Ultrasonic Vocalizations by Rat Pups: The Primary Importance of Ambient Temperature and the Thermal Significance of Contact Comfort

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We investigated the effects of isolation, huddling, and air temperature on ultrasound production by rat pups. Experiment 1 showed that ultrasound production by 8- to 9-day-olds was minimal at thermoneutrality and increased in response to small deviations of air temperature on either side of the thermoneutral zone. Experiments 2 and 3 showed that suppression of ultrasound production by contact with littermates is consistent with the thermal consequences of huddling. Experiment 4 showed that, contrary to previous conclusions, ultrasound production is not independent of ambient temperature in pups older than 10 days of age. Taken as a whole, these experiments emphasize (1) the importance of ambient temperature for the elicitation of ultrasound by rat pups of all ages studied, (2) the importance of thermal factors in the suppression of ultrasound by littermate contact, and (3) the manner in which different methods can change interpretations of the behavior and physiology of infant rats.

Since their discovery in the 1950s, ultrasonic vocalizations by rat pups have been widely interpreted as distress calls that attract maternal attention and elicit retrieval of pups to the nest (Allin & Banks, 1972; Kehoe & Blass, 1986; Hofer & Shair, 1978, 1980). There are numerous reports that removal and isolation from the nest are effective stimuli for ultrasound production (Okon, 1970a, 1970b, 1971; Allin & Banks, 1971). Conversely, isolation-induced ultrasound is suppressed if a

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pup is presented with an object with which it will huddle, such as a sibling or a warm, furry model (Hofer & Shair, 1978). Such findings support the notion that ultrasound production is a measure of separation distress.

Much confusion, however, still surrounds the question of proximate stimuli for ultrasound production. Specifically, when a pup is removed from the nest by an experimenter and isolated in a "novel" chamber that is not maintained at thermoneutrality, is ultrasound emitted in response to isolation per se, to the handling procedure, to cold exposure, to the unfamiliarity of the new context, or to some combination of these factors? Similarly, for example, when the presence of a sibling suppresses isolation-induced ultrasound, is this suppression the result of a form of social calming referred to as "contact comfort" or do these social conditions merely contain a set of more basic physical or physiological factors?

Answering these questions is difficult, in part because of the varied experimental procedures used by different investigators, especially as regards the thermal properties of the isolation chamber. For example, Hofer and Shair (1987) tested pups at room temperature (22°C), while Kehoe and Blass (1986) tested pups in a 32°C heated chamber. In many cases, pups have been tested while in contact with surfaces that promote conductive heat exchange and thus make identification and control of the thermal stimulus nearly impossible. For example, Allin and Banks (1971) tested their pups in glass flasks suspended in a temperature-controlled water bath and Oswalt and Meier (1975) tested their pups in stainless steel bowls that were lined with bedding in some, but not all, conditions.

Methodological difficulties surrounding investigations of pup ultrasound multiply when the dimension of development is added. Although it is accepted that ultrasound emission by rat pups up to 10 days of age is highly responsive to thermal factors, ultrasound emission by pups older than 10 days has been considered to be independent of ambient temperature (Hofer & Shair, 1978). This conclusion, however, is based on experiments (i.e., Allin & Banks, 1971) that were only 4 1/2 min long and thus did not control for the increased thermal inertia of older pups that are both larger and insulated with fur.

The numerous methodological differences and difficulties (e.g., as regards ambient temperature, convective vs. conductive heat exchange, short test durations) among these studies in the literature confuse the process of interpretation and make it difficult to focus clearly on the issues of thermal and social factors that might regulate ultrasound production in rat pups. In some of our recent experiments (Blumberg & Alberts, 1990), we noted that pups placed individually in a chamber maintained at a thermoneutral temperature (35°C) stopped vocalizing within a few min; these pups soon appeared asleep. This suggested to us that isolation per se does not evoke a long-lasting ultrasonic response, at least not at a thermoneutral temperature. Nevertheless, the same rat pups vigorously produced ultrasound when air temperature was decreased to 20°C.

Thus, we decided to investigate systematically the effects of isolation, huddling, and air temperature on ultrasound production. We demonstrate that, contrary to previous conclusions, ultrasound production is not independent of ambient temperature at any age tested, and that the diminution of ultrasound production by contact with littermates is consistent with the thermal consequences of huddling.

Experiment 1: Ultrasonic Emission by 8- to 9-Day-Old Rats is Sensitive to Small Incremental Changes in Ambient Temperature

Although it is generally accepted that ultrasound production by pups younger than 10 days of age is dependent on ambient temperature (e.g., Hofer & Shair, 1978), there is little information regarding the sensitivity of ultrasound production to small increments in ambient temperature. Okon (1971) tested pups at four different ambient temperatures, from 2–33°C, but this wide range of temperatures with few intermediate values tells little about pups' sensitivity to temperature or temperature change.

In contrast to investigators of rat pup ultrasound, physiologists have tended to use relatively small increments in ambient temperature, both inside and outside the pups' thermoneutral zone, to test the sensitivity of an infant rat's metabolic responses. Spiers and Adair (1986), for instance, have shown that 10-day-old rats increase oxygen consumption to more than 100% over thermoneutral values as ambient temperature decreases just 10°C. Such sensitivity in metabolic response to ambient temperature might be reflected in levels of ultrasound production if, as we have previously hypothesized, ultrasound emission is causally related to thermogenic effort and respiratory activation (Blumberg & Alberts, 1990, 1991a, 1991b).

Experiment 1 was designed to provide systematic information on the effect of ambient temperature on ultrasonic vocalizations. Ambient temperature was manipulated in six increments of about 3°C. These increments covered a range that bracketed the thermoneutral zone of the 8- to 9-day-old subjects we tested. Thus, we have included ambient temperatures above thermoneutrality (i.e., 35°C) in this experiment. Allin and Banks (1971) also tested pups at a temperature above thermoneutrality (i.e., 40°C) but ultrasound production was not significantly increased in the heat; however, as we discussed above, their methods may have masked differences in ultrasound production at these ambient temperatures.

Method

Subjects

Seventy-two rat pups from 49 litters were used. Rats were bred from Sprague-Dawley stock originally obtained from Charles River (Portage, Michigan) and were born in the Animal Behavior Laboratory colony at Indiana University. On the day of testing all pups were 8–9 days of age. The pups were raised in litters that were culled to 8 pups within 3 days after birth (day of birth = Day 0). Litters and their mothers were housed in standard laboratory cages (48 × 20 × 26 cm) in which food and water were available ad libitum. All animals were maintained on a 16 : 8 hr light/dark schedule with lights on at 7:00 a.m.

Apparatus

The test chamber consisted of a glass cylinder (12 cm inside diameter × 23 cm length) open at one end. The cylinder was surrounded by a clear glass water jacket

with two nozzles through which water could be circulated. The two nozzles were connected via tubing to a water circulator in which the water was maintained at a fixed temperature.

