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Thermogenesis, Myoclonic Twitching, and Ultrasonic Vocalization in Neonatal Rats During Moderate and Extreme Cold Exposure

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Physiological and behavioral responses of 2- and 7–8-day-old rats were monitored during moderate and extreme cold exposure. During moderate cold exposure (30 °C ≤ air temperature ≤ 32.5 °C), pups at both ages increased heat production, maintained an elevated interscapular temperature, and maintained baseline levels of myoclonic twitching, a behavior commonly associated with active sleep. During extreme cold exposure (21 °C ≤ air temperature ≤ 25 °C), pups at both ages continued producing metabolic heat, but now exhibited pronounced decreases in interscapular temperature and decreased rates of myoclonic twitching. Furthermore, the 7–8-day-old pups exhibited significant increases in ultrasound production, and males vocalized more than females. These results suggest the presence of a narrow subthermoneutral zone in neonates in which nonshivering thermogenesis is regulated and sleep-related behaviors are protected.

When exposed to a cold environment, newborn rats exhibit a number of physiological and behavioral responses. Their physiological response is largely directed toward activation of brown adipose tissue (BAT), a thermogenic organ located primarily, but not exclusively, in the interscapular region (Smith, 1964). Behaviorally, pups use their mother and littermates to stay warm; in the huddle, pups shuttle between the surface and the core of the huddle and, by doing so, minimize metabolic expenditure (Alberts, 1978). When outside the nest, however, stimulation of maternal retrieval responses is important and, within a week after birth, pups emit an ultrasonic vocalization when cold that is an effective stimulus for maternal retrieval of pups to the nest (Allin & Banks, 1972). This signal has been hypothesized to be the acoustic by-product of underlying respiratory processes (Blumberg & Alberts, 1990).

The interactions between the physiological and behavioral responses of newborn rats to thermal challenge are not well understood. As an initial examination, we report here on the differential effects of two levels of cold exposure on pups' BAT heat production and ultrasound production. In addition, because of the predominance of active sleep behaviors during the perinatal period and their hypothesized developmental significance (Blumberg & Lucas, 1996; Jouvet-Mounier, Astic, & Lacote, 1970; Roffwarg, Muzio, & Dement, 1966), we monitored myoclonic twitching of the limbs and tail, a pronounced neonatal behavior that has also been relied upon as the exclusive indicator of active sleep in newborn rats (Gramsbergen, Schwartze, & Prechtl, 1970; Jouvet-Mounier et al., 1970). Finally, rat pups were tested at two ages (i.e., at 2 and 7–8 days of age) because it is known that at least one of our behavioral measures (i.e., ultrasound production) develops significantly over that time span (Okon, 1971).

To explain the selection of the two levels of cold exposure used here, it is first necessary to describe the thermoregulatory responses of pups over a range of air temperatures. Specifically, at thermoneutral air temperatures (defined as the range of air temperatures within which metabolic rate is minimal), pups do not exhibit BAT heat production. But below thermoneutral temperatures, pups exhibit nonshivering thermogenesis that increases in a graded fashion as air temperature decreases. For example, in 5-day-olds, heat production is inversely related to air temperatures between 28 °C and 35 °C (Spiers & Adair, 1986). Below this range of air temperature, metabolic responses become highly irregular across individual pups, indicating that the system is being stressed beyond its capacity. Therefore, we can identify two subthermoneutral zones in neonatal rats: First, a zone of moderate cold exposure in which heat production increases monotonically in response to decreasing air temperature; and second, a zone of extreme cold exposure in which heat production no longer increases significantly in response to greater thermal challenge. We then asked: For the pups at both ages, what are the differential consequences of these moderate and extreme levels of cold exposure for thermoregulation, myoclonic twitching, and ultrasound production.

Method

Subjects

Fifteen 2-day-old and fifteen 7–8-day-old rat pups of both sexes were used. All pups were born to Harlan Sprague-Dawley females in the animal colony at the University of Iowa. The pups were raised in litters that were culled to 8 pups within 3 days after birth (day of birth = Day 0). Litters and mothers were raised in standard laboratory cages (48 × 20 × 26 cm) in which food and water were available ad libitum. All rats were maintained on a 12-hr light–dark schedule with lights on at 6 a.m.

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Test Environment

The experiment was conducted by placing individual pups inside a double-walled glass chamber (height = 17 cm; i.d. = 12.5 cm) constructed in the glass shop at the University of Iowa. The double-walled design allowed for the passage of water through the walls of the chamber; by controlling the temperature of the water with a water circulator, air temperature inside the chamber could also be controlled. Three access holes in the side of the chamber and a sealed Plexiglas top allowed for the passage of air into and out of the chamber as well as the passage of thermocouple wires and an ultrasonic microphone.

A round platform constructed of polyethylene mesh was fitted inside the chamber. When placed on the platform, the pup could move freely on the platform's surface (diameter = 12 cm). A small wall around the platform, also constructed of polyethylene, prevented the pups from making contact with the glass walls of the chamber. The mesh allowed for the movement of air from the bottom of the chamber (where it entered) to the top of the chamber (where it was drawn for analysis of its oxygen content). Finally, the height of the platform was approximately half the height of the chamber, that is, 8 cm.

Temperature Measurements

Physiological and air temperatures were measured using chromel-constantan thermocouples (Omega, Stamford, CT). Electrical signals from the thermocouples were subjected to cold-junction compensation and fed into a computerized data acquisition system (National Instruments, Austin, TX). All thermocouples were calibrated before the experiment in a temperature-controlled water bath using a mercury thermometer accurate to within 0.1 °C. Air temperature within the metabolic chamber was measured using two thermocouples located beneath the platform; the two air temperatures were averaged upon acquisition by the computer. The two physiological temperatures were attained by attaching thermocouples to the skin surface using collodion as an adhesive. Both thermocouples were attached on the midline. One thermocouple was attached in the interscapular region above the brown fat pad, thus providing a measure of interscapular temperature (hereafter designated T_{is}). The second thermocouple was attached in the lumbar region, thus providing a measure of back temperature (hereafter designated T_{back}). We derived a relative measure of BAT heat production by subtracting T_{back} from T_{is} (hereafter designated $T_{is} - T_{back}$).

