddling mammals. This method allowed us to determine energy expenditure of pups individually, over 2 days of growth, and without disturbance of their huddling behavior. This method has rarely been used in such a context (2, 34). Thanks to a multidisciplinary approach, we were able to estimate all components of the pups’ energy expenditure to show that the cost of thermoregulation was a direct function of the huddling group size. Moreover, this study is the first to investigate the impact of the heat-buffering effect of huddling on the body temperature and activity rhythms, which should enhance survival and suckling competitiveness.

Effect of Huddling on Energy Allocated to Growth and Growth Components

Effect of huddling on energy allocation to growth vs. thermoregulation. According to the energy balance equation, MEI represents the sum of TEE and energy stored in fat mass and

![Fig. 6. Changes in physical activity and body temperature (compared with the mean physical activity and the mean body temperature calculated between 2 suckling events) and milk intake as a percentage of body wt at day 1 (%). Bars that share no common symbol differ significantly. Significances for activity: after suckling (P = 0.002), 2 h after (P = 0.01). Significances for temperature: 3 h before suckling (P < 0.001), before (P < 0.001), after (P = 0.002), 2 h after (P = 0.016), 6 h after (P = 0.02).

Fig. 7. Plasma corticosterone, T3, and T4 levels for pups in groups of 8, 4, 2, and 1. Bars that share no common symbol differ significantly. Plasma corticosterone levels: P = 0.67 (G8, n = 12; G4, n = 8; G2, n = 7; G1, n = 11). Plasma T3 levels: P = 0.02 (G8, n = 13; G4, G2, and G1, n = 7). Plasma T4 levels: P = 0.09 (G8, n = 13; G4, G2, and G1, n = 7).
fat-free mass. In the TE period, pups had a MEI and a TEE (expressed per g body wt), respectively, 38 and 34% lower than when in the TI period. This reflects the complete lack of insulation of pups at birth and their high surface-to-volume ratio, which is consistent with our results about milk conversion efficiency. Moreover, calculated MEI was not different between groups, implying that pups in each group had the same amount of energy to allocate either to growth or to thermoregulation. However, when TI, huddling pups in G8 had a TEE reduced by 40% compared with pups in G1, and when TE by 32%. Huddling thus provides a reduction in TEE, which is more important when rabbit pups are TI. In the same way, Alberts (1) found that huddling newborn rats in a group of eight (without considering their thermoregulatory status) allowed an average 34% reduction in metabolic rate. Moreover, to determine that this extra energy expenditure is due to extra thermoregulatory costs, we examined all TEE components. TEE is composed of resting costs as a function of fat-free mass, postprandial thermogenesis as a function of milk intake, physical activity, and other costs such as thermoregulation and growth. The variations in TEE comparing huddling and isolated pups cannot totally account for the differences in postprandial thermogenesis costs, even if the difference in stored tissue is taken into account, because 1) milk intake showed no major differences between groups and 2) these costs represent a small percentage of TEE (~10%). The greater TEE for G1 pups compared with huddling pups in G8 cannot be explained by a higher activity, as pups in G8 showed a physical activity significantly higher than for pups in G4, G2, and G1, both when TI and when TE. Furthermore, plasma corticosterone levels of pups, equivalent to those in previous studies (42, 48), were not significantly different between groups. Because corticosterone is a major indicator of stress, we may conclude that the differences in TEE between pups alone and those in a group are unlikely due to extra heat production through stress. In parallel, pups in G1, showing the highest TEE, maintained a higher heat production than pups in G4 and G8, as their T3 plasmatic concentrations, enhancing thermogenesis, were significantly higher. These data all together confirm that huddling allows the pups to reduce their thermoregulatory costs, which consequently leads to a reduction in their energy expenditure. Energy saved by huddling pups could be allocated into growth instead of thermoregulation, especially when pups were TI.