Air temperature within the chamber (T_{air}) was measured, with a resolution of 0.25°C, using an ambient temperature probe (Yellow Springs Probe #405) connected to a telethermometer (Yellow Springs). Air temperature was recorded by hand at the beginning of each min of the test.

Ultrasonic vocalizations were detected using a microphone placed inside the test chamber. The microphone was connected to a "bat detector" (QMC, Ltd., London, U.K., Model S100) tuned to a range centered on 40 kHz. Occurrence of ultrasonic vocalization was collected every sec using a computerized data acquisition system (OmegaLog, Omega Engineering, Inc., Stamford, CT). To quantify the vocalization data, the observer pressed a button every time a vocalization was detected. Pressing the button activated a counter in the computer which indicated that a vocalization had been detected during that second. After the test, the number of 1-s bins in which an ultrasonic pulse had been detected was counted for each min of the test. These data are presented as estimated percentage of time spent vocalizing.

Procedure

On the day of testing, a cage containing a litter and its mother was transferred from the colony room to the testing room. A pup was removed from the cage and placed as quickly as possible into the temperature-controlled test chamber. Conductive heat exchange was minimized in two ways: First, the glass chamber was lined with polyethylene mesh and second, the experimenter wore a rubber glove while transferring the pup.

After a pup was transferred, the experimenter noted the time and began recording the occurrence of ultrasound production. Each trial lasted 6 min, after which the pup was removed from the chamber, weighed, and its sex was determined. In addition, the data from any given pup were included only if the pup had been fed recently, as evidenced by the presence of a milk band.

Each pup was exposed to one of six ambient temperatures: 25, 28, 32, 35, 38, and 41°C. The ranges of air temperatures for each ambient temperature condition across all tests are as follows: "25°C": 24.5–26°C; "28°C": 27.5–28.75°C; "32°C": 31.5–32.5°C; "35°C": 34–35.25°C; "38°C": 37–38.5°C; "41°C": 40–41.25°C. Twelve pups, 6 of each sex, were tested at each of these six temperatures. No more than 2 pups from any litter were tested; if 2 pups did come from the same litter, they were tested on separate days and at different ambient temperatures.

Statistical Analysis

Data are presented as Mean ± *SEM*. Differences between groups were tested using analysis of variance. Post-hoc analyses are considered significant when $p < 0.05$. Statistical calculations were performed using Statview II on the Macintosh computer.

Results

During the 6-min tests, there were some fluctuations in the air temperatures, typically of a magnitude no greater than 1°C within a given test. Body weights of the pups ranged from 8.9–25.3 g. Excluding the 2 smallest pups with body weights of 8.9 and 11.0 g, the range was 15.1–25.3 g. Because the 2 runts exhibited rates of ultrasound production within the range of the other pups at those air temperatures, their data were not discarded. Finally, there were no significant differences between the air temperature groups with respect to body weight, $F(1,65) = 0.869$, ns.

A two-factor analysis of variance was used to determine whether air temperature and sex significantly affected vocalization rates following the transfer of pups from the nest to the testing chamber. The effect of air temperature on vocalization rate was significant, $F(5,60) = 6.604$, $p < 0.0001$, but the effect of sex was not, $F(1,60) = 1.703$, ns. Because the effect of sex was not significant, the data were collapsed for the analyses that follow, which are presented for each of the 6 min of the test.

Table 1 presents the mean percentage of time vocalizing during each of the 6 min following isolation at each of the air temperatures tested. Although air temperature did not significantly affect vocalization rates during the first min follow-

Table 1
Percentage of Time Vocalizing during each of the 6 Min Following Isolation at each of the six Ambient Temperatures Tested in Experiment 1

	Ambient Temperature (°C)					
Minute	25	28	32	35	38	41
1	57.9 (8.3)	55.4 (7.0)	38.0 (9.6)	32.6 (9.0)	45.8 (10.0)	44.0 (9.2)
2	**60.0*** (7.8)	**58.2*** (6.2)	35.0 (10.0)	28.5 (9.0)	31.0 (9.8)	34.3 (8.5)
3	**72.6*** (6.8)	**60.2*** (6.7)	30.1 (9.3)	22.0 (8.7)	29.4 (10.2)	33.2 (9.4)
4	**71.5*** (7.4)	**57.6*** (8.4)	15.1 (6.5)	12.1 (6.3)	28.3 (9.8)	**42.4*** (8.5)
5	**73.6*** (5.7)	**47.5*** (10.4)	10.6 (7.0)	9.0 (6.2)	25.6 (9.9)	**47.1*** (9.3)
6	**68.0*** (7.1)	**37.2*** (8.8)	7.9 (4.4)	8.2 (4.7)	**31.8*** (9.0)	**40.0*** (8.5)

*Bold values are significantly different from the value at 35°C.
Twelve animals were tested at each temperature.
A one-factor ANOVA was significant, $p < 0.05$, at each min except Min 1.

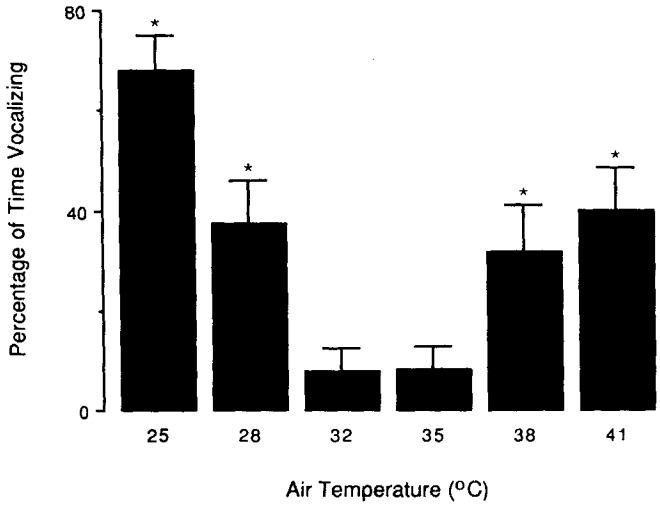

Fig. 1. Percentage of time vocalizing during the 6th min following isolation from the nest at each of the six ambient temperatures in Experiment 1. Asterisks indicate that vocalization rate was greater than at 35°C and 32°C, $p < 0.05$.

ing isolation, $F(5,66) = 1.199$, ns, it did significantly affect vocalization rates for each of the remaining 5 min (Min 2: $F(5,66) = 2.657$, $p < 0.03$; Min 3: $F(5,66) = 5.505$, $p < 0.0003$; Min 4: $F(5,66) = 9.019$, $p < 0.0001$; Min 5: $F(5,66) = 9.149$, $p < 0.0001$; Min 6: $F(5,66) = 9.494$, $p < 0.0001$). Moreover, by Min 6 and as shown in Table 1 and Figure 1, vocalization rates were significantly higher at 25, 28, 38, and 41°C than at 35°C, thus indicating a U-shaped function relating ultrasound production and air temperature. Finally, the vocalization rate at 35°C did not differ significantly from the vocalization rate at 32°C for any of the 6 min of the test.