Oxygen Consumption Measurements

Compressed air passed through a two-stage regulator and was split into two lines. One line entered a digital flowmeter (Omega, Stamford, CT), was humidified, and then circulated through the metabolic chamber at 300 ml/min. After passing through the chamber, the exhaust air was dried and then drawn through one of two channels of an electrochemical oxygen analyzer (Ametek, Pittsburgh, PA). The second line of air travelled directly from the air cylinder to the second channel of the oxygen sensor. Oxygen concentration in each airstream was measured simultaneously. The difference in percentage oxygen between the chamber's effluent airstream and the nonrespired airstream reflects oxygen consumption by the pup. Specifically, by knowing the air flow rate (i.e., 300 ml/min) and the pup weight, oxygen consumption can be calculated. All oxygen consumption values are presented as ml O_2/min/100 g.

Ultrasonic Vocalizations

Ultrasonic vocalizations were detected using a microphone sealed inside the lid of the metabolic chamber. The microphone was connected to a "bat detector" (QMC, Ltd., London, England, Model SM100) tuned to a ±5 kHz range centered on 40 kHz. It was sometimes necessary to vary the tuning of the bat detector in order to detect the maximum number of ultrasonic pulses. The rate of ultrasonic vocalization was measured by listening for the vocalization and pushing a button every time a pulse was detected. In turn, the button push was detected by the data acquisition system and a counter was incremented. Data were quantified by determining whether or not an ultrasonic pulse was detected within a 1-s bin.

Twitching Behavior

An experienced observer was easily able to monitor twitching movements through the glass wall of the metabolic chamber. A *twitch* was defined as a phasic, rapid, and independent movement of the fore limbs, hind limbs, or tail (Blumberg & Lucas, 1994; Gramsbergen et al., 1970). Typically, pups exhibited twitching movements while their heads rested on the substrate. Limb movements indicative of an awake animal (e.g., stepping, stretching) were easily distinguished from twitching movements. Data were quantified by determining whether or not a twitch was detected within a 1-s bin.

Procedure

On the day of testing, a pup was removed from its cage and placed inside an incubator maintained at 35–36 °C. All test pups had fed recently, as evidenced by the presence of milk visible through their abdominal skin. After the two thermocouples were attached, the pup was placed inside the metabolic chamber maintained within the pups' thermoneutral zone (35–36 °C). The pup was given at least 45 min (2-day-olds: 49.2 ± 2.0 min; 7–8-day-olds: 50.9 ± 2.4 min) to acclimate to the chamber.

The test began by initiating second-by-second data collection onto computer disk. The initial baseline period lasted 10 min at the thermoneutral temperature. After these baseline data were taken, T_{air} was decreased. For the 2-day-olds, T_{air} was either decreased to 32.5 °C or 25 °C. For the 7–8-day-olds, T_{air} was either decreased to 30 °C or 21 °C. On the basis of our own work as well as that of others (e.g., Spiers & Adair, 1986), we selected moderate air temperatures for the 2- and 7–8-day-olds (i.e., 32.5 °C and 30 °C, respectively) for their ability to elicit a significant increase in heat production. In contrast, we selected extreme air temperatures at each age (i.e., 25 °C and 21 °C, respectively) on the basis of their being well beyond the air temperature range that elicits a maximal increase in oxygen consumption.

Occasionally, especially at the colder temperatures, pups became active and tangled their thermocouple wires. If this tangling began to disrupt the pup's behavior, the chamber was opened and the wires were untangled. This occurred once with an 8-day-old and three times with 2-day-olds. When our measures were disrupted by this procedure, the affected data were deleted from analysis. This was especially true for oxygen consumption because of the time required for the system to restabilize after the chamber was opened.

Physiological measurements and event data were recorded for 1 hr after air temperature was changed; thus, the entire test lasted 70 min. After the test, the pup was removed from the chamber, its thermocouples were removed and it was returned to its home cage. A littermate of the same sex was then tested at the other air temperature. The ordering of tests was counterbalanced.

Data Analysis

Data were imported to Statview 4.1 for the Macintosh for statistical analysis. The effects of air temperature and time on each recorded variable were tested for significance using a repeated-measures analy-

sis of variance (ANOVA). Where appropriate, post hoc unpaired and paired t tests were used to test for differences between groups and between successive time periods within groups, respectively; the significance level was set at $p < .05$. In all cases, means are presented with their standard errors.

Results

2-Day-Olds

At the time of testing, mean body weight for the twelve 2-day-olds was 8.2 ± 0.3 g (range: 7.2–10.1 g). Because the size of any object affects its rate of heat loss (Schmidt-Nielsen, 1975), it is important that there were no significant body weight differences between pups in the moderate and extreme groups, $t = .17$, $df = 10$. Body weights also did not differ significantly between the male and female pups, $t = .67$, $df = 10$.

The data were analyzed by dividing the test into seven 10-min periods. Figure 1 presents the data for the six variables in the test. Panel A in Figure 1 shows the two air temperature conditions. In the moderate condition, air temperature averaged 32.5 °C by the 40th min of the test. In the extreme condition, air temperature averaged 25.5 °C by the 40th min of