Effect of huddling on growth components. Huddling only affected the energy stored as fat: pups in G1 had 20 and 115% less fat mass when TI and TE, respectively, compared with pups in G8. G4 pups accrued more fat mass. This could be due to the greater physical activity of pups in G8 compared with pups in G4. However, although those differences were seen for body mass and fat-free mass, they did not reach significance, possibly because of a high interindividual variability. The fact that huddling pups accrued more fat mass may favor their insulation and thus their thermoregulatory capacities, enhancing their survival.

Effect of number of huddling pups and thermoregulatory status on saved energy. TEE increased with the decreasing number of pups per group, as noted in previous studies (1, 4, 6, 10, 11, 17, 39). However, the influence of the number of pups while huddling was dependant on the pups’ thermoregulatory capacities. Four was the minimum number of grouped pups to maintain a low metabolic heat production when TI, whereas two was the minimum when TE. From an evolutionary point of view, an optimal number of rabbit pups in a litter may be needed to accrue the litter fitness (16). A greater number of pups reduces thermoregulatory costs, while it may conversely increase the intralitter competition for milk. In this study, all pups were competing with six to eight pups, even if they were placed in G1, G2, G4, and G8 groups. It appears that the optimal number of pups, considering the thermal challenge we chose (ambient temperature in the room was 23–24°C), was four pups. This may vary with thermal conditions in rabbit burrows, depending on weather conditions or geographical range.

Effect of Huddling on Pups’ Body Temperature Regulation and Competitiveness

Consistent with previous studies, we showed that mean body temperatures of rabbit pups increased along growth (32) and that huddling pups maintained a higher body temperature compared with isolated ones (1, 5). We moreover showed that huddling was critically important for the maintenance of body temperature during the first days of growth, when pups are TI: G1 and G2 pups maintained a body temperature lower than that of pups in G4 and G8, whereas no differences were noted when TE.

Furthermore, the constant monitoring of body temperature of pups along their growth allowed us to focus on the importance of huddling in case of a failed suckling event. Body temperatures of pups in G2 and G1 showed drops down to 34.3°C when they failed a suckling event, in contrast to the body temperatures of G4 and G8 pups, which were maintained. Healthy pups can, and often do, miss a feed and survive (16, 24). However, we showed that when pups alone failed a suckling event, their body temperature was no longer maintained. This may jeopardize their growth, as hypothermia reduces biochemical activities. Huddling can thus be seen as a strategy to increase fitness of the group by limiting the impact of failed suckling events.

To go further, we examined the impact of huddling on the pups’ competitiveness. Female rabbits only visit their pups for a few minutes once a day for nursing: their survival then depends on the tight synchronization of the two parties. Similar to other studies, we showed that body temperatures and physical activity of rabbit pups before and after suckling showed a significant rise (30–32). It was previously hypothesized that the pups’ anticipatory behavior gives them the advantage to suckle more milk (15, 25, 32). Here we show that before suckling, pups in G1 were the only ones to show a slight drop in their body temperature, while pups in the other groups showed a rise in their body temperature. Nevertheless, the activity of pups in G1 before suckling showed a similar rise compared with the other groups. However, milk intake from day 1 to 5 as a percentage of pups’ weight was significantly lower for pups in G1 than for pups in G8. Huddling then helps pups to facilitate their rise in body temperature before suckling, to be more competitive during the nursing bout. This appears to be particularly important during the first days of their life, when TI.
Conclusion

The multidisciplinary approach of this study provided us new insights into huddling and its underlying physiological mechanisms in a newborn mammal. Huddling can be seen as a behavior necessary for an altricial mammal to reduce its thermoregulatory costs until it has acquired enough isolative fat mass and a sufficient surface-to-volume ratio. Huddling is furthermore critical for TI pups 1) to maintain their body temperature in case of food shortage (i.e., a missed suckling) and 2) to favor their anticipatory behavior, increasing their competitiveness. We suggest that huddling helps altricial pups in the acquisition of their thermoregulatory capacities, through energy allocation to fat mass and increased competitiveness.

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