The effect of air temperature on vocalization rate is illustrated even more clearly by analyzing individual data. During the 6th min of the test at 35°C, 4 of 12 pups did not emit a single pulse and 5 pups emitted only two pulses; the remaining 3 pups at 35°C vocalized for 10%, 13.3%, and 58% of the time. In contrast, during the 6th min at 25°C, all 12 pups vocalized at least 38% of the time and 7 of these pups vocalized at least 70% of the time. Thus, 75% of pups were virtually silent by the 6th min at 35°C while all pups vocalized at high rates at 25°C.

Discussion

The results of Experiment 1 show that ultrasound production following the isolation of 8- to 9-day-old pups from the nest is highly sensitive to the ambient temperature of the environment to which the pups are transferred. By the 3rd min of isolation, ultrasound production was significantly greater at an ambient temperature only 4°C cooler, and by the 6th min of isolation, ultrasound production was also significantly greater at ambient temperatures 3°C and 6°C above thermoneutrality.

Interestingly, the relationship between ambient temperature and ultrasound production shown in Figure 1 resembles that usually found between ambient temperature and metabolic rate. Endothermic animals generally show minimal metabolic rates within a range of ambient temperatures that is, by definition, the *thermoneutral zone*. The precise range of temperatures that constitute thermoneutrality varies with age, size, and other factors. Thermoneutrality for rat pups of the ages used in Experiment 1 is typically reported to be between 34°C and 36°C (Conklin & Heggeness, 1971; Spiers & Adair, 1986; Taylor, 1960). In our very brief 6-min test, ultrasound production was lowest in pups exposed to 32°C and 35°C. Furthermore, ultrasound production rate, like metabolic rate, showed a graded pattern of increase with decreasing ambient temperatures *and* a corresponding increase with increasing ambient temperatures. This U-shaped pattern of ultrasound production with ambient temperature is consistent with our hypothesized link between metabolic effort and ultrasound production (Blumberg & Alberts, 1990). Of course, simultaneous measurement of ultrasound production, respiration, and metabolic rate at different ambient temperatures is needed to further support this suggested connection.

As we suspected, transfer to a thermoneutral environment elicited much lower levels of ultrasound production than did transfer to a cool environment. Nonetheless, 25% of the pups at thermoneutrality vocalized at least 10% of the time during the 6th min of the test. One might conclude that non-zero levels of ultrasound after 6 min at thermoneutrality indicate that at least some ultrasound production is independent of thermal factors. Such a conclusion would be supported if vocalizing continued or increased beyond the 6th min of testing at thermoneutral ambience. We test this possibility, as well as the effect of contact with littermates on ultrasound production, in the next experiment.

Experiment 2: Distinguishing between Thermal and Social Influences on Ultrasound Production by 8- to 9-Day-Old Rats

The results of Experiment 1 demonstrated that even small differences in ambient temperature can significantly affect ultrasound production in a graded, orderly manner. The power of thermal stimuli to regulate pup ultrasound, however, does not rule out the possibility that social cues such as those involved in contact with a littermate can also decrease ultrasound production at this age, as it can in 2-week-old pups (Hofer & Shair, 1978). One purpose of Experiment 2 was to determine whether the presence of a littermate can affect the rate of ultrasound production by young pups.

In the introduction to this paper, we alluded to two competing hypotheses regarding the mechanism by which littermate contact attenuates ultrasound production. The first interpretation states that contact behavior reduces ultrasound production through the effects of *nonthermal, social* stimuli (Hofer & Shair, 1980) that serve to calm or comfort the infant and thus alleviate the aversive aspects of isolation. The second interpretation states that contact behavior reduces ultrasound production through the thermal consequences of huddling (Alberts, 1978). Huddling by rat pups has powerful thermoregulatory consequences. Alberts (1978) has shown that huddling reduces heat loss and that pups' metabolic rate decreases with increasing number of pups in a huddle. These effects can be

explained by the reduced surface-to-mass ratio created by huddling (and the corresponding diminution of heat loss) as well as the insulative benefits of contact.

Another purpose of this experiment was to extend the duration of observations made in Experiment 1. We tested pups for 30 min, rather than 6 min, to determine the longer-term effects of ambient temperature and littermate contact on ultrasound production.

Method

Subjects

Thirty-two pups from 8 litters were used. Pups were from the same stock and were reared as the pups described in Experiment 1. On the day of testing, all pups were 8–9 days of age.

Apparatus

The test chamber was used as described in Experiment 1. T_{air} and ultrasound production were monitored continuously and were recorded by the data acquisition system on a sec-to-sec basis.

Procedure

On the day of testing, 2 pups were removed from a litter and placed inside a polyethylene cage. In this experiment, pups either were placed individually into two separated compartments ("singletons"; each singleton compartment had dimensions of 8 × 4 × 8 cm) or were placed together inside an undivided compartment ("pairs"; the pair compartment had dimensions of 8 × 7 × 8 cm). When transferring the pups from the nest into the cage the experimenter wore rubber gloves to reduce conductive heat exchange between the experimenter's hand and the pup. After the pups were inside the cage, the cage was slipped inside the test chamber and the test began. The entire transfer procedure from nest to test chamber took approximately 15 s.

There were four conditions in this experiment. Singletons and pairs were tested for 30 min when T_{air} was either 20°C or 35°C. For the "20°C condition", T_{air} ranged from 17.9–22.7°C. For the "35°C condition", T_{air} ranged from 33.9–35.9°C. Four trials were conducted for each condition and no more than 4 pups (2 trials × 2 pups) were tested from a single litter. Ordering of trials was random. When the 30-min test was over, the pups were removed from the cage, weighed, and sexed. Trials were counted only if the pups had suckled recently, as indicated by the presence of milk bands. Pups were then returned to their mother.

Statistical Analysis

Data are presented as Mean ±*SEM*. Differences between groups are tested using a two-factor analysis of variance. Statistical calculations were performed using Statview II on the Macintosh computer.

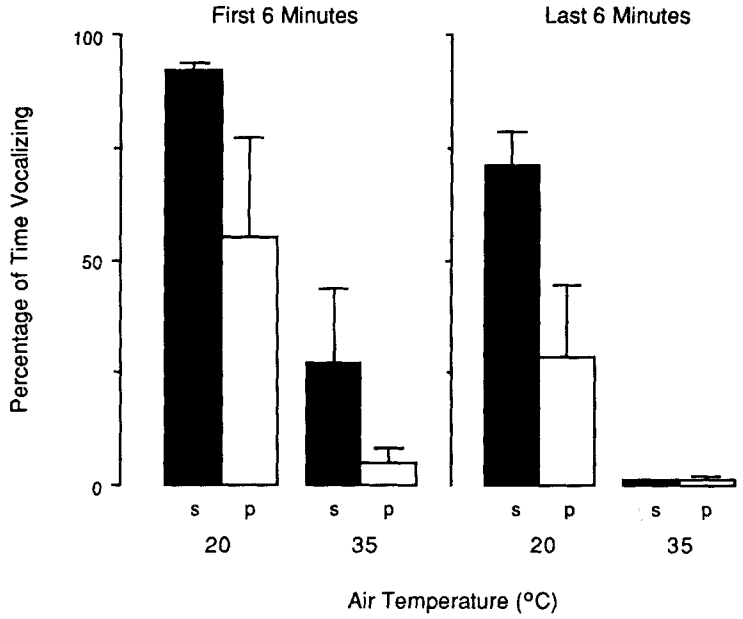

Fig. 2. Percentage of time spent vocalizing for the first 6 min (left panel) and last 6 min (right panel) of the 30-min "isolation" test for the four groups in Experiment 2. Two pups were either separated from each other (s: "singletons") or not separated (p: "pairs"). Air temperature was either 20°C or 35°C for the duration of the test. (Mean ± *SEM*)

Results

There were no significant differences between groups with respect to body weight, $F(3,15) = 0.68$, ns, or sex, $F(1,15) = 1.235$, ns. At 20°C, the singletons emitted ultrasonic vocalizations during 82.2 ± 5.2% of the eighteen hundred 1-s bins of the experiment. Pairs of pups at 20°C were vocal during 39.0 ± 17.7% of the half-hour test. At 35°C, ultrasound rates were 10.2 ± 7.4% and 3.4 ± 1.4% for singletons and pairs, respectively. Thus, pairs vocalized less than singletons at both temperatures.

To evaluate some of the temporal aspects of ultrasound emission following removal from the nest, we compared the pups' vocalization rates during the initial 6 min after removal (i.e., Min 1–6) with the final 6 min of the test (i.e., Min 25–30). Even within the first 6 min, the powerful effect of ambient warmth can be seen in the reduced rate of ultrasound production in singletons at 35°C relative to 20°C. The left panel of Figure 2 presents the mean percentage of time spent vocalizing over the first 6 min of the 30-min test for the two groups (i.e., singletons and pairs) at the two ambient temperatures. Analysis of variance revealed significant main effects for T_{air}, $F(1,12) = 22.412$, $p < 0.0005$, and group, $F(1,12) = 5.826$, $p < 0.05$. The interaction was not statistically significant, $F(1,12) = 0.395$, ns.

The right panel of Figure 2 presents the mean percentage of time spent vocalizing over the last 6 min of the 30-min test for the two groups at the two ambient temperatures. The singletons and pairs at 20°C still vocalized at high rates al-

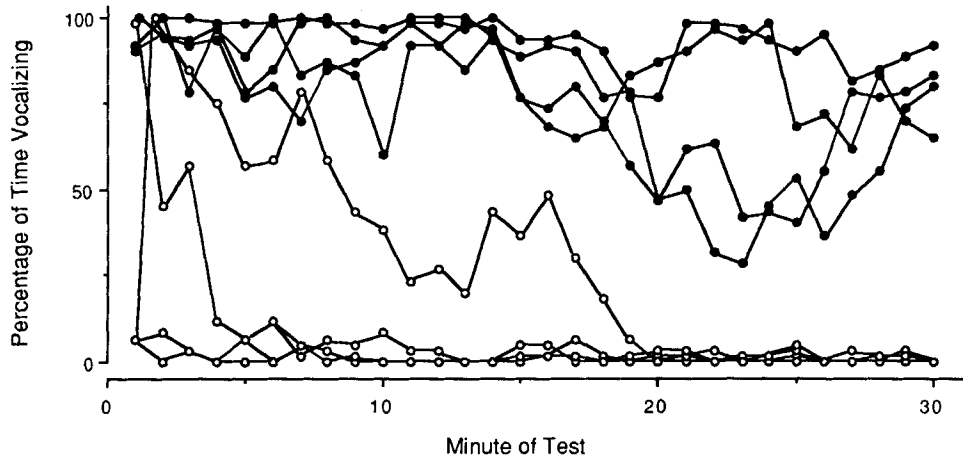

Fig. 3. Individual records of percentage of time spent vocalizing over the 30 min of isolation from the nest in Experiment 3. Filled circles: data for four sets of 2 singletons exposed to 20°C. Open circles: data for four sets of 2 singletons exposed to 35°C.

though, again, the pairs vocalized less than the singletons. In striking contrast, both the singletons and pairs were virtually silent during these 6 min, emitting ultrasounds only 1–2% of the time. Analysis of variance revealed significant main effects for T_{air}, $F(1,12) = 39.619$, $p < 0.0001$, and group, $F(1,12) = 7.463$, $p < 0.05$, as well as the interaction, $F(1,12) = 7.894$, $p < 0.05$. As suggested by Figure 2, singletons at 35°C vocalized predominantly at the beginning of the 30-min test.

Presentation of the singletons' individual data provides a better impression of the effect of ambient temperature on ultrasound production. Figure 3 presents the data for singletons exposed to 35°C (open circles) and 20°C (filled circles). Of the four trials of singletons exposed to 35°C, vocalization rates were very low (< 10%) in two of them for the duration of the test. In one case, vocalizing decreased from a high level (98%) at Min 1 to a low level (0%) at Min 6. In another trial at 35°C, vocalizing during Min 1 was very low, jumped to a high rate at Min 2, and then decreased slowly to 0% at Min 20. In contrast, for all four sets of singletons exposed to 20°C, rates of vocalization were high for the entire 30 min of the test.

Discussion

The results of the present experiment demonstrate that contact with a littermate reduces ultrasound production by 8- to 9-day-old pups both at a thermoneutral (35°C) and cold (20°C) ambient temperature. Nevertheless, pairs at 20°C vocalized more than did singletons at 35°C; this was evident especially towards the end of the 30-min test. Thus, with respect to the suppression of ultrasound, a thermoneutral ambience is a more effective suppressor of ultrasound than is the presence of a single littermate in the cold.

We know from Experiment 1 that, by the 2nd min of isolation, air temperature is a significant factor in the elicitation of ultrasound and that relatively modest increments in ambient temperature (e.g., 4°C) can have significant effects on rate of ultrasound production. We also know that huddling, even among only two

pups, provides significant thermal protection in the cold (Alberts, 1978). Therefore, it is reasonable to conclude that at least some of the reduction in ultrasound production caused by contact with a littermate is due to the thermal benefits of huddling.

Figure 3 shows that two of the four sets of singletons at 35°C vocalized during the first 6 min of the test despite our efforts to reduce conductive heat losses during the transfer procedure. Nonetheless, these efforts would not have eradicated other sources of temperature stimulation such as the convective heat losses related to the transfer of pups through room air. (These effects would be exacerbated if, at the time of removal from the nest, the selected pup was close to or below its lower critical temperature for heat production; in other words, the fact that some pups vocalize very little and some pups vocalize a lot following transfer may result in part from different local thermal conditions in the nest.) Furthermore, the transfer procedure inevitably involves tactile stimulation of the pup which has been shown to elicit ultrasound (Okon, 1970a). If these thermal and tactile factors could be eliminated from the transfer procedure, then it may be possible to reduce even further the already-low levels of ultrasound production by singletons at 35°C.

On the other hand, pairs vocalized less than singletons at 35°C; thus, littermate contact appears to have decreased the stimulatory effect of the transfer procedure on ultrasound production. By the end of the 30-min test, however, both singletons and pairs at 35°C were nearly silent, while pairs at 20°C were still vocalizing 28% of the time. Clearly, any nonthermal calming effect caused by littermate contact (1) is of value for a relatively short period of time, and (2) cannot override the stimulatory effect of a cool ambience, even after 30 min.

Experiment 3: Cold-Induced Ultrasound Production in 8- to 10-Day-Old Rats as a Function of Huddling

In Experiment 2, we showed that huddling with another littermate diminishes ultrasound production following a transfer procedure to an ambient temperature at or below thermoneutrality. Even though the results of Experiment 2 are consistent with the hypothesis that littermate contact diminishes ultrasound production at least partly through its thermal effects, the nature of the transfer procedure makes it difficult to argue definitively that only thermal factors were involved. However, after a pup has been isolated at a thermoneutral temperature and has been given sufficient time to become quiescent, merely reducing ambient temperature will reinstate high levels of ultrasound production (Blumberg & Alberts, 1990). Because pups are not handled before being exposed to cold, ultrasound production elicited by this procedure is more clearly the result of thermal stimulation. Thus, under these conditions, any suppression of ultrasound production that might result from littermate contact could be more easily interpreted as resulting from the insulative benefits of littermate contact.

Experiment 3, then, was designed to determine the effect of huddling on ultrasound production. Pups were tested in compartments that either prevented huddling or allowed huddling among 2 or 4 littermates. Ultrasound production was monitored throughout the test, that is, at thermoneutrality for 10 min post habituation and during the ensuing cold phase.

Method

Subjects

Forty-eight rat pups from 12 litters were used. Pups were from the same stock and were reared as the pups described in Experiment 1. On the day of testing all pups were 8–10 days of age.

Apparatus

The test chamber was used as described in Experiment 1. For this experiment, the two nozzles of the glass test chamber were connected via tubing to one of two water circulators in which the water was maintained at different temperatures. By changing the supply of water to the test chamber from one circulator to another, it was possible to change rapidly the air temperature within the chamber. This double-walled glass design also permitted visual access to the subject. As in Experiment 2, T_{air} and ultrasound production were monitored continuously and were recorded by the data acquisition system on a sec-to-sec basis.

Procedure

On the day of testing, 4 pups from a single litter were placed inside a rectangular cage constructed of polyethylene mesh (15 × 8 × 8 cm). The cage was modified for the three conditions of the experiment. In the "singletons" condition, 4 pups were placed inside four individual and equal-sized compartments within the cage. These compartments, separated by polyethylene mesh, prevented the pups from making physical contact, although the mesh undoubtedly permitted olfactory exchanges. In the "pairs" condition, two pairs of pups were placed inside the cage with a single mesh divider between them. In this case, each member of a pair was able to make physical contact with the other member but the two pairs were not able to make physical contact. In the "quad" condition, the 4 pups were placed in an undivided cage; in this case, all 4 pups were able to make physical contact with each other.

Four trials were run in each of the three conditions and all 12 trials came from 12 separate litters. Each group of 4 pups was composed of 2 males and 2 females. Pups were used only if they had been recently fed, as evidenced by the presence of milk bands.

After placing the pups in the cage, the cage was slipped inside the test chamber. T_{air} was maintained at the thermoneutral temperature of 35°C (Spiers & Adair, 1986). The pups remained in the test chamber for 30–40 min, after which time data collection began. For 10 min, T_{air} remained at 35°C and ultrasound production was monitored. After this 10-min baseline period, cold water was circulated through the water-jacket of the test chamber. Rate of temperature decline is described more precisely below, but averaged about 17°C during the first 10 min of the cooling phase of the test. As T_{air} decreased, ultrasound production was monitored continuously until the pups reached a criterion of a minimum of 70% vocalizing for 5 consecutive min (i.e., at least 42 of 60 one-sec bins for each of 5 consecutive min). It is important to note that use of this criterion is not compro-

mised by the varied groupings of pups. That is, because 4 pups were always monitored, regardless of group composition, the percentage of time spent vocalizing applied equally across conditions. When the criterion was met, the pups were removed from the chamber and returned to their mother.

Statistical Analysis

Data are presented as Mean ± *SEM*. Differences between groups were tested using a one-factor analysis of variance. Statistical calculations were performed using Statview II on the Macintosh computer.

Results

During the 10-min baseline period, T_{air} remained between 34.7 and 35.5°C for all trials. During this time, ultrasound production was virtually absent. Specifically, none of the pup groups vocalized more than 1.5% of the time during this 10-min period (i.e., during no more than 9 of 600 one-sec bins). As expected, there were no differences between the singletons, pairs, and quads with respect to baseline ultrasound production, $F(2,11) = 1.156$, ns.

After the 10-min baseline period, T_{air} was reduced using an identical procedure for all three groups. By Min 20 of the test, T_{air} had decreased to approximately 18°C. By Min 30, T_{air} had decreased to 12°C, and by Min 40, it had decreased to 9°C. The lowest temperature to which pups were exposed was 6°C.

Although all the pups were exposed to similar decreases in T_{air}, rates of ultrasound production varied as a function of available contact behavior. Overall, the time to reach criterion levels of ultrasound production increased with huddle size. Following the 10-min baseline period, singletons required 11.2 ± 1.9 min to reach criterion, pairs required 27.2 ± 6.5 min, and quads required 57.5 ± 9.4 min. Analysis of variance indicated a significant effect of huddle size on the min at which criterion levels of vocalization were obtained, $F(2,11) = 16.571$, $p < 0.001$). Moreover, with one exception, there was no overlap between singletons, pairs, and quads with respect to time required to reach criterion levels of ultrasound emission.

Figure 4 presents data for three representative trials involving 4 singletons, 2 pairs, and 1 quad. During the initial 10 min of baseline recording, ultrasound production was essentially absent in the three groups. Then, as T_{air} decreased, singletons were the first to show pronounced vocalization rates; at Min 21 these pups reached the criterion level of vocalization, i.e., they were vocalizing for at least 70% of each min for 5 consecutive min. At the equivalent time and temperature that singletons were vocalizing vigorously (i.e., vocalizing greater than or equal to 70% of the time), pups in pairs had begun to show noticeable increases in ultrasound emission. These pairs of pups reached the criterion level at Min 39, after an additional 18 min of ambient temperature decline. The quads were nearly silent for over 50 min despite the continuous decline in ambient temperature and did not reach the criterion level until Min 68. (We should restate that although the availability of huddling differed between groups, each group tested contained the same number of potential vocalizers, i.e., 4.)

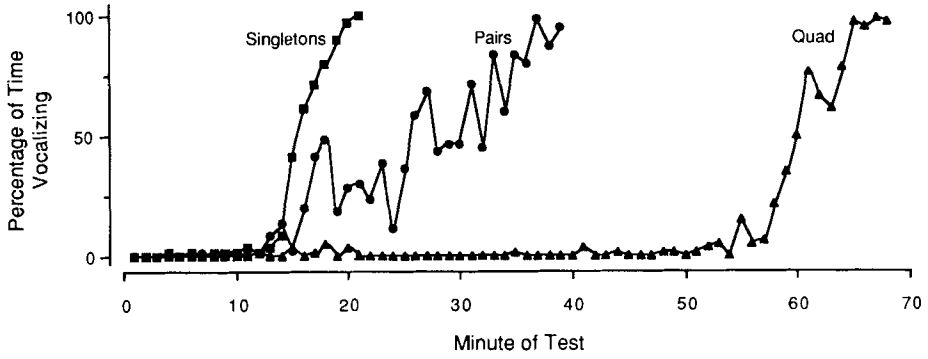

Fig. 4. Percentage of time vocalizing before and after cold exposure for three representative trials in Experiment 3. Each trial involved 4 pups: The pups were either separated into four separate compartments ("singletons"), separated into two separate compartments ("pairs"), or not separated ("quad"). For the first 10 min of the test, pups were exposed to a thermoneutral ambient temperature (35°C). Thereafter, air temperature was decreased.

Discussion

The results of the present experiment can be considered in at least two ways: First, the delayed onset of ultrasound production by pups allowed to form huddles of two or four littermates could be attributed to "contact comfort" and other social cues. This could be described as a "social explanation" for reduced ultrasound production, according to which a pup emits more or less ultrasound depending on the presence of specific social stimuli or the general similarity of the testing environment to home-cage conditions (Hofer & Shair, 1980). Clearly, the power of this explanation is limited because, under the conditions of this experiment, the pups in all three groups were virtually silent at 35°C before air temperature was decreased despite being out of contact with their mother and nest.

An alternative explanation for delayed onset of ultrasound production emphasizes thermal cues in the elicitation of ultrasound (Allin & Banks, 1971). As stated above, this "thermal explanation" posits that the presence of littermates with whom contact could be made reduced the surface area of each pup that was exposed to the cooling air. Rat pups use the bodies of littermates to regulate their own body's and huddle's exposed surface area in relation to ambient temperature (Alberts, 1978). We noted in this experiment that the pairs and quads made contact and formed huddles as T_{air} decreased; of course, the singletons were prevented from making such contact.

In comparing the "social" and "thermal" explanations of the present results, we must stress that the quads vocalized as much as the singletons; it simply took a longer time for the vocalizing to begin and reach criterion levels. Thus, the social perspective would require us to posit that the effects of contact comfort diminished as T_{air} decreased, or that the social benefits of huddling were suppressed by the cold. Clearly, the more parsimonious explanation for the delayed onset of ultrasound production seen in this experiment is that huddling during cold exposure enhances insulation and reduces the exposed surface area of each pup and thus delays, but does not suppress, the activational effects of decreasing tempera-

ture. Of course, we should note that the "social" and "thermal" perspectives are not mutually exclusive.

Experiment 4a: Ultrasound Production by 12- to 13-Day-Old Rats is Not Independent from Ambient Temperature

The findings of the first three experiments, emphasizing the importance of thermal factors for ultrasound emission, can be used to reinforce the prevailing opinion that, up to 10 days of age, ultrasound emission by rat pups is stimulated and modulated primarily by thermal factors. It has been argued, however, that after 10 days of age ultrasound emission becomes independent of thermal cues as other stimulus modalities gain in importance (see Hofer & Shair, 1978). Thus, when huddling suppresses ultrasonic emissions by 2-week-old pups, the effect is attributed to the contact comfort offered by huddling with littermates, independent of the thermal insulation that such contact provides (Hofer & Shair, 1978, 1980). This suggests that the stimulus control of ultrasonic vocalization changes during development.

Another possible explanation is that dependence of ultrasound emission on ambient temperature has been overlooked in the 2-week-old animals because of limitations imposed by experimental procedures. Specifically, it is clear that the severity and duration of exposure to subthermoneutral temperatures can regulate the onset and probability of ultrasound production. Thus, in the present experiment, we investigate the effect of ambient temperature on ultrasound emission by 12- to 13-day-old rats. Our expectation is that longer tests and improved control of the thermal environment will allow us to detect thermal influences on ultrasound production, even in the older pups.

Method

Subjects

Sixteen female rat pups from eight litters were used. Rats were from the same stock and were housed and reared in the same way as those in Experiment 1. On the day of testing all pups were 12–13 days of age.

Apparatus

The test chamber, detection and recording of ultrasound, and recording of air temperature were the same as in Experiment 1.

Procedure

Pups were tested as in Experiment 1. Briefly, a pup was transferred from its home cage to the testing chamber, at which time data collection began. Again, conductive heat exchange was minimized by lining the glass chamber with polyethylene mesh and by the use of a rubber glove during the transfer.

In this experiment each trial lasted 20 min. Each pup was exposed either to a chamber air temperature of 20°C or 32–33°C. Air temperatures of 32–33°C are

considered to be at the lower border of the thermoneutral zone for pups 12–13 days of age (Spiers & Adair, 1986). For the "20°C condition," air temperature ranged from 19–23.5°C across all min of all tests but ranged from 19–21.5°C 84% of the time. For the "32°C condition," air temperature ranged from 30–35°C across all min of all tests but ranged from 32–33°C 88% of the time.

A paired design was used. When the pups in a litter were 12 days old, one of the pups was tested. The next day, another pup from the same litter was tested at the other ambient temperature. Ordering of ambient temperatures was counterbalanced. No more than 2 pups from any given litter were tested. Finally, it was not possible to check for nutritive state of the pups because of the difficulty of detecting milk bands in these older and furrier pups.

Statistical Analysis

Data are presented as Mean ± *SEM*. Differences between temperature groups were tested using a paired t test. Statistical calculations were performed using Statview II on the Macintosh computer.

Results and Discussion

Body weights of the 16 pups in the experiment ranged from 24.6–36.7 g with no significant differences in body weights between the two temperature groups, paired $t = 0.374$, ns.

Figure 5 presents vocalization rates for pups exposed to air temperatures of 20°C and 32°C for each of the four 5-min blocks of time during the test. Vocalization rates were significantly greater at 20°C than at 32°C during each of the 5-min blocks except Min 1–5 (Min 1–5: paired $t = 2.298$, $p < 0.06$; Min 6–10: paired $t = 3.387$, $p < 0.02$; Min 11–15: paired $t = 2.857$, $p < 0.03$; Min 16–20: paired $t =$

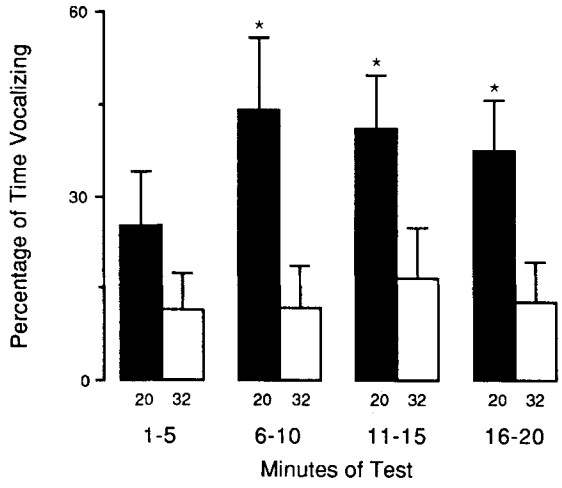

Fig. 5. Percentage of time vocalizing during the four 5-min blocks at either 20°C or 32°C for the 12- to 13-day-old pups in Experiment 4a. Asterisks indicate significantly greater vocalization rates relative to paired controls, $p < 0.05$. Note the expanded y axis.

2.767, $p < 0.03$). Although vocalization rates during Min 1–5 were not significantly greater at the air temperature of 20°C than at 32°C, analysis of each of the first 5 min shows that vocalization rates were greater at the colder temperature by Min 4, paired $t = 3.012$, $p < 0.02$.

The vocalization rates of the 8 pups at 32°C were highly variable. For example, as shown in Figure 5, the mean vocalization rate at 32°C during Min 16–20 of the test was 12.5%. Of the 8 pups tested, 4 did not emit a single pulse over these 5 min and 1 pup emitted only two pulses. However, the remaining 3 pups vocalized 18%, 29%, and 51% of the time. [Perhaps ultrasound production could have been reduced even further had we used an ambient temperature more securely within the thermoneutral temperature range of pups of this age (see Spiers & Adair, 1986)]. In contrast, during Min 16–20 at 20°C, all 8 of the pups vocalized at least 3% of the time and 7 of these pups vocalized at least 20% of the time.

The present results demonstrate that ultrasound production is not independent of ambient temperature in 12- to 13-day-old rats. In the next experiment, we examine this same issue in 18- to 19-day-old rats.

Experiment 4b: Ultrasound Production by 18- to 19-Day-Old Rats is Not Independent from Ambient Temperature

Although ultrasound production by 2-week-old pups, like that of 8- to 10-day-old pups, is dependent to a large degree on ambient temperature, it is possible that older pups lose this dependence on ambient temperature. This may be true especially of pups older than 15 days, whose eyes have opened and who are now able to locomote more freely about their environment. On the other hand, it has been reported that as pups get older it becomes more difficult to elicit significant levels of ultrasound from them (Okon, 1971; Allin & Banks, 1971). Unfortunately, these earlier studies were limited by methodological difficulties that have been described above. Thus, in the present experiment we determine whether ultrasound production by 18- to 19-day-old rats shows the dependence on ambient temperature characteristic of younger pups. As in Experiment 4a, we exposed pups to a thermoneutral air temperature (32°C; see Spiers & Adair, 1986) and a cold air temperature (15°C).

Method

All methods were identical to those in Experiment 4a except that 18- to 19-day-old pups ($n = 16$) were used. Furthermore, pups were exposed either to a thermoneutral ambient temperature of 32°C or a cold ambient temperature of 15°C. Each test lasted 25 min.

For the "32°C condition," air temperature ranged from 31.5–33.5°C across all min of all tests. For the "15°C condition," air temperature ranged from 12.5–19.5°C across all min of all tests. However, these fluctuations in the cold air temperature are deceiving because the older and more mobile pups often crawled over or near the air-temperature probe during the test, resulting in an apparent increase in air temperature. Therefore, it is useful to know that air temperature at the beginning of the test ranged from 12.5–15.5°C.

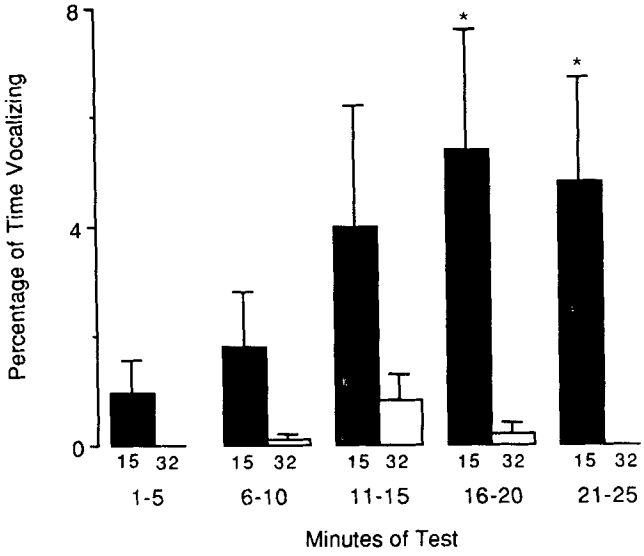

Fig. 6. Percentage of time vocalizing during the five 5-min blocks at either 15°C or 32°C for the 18- to 19-day-old pups in Experiment 4b. Asterisks indicate significantly greater vocalization rates relative to paired controls, $p < 0.05$. Note the expanded y axis.

Results and Discussion

Body weights of the 16 pups in the experiment ranged from 33.6–47.6 g. Moreover, there were no significant differences in body weight between the two temperature groups, paired $t = 0.810$, ns.

Figure 6 presents vocalization rates for pups exposed to air temperatures of 15°C and 32°C for each of the five 5-min blocks of time during the test. Vocalization rates were not significantly greater at 15°C than at 32°C except during Min 16–20 and Min 21–25 (Min 1–5: paired $t = 1.477$, ns; Min 6–10: paired $t = 1.628$, ns; Min 11–15: paired $t = 1.689$, ns; Min 16–20: paired $t = 2.439$, $p < 0.05$; Min 21–25: paired $t = 2.470$, $p < 0.05$). A min-by-min analysis indicated that vocalization rate became significantly higher at 15°C than at 32°C by Min 18, paired $t = 2.758$, $p < 0.03$.

Six of the pups isolated at 32°C did not emit a single ultrasonic pulse during the entire 25 min of the test, compared to only 2 of the pups isolated at 15°C. In addition, concentrating on Min 21–25 of the test, not a single ultrasonic pulse was detected from the pups isolated at 32°C. In contrast, there was a great deal of variability in the responses of the pups isolated at 15°C: 3 of the pups also did not vocalize at all, 1 pup emitted one pulse over the 5 min, and the remaining pups vocalized for 11.7%, 13.0%, 6.7%, and 6.7% of the time.

Previous research has led to the understanding that it is difficult to elicit ultrasonic vocalizations from rat pups as they get older, even at extremely cold (2°C) temperatures (Hofer & Shair, 1978; Okon, 1971). Experiments 4a and 4b show that extreme cold is not necessary to elicit ultrasound production from 2- to 3-week-old pups. Rather, less extreme air temperatures are sufficient provided

that pups are tested for a period of time long enough to allow the cold temperatures to effect thermal changes in the larger and better-insulated older pups. That the thermal inertia of rat pups increases with age is evidenced by the fact that 12- to 13-day-olds required only 4 min to vocalize significantly more at 20°C than at 32°C, while 18- to 19-day-olds required 18 min to vocalize significantly more at 15°C than at 32°C. It now seems reasonable to conclude that at no age tested thus far is ultrasound production by rat pups independent of ambient temperature.

General Discussion

The results of the four experiments in the present paper show that (a) relatively small (3–4°C) temperature increments surrounding the thermoneutral zone of 8- to 9-day-old pups are associated with significant increases in ultrasound production (Experiment 1), (b) a thermoneutral ambient temperature more effectively suppresses ultrasound production than does the presence of a single littermate (Experiment 2), (c) the latency to vocalize and the severity of the ultrasound-inducing temperature decrease are directly related to the ability of pups to reduce heat loss by huddling (Experiment 3), and (d) cold-induced ultrasonic vocalizations do not disappear with maturation beyond 10 days of age (Experiment 4).

Some of the methodological "details" introduced into these experiments, namely reduction of conductive heat exchange during handling, short transfer times, pretest habituation periods, temperature change without physical transfer, small increments of temperature challenges, and better control over convective versus conductive heat exchange emphasize an infant rat's extreme sensitivity to thermal perturbations. We suspect that residual levels of ultrasound production are related to nonthermal tactile stimulation and inadvertent convective heat loss during a brief transfer. This suspicion is reinforced by observations made with an infrared thermography system that provides a visual display of a pup's body surface temperature profile (Blumberg, Efimova, & Alberts, unpublished). We observed localized metabolic heat production in the interscapular region (that overlies a major depot of brown fat) in rat pups that had been transferred from their nest to a thermoneutral environment; moreover, as these pups reached a thermal steady state and heat production decreased, so too did ultrasound production decrease. Whether these pups were exhibiting heat production in the nest and/or whether some aspect of the transfer procedure was responsible for stimulating heat production remains to be determined. Regardless, it is clear that one cannot be certain that a pup transferred from its nest to a thermoneutral environment is experiencing thermal comfort unless the pup's physiological responses are measured.

In emphasizing thermal factors in the elicitation of ultrasound production and in the suppression of ultrasound by littermate contact, we have deemphasized the importance of distress. Of course, it is still possible that cold air temperatures cause distress and littermate contact alleviates or delays the distress through a nonthermal mechanism.

We know, however, that cold air temperatures have physiological consequences (such as increased oxygen consumption, heat production, and respiratory rate) that do not involve distress as a causative factor. We have hypothesized a

connection between these physiological consequences of cold exposure and ultrasound production (Blumberg & Alberts, 1990, 1991b). During cold exposure, ultrasound production by rat pups begins contemporaneously with increases in metabolic heat production and ventilation (Blumberg & Alberts, 1990). Moreover, ultrasound production is diminished by manipulations, such as milk deprivation and hypoxia, that suppress activation of metabolic heat production during cold exposure (Blumberg & Alberts, 1991b).

The contemporaneous onset of ultrasound production and metabolic heat production during cold exposure inspired a reinterpretation of ultrasound. Specifically, we noted that ultrasound production by rat pups shares a number of similarities with the audible grunting of human infants with respiratory distress syndrome (Blumberg & Alberts, 1990). These grunts are known to be acoustic by-products of a respiratory mechanism called laryngeal braking, a mechanism that literally brakes expiration, increases intrathoracic pressure, and enhances oxygen uptake in the lungs (Davis & Bureau, 1987; England, Kent, & Stogryn, 1985). Thus, we contend that ultrasonic vocalizations are acoustic by-products of laryngeal braking and that rat pups produce ultrasounds during cold exposure because their respiratory system has become activated. Any communicatory benefits that might accrue to the ultrasounding rat pup could be considered an exaptation, that is, a nonadapted response that nonetheless may have come to have survival value (Gould & Vrba, 1982).

As described above, during cold exposure the activation of laryngeal braking complements the contemporaneous increase in metabolic heat production; heat production requires oxygen and laryngeal braking enhances gas exchange in the lungs. However, cold exposure is not necessary for laryngeal braking because cold exposure is not the only context in which the respiratory system becomes activated and in which laryngeal braking is likely to occur. In fact, activation of the respiratory system is modulated by a number of factors including sleep state, metabolism, and temperature (Johnson, 1985). Thus, it is possible that high ambient temperatures elicited ultrasound production in Experiment 1 because metabolic rate and respiratory drive were increased. Direct measurement of metabolic rate, respiratory rate, and ultrasound production is required to clarify this issue.

It should also be emphasized that laryngeal braking may enhance oxygen uptake in the lungs during cold exposure and yet still occur under other circumstances (i.e., heat exposure, tactile stimulation) where its functional value is questionable. In other words, the fact that ultrasound production is emitted during circumstances that do not appear to relate to cold exposure says little about the functional value of ultrasound (and thus laryngeal braking) during cold exposure. More generally, it is conceivable that nonspecific arousing stimuli can elicit laryngeal braking (and ultrasound production) without diminishing the importance of laryngeal braking during cold exposure.

Our hypothesis that ultrasonic vocalizations are acoustic by-products of laryngeal braking suggests that more rigorous physiological analyses are warranted during experiments that attempt to manipulate sound production by neonates. For example, the conventional interpretation of ultrasonic vocalizations as distress calls has suggested to experimenters that endogenous opioid systems may mediate the distress associated with isolation and thus the production of ultrasound. In support of this suggestion, Kehoe and Blass (1986) found that morphine depressed

and naltrexone (an opioid antagonist) enhanced ultrasound production by 10-day-old rats during isolation. Thus, it is possible that opioids affect ultrasound production via their mediation of pain and distress responses.

Although opioids are indeed associated with the mediation of pain and distress, especially in adults, they have also been implicated in the modulation of breathing responses of mammalian neonates. Specifically, opioid agonists depress breathing and opioid antagonists (such as naltrexone) activate breathing (for a review, see Moss & Inman, 1989), results that are consistent both with the findings of Kehoe and Blass as well as the interpretation of ultrasound as a by-product of laryngeal braking. Moreover, opioids are found in respiratory-related brain regions at higher levels in neonates than in adults (Moss & Inman, 1989), suggesting that caution should be exercised when drawing analogies between adults and neonates with regard to the function of neurochemicals. Thus, we believe that the current debate over the role of opioids in the mediation of ultrasound production (see Winslow & Insel, 1991) must include careful consideration of the effects of these drugs on the respiratory system.

Questions of nonthermal social stimuli of ultrasound production, such as those presumed to be involved in "contact comfort," remain unresolved. Our findings that relatively small temperature variations can initiate and modulate ultrasound reveal previously unappreciated responsivity in rat pups to thermal factors in their environment. Some of these results suggest that regional insulation or conductive warming, such as that derived from social contact, might account for the reduction of ultrasound production by the presence of social companions. Nevertheless, these considerations do not rule out tactile or olfactory modulation of ultrasonic vocalization. We believe, however, that further study of the production, development, neuropharmacology, and significance of ultrasound must take into account the respiratory mechanics and thermal physiology of newborn rats.

Notes

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