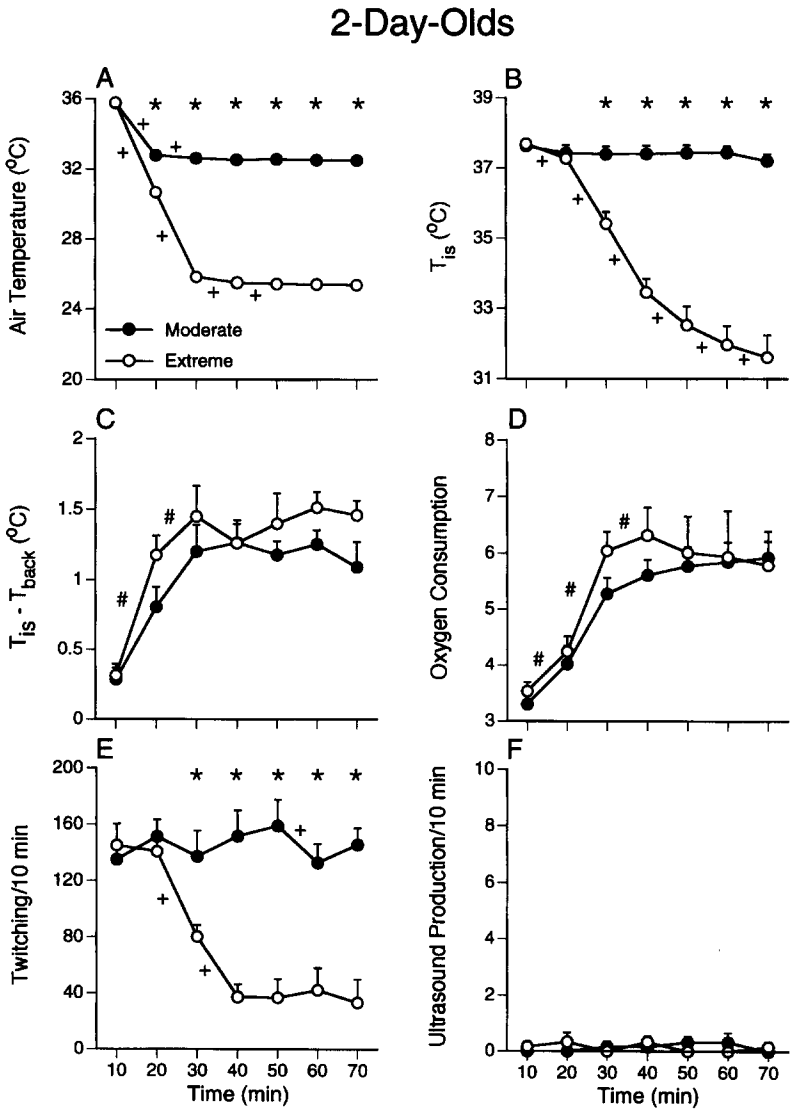

Figure 1. Air temperature (panel A), interscapular temperature (T_{is}; panel B), interscapular minus back temperature ($T_{is} - T_{back}$; panel C), oxygen consumption (ml O_2/100 g/min; panel D), twitching (panel E), and ultrasound production (panel F) for the 2-day-old pups in the moderate (filled circles) and extreme (open circles) groups. Thermal and metabolic values (panels A–D) represent data at the end of each 10-min period. Behavioral values (panels E–F) represent number of 1-s bins in which the behavior was detected during the preceding 10-min period. *M* + *SEM*. $n = 6$ for all data points except $n = 5$ for the extreme group in the oxygen consumption plot (Panel D) at 40 min, 50 min, and 60 min. *Significant ($p < .05$) difference between extreme and moderate groups using unpaired t test. +Significant ($p < .05$) difference between adjacent points using paired t test. #Significant ($p < .05$) difference between adjacent points using paired t test on collapsed data.

the test. As expected, repeated-measures ANOVA indicated significant main effects of group, $F(1, 60) = 15162.0$, $p < .0001$, and time, $F(6, 60) = 10106.3$, $p < .0001$, and a significant Group × Time interaction, $F(6, 60) = 3365.7$, $p < .0001$.

Repeated-measures ANOVAs were used to test for differences in T_{is}, $T_{is} - T_{back}$, oxygen consumption, twitching, and ultrasound production. For T_{is}, there were significant main effects of group, $F(1, 60) = 54.062$, $p < .0001$, and time, $F(6, 60) = 132.0$, $p < .0001$, and a significant Group × Time interaction, $F(6, 60) = 115.8$, $p < .0001$. Across the entire test, T_{is} decreased approximately 0.4 °C in the high group as compared with 6.1 °C in the Extreme group (Figure 1, panel B). Moreover, pups in the moderate group were able to stabilize T_{is} whereas pups in the extreme group exhibited continually decreasing T_{is} throughout the test.

Pups in the moderate and extreme groups responded to the decreases in air temperature by increasing BAT heat production (Figure 1, panel C), as measured by $T_{is} - T_{back}$. Repeated-measures ANOVA indicated a significant main effect of time, $F(6, 60) = 33.9$, $p < .0001$, but not group, $F(1,60) = 1.62$; the Group × Time interaction also was not significant, $F(6, 60) = 1.29$.

In three instances for 3 pups in the extreme group, it was necessary to open the chamber and untangle the thermocouple wires. This led, in turn, to the loss of 3 data points for oxygen consumption. These points were filled by averaging the two values for the time points preceding and following the missing values and then, when the repeated-measures ANOVA was performed, reducing the degrees of freedom accordingly. This analysis of oxygen consumption (Figure 1, panel D) indicated a significant main effect of time, $F(6, 57) = 62.34$, $p < .0001$, but neither the group effect, $F(1, 57) = .39$, nor the Group × Time interaction, $F(6, 57) = 1.23$, was significant. Thus, the greater thermal challenge posed by the extreme condition did not elicit greater thermal responses from the pups, as measured either by $T_{is} - T_{back}$ or oxygen consumption. This indicates that the moderate air temperature elicited a maximal metabolic response.

Amount of twitching per 10-min interval was significantly affected by air temperature (Figure 1, panel E). Repeated-measures ANOVA indicated significant main effects of group, $F(1, 60) = 20.1$, $p < .005$, and time, $F(6, 60) = 14.4$, $p < .0001$, and a significant Group × Time interaction, $F(6, 60) = 17.5$, $p < .0001$. There was no affect of cold exposure on twitching by pups in the moderate group, whereas twitching by pups in the extreme group decreased significantly during the test.

Few ultrasonic vocalizations were emitted by 2-day-old pups in either group (Figure 1, panel F), which is consistent with the findings of others (e.g., Okon, 1971). Specifically, pups in the moderate and extreme groups combined emitted ultrasonic vocalizations during only 1.0 ± 0.4 1-s bins throughout the entire test. As expected, repeated-measures ANOVA did not indicate significant main effects of group, $F(1, 60) = 0.00$, or time, $F(6, 60) = .3$, or a significant Group × Time interaction, $F(6, 60) = 1.5$.

