Influence of juvenile housing conditions on the ventilatory, thermoregulatory, and endocrine responses to hypoxia of adult male rats

Sébastien Fournier, Vincent Joseph, and Richard Kinkead

Department of Pediatrics, Centre de Recherche Hospitalier Universitaire de Québec, Université Laval, Québec, Canada

Submitted 25 March 2011; accepted in final form 12 May 2011

Fournier S, Joseph V, Kinkead R. Influence of juvenile housing conditions on the ventilatory, thermoregulatory, and endocrine responses to hypoxia of adult male rats. J Appl Physiol 111: 516–523, 2011. First published May 19, 2011; doi:10.1152/japplphysiol.00370.2011.—“Extreme” housing conditions, such as isolation (single housing) or crowding, are stressful for rats, and their deleterious impact on behavior is well documented. To determine whether more subtle variations in housing can affect animal physiology, the present study tested the hypothesis that the hypoxic ventilatory response (HVR) of adult male rats housed in pairs during the juvenile period (postnatal day 21 to adulthood) does not differ from that of animals housed in triads. Because neonatal stress augments the neuroendocrine responsiveness to stress and HVR, experiments were performed both on “control” (undisturbed) animals and rats subjected to neonatal maternal separation (NMS; 3 h/day, postnatal days 3–12). At adulthood, ventilatory activity was measured by whole body plethysmography under normoxic and hypoxic conditions (inspired fraction of O2 = 0.12; 20 min). The ventilatory and body temperature responses to hypoxia of rats raised in triads were less than those of rats housed in pairs. For the HVR, however, the attenuation induced by triad housing was more important in NMS rats. Triad housing decreased “basal” plasma corticosterone, but increased estradiol and testosterone levels. Much like the HVR, housing-related decrease in corticosterone level was greater in NMS than control rats. We conclude that modest changes in housing conditions (pairs vs. triads) during the juvenile period can influence basic homeostatic functions, such as temperature, endocrine, and respiratory regulation. Housing conditions can influence (even eliminate) the manifestations of respiratory plasticity subsequent to deleterious neonatal treatments. Differences in neuroendocrine functions likely contribute to these effects.

Control of breathing; maternal separation; environment; chemoreflex; temperature regulation; hormone
poses to respiratory instability during sleep (12, 30, 53), understanding the impact of external conditions such as housing on this physiological function may provide valuable insight into the environmental determinants of health.

METHODS

Experimental Animals

Experiments were performed on 157 Sprague-Dawley male rats aged between 10 and 16 wk. Experiments were first performed on 14 males housed in pairs. This housing condition is standard in our facilities. Since data obtained from these animals were nearly identical to those reported previously in our laboratory (14, 20), data from 83 males housed in pairs (see details below) from these earlier studies were added to the current data set to reduce and optimize use of laboratory animals. The remaining 60 animals were housed in triads. On the day of surgery (see below), rats weighed between 422 and 698 g. All rats were born and raised in our animal care facility. Animals were supplied with food and water ad libitum and maintained in standard care conditions (21°C, 12:12-h dark-light cycle; lights on at 0600 and off at 1800). Laval University Animal Care Committee approved the experimental procedures, and the protocols were in accordance with the guidelines detailed by the Canadian Council on Animal Care.

Mating and Housing Conditions

Nulliparous females were mated and typically delivered between 12 and 18 pups. Two days after delivery, litters were normalized to 12 pups, when necessary, with an equal number of males and females whenever possible. Results obtained from these animals were compared with those subjected to neonatal stress (see below). To constitute proper controls (20, 52), pups were undisturbed and continuously remained with the dam from postnatal days 3–12. The litter was placed in a larger cage where animal care technicians’ attention could be dismissed for 10 consecutive days so as to leave animals completely free from human interactions. Standard care (including weekly bedding change) was reinstated at the end of the protocol. Pups were weaned on postnatal day 21 and were housed either in pairs or triads. Cage size was the same for both groups (length: 47 cm, width: 25.5 cm, height: 20 cm; density: 12 dm³ or 8 dm³/rat). Each cage was supplemented with a black PVC pipe 3 in. in diameter. Rats were so reared until adulthood (10–16 wk), at which time the surgical inter-

Wb Profiles

Over time, rats housed in pairs usually develop a social hierarchy, such that one animal establishes itself as a dominant, whereas the other becomes subordinate. Dominance is typically associated with an increased food consumption and weight gain compared with the other. To determine whether housing affects this pattern, individual Wb were recorded on the day of surgery; data were compared between groups.

Neonatal Maternal Separation

Litters selected for exposure to neonatal stress were subjected to our standard NMS protocol from postnatal days 3–12 (20, 28). Briefly, the entire litter of pups was separated from the dam, placed in a temperature (32°C) and humidity (45%) controlled incubator, and isolated from each other by opaque Plexiglas compartments. The NMS protocol was performed from 0900 to 1200 on each of postnatal days 3–12.

Anesthesia and Surgical Procedures

Telemetry. At adulthood, rats received a surgical intervention to implant a fixed telemetric probe transponder (E-mitter, Mini Mitter, Bend, OR) to measure core Tb during ventilatory measurements. Rats were anesthetized with isoflurane (3% in air). The probe was inserted in the peritoneum and sutured behind the internal wall of the abdominal cavity, according to our standard procedure (14, 19, 38). At the end of the surgery, rats received subcutaneous injections of an antibiotic (Baytril 5 mg/kg), an analgesic (Buprenorphine 0.02 mg/kg), and fluids (5 ml lactated Ringer). Administration of antibiotics and fluids was repeated 24 and 48 h postsurgery. As rats recovered rapidly, supplemental analgesic was not necessary. Note that, in some animals from previous studies, Tb was measured using a rectal probe before and after hypoxia. Comparison of the results between studies revealed no significant effect of the procedure (and surgery) used for Tb measurement.

Blood Sampling and Hormone Analyses

During isoflurane anesthesia, a 2.5-ml blood sample was withdrawn from the jugular vein immediately after surgery and before postoperative treatment. Samples for CORT analysis were placed in 2 Microvette 500 K+, EDTA tubes, and samples for analysis of testosterone, progesterone, and estradiol level were placed in one serum-gel clotting activator Microtube (Sarstedt). Serum-gel tubes were kept at room temperature for 30 min before centrifugation (15,000 rpm, 4°C for 5 min). After centrifugation, blood plasma was collected and placed in a −80°C freezer until assayed. Immediately after ventilatory measurements, rats were deeply anesthetized with ketamine (Rogarsetic; 80 mg/kg) and Xylazine (Rompun; 10 mg/kg). At this dose, rats typically lose consciousness and reach a surgical plane of anesthesia within minutes, and another 2.5-ml blood sample was obtained by intracardiac puncture. This posthypoxic sample was handled as the same as baseline (normoxic) blood and stored in the −80°C freezer until total CORT, testosterone, progesterone, and estradiol assays were performed. Analysis of CORT was performed in our laboratory, as we have done previously (14, 20), using the Correlate EIA ELISA kits (Assay Design, Ann Arbor, MI) and a microplate spectrophotometer (μ-Quant, Bio-Tek Instruments, Winooski, VT). CORT concentrations were calculated from the parameters of the standard curve linearized by a log-log transformation. Analyses of testosterone, estradiol, and progesterone were performed by the clinical biochemistry laboratory of our hospital using an electrochemiluminescence immunoassay test and read by the Eclecsys 1010/2010 modular analyzer (Roche Canada, Mississauga, ON, Canada).

Ventilatory and Metabolic Measurements

Ventilatory measurements were performed using a whole body, flow-through plethysmography system (model PLY3223, Buxco Electronics, Sharon, CT), according to a protocol previously described (14, 19–21, 27). Briefly, the rat was placed unrestrained in a 4.5-liter Plexiglas experimental chamber and allowed to calm and acclimate before launching measurements. This period typically lasted between 30 and 60 min. The breathing frequency (f), tidal volume (VT), minute ventilation (Ve), and oxygen consumption (Vo2) were all recorded using data acquisition software (IOX, EMKA Technologies, Falls Church, VA). The flow rate of air going in and out of the chamber was maintained between 2.0 and 2.5 l/min using a push-flow regulator pump (PLY 1020; Buxco Research). Inspired O2 levels were monitored using a portable O2 analyzer (TED-60-T; Teledyne Analytical Instruments). Excurrent gas was passed through a drying column (drierite) before entering the cell of a high-precision O2 analyzer (AEI technologies; model S-3A, Ametek, Pittsburg, PA).

In all animals, basal ventilatory activity was first recorded while the rat was breathing room air (normoxia) for 10 min, immediately followed by a 20-min period of moderate hypoxia (inspired fraction of
Variability was not affected by housing (Table 1; $P > 0.23$ for both variables). However, the intracage weight variation was calculated before experimentation. *Main effect of housing (ANOVA), $P < 0.05$.

$O_2 = 0.12$). Specifically, baseline measurements of ventilatory variables were obtained by averaging the last 10 min of stable recording, whereas a 5-min average was taken for each variable at the end of hypoxic exposure. Recordings were made between 0900 and 1300 to minimize fluctuations associated with circadian rhythms. Also, during ventilatory measurements, the barometric pressure, $T_b$, chamber temperature, and humidity were all recorded for subsequent calculation of $V_T$ expressed in milliliters (BTPS) per 100 grams of $W_b$, according to the equations provided by Drobrough and Fenn (13). Given the wide range of $W_b$ among animals, this variable was included as a covariate in the ANOVA. Given its significant effect on some respiratory variables, calculations of $V_E$ and $V_O_2$ were done anew using allometric correction factors, according to the equations described by Mortola et al. (40). These equations are described in Table 2 legend. Since there is no equation for allometric correction of $V_T$ per se, these were inferred from corrected $V_E$ values.

**Statistical Analysis**

The effects of different housing conditions (pairs vs. triads) and treatment (control vs. NMS) on $W_b$, plasma hormone levels, and normalized ventilatory data were compared using a two-way ANOVA. Absolute (non-normalized) respiratory data were also analyzed using a three-way ANOVA (hypoxia, housing, and stress). The $W_b$ of cage mates was averaged, and the standard deviation calculated. These values were then used to calculate the coefficient of variation for $W_b$, as an index of dominance among littermates. The coefficient of variation was calculated by dividing the standard deviation by the average.

All statistical analyses were done using Statview 5.0 (SAS Institute, Cary, NC). A repeated-measures design was used when appropriate. ANOVA was followed by Fisher’s post hoc test when $P \leq 0.05$. $P$ values reported in the text are results of ANOVA. Results from post hoc tests are displayed in Figs. 1–6. Data are reported as means $\pm SE$.

**RESULTS**

**Effects of Housing and Neonatal Stress on Body Weight and Plasma Corticosterone Levels**

$W_b$ profiles. Measures of individual $W_b$ before experimentation show that rats housed in triads were older and thus heavier than those housed in pairs (Table 1; housing effect: $P < 0.0001$ for both variables). However, the intracage weight variability was not affected by housing (Table 1; $P = 0.23$). None of the weight parameters or age was affected by NMS (Table 1; $P > 0.05$). Analysis of covariance suggests that age was the strongest determinant of $W_b$ ($P < 0.0001$).

Plasma corticosterone. $CORT$ levels measured in rats housed in triads were generally lower than those housed in pairs. This effect was more important in NMS rats in which $CORT$ levels are typically greater than controls (Fig. 1; housing $\times$ stress: $P = 0.03$). Plasma $CORT$ levels measured at the end of the hypoxic protocol were higher than baseline (Fig. 1; hypoxia effect: $P = 0.0001$). However, this response was not influenced by housing or NMS (hypoxia $\times$ stress: $P = 0.68$; hypoxia $\times$ housing: $P = 0.23$).

**Ventilatory and Metabolic Variables at Rest and in Response to Hypoxia**

**Baseline conditions.** During normoxia, $V_E$ was not influenced by housing or neonatal stress (Table 2: $P = 0.36$ and $P = 0.31$, respectively). Since analysis of covariance indicates that $W_b$ was a strong determinant of baseline $V_E$ ($P < 0.0001$), allometric correction was performed. Allometric baseline $V_E$ of rats housed in pairs was lower than those housed in triads (Table 2; housing effect: $P = 0.005$). Compared with controls, baseline $f$ was lower in NMS rats (Table 2; stress effect: $P = 0.005$); $f$ was not influenced by housing ($P = 0.4$). There is no allometric correction factor for $V_T$ per se. This variable is also greatly influenced by $W_b$ ($P < 0.0001$); allometric correction for $V_T$ was calculated from corrected $V_E$ to confirm that this variable is responsible for the higher corrected $V_E$ reported for rats housed in triads (Table 2; housing effect: $P = 0.003$).

Basal $T_b$ was similar across all groups (Table 2; housing effect: $P = 0.16$; stress effect: $P = 0.29$). $V_O_2$ of NMS rats was greater than that of controls (Table 2; stress effect: $P = 0.02$). Allometric correction did not affect this result (stress effect: $P = 0.02$). Consequently, the convective requirement ratio ($V_E/V_O_2$) of rats housed in triads was greater than that of those housed in pairs (Table 2; housing effect: $P = 0.03$).

**Hypoxic ventilatory response.** Hypoxia increased $V_E$ in all groups, but this response was reduced significantly when rats were housed in triads (hypoxia $\times$ housing: $P = 0.005$; absolute data not shown). As reported previously, NMS augmented the HVR (hypoxia $\times$ stress: $P = 0.03$; absolute data not shown). Expressing these responses as a percent change from baseline confirmed these results (Fig. 2; housing effect: $P = 0.004$). Performing these analyses on data subjected to allometric corrections was not appropriate. ANOVA was followed by Fisher’s post hoc test when $P \leq 0.05$. No pairwise comparisons were performed when corrected $V_E$ was used due to the significant effect of housing (ANOVA). The histograms compare results from animals subjected to neonatal maternal separation (NMS) or undisturbed (control) and then housed in pairs (open bars) or triads (solid bars). Values are means $\pm SE$. ANOVA results reported in the figure are for the entire data set. Post hoc pairwise comparisons were performed only when warranted by ANOVA. Value significantly different from *pairs and †control ($P < 0.05$).
Neither housing nor NMS affected the $\dot{V}O_2$ response to hypoxia. 
correction factors were obtained from Mortola et al. (40): $\dot{V}E$ baseline; housing effect: 
were raised in either pairs or triads. *Main effect of housing (ANOVA), 
requirement ratio. Measurements were performed in rats subjected to neonatal maternal separation or undisturbed over the same time period. In each group, rats 
are no allometric correction equations for VT, allometric values for this variable were calculated by dividing allometric $\dot{V}E$ by breathing frequency. 
responses did not differ between groups (Fig. 2; housing effect: 
processes by NMS and housing; the highest levels were observed 
baseline / Housing and O2 consumption responses. $T_h$ and 
response decreased during hypoxia (hypoxia effect: $P < 0.0001$ for both; absolute data not shown). At the end of the protocol, the $T_h$ decrease observed in rats housed in triads was significantly lower than that of those housed in pairs (Fig. 3A; housing effect: $P < 0.0001$); however, NMS had no effect on this aspect of the response (Fig. 3A; stress effect: $P = 0.45$). 
Neither housing nor NMS affected the $\dot{V}O_2$ response to hypoxia (Fig. 3B; housing effect: $P = 0.87$; stress effect: $P = 0.15$); similar results were obtained following allometric correction (data not shown).

Hypoxia increased $\dot{V}E/\dot{V}O_2$ (data not shown; hypoxia effect: $P < 0.0001$). Analysis of the hypoxic data show that NMS augmented $\dot{V}E/\dot{V}O_2$ of rats housed in pairs by 27%, but not in triads (Fig. 3C; housing × stress: $P = 0.05$).

Hormonal Profile

Testosterone. Baseline testosterone levels were augmented both by NMS and housing; the highest levels were observed in NMS males housed in triads (Fig. 4; housing × stress: $P = 0.006$). Hypoxia affected testosterone levels; however, the response was influenced by housing conditions and NMS (Fig. 4; hypoxia × housing × stress: $P = 0.05$). Specifically, hypoxic exposure had no effect on testosterone levels of control animals, regardless of their housing conditions. In NMS rats, however, testosterone levels measured at the end of hypoxia were higher than baseline when animals were housed in pairs. When NMS rats were housed in triads, hypoxia reduced plasma testosterone levels below baseline.

Estradiol. By comparison with rats housed in pairs, baseline estradiol levels were greater in rats housed in triads; this effect of housing was greater in NMS rats (Fig. 5; housing × stress: $P = 0.001$). Exposing rats to hypoxia had opposite effects on estradiol levels, depending on whether rats were housed in pairs or triads (Fig. 5; hypoxia × housing: $P < 0.0001$). Specifically, hypoxia increased estradiol levels of rats housed in pairs, whereas a decrease was observed when rats were housed in triads.

Progesterone. Baseline levels of progesterone did not differ between groups (Fig. 6; housing effect: $P = 0.18$; stress effect: $P = 0.71$). Furthermore, hypoxia did not affect plasma progesterone levels significantly (Fig. 6; hypoxia effect: $P = 0.08$).

DISCUSSION

The present experiment tested the hypothesis that augmenting the number of rats per cage from two to three during the
juvenile period does not affect the HVR measured at adulthood. Based on that premise, results showing that housing rats in triads significantly reduces the ventilatory and temperature responses to hypoxia were not anticipated. As we discuss below, hormone measurements and the use of NMS rats, which are highly sensitive to stressful (and enriched) environments, indicate that triad housing reduces basal HPA axis activity and may be viewed as a form of enrichment. Consequently, the different physiological phenotypes reported in this study are likely related to housing-mediated changes in hypothalamic function. These effects may be indirect (via changes in hormone levels) and/or direct, owing to the numerous connections that exist between the PVN and respiratory neurons. The significant impact of housing on this highly social species raises important methodological issues, as it may interfere with manifestations of physiological plasticity arising from neonatal challenges, such as chronic or intermittent hypoxia or drug administration.

**Interactions Between Gonadotropic and Corticotropic Axes and the Control of Breathing**

Housing had remarkable effects on the “basal” hormonal profile of rats. While estradiol and progesterone are generally viewed as ovarian hormones, the adrenals, testes, adipose tissues, and even the skin are also important sources of these.

**Fig. 3. Effects of neonatal stress and housing on the decrease in body temperature and oxygen consumption ($V_O^2$) following hypoxia.**

A: body temperature was measured during normoxia (baseline) and following 20-min exposure to moderate hypoxia (12% $O_2$) and then expressed as the absolute difference (delta) between hypoxia and baseline. B: $V_O^2$ was measured at the same times, but the response to hypoxia is expressed as a percent change from baseline. C: convective requirement ratio ($V_E/V_O^2$) measured at the end of hypoxia. Data were obtained from rats subjected to NMS or undisturbed (control) and then raised in pairs (open bars) or triads (solid bars). Values are means ± SE. ANOVA results reported in the figure are for the entire data set. Post hoc pairwise comparisons were performed only when warranted by ANOVA.

**Fig. 4. Resting (normoxic) and hypoxic plasma testosterone levels.** Blood samples were obtained from rats breathing room air (normoxia) or following exposure to hypoxia (12% $O_2$; 20 min) during ventilatory measurements with plethysmography. The histograms compare results from animals subjected to NMS or undisturbed (control) and then raised in pairs (open bars) or triads (solid bars). Values are means ± SE. ANOVA results reported in the figure are for the entire data set. Post hoc pairwise comparisons were performed only when warranted by ANOVA. *Value significantly different from *pairs, †control, and §normoxia ($P < 0.05$).

**Fig. 5. Resting (normoxic) and hypoxic plasma total estradiol levels.** Blood samples were obtained from rats breathing room air or following exposure to hypoxia (12% $O_2$; 20 min) during ventilatory measurements with plethysmography. The histograms compare results from animals subjected to NMS or undisturbed (control) and then raised in pairs (open bars) or triads (solid bars). Values are means ± SE. ANOVA results reported in the figure; the statistical interaction between housing and stress was detected during normoxia only. The interaction between hypoxia and housing was for the entire data set. Post hoc pairwise comparisons were performed only when warranted by ANOVA. *Value significantly different from *pairs and §normoxia ($P < 0.05$).
tory consequence of NMS in adult male rats (19, 20, 26, 28),

Augmentation of the HVR is the best documented “respiratory activity