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# Thermogenic, Respiratory, and Ultrasonic Responses of Week-Old Rats across the Transition from Moderate to Extreme Cold Exposure

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**ABSTRACT:** Previously, it was reported that week-old rats exposed to air temperatures that elicited submaximal levels of heat production (designated moderate cold exposure) remained asleep and did not vocalize (Blumberg & Stolba, 1996). In contrast, pups exposed to air temperatures that elicited maximal levels of heat production (designated extreme cold exposure) woke up and emitted ultrasonic vocalizations. We now report on the physiological and behavioral responses of pups in the transitional region between moderate and extreme air temperatures. Small decreases in air temperature across the transition resulted in pronounced decreases in physiological temperature and concomitant increases in ultrasound production. In a second experiment, it was shown that during moderate cold exposure respiratory frequency increased as air temperature decreased but, as extreme air temperatures were reached, respiratory frequency was maximized as ultrasound production began. The results from these two experiments illustrate how air temperatures that differ by as little as 2°C can differentially modify the physiological and behavioral responses of neonates. © 1997 John Wiley & Sons, Inc. *Dev Psychobiol* 30: 181–194, 1997

**Keywords:** rat; neonate; thermoregulation; brown fat; ultrasound; respiration; laryngeal breaking

When neonatal rats are exposed to air temperatures below thermoneutrality they initiate nonshivering thermogenesis by brown adipose tissue (BAT), a heat-producing organ that is concentrated largely in the interscapular region (Smith, 1964). In recent years, some progress has been made in delineating the behavioral correlates of BAT thermogenesis as well as the consequences of

thermal challenges that exceed the thermogenic capabilities of the neonate. For example, it has recently been shown that newborn and week-old pups exposed to moderately cold air temperatures (as defined by the elicitation of significant but submaximal levels of heat production) exhibit the same rates of myoclonic twitching (a measure of active sleep; see Blumberg & Lucas, 1996) as when they are at high air temperatures above the threshold for BAT activation (Blumberg & Stolba, 1996). Moreover, only when heat production has been maximized and air temperature is decreased further do pups “wake up” and, in the case of week-old pups,

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begin emitting ultrasonic vocalizations. Thus, it was suggested that there exist three air temperature “zones” that are associated with reliable thermoregulatory and behavioral responses. Within the first such zone, air temperature is high enough such that BAT thermogenesis is not activated and, in addition, pups exhibit high rates of myoclonic twitching and do not vocalize. Within a zone of moderate air temperatures, nonshivering thermogenesis increases in proportion to the decline in air temperature, pups continue to exhibit high rates of myoclonic twitching but still do not vocalize. Finally, within a zone of extreme air temperatures, pups can increase BAT thermogenesis no further, they “wake up” and begin emitting the vocalization.

In the experiment of Blumberg and Stolba (1996), week-old pups were tested at only two air temperatures: a moderate air temperature of 30°C that elicited submaximal heat production, and an extreme air temperature of 21°C that was sufficiently cold to elicit maximal heat production. No attempt was made in that experiment to examine the behavioral and physiological responses across a finer continuum of air temperatures. Therefore, in the present experiment, we exposed pups to air temperatures of 27°C, 25°C, and 23°C (hereafter designated as the Moderate, Intermediate, and Extreme conditions, respectively), and monitored thermal and metabolic responses and ultrasound production throughout a 90-min period.

At the end of this 90-min cooling period, pups in the Moderate and Intermediate groups experienced a second air temperature drop, to 23°C, while the air temperature for pups in the Extreme group remained at 23°C. This second cooling period lasted 30 min and was used to determine whether small, additional decreases in air temperature would increase ultrasound production in the Moderate and Intermediate pups.

## EXPERIMENT 1: RATES OF ULTRASOUND PRODUCTION INCREASE ACROSS THE TRANSITION FROM MODERATE TO EXTREME COLD EXPOSURE

### METHOD

#### Subjects

Twenty-four 7- to 8-day-old male rat pups from 17 litters were used; no more than 3 pups were

used from a single litter and pups from the same litter were always assigned to different experimental groups. 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 three 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 animals were maintained on a 12:12 hr light:dark schedule with lights on at 6:00 A.M.

### Test Environment

Individual pups were placed 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 ( $T_{\text{air}}$ ) 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).

### 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.  $T_{\text{air}}$  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 ( $T_{is}$ ). The second thermocouple was attached in the lumbar region, thus providing a measure of back temperature ( $T_{back}$ ). We derived a measure of BAT heat production by subtracting  $T_{back}$  from  $T_{is}$  ( $T_{is}-T_{back}$ ; see Heim & Hull, 1966).

### Oxygen Consumption Measurements

Compressed air passed through a two-stage regulator and was split into two lines. One line entered a digital flow meter (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/100g/min. We did not correct oxygen consumption for respiratory exchange ratios less than unity, which can lead to a systematic underestimation of oxygen consumption by approximately 5% during normoxia in newborn rats (Frappell, Dotta, & Mortola, 1992).

### 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, U.K., Model SM100) tuned to a  $\pm 5$  kHz range centered on 40 kHz. It was sometimes necessary to vary the tuning of the bat detector in order to maximize the clarity of the signal. 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.

### 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 were in a postabsorptive state, 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 at approximately 35.5°C. The pup was given at least 45 min (range: 45–54 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 this baseline period,  $T_{air}$  was decreased to 27°C, 25°C, or 23°C (i.e., the Moderate, Intermediate, and Extreme conditions, respectively). After 90 min, pups in the Moderate and Intermediate groups experienced a second drop in air temperature to 23°C for another 30 min; pups in the Extreme group did not experience a second decrease but were monitored for this 30-min period.

After the test, the pup was removed from the chamber, its thermocouples were removed, and it was returned to its home cage. The oxygen consumption system was allowed to rezero to verify that there had been minimal drift in the system over the course of the test.

### Data Analysis

Data were imported into StatView 4.5 for the Macintosh for statistical analysis. First, repeated measures ANOVAs were used to test for significant differences in our thermal and metabolic measures. Then, where appropriate, one-factor ANOVAs were used to test for differences between groups at selected time points, and Scheffe's  $F$  test was used for post hoc comparisons. Finally, paired  $t$  tests were used to determine whether a variable for a particular group changed over the course of the second cooling period.

Because ultrasonic vocalization data do not distribute normally, nonparametric statistics were used. Specifically, the Wilcoxon matched-pairs signed-ranks test was used to test for changes across time within groups. The Mann-Whitney  $U$  test was used to perform planned comparisons as

to whether, for each time period, pups exhibited higher vocalization rates in the Extreme and Intermediate groups as compared with the Moderate group. For both nonparametric tests, alpha was set at .05 and was adjusted for multiple tests by dividing it by the number of pairwise comparisons; accordingly, alpha for all Wilcoxon matched-pairs signed-ranks tests was set at .0125 and alpha for all Mann-Whitney *U* tests was set at .025. In addition, one-tailed tests were used because, based on a previous study using similar procedures (Blumberg & Stolba, 1996), we expected ultrasound production to increase in relation to precooling baseline levels and in relation to the low ultrasound production levels expected from the Moderate pups. Finally, all means are presented with their standard errors.

## RESULTS AND DISCUSSION

### Body Weight

At the time of testing, mean body weight for the twenty-four 7- to 8-day-olds was  $18.5 \pm 0.3$  g (range: 15.5–21.6 g). Body weights did not differ significantly between pups in the three groups,  $F(2,21) = .56$ .

### Thermal and Metabolic Responses

The data were analyzed by dividing the test into one 10-min baseline period and four 30-min post-baseline periods. Figure 1 presents the data for the four thermal and metabolic variables. Figure 1A shows the three air temperature conditions. After 30 min of cooling,  $T_{\text{air}}$  averaged 26.7°C in the Moderate group, 25.0°C in the Intermediate group, and 23.2°C in the Extreme group. After the initial 90-min cooling period, pups in the Moderate and Intermediate groups experienced a second air temperature decrease for 30 min. At the end of this second cooling period,  $T_{\text{air}}$  averaged 22.9°C for the three groups.

Repeated measures ANOVA for  $T_{\text{air}}$  indicated significant main effects of group,  $F(2,84) = 129.35$ ,  $p < .0001$ , and time,  $F(4,84) = 157,560.78$ ,  $p < .0001$ , and a significant Group x Time interaction,  $F(8,84) = 2,141.72$ ,  $p < .0001$ . As shown in Figure 1A,  $T_{\text{air}}$  differed significantly between groups at the 30-, 60-, and 90-min time points,  $305.63 \leq F(2,21) \leq 358.70$ ,  $p < .0001$ . All post-hoc pairwise comparisons were statistically significant.

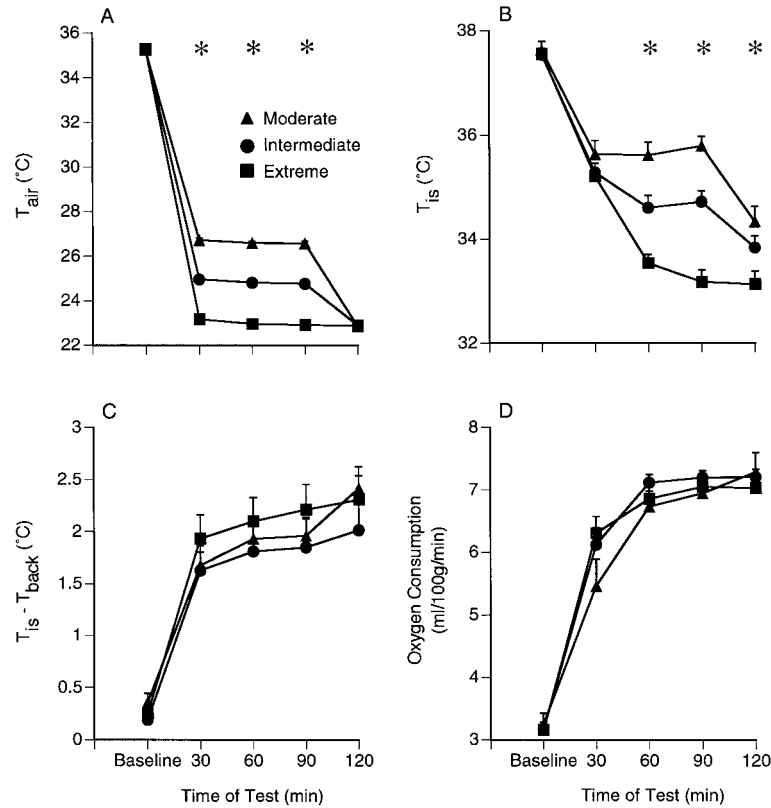
Repeated measures ANOVA for  $T_{\text{is}}$  indicated

significant main effects of group,  $F(2,84) = 13.61$ ,  $p < .0005$ , and time,  $F(4,84) = 416.69$ ,  $p < .0001$ , and a significant Group x Time interaction,  $F(8,84) = 19.25$ ,  $p < .0001$ . As indicated in Figure 1b,  $T_{\text{is}}$  did not differ between groups at the end of baseline and at the 30-min time point but did differ significantly at all subsequent time points,  $5.50 \leq F(2,21) \leq 40.40$ ,  $p < .05$ . Moreover, all post-hoc pairwise comparisons were significant at 60 and 90 min but, at 120 min, only the Moderate and Extreme groups differed from each other.

Repeated measures ANOVA for  $T_{\text{is}}-T_{\text{back}}$  only indicated a significant main effect of time,  $F(4,84) = 257.47$ ,  $p < .0001$ . Therefore, no further tests of  $T_{\text{is}}-T_{\text{back}}$  were performed. For oxygen consumption, repeated measures ANOVA indicated a significant main effect of time,  $F(4,84) = 368.74$ ,  $p < .0001$ , and a significant Group x Time interaction,  $F(8,84) = 2.24$ ,  $p < .05$ . As shown in Figure 1d, at no time point did oxygen consumption differ significantly between the three groups.

Paired *t* tests were used to determine the effect of the second cooling period on  $T_{\text{is}}$  in relation to its value at 90 min; the reader should recall that air temperature for pups in the Extreme group did not change over this period. There was a significant decrease in  $T_{\text{is}}$  over the 30-min period for the Moderate group, paired  $t = 8.56$ ,  $df = 7$ ,  $p < .0001$ , and for the Intermediate group, paired  $t = 12.80$ ,  $df = 7$ ,  $p < .0001$ , but not for the Extreme group, paired  $t = .72$ ,  $df = 7$ .

Paired *t* tests were similarly used to determine the effect of the second cooling period on  $T_{\text{is}}-T_{\text{back}}$  and oxygen consumption. As stated above,  $T_{\text{is}}-T_{\text{back}}$  provides a relative measure of BAT thermogenesis, with an increasing value of  $T_{\text{is}}-T_{\text{back}}$  suggesting increasing BAT thermogenesis (Blumberg & Alberts, 1990; Dawkins & Hull, 1964). Analysis of  $T_{\text{is}}-T_{\text{back}}$  revealed small but significant increases for pups in the Moderate group, paired  $t = 4.64$ ,  $df = 7$ ,  $p < .005$ , and in the Intermediate group, paired  $t = 2.92$ ,  $df = 7$ ,  $p < .05$ , but not in the Extreme group, paired  $t = 1.43$ ,  $df = 7$ . Because small relative changes in  $T_{\text{is}}$  and  $T_{\text{back}}$  can occur independently of changes in BAT thermogenesis (e.g., postural adjustments resulting in mechanical displacement of the thermocouples), increases in  $T_{\text{is}}-T_{\text{back}}$  can only reliably indicate increased BAT thermogenesis when accompanied by increased oxygen consumption. Thus, if these small increases in  $T_{\text{is}}-T_{\text{back}}$  for the Moderate and Intermediate pups reflected increased BAT thermogenesis, then oxygen consumption should have also increased during the second cooling period. In fact, however,



**FIGURE 1**  $T_{air}$  (A),  $T_{is}$  (B),  $T_{is}-T_{back}$  (C), and oxygen consumption (D) for the 7- to 8-day-old pups in the Moderate (triangles), Intermediate (circles), and Extreme (squares) groups. Values represent data at the end of the 10-min baseline period and at the end of the four subsequent 30-min periods. Mean + SEM.  $n = 8$  for all data points. \*significant difference between groups using a one-factor ANOVA.

oxygen consumption did not increase significantly in these two groups, Moderate: paired  $t = 1.88$ ,  $df = 7$ ; Intermediate: paired  $t = .02$ ,  $df = 7$ . These results suggest that pups in the Moderate and Intermediate groups had already increased metabolic heat production to maximal or near-maximal levels through 90 min of the first cooling period, and thus could not increase it further during the second cooling period.

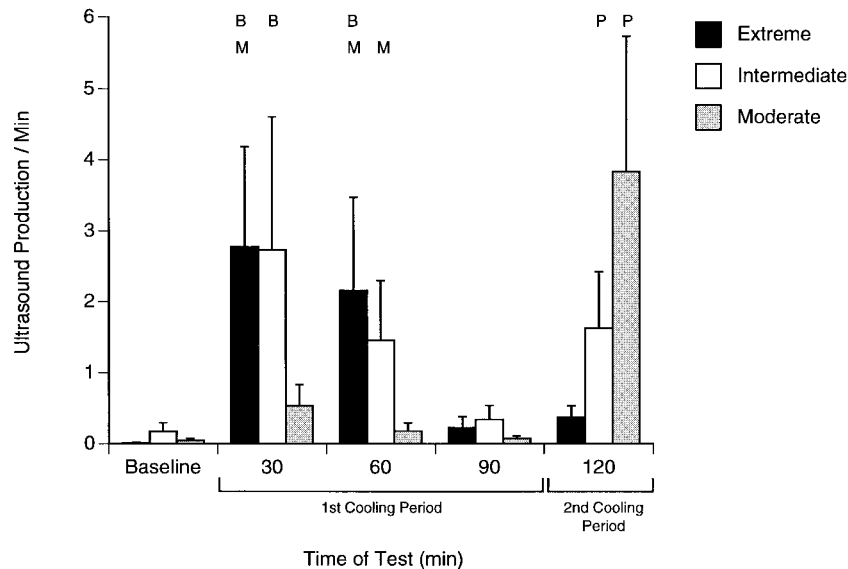
### Ultrasound Production

Figure 2 presents mean rates of ultrasound production per min for the 10-min baseline period and each of the subsequent 30-min periods. It is apparent that the pups in the Extreme and Intermediate groups tended to vocalize more than did the pups in the Moderate group, especially during the first 60 min of cold exposure.

The Wilcoxon matched-pairs signed-ranks test was used to determine whether ultrasound produc-

tion increased during the first cooling period in relation to the baseline period. Pups in the Extreme group exhibited significant increases in ultrasound production during the first and second 30-min time periods, 30 Min:  $z = 2.52$ ,  $p < .01$ ; 60 Min:  $z = 2.38$ ,  $p < .01$ . In the Intermediate group, ultrasound production was significantly higher during the first 30-min time period,  $z = 2.31$ ,  $p = .01$ , but only approached statistical significance during the second 30-min time period,  $z = 1.69$ ,  $p < .05$ . In contrast, at no time during the first cooling period did pups in the Moderate group exhibit significant increases in ultrasound production in relation to baseline.

Figure 2 shows that during the first and second 30-min time periods, pups in the Extreme group vocalized significantly more than did pups in the Moderate group, 30 Min:  $U = 52.0$ ,  $z = 2.10$ ,  $p < .02$ ; 60 Min:  $U = 56.0$ ,  $z = 2.53$ ,  $p < .01$ . Similarly, pups in the Intermediate group vocalized significantly more than did pups in the Moder-



**FIGURE 2.** Ultrasound production per min during the 10-min baseline period and four subsequent 30-min periods for pups in the Moderate, Intermediate, and Extreme groups. Data represent the mean number of 1-s bins in which an ultrasonic vocalization was detected. <sup>B</sup> significant difference in relation to baseline using the Wilcoxon matched pairs signed ranks test. <sup>M</sup> significant difference in relation to the Moderate group using the Mann-Whitney *U* test. <sup>P</sup> significant difference in relation to the previous period using the Wilcoxon matched-pairs signed-ranks test.  $n = 8$  for all data points.

ate group during the second 30-min time period,  $U = 51.5$ ,  $z = 2.05$ ,  $p = .02$ , although the difference only approached statistical significance for the first 30-min time period,  $U = 47.5$ ,  $z = 1.63$ ,  $p = .05$ .

Figure 2 also shows that pups in the Moderate group increased ultrasound production during the second cooling period in relation to the previous 30-min period,  $z = 2.53$ ,  $p < .01$ ; this was also true of pups in the Intermediate group,  $z = 2.37$ ,  $p < .01$ . In contrast, pups in the Extreme group did not experience a change in  $T_{\text{air}}$  during that period and also did not exhibit increased ultrasound production. Despite these results, however, during the second cooling period ultrasound production by pups in the Moderate group was not significantly greater than that of pups in the Intermediate group, and only approached significance in comparison to pups in the Extreme group,  $U = 50.0$ ,  $z = 1.90$ ,  $p < .03$ .

Thus, it is apparent that rates of ultrasound production increased during the second cooling period for pups in the Moderate group. As further evidence, consider that 6 of the 8 pups in this group vocalized more during this final 30-min period than in the previous 90 min combined; this was not true for any pup in the Extreme group. Thus, for the Moderate pups, the 4°C drop from 27°C to 23°C

elicited more ultrasound production than did the initial 8°C drop from 35°C to 27°C. This suggests that it was not the change in  $T_{\text{air}}$ , *per se*, but rather the thermoregulatory consequences of the change in  $T_{\text{air}}$  that resulted in increased ultrasound production during the second cooling period.

The effect of the second cooling period on ultrasound production by pups in the Intermediate group is not very informative because pups in this group had already exhibited significant amounts of ultrasound during the initial decrease of  $T_{\text{air}}$ . Nonetheless, even though the  $T_{\text{air}}$  decrease in this group had a smaller impact on  $T_{\text{is}}$  both in terms of amount and rate of cooling, 5 of 8 pups exhibited notable increases in ultrasound production during the second cooling period.

The present data also inform our understanding of the neurochemical basis of ultrasound production. Specifically, 9 of the 16 pups in the Moderate and Intermediate groups exhibited ultrasound production during the first 30 min of cooling and then were relatively quiet from 60 to 90 min after cooling began. Then, as stated earlier, when  $T_{\text{air}}$  was decreased a second time to 23°C, many of these pups initiated high levels of ultrasound production again (see Figure 2). Such effects of multiple air temperature drops have been seen before

(e.g., Blumberg & Alberts, 1990). These findings are not consistent with the hypothesis that ultrasound production ceases during prolonged cold exposure due to the expenditure of modulatory neurochemicals (e.g., Carden, Tempel, & Hofer, 1995); if such critical neurochemicals were truly exhausted, then a second decrease in  $T_{\text{air}}$  should not be capable of eliciting more ultrasound production.

It should be noted that rates of ultrasound emission in the present experiment appear low compared to rates reported by other investigators (e.g., Kehoe & Blass, 1986; Hofer & Shair, 1980). It is important to keep in mind, however, how the present methods differ from those used elsewhere. First, the extreme  $T_{\text{air}}$  of 23°C used here results in ultrasound production rates that are lower than those seen using identical procedures but at air temperatures just 2°C lower (see Figure 4 and discussion below). Second, the ultrasound data presented here are binned and averaged over 30 min, not counted individually during relatively brief tests; shorter test periods lead to higher average values because ultrasound production is not evenly distributed over time. Finally, our cold exposure procedure is less abrupt than that used by others in which pups are removed from the nest using bare hands and immediately tested in an open box; such a procedure involves stimuli not found here, such as conductive cooling of the skin. It is also true that these isolation procedures involve the instantaneous removal of nonthermal stimuli associated with the pup's social environment, such as olfactory and tactile cues from the mother and littermates. Some disagreement continues to exist, however, as to the relative significance of thermal and nonthermal stimuli for ultrasound production (Blumberg, Efimova, & Alberts, 1992; Hofer, Brunelli, & Shair, 1993).

The data from the present experiment and those of Blumberg and Stolba (1996) can be combined to provide a more complete picture of the week-old pup's thermogenic and ultrasonic responses to cold exposure. In that previous report, a new method of visualizing an individual pup's thermogenic responses was introduced. That method entails the construction of a state space in which oxygen consumption is plotted against  $T_{\text{is}}$ . Figure 3A illustrates this graphical method for a pup from that previous report. First, at point *a*,  $T_{\text{air}}$  is approximately 35°C and the pup exhibits a high value of  $T_{\text{is}}$  and a low value of oxygen consumption. Then, as  $T_{\text{air}}$  decreases toward 30°C, the pup displays a horizontal trajectory toward

point *b*; oxygen consumption has not yet increased. Finally, as BAT heat production is initiated, the pup's trajectory moves in a vertical direction toward point *c* as  $T_{\text{is}}$  is maintained within a narrow range and oxygen consumption reflects the increased heat production.

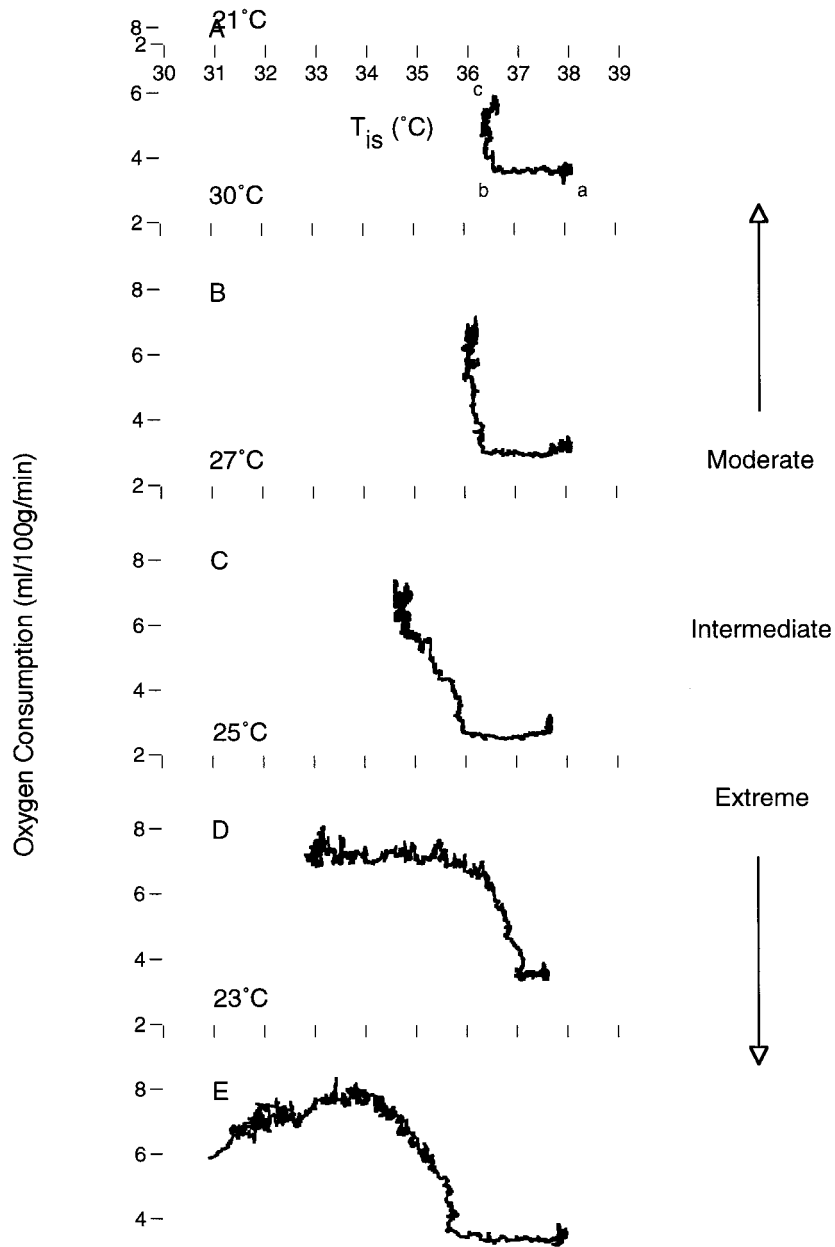
Figure 3B depicts the trajectory for a pup in the Moderate group in the present experiment. The trajectory's pattern is fundamentally the same as that described above, including the vertical progression from *b* to *c*. The trajectory in Figure 3C is that of a pup in the Intermediate group; this trajectory deviates from that above it in that the vertical arm of the trajectory is skewed toward lower values of  $T_{\text{is}}$ . In Figure 3D the skewing of  $T_{\text{is}}$  is greater and, in Figure 3E, oxygen consumption even decreases as thermogenic capacity is exceeded. Thus, pups at a moderate  $T_{\text{air}}$  characteristically exhibit trajectories that are suggestive of a capability for regulating  $T_{\text{is}}$  (or a correlate) and, as  $T_{\text{air}}$  decreases toward extreme values, there is a failure in this capability as indicated by decreasing  $T_{\text{is}}$  as BAT thermogenesis can no longer compensate for greater levels of cold exposure.

The combined ultrasound data from the two studies are presented in Figure 4. This figure presents, on the *y* axis, cumulative ultrasound production through 60 min of cooling to the temperature given on the *x* axis. (Blumberg & Stolba, 1996, followed pups only through a 60-min cooling period, requiring that the present 90 min of data be truncated.) This figure demonstrates a clear trend from little or no ultrasound production at moderate air temperatures to high levels of ultrasound production at extreme air temperatures.

The above results show that at the transition between moderate and extreme air temperatures where BAT thermogenesis is maximized, rates of ultrasound production increase. Because the respiratory system plays fundamental roles in both the supply of oxygen to BAT as well as the production of ultrasound, we next conducted a preliminary examination of respiratory responses to cold challenge under thermal conditions similar to those used in Experiment 1.

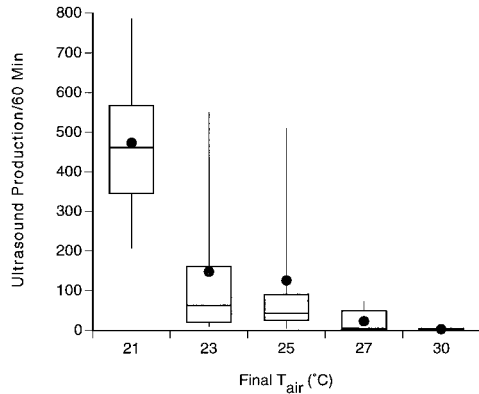
## EXPERIMENT 2: RESPIRATORY ADJUSTMENTS DURING MODERATE AND EXTREME COLD EXPOSURE

Heat production by BAT is sensitive to the availability of oxygen (Blumberg & Alberts, 1991; Heim & Hull, 1966), thus making it likely that the



**FIGURE 3.** State-space diagrams for individual week-old male pups in which oxygen consumption is plotted against  $T_{is}$ .  $T_{air}$  at the beginning of the test (e.g., point *a*) was always above 35°C. Temperatures at the lower left corner of each plot indicates the final  $T_{air}$  for that test. Data for plots A and E are from Blumberg and Stolba (1996); the other plots are from the present experiment. See text for discussion.





**FIGURE 4.** Box plots depicting cumulative ultrasound production through 60 min of cooling to 21°C, 23°C, 25°C, 27°C, and 30°C. Data for air temperatures of 30°C ( $n = 3$ ) and 21°C ( $n = 6$ ) are from Blumberg & Stolba (1996); only data from male pups are included in this figure. All other data ( $n = 8$ ) are from the present experiment. Data represent the number of 1-s bins in which an ultrasonic vocalization was detected. The top, middle, and bottom horizontal lines of the box represent the 75th, 50th (median), and 25th percentiles, respectively. The thin vertical lines above and below the box represent the 90th and 10th percentiles, respectively. Filled circles are means.

cold-exposed pup adjusts respiratory activity in order to sustain BAT thermogenesis. Therefore, given the results of Experiment 1, we might expect the pup to increase respiratory rate as  $T_{\text{air}}$  decreases and BAT thermogenesis increases. If so, then the limits on maximal oxygen consumption observed in extreme air temperatures may be accompanied by limits on respiratory frequency as well. Thus, in the present preliminary experiment, we monitored the respiratory system during moderate and extreme cold exposure to examine the relationship between respiratory frequency, oxygen consumption, the emission of ultrasound, and the incidence of laryngeal braking.

## Method

### Subjects

Four 7-day-old male rat pups from four litters were used. All pups were raised as in Experiment 1.

### Test Environment

The experiment was conducted using the same apparatus as in Experiment 1.

## Physiological and Behavioral Measurements

$T_{\text{air}}$ ,  $T_{\text{is}}$ , oxygen consumption, and ultrasound production were measured and monitored as in Experiment 1, except ultrasound production was not quantified. Thermal and metabolic measures were acquired by the computer at a rate of four times per min.

## Respiratory Measurements and Analysis

Respiratory rate was measured using strain-gauge plethysmography, as described previously (Blumberg & Alberts, 1990). Briefly, closed (diameter = 1.6 cm) or open (length = 4.5 cm) mercury-filled strain gauges were secured around a pup's midsection using collodion as an adhesive. The strain gauge was connected to a plethysmograph (Model 273, Parks Medical Electronics, Aloha, OR) whose output was then fed into the computerized data acquisition system described in Experiment 1. Respiratory data were collected at a rate of 200 times per second. Tidal volume was not determined because the amplitude of respirations cannot be reliably measured using strain-gauge plethysmography for long tests involving free-moving animals (Porges & Byrne, 1992).

Respiratory data were imported into Data Desk 4.1 for analysis. A scatterplot of the data was produced for each session, and each point in the scatterplot could be linked to a row number that represented the passage of 1/200 s. The row numbers corresponding to successive respiratory peaks were then determined. In general, for determinations of respiratory rate, clear respiratory records were only attainable when pups were behaviorally quiescent; gross body movements contaminated data such that respiratory records were unreadable. In addition, an effort was made to include only those respiratory cycles for analysis that exhibited uninterrupted inhalations and exhalations, that is, that were not disrupted by laryngeal activity. Laryngeal brakes were identified from the occurrence of shoulders on the expiratory portion of the respiratory record (Andrews, Symonds, & Johnson, 1991). Unless otherwise indicated, mean respiratory frequency was determined from 40 respiratory cycles for each episode of respiratory data collection. Periods of respiratory data collection ranged from 2 to 7 min.

## Procedure

On the day of testing, a postabsorptive pup was removed from its cage, placed inside an incubator

maintained at 35–36°C, and the strain gauge and interscapular probe were attached. Afterward, the pup was placed inside the metabolic chamber maintained at approximately 35.5°C and given at least 45 min to acclimate to the chamber.

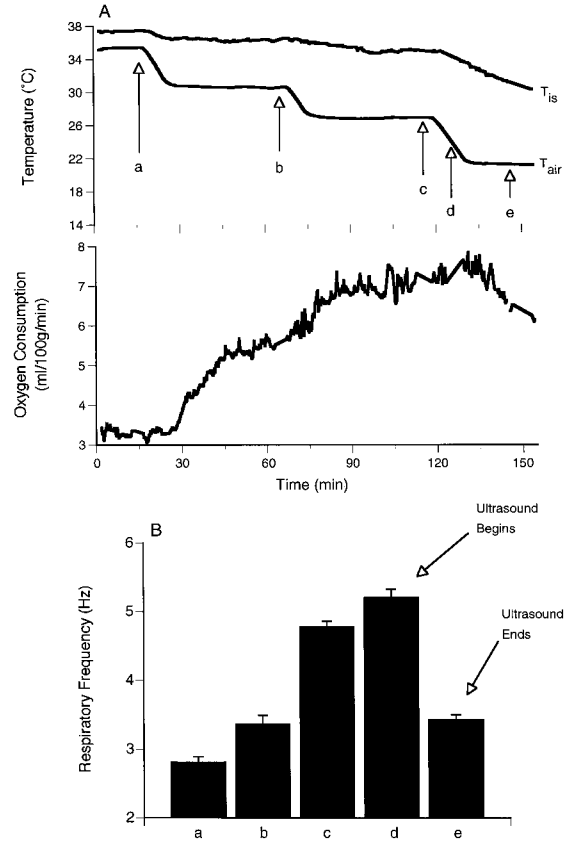
In this experiment, we were not able to collect respiratory data simultaneously with the thermal and metabolic data; thus, we interrupted the acquisition of the thermal and metabolic data with brief periods of respiratory data collection. Each test session began by initiating data collection of thermal and metabolic measures onto computer disk. The initial baseline period lasted at least 10 min at 35.5°C. After this baseline period, respiratory data were collected for a period of time sufficient to ensure the acquisition of at least 40 measurable interbreath intervals. In addition, while respiratory data were acquired, ultrasound production was monitored and the amount of active sleep, based on the occurrence of myoclonic twitching in the limbs and tail (Blumberg & Stolba, 1996), was noted. After acquisition of baseline respiratory data, thermal and metabolic data acquisition was resumed and  $T_{\text{air}}$  was decreased to 30.5°C. After a period of at least 45 min, respiratory measurements were once again acquired, after which  $T_{\text{air}}$  was decreased to 27°C. Again, after a period of at least 45 min, respiratory data were acquired.

After this third period of respiratory measurement,  $T_{\text{air}}$  was decreased toward 21°C. At this point, ultrasound production was monitored continuously. When the initiation of ultrasound was detected, respiratory measurements were resumed until adequate data had been acquired. Then, thermal and metabolic measurements were resumed until the pup had substantially decreased its rate of ultrasound emission; at that time (range: 23–56 min after the last temperature drop), a final respiratory measurement was taken followed by a final acquisition of thermal and metabolic data.

After the test, the pup was removed from the chamber, the thermocouple and strain gauge were removed, and it was returned to its home cage. The oxygen consumption system was allowed to rezero to verify that there had been minimal drift in the system over the course of the test.

## RESULTS AND DISCUSSION

Figure 5 presents the data for one of the four pups in this experiment. Figure 5A shows the independent variable,  $T_{\text{air}}$ , as it decreases in a step-wise fashion from approximately 35.5°C to 21°C. The



**FIGURE 5.**  $T_{\text{air}}$ ,  $T_{\text{iss}}$ , and oxygen consumption (A), and mean respiratory frequency (+ SEM; B) for an individual pup in Experiment 2. The arrows with accompanying letters (a through e) indicate times when respiratory measures were acquired; these letters correspond to the letters on the x axis of the histogram.

letters and accompanying arrows indicate the times when respiratory measures were taken. This figure also shows that interscapular temperature decreased slowly throughout the test until the final air temperature drop, at which point interscapular temperature's decline accelerated. Finally, Figure 5A shows that oxygen consumption was minimal at the beginning of the test and increased progressively as  $T_{\text{air}}$  decreased. After the third drop in  $T_{\text{air}}$ , however, oxygen consumption failed to increase further and even began to decline, which is consistent with the observed rapid decrease in interscapular temperature.

Figure 5B presents the respiratory frequency of the pup at each stage of the experiment. The letters on the x axis of the histogram correspond to the letters in Figure 5A. It can be seen that just as oxygen consumption progressively increased as  $T_{\text{air}}$  decreased, so was there a progressive increase in respi-

**Table 1.  $T_{\text{air}}$ ,  $T_{\text{is}}$ , Oxygen Consumption ( $\text{VO}_2$ ), and Respiratory Measures for the 4 Individual Pups in Experiment 2.  $T_{\text{air}}$ ,  $T_{\text{is}}$ , and  $\text{VO}_2$  are Means from Two Data Points Taken Just Before and Just After the Acquisition of Respiratory Data (in Three Cases, Only One Data Point was Available for  $\text{VO}_2$ ). Respiratory Frequency was Calculated from Unobstructed Respirations Only. Ultrasound Production was Monitored During Each Session; When a Numerical Value is Given, it Indicates the Number of Pulses Detected During a Recording Session. Incidence of Laryngeal Braking was Assessed by Determining the Percentage of Respirations in Which Clear Shoulders (i.e., Brakes) were Observed on the Expiratory Curve**

Pup	$T_{\text{air}}$ (°C)	$T_{\text{is}}$ (°C)	$\text{VO}_2$ (ml/100g/min)	Respiratory Frequency (Hz) (SEM) ( $n = 40^\dagger$ )	Ultrasound Production	% of Respirations with Evidence of Laryngeal Braking ( $n = 100^\ddagger$ )
1	35.4	37.5	3.32	2.81 (.08)	0	5
	30.6	36.3	5.59	3.36 <sup>(36)</sup> (.13)	0	3 <sup>(58)</sup>
	26.9	35.0	7.19	4.79 (.08)	0	12 <sup>(97)</sup>
	23.8*	34.3	7.35	5.20 (.12)	high	47
	21.2	30.7	6.38	3.43 (.07)	low	28 <sup>(74)</sup>
2	35.4	36.7	3.52	2.26 (.09)	0	11
	30.5	36.0	6.06	2.60 (.06)	0	23
	26.8	34.5	7.60	3.51 (.04)	0	10
	21.7*	32.5	7.82	3.64 (.06)	high	40
	21.2	30.8	7.30	2.99 (.03)	2	16
3	35.4	37.4	2.67	2.44 (.11)	0	3
	30.6	36.5	4.95	3.24 (.05)	0	10
	26.8	36.2	6.78	3.44 (.06)	0	18
	22.4*	35.5	7.08	5.54 (.16)	high	47
	21.2	33.9	6.89	3.66 (.07)	0	18
4	35.4	36.8	3.33	2.72 (.20)	0	7
	30.7	37.0	6.00	3.22 (.06)	1	13
	26.8	35.5	6.73	3.75 (.05)	0	13
	22.1*	33.8	6.66	3.97 (.10)	high	48
	21.2	31.6	4.70	3.09 (.06)	low	18

\*Data collected as  $T_{\text{air}}$  was still decreasing.

†Superscript indicates  $n$  when less than 40.

‡Superscripts indicate  $n$  when less than 100.

ratory frequency. When pups initiated ultrasound production, respiratory frequency was at its maximum level. Then, when ultrasound production ended, respiratory frequency fell in conjunction with the previously described decreases in oxygen consumption and interscapular temperature.

Table 1 presents the physiological and behavioral data for all 4 pups at each stage of the experiment (Pup 1 was presented in Figure 5.) It can be seen that as  $T_{\text{air}}$  decreased from 35.5°C to 27°C, oxygen consumption and respiratory frequency increased while  $T_{\text{is}}$  decreased slowly. Then, when  $T_{\text{air}}$  was decreased a third time and as pups began to vocalize, oxygen consumption and respiratory frequency attained maximal values. Finally, as ultrasound production subsided and  $T_{\text{air}}$  had stabilized at 21°C, relatively depressed levels can be seen for  $T_{\text{is}}$ , oxygen consumption, and respiratory

frequency. In general, active sleep predominated at moderate temperatures and fell off substantially at extreme temperatures, which is consistent with previous findings (Blumberg & Stolba, 1996).

Table 1 indicates a tight coupling between oxygen consumption and respiratory frequency. In fact, for the four air temperatures at which the 4 pups were allowed to stabilize (i.e., 35.5°C, 30.5°C, 27°C, and 21°C), the correlation between oxygen consumption and respiratory frequency was statistically significant,  $r = .71$ ,  $n = 16$ ,  $p < .005$ .

In order to estimate the incidence of laryngeal braking at different air temperatures, 100 randomly selected respiratory cycles for each recording episode were assessed for the presence or absence of laryngeal braking; when 100 cycles were not available, we assessed as many as were

available. Only respirations that exhibited clear and pronounced shoulders on the expiratory portion of the cycle were counted as indicative of laryngeal braking (Andrews et al., 1991). Table 1 shows that laryngeal braking occurred at all stages of the experiment, although the relative frequency of braking differed at different air temperatures. Specifically, it appears that laryngeal braking increased in frequency as  $T_{\text{air}}$  decreased to 27°C but, when  $T_{\text{air}}$  was decreased further and ultrasound production was detected, there was a pronounced increase in the incidence of laryngeal braking. Finally, as  $T_{\text{air}}$  stabilized at 21°C and ultrasound production subsided, the incidence of laryngeal braking decreased as well, although not down to baseline levels.

Because an ultrasonic vocalization is associated with constriction of the larynx and the prolongation of expiration (Blumberg & Alberts, 1990; Roberts, 1972), it could be considered to be an audible laryngeal brake. We also know, however, that many instances of laryngeal braking are not accompanied by sound production; that is, they are inaudible. For example, in Experiment 2, pups that were not vocalizing exhibited many instances of laryngeal braking (see Table 1). Moreover, when pups were vocalizing, we also detected many instances of inaudible laryngeal brakes, which is consistent with the observations of Roberts (1972). Although we did not attempt to distinguish between audible and inaudible brakes in this experiment, it will be important to ascertain their temporal association at different air temperatures; if a temporal association between audible and inaudible laryngeal braking were found, it would suggest that the two are related processes. Only further experimentation can adequately address this question.

## GENERAL DISCUSSION

The results from these two experiments illustrate how the physiological and behavioral responses of neonates are modulated across a continuum of air temperatures. Specifically, Experiment 1 showed that at a moderately cold air temperature of 27°C, pups emitted ultrasound infrequently while still exhibiting signs of regulatory control over BAT thermogenesis. When  $T_{\text{air}}$  was decreased to 23°C, however, BAT thermogenesis increased no further,  $T_{\text{is}}$  decreased more quickly, and rates of ultrasound production increased. In addition, as shown in Experiment 2, respiratory rate was associated

with the metabolic demands of the pups (see Andrews et al., 1991; Johnson, 1985). Specifically, respiratory rate increased in lock step with BAT thermogenesis as air temperature decreased from 35°C to 27°C; moreover, similar to BAT thermogenesis, further decreases in  $T_{\text{air}}$  did not result in higher respiratory rates.

The observed upper limit to heat production may in part be due to limitations in oxygen transfer to BAT (Dotta & Mortola, 1992). As stated above, respiratory rate was maximized at the transition between moderate and extreme air temperatures. Given that respiratory rate was reaching maximum levels as  $T_{\text{air}}$  decreased to 23°C, it may be significant that the pups altered their pattern of respiration, as indicated by increased levels of laryngeal braking and ultrasound production. Then, after prolonged exposure to a  $T_{\text{air}}$  of 23°C, pups may have downregulated heat production, as suggested by pronounced decreases in oxygen consumption, respiratory rate, and  $T_{\text{is}}$ ; moreover, the pups' respiratory pattern reverted to the unobstructed symmetrical patterns typical of respiration at moderate air temperatures.

In sum, these results suggest that the effects of moderate and extreme cooling can be differentiated across a number of physiological and behavioral dimensions. Moreover, these dimensions are dependent to some degree on the age of the animal. For example, Blumberg and Stolba (1996) showed in 2-day-old and week-old pups that levels of myoclonic twitching remained high at a moderate  $T_{\text{air}}$  and decreased substantially at an extreme  $T_{\text{air}}$ . But, as would be expected from previous work on the development of thermoregulation in rat pups (e.g., Spiers & Adair, 1986), the transition between moderate and extreme air temperatures occurs at a higher air temperature in 2-day-olds. In addition, and in contrast to older pups, 2-day-olds emitted very little ultrasound during cold exposure, suggesting the possibility of different respiratory responses to cold at this age. Thus, extending the present results to both younger and older pups will require careful attention to the thermal environment and the relative maturity of contributing physiological systems.

Pronounced changes in thermal and metabolic physiology, respiratory patterning, ultrasound production, and sleep/wake behaviors all accompany the transition from moderate to extreme cooling. At this time, it is perhaps easiest to point to the maximization of BAT thermogenesis and oxygen consumption, and the ensuing accelerated decrease in interscapular temperature as precipi-

tating influences on the changes in respiration, vocalization, and arousal. The interdependence of all of these factors makes it difficult to ascertain which are of primary or secondary importance.

The high incidence of laryngeal braking and ultrasound production as extreme air temperatures are reached and respiratory rate is maximized remains an unexplained phenomenon. It is also not clear why pups decrease rates of laryngeal braking and ultrasound production during prolonged cold exposure. Although Experiment 2 suggests the possibility that pups rely more on laryngeal braking and emit more ultrasound when respiratory rate has been maximized, we and others (Hofer & Shair, 1992; Roberts, 1972) have observed ultrasound production at a variety of respiratory frequencies. In fact, there is still some disagreement among respiratory physiologists as to the conditions that promote laryngeal braking in neonates. For example, while some consider laryngeal braking to be one aspect of a newborn breathing strategy that includes high breathing rates and post-inspiratory activity of the inspiratory muscles (Mortola, 1984, 1985), others report recruitment of laryngeal braking during low breathing rates (Andrews et al., 1991). Although the resolution of this problem awaits, it is becoming apparent that ultrasound production and laryngeal braking result from complex interactions between systems that promote oxygen use and systems that provide oxygen delivery.

## NOTES

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