7–8-Day-Olds

At the time of testing, mean body weight for the twelve 7–8-day-olds was 18.3 ± 0.7 g (range: 15.4–22.8 g). Body weights did not differ significantly between pups in the moderate and extreme groups, $t = .49$, $df = 10$, nor did they differ significantly between the male and female pups, $t = 1.38$, $df = 10$.

The data were analyzed by once again dividing the test into seven 10-min periods. Figure 2 presents the data for the 6 variables in the test. Panel A in Figure 2 shows the two air temperature conditions. In the moderate condition, air temperature averaged 30.2 °C by the 40th min of the test. In the extreme condition, air temperature averaged 20.9 °C by the 40th min of the test. As expected, repeated-measures ANOVA indicated significant main effects of group, $F(1, 60) = 14946.8$, $p < .0001$, and time, $F(6, 60) = 6{,}847.9$, $p < .0001$, and a significant Group × Time interaction, $F(6, 60) = 2187.1$, $p < .0001$.

Repeated-measures ANOVAs were used to test for differences in T_{is}, $T_{is} - T_{back}$, oxygen consumption, twitching, and ultrasound production. For all these variables but one, there were significant main effects of group, $F(1, 60) = 5.3 - 50.4$, $p < .05$, and time, $F(6, 60) = 3.7 - 113.6$, $p < .005$, and significant Group × Time interactions, $F(6, 60) = 3.7 - 67.2$, $p < .005$. The one exception was that the main effect of group for amount of ultrasound production only approached significance, $F(1, 60) = 4.2$, $p < .07$. As presented in panels B–F in Figure 2, post hoc unpaired t-tests indicated significant differences between the two groups for the majority of post-baseline time periods.

Across the entire test, T_{is} decreased approximately 1 °C in the moderate group as compared with 6 °C in the extreme group (Figure 2, panel B). Moreover, for pups in the moderate group, T_{is} stabilized and even increased slightly toward the end of the test. For pups in the extreme group, however, T_{is} decreased continually throughout the test.

The stabilization of T_{is} by pups in the moderate group was accomplished by increases in BAT heat production (Figure 2, panel C) and resulted in concomitant increases in oxygen consumption (Figure 2, panel D). These increases in heat production were not, however, the maximum attainable. On the contrary, the greater thermal challenge posed to pups in the extreme group elicited increased levels of BAT heat production (Figure 2, panel C) and oxygen consumption (Figure 2, panel D). We estimate that the thermal challenge posed to pups in the moderate group was only half that required to elicit a maximal metabolic response. In fact, current findings in our laboratory are indicating that the $T_{is} - T_{back}$ and oxygen consumption data would have been more similar across the two conditions (as they were for the 2-day-olds) had we decreased air temperature in the moderate group to approximately 27 °C, rather than 30 °C.

The two air temperature conditions elicited different behavioral responses. First, as with the 2-day-olds, 7–8-day-old pups in the moderate group continued to exhibit twitching throughout the test (Figure 2, panel E), whereas twitching by pups in the extreme group decreased significantly during the test.

Second, pups in the moderate group emitted ultrasonic vocalizations during only 6.3 ± 3.6 1-s bins throughout the entire test (Figure 2, panel F). Thus, increasing BAT thermogenesis alone is not a sufficient stimulus for reliably eliciting the vocalization. In contrast, pups in the extreme group emitted ultrasonic vocalizations during 254 ± 120.7 1-s-bins

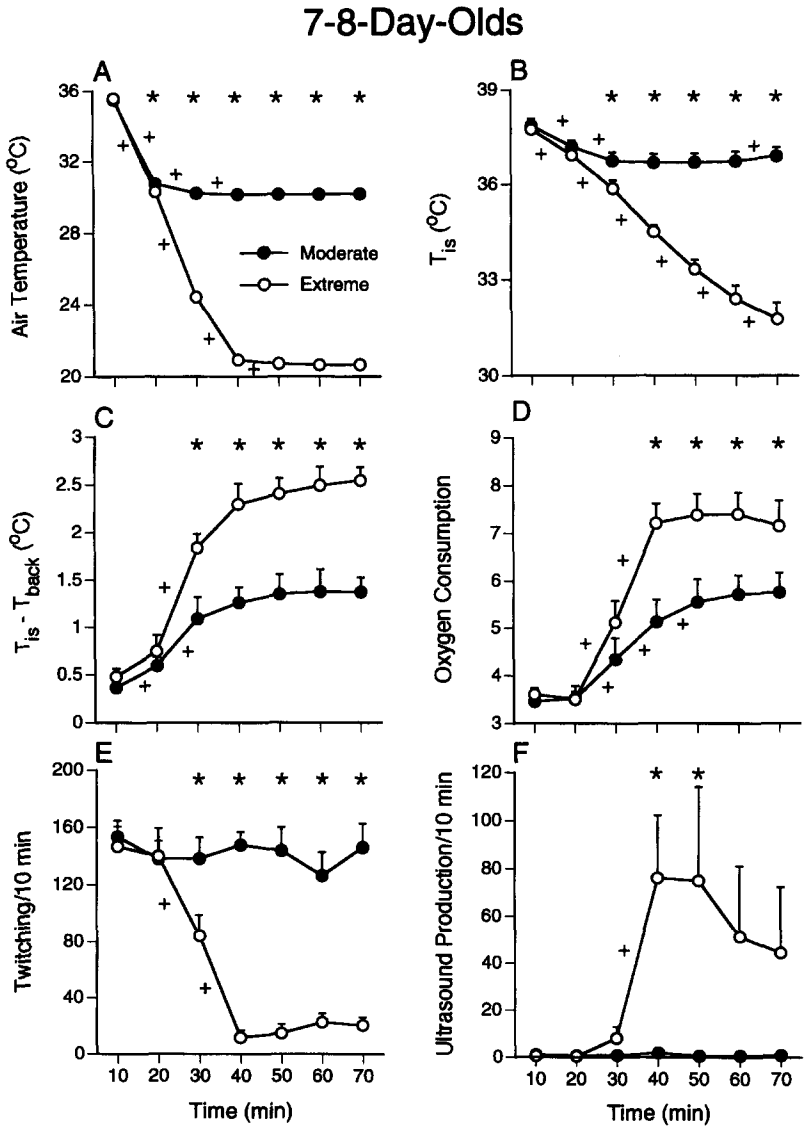

Figure 2. Air temperature (panel A), interscapular temperature (T_{is}, panel B), interscapular minus back temperature ($T_{is} - T_{back}$, panel C), oxygen consumption (ml O_2/100 g/min; panel D), twitching (panel E), and ultrasound production (panel F) for the 7–8-day-old pups in the moderate (filled circles) and extreme (open circles) groups. Thermal and metabolic values (panels A–D) represent data at the end of each 10-min period. Behavioral values (panels E–F) represent number of 1-s bins in which the behavior was detected during the preceding 10-min period. *M* + *SEM*. $n = 6$ for all data points. *Significant ($p < .05$) difference between extreme and moderate groups using unpaired *t* test. +Significant ($p < .05$) difference between adjacent points using paired *t* test.

throughout the entire test. The enormous variability in ultrasound production appeared to be related to the sex of the pups. Specifically, while the 3 female pups in the extreme group vocalized during only 59.3 ± 11.8 1-s intervals (range: 46–83 1-s intervals) throughout the test, the 3 male pups vocalized during 450.3 ± 185.6 1-s intervals (range: 194–811 1-s intervals).

To further analyze this apparent sex difference in ultrasound production, additional 8-day-old pups were tested. Specifically, 6 pups from 4 litters were tested using the same protocol established for pups in the extreme condition. Thus, the following analysis included these 6 pups and the 6 pups previously tested (mean body weight for all 12 pups: 17.5 ± 0.6 g; range: 14.3–22.8 g). The body weights for the male (18.1 ± 1.0 g) and female (17.0 ± 0.6 g) pups did not differ significantly, $t = 0.846$, $df = 10$.

The males and females experienced identical drops in air temperature (Figure 3, panel A). Repeated-measures ANOVA indicated a significant main effect of time, $F(6, 60) = 3577.6$, $p < .0001$, but there was no main effect of sex, $F(1, 60) = .00$, nor was the Group × Time interaction significant, $F(6, 60) = .26$.

When the 6 males and 6 females were compared with

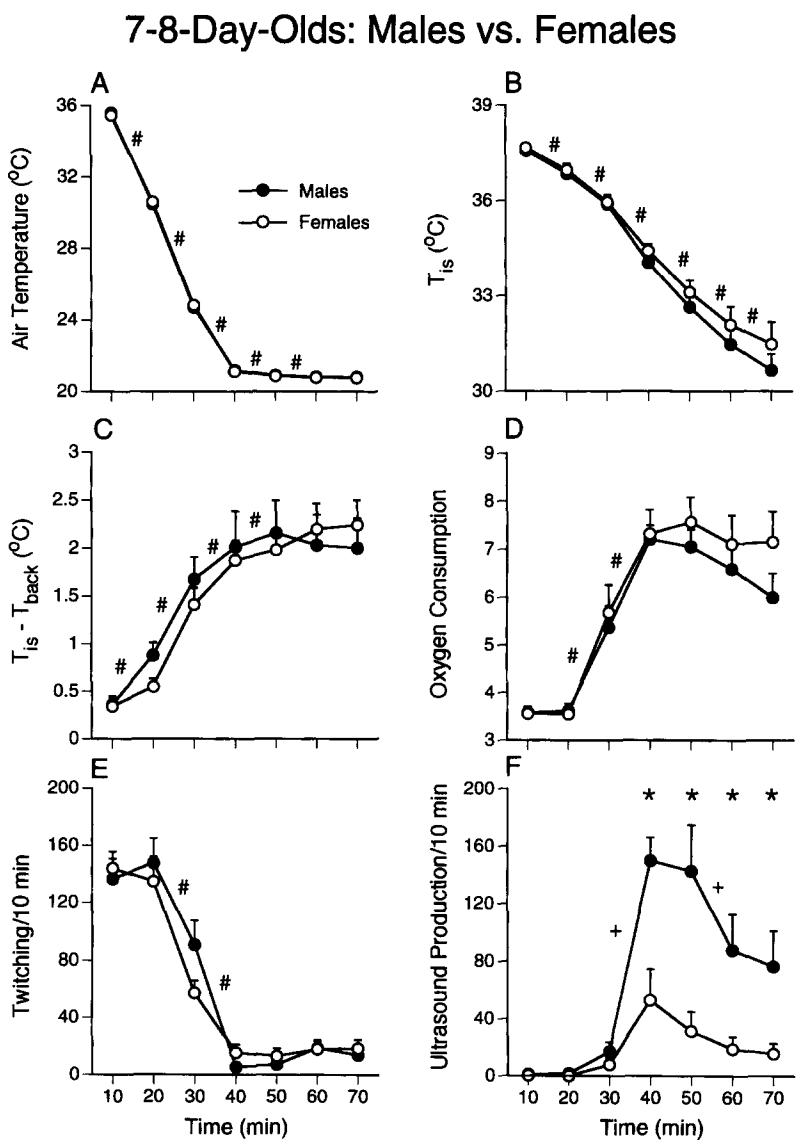

Figure 3. Air temperature (panel A), interscapular temperature (T_{is}, panel B), interscapular minus back temperature ($T_{is} - T_{back}$, panel C), oxygen consumption (ml O_2/100 g/min; panel D), twitching (panel E), and ultrasound production (panel F) for the 7–8-day-old male (filled circles) and female (open circles) pups. Thermal and metabolic values (panels A–D) represent data at the end of each 10-min period. Behavioral values (panels E–F) represent number of 1-s bins in which the behavior was detected during the preceding 10-min period. *M* + *SEM*. $n = 6$ for all data points except $n = 5$ for both groups in the oxygen consumption plot. *Significant ($p < .05$) difference between extreme and moderate groups using unpaired *t* test. +Significant ($p < .05$) difference between adjacent points using paired *t* test. #Significant ($p < .05$) difference between adjacent points using paired *t* test on collapsed data.

respect to ultrasound production, the sex difference was confirmed (Figure 3, panel F). Repeated-measures ANOVA indicated significant main effects of sex, $F(1, 60) = 12.4, p < .01$, and time, $F(6, 60) = 20.8, p < .0001$, as well as a significant Group × Time interaction, $F(6, 60) = 6.7, p < .0001$. Males emitted ultrasound during 194–811 1-s intervals during the test while females emitted the vocalization during 46–332 1-s intervals. Three of the 6 males vocalized during 500 or more intervals whereas none of the females did.

None of the three physiological measures was different between the sexes (Figure 3, panels B–F). Once again, although repeated-measures ANOVA of T_{is} and $T_{is} - T_{back}$ indicated significant main effects of time, $F(6, 60) = 60.0–196.8, p < .0001$, the main effects of sex, $F(1, 60) = .07–.88$, and the Group × Time interactions, $F(6, 60) = .66–1.29$, were not significant.

For 1 male and 1 female, the oxygen consumption system indicated technical problems and so the data for these 2 rats

were discarded. Repeated-measures ANOVA for the remaining 5 pairs of data indicated a significant main effect of time, $F(6, 48) = 54.7, p < .0001$, but not of sex, $F(1, 48) = .61$; the Group × Time interaction also was not significant, $F(6, 48) = .92$.

Finally, sex did not influence twitching. Repeated-measures ANOVA once again revealed a significant main of time, $F(6, 60) = 76.7, p < .0001$, but neither the main effect of sex, $F(1, 60) = .16$, nor the Group × Time interaction, $F(6, 60) = 1.3$, was significant.

Discussion

The aim of the present experiment was to examine the interactions between the physiological and behavioral responses of infant rats to moderate and extreme cold exposure. The findings can be outlined as follows: (a) Pups at both ages maintained stable and elevated interscapular temperatures at the moderate, but not extreme, air temperatures; (b) pups at both ages exhibited baseline levels of myoclonic twitching at the moderate, but not extreme, air temperatures; (c) at both ages, high rates of myoclonic twitching were compatible with BAT heat production and the maintenance of interscapular temperature; (d)7-8-day-olds, but not 2-day-olds, emitted the ultrasonic vocalization but only during extreme cold exposure; and (e) 8-day-old male pups vocalized more than female pups. These issues are addressed below.

Is heat production regulated? Although BAT's only known function is to produce heat, and although BAT normally reaches its peak development during the first week postpartum (Nedergaard, Connolly, & Cannon, 1986), it nonetheless does not produce enough heat to maintain a constant colonic temperature even during relatively mild cold exposure (Conklin & Heggeness, 1971). This failure has led investigators to conclude that rat pups are born without homeostatic control of body temperature, and that this control does not develop until pups become more effective at retaining heat (Hahn, 1956; Spiers & Adair, 1986; Taylor, 1960).

Although colonic temperature (where body temperature is typically measured) can arguably be used to assess the capabilities of the adult's thermoregulatory system, it is not necessarily the case that the newborn's thermoregulatory system should be judged by the same criterion. Indeed, colonic temperature is not a good metric for assessing the regulation of heat production by BAT for the following reason: the anatomical location of BAT and the organization of its blood supply indicate that heat is directed toward cervical spinal cord, heart, and lungs, not the colon (Smith, 1964). Therefore, if we want to understand the regulatory control of this thermogenic organ, we should measure its heat production at the source, not at some distant site.

By demonstrating that interscapular temperature is stabilized at a high temperature during moderate cold exposure (panel B in Figures 1 and 2), the present experiment provides preliminary evidence that rat pups are able to regulate heat production by BAT. Demonstrating regulation, however, requires the observation of thermoregulatory responses across a finer continuum of air temperatures than was used here. To assess regulation, however, it is also informative to examine the real-time dynamics of thermoregulatory processes in individual pups. These dynamics can be visualized by constructing a thermoregulatory state space in which oxygen consumption (or, alternatively, $T_{is} - T_{back}$) is plotted against interscapular temperature. What results is an individual pup's trajectory through the state space as air temperature changes. Figure 4 presents such state-space diagrams for 4 individual pups—two 2-day-olds and two 8-day-olds exposed to either moderate or extreme cold.

First, consider the 2-day-old in the moderate condition. The cluster of data at Point A denotes the 10-min baseline period at the thermoneutral temperature: T_{is} is high (approximately 37.5 °C) and oxygen consumption is low. As air temperature decreases, the pup exhibits a vertical trajectory to point B, where a new steady state is reached. This vertical trajectory indicates the maintenance of a stable value for T_{is} as air temperature decreases and oxygen consumption increases. The diagram for the 8-day-old indicates a similar process, except that T_{is} decreases to a critical temperature at approximately 37 °C before its vertical trajectory begins.

The state-space diagrams for pups in the extreme condition show that the 2-day-old pup passes through a similar trajectory from points A to B, including the vertical component. Beyond point B, however, as air temperature continues to drop, T_{is} begins decreasing as well; apparently, oxygen consumption can no longer be increased sufficiently to maintain T_{is} at a stable level. The 8-day-old pup also traverses through the state space to point B in a similar fashion, after which metabolic heat production can no longer maintain a stable T_{is}.

These data support the notion that the conventional standards for assessing thermoregulatory ability (i.e., the ability to maintain colonic temperature at a given level in the cold) do not tell the whole story in the neonate. Rather, the appropriate measure for assessing regulation and the mechanisms underlying it is one related to the production of heat at its source (i.e., interscapular temperature) at air temperatures that challenge but do not overwhelm the animal.

Interactions Between Ambient Temperature, Myoclonic Twitching, and BAT Heat Production

Jouvet-Mounier et al. (1970) reported that newborn rats spend 80% of their time in active sleep, as compared with approximately 5% in adults. To measure active sleep in neonates, these investigators were forced to rely exclusively on the occurrence of myoclonic twitching as their measure of active sleep because other standard measures used in adults (e.g., rapid eye movements, desynchronized EEG) are not expressed at the earliest postnatal ages (see also Blumberg & Lucas, 1996). Moreover, because quiet sleep is not observed until after 10 days of age (Jouvet-Mounier et al., 1970), newborns typically cycle between periods of myoclonic twitching and periods of "awake" behaviors such as locomotion and stretching (Blumberg & Lucas, 1994). Thus, one can posit myoclonic twitching as an indicator of a behavioral state, and then ask whether this behavioral state is similar to active sleep as defined in adults with regard to the effect of ambient temperature and the production of heat by BAT.

With regard to the first question, Szymusiak & Satinoff (1981) found that active sleep in adults rats (as determined

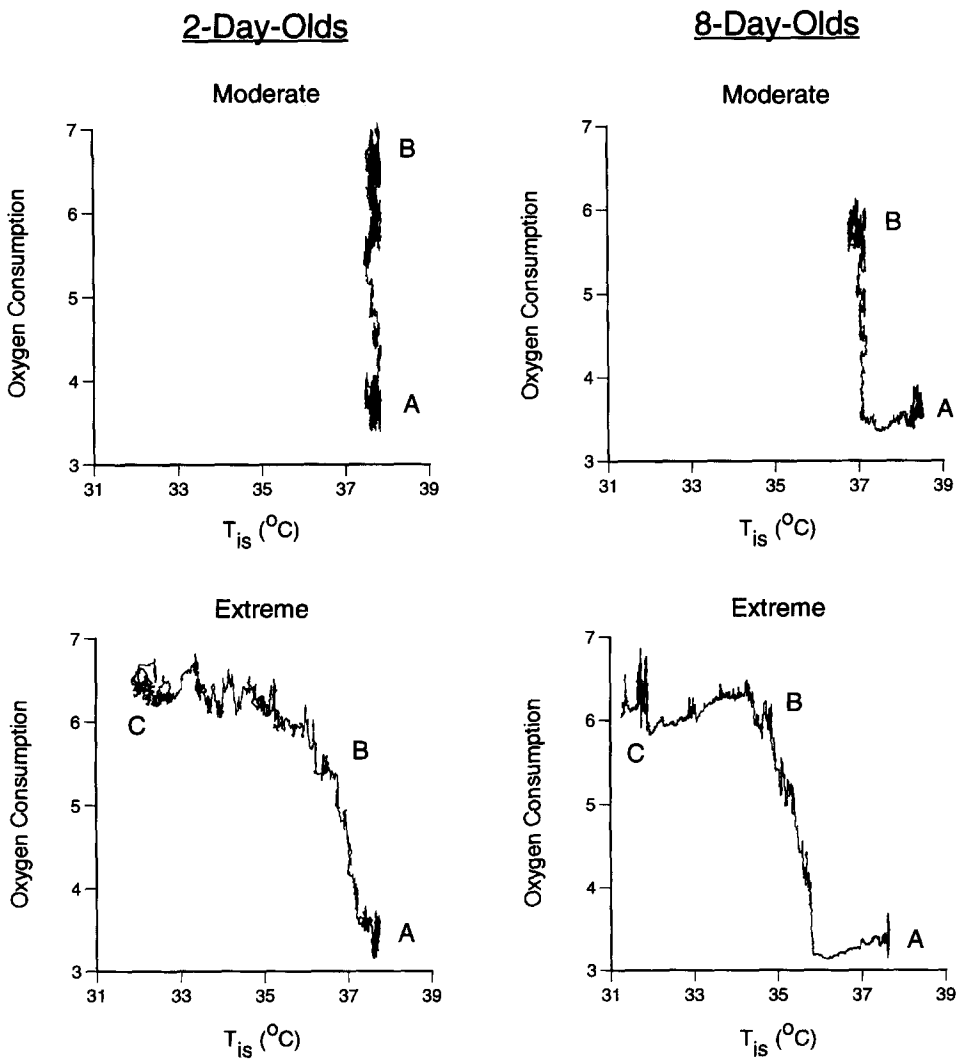

Figure 4. State-space diagrams for individual 2- and 8-day-old pups, in which oxygen consumption (ml O_2/100 g/min) is plotted against interscapular temperature (T_{is}). For pups in the moderate group, point A represents the baseline period where air temperature is within the pup's thermoneutral zone. As air temperature decreases, T_{is} moves through a trajectory that has a pronounced vertical component and restabilizes at point B. For pups in the extreme group, the trajectory from point A to point B is similar but, because of the further decrease in air temperature, T_{is} decreases beyond point B to point C.

from electroencephalographic and electromyographic recordings) is extremely sensitive to ambient temperature changes within the thermoneutral zone. In contrast, we have found here that myoclonic twitching in neonates occurred at equally high rates at thermoneutral as well as moderately cold air temperatures, while pups at extreme temperatures became behaviorally aroused and, thus, exhibited reduced rates of twitching. This finding suggests that ambient temperature plays a permissive role in the expression of myoclonic twitching in neonates, and once again illustrates the need for caution when comparing behavioral states defined by different components at different ages (Blumberg & Lucas, 1996).

With regard to the second question, one of the defining features of active sleep in adults is an inhibition of thermoregulatory responses, a phenomenon that has been clearly demonstrated in a number of species for such thermoregulatory responses as shivering, panting, and sweating (Parmeggiani, 1977; Walker, Walker, Harris, & Berger, 1983). Nonshivering thermogenesis has also been examined during active sleep in adult rats (Calasso, Zantedeschi, & Parmeggiani, 1993), 5–6-week-old rabbits (Franzini et al., 1986), and adult golden hamsters (Tegowska & Narebski, 1980); these studies suggest that BAT heat production is suppressed during active sleep, although such suppression is harder to observe in larger animals (that have higher thermal inertia) than in smaller animals.

There are a number of reasons why the present results do not allow us to state definitively whether BAT heat production is or is not inhibited during active sleep in newborns. First, the rapidity with which newborns cycle through behavioral states

may be too fast to see changes in BAT heat production without directly measuring changes in the neural activation of BAT. Second, there is once again the question of how we define active sleep in neonates. Thus, the present experiment most directly addresses the issue of whether myoclonic twitching is compatible with the maintenance of BAT heat production in the cold.

At this time, it can be concluded that high rates of myoclonic twitching do not interfere with a pup's ability to maintain BAT heat production in a moderately cold environment. This conclusion arises most clearly from the data for the 2-day-old pups, whose small size and poor insulation results in their having very low thermal inertia. Nonetheless, even as these pups twitched at high rates in the moderately cold environment, they exhibited BAT heat production. Indeed, the data in Figure 1 show that the 2-day-old pups in the two groups exhibited similar levels of heat production (panels C and D) even though the pups in the extreme condition were twitching significantly less and were in a colder environment. If the behavioral state associated with myoclonic twitching was incompatible with BAT heat production, the pups at the extreme temperature should have exhibited increases in BAT heat production as their rates of twitching decreased and the thermal challenge increased.

Given that shivering and myoclonic twitching appear to be obviously incompatible behaviors, the finding that myoclonic twitching and nonshivering thermogenesis are not incompatible suggests an interesting possibility. First, it should be noted that shivering thermogenesis is undeveloped in relation to nonshivering thermogenesis in rats (Taylor, 1960) and guinea pigs (Brück & Wünnenberg, 1966). Moreover, it has been demonstrated in neonatal guinea pigs that heat production by BAT inhibits shivering thermogenesis by directly warming the underlying cervical spinal cord (Brück & Wünnenberg, 1970). Thus, we hypothesize that when the maintenance of interscapular temperature breaks down during the transition from moderate to extreme cold exposure, the decrease in interscapular temperature, and thus cervical spinal cord temperature, acts as a stimulus to elicit a behavioral change from myoclonic twitching to behavioral arousal. Whether pups increase shivering during extreme cold exposure, or when BAT heat production is experimentally inhibited, is an open question.

Ultrasound Production: Developmental Changes and Sex Differences

Under normal conditions during cold exposure, it has been shown (Blumberg & Alberts, 1990) and confirmed (Hofer & Shair, 1991) that rat pups initiate ultrasound emission contemporaneously with BAT activation. But, it has also been shown that BAT activation is neither necessary nor sufficient for ultrasound production (Hofer & Shair, 1991). The present results support this finding in that 8-day-olds in the moderate group did not vocalize despite their exhibiting significant increases in BAT heat production.

That the 8-day-olds vocalized as they exhibited lower rates of myoclonic twitching might suggest that pups simply vocalize when they are behaviorally active. There are a number of observations that mitigate against this conclusion. First, the 2-day-olds were virtually silent during extreme cold exposure even as they exhibited locomotor and other waking behaviors. Second, the 8-day-old males emitted significantly more ultrasound than the females despite there being no difference in their twitching behavior (a sex difference in ultrasound production has also been reported by others but using different experimental conditions; Naito & Tonuoe, 1987). Finally, it can be readily observed that pups that are handled vigorously (and are thus awake) in a warm environment do not ultrasound (in contrast, handling in cold conditions does elicit ultrasound; Okon, 1970). Thus, the ultrasonic vocalization is not merely a sign of an awake pup.

At least three competing perspectives can be identified regarding the functional significance of ultrasound and its relation to its physiological correlates. The traditional view of ultrasound production holds that it is a communicatory act directed toward the stimulation of maternal retrieval responses (Allin & Banks, 1972). A second perspective holds that although the vocalization may have the effect of stimulating maternal retrieval, it is nonetheless an acoustic by-product of a respiratory maneuver that helps to maintain lung volume during periods of respiratory activation and high oxygen demand (Blumberg & Alberts, 1990, 1992). This respiratory maneuver, called *laryngeal braking,* is one of two mechanisms by which newborns maintain elevated lung volume (the second mechanism is the postinspiratory activation of the inspiratory muscles; Mortola, 1985). Finally, a third perspective is a weaker version of the second. This perspective does not view the vocalization as an acoustic by-product of respiration but does posit that the respiratory system must be in an activated state (e.g., as a result of cold exposure) in order for the vocalization to be emitted efficiently (see Speakman & Racey, 1991, for a discussion of efficient echolocation in bats during flight).

Although not designed to test hypotheses regarding the functional significance of the vocalization, the present experiment does raise some important questions. Specifically, Figure 3 illustrates that males and females exhibited identical physiological responses to cold exposure even as they showed sizable difference in rates of ultrasound production. The cause of this sex difference is not obvious. For example, it could be hypothesized that males are more anxiety-prone than females, but such a hypothesis cannot be independently evaluated in this study (or perhaps in any study) because ultrasound production is itself a putative marker for anxiety (e.g., Winslow & Insel, 1991). Alternatively, male and female respiratory systems may differ such that males rely more on laryngeal braking and females rely more on postinspiratory activity of the inspiratory muscles. (In this regard, it should be noted that sex differences in newborn respiratory control have been documented: For example, Scott, Inman, & Moss, 1990), found that male piglets were less able than females to properly coordinate activation of the muscles of the larynx and diaphragm.) The relative advantages and disadvantages of these two respiratory mechanisms (i.e., laryngeal braking and postinspiratory activity of the inspiratory muscles) are not yet known, nor do we know which conditions favor the use of one mechanism over the other. We also have little information regarding the energetics of ultrasound production. Such informa-

tion will be necessary, however, if we are to understand the relation between ultrasound production and respiratory function and thus be able to evaluate different ultrasound hypotheses.

In summary, the present results suggest that rat pups are capable of regulating BAT heat production within narrow subthermoneutral air temperature ranges. At these moderate air temperatures, neonates continue to exhibit myoclonic twitching, suggesting that brown adipose tissue protects sleep-related behaviors; moreover, 8-day-olds do not vocalize at these moderate air temperatures. It is only at extreme air temperatures that regulation fails and pups cease to twitch and, in the 8-day-olds, vocalize. The proximate stimulus to arousal and vocalization could be a decrease in cervical spinal cord temperature directly or perhaps hypothermia-induced changes in oxygen transfer or ventilation. Identification of the stimuli that evoke behavioral arousal and further understanding of the mechanics of respiratory control are necessary to continue elucidating the interactions among the newborn's physiological and behavioral responses to varying thermal conditions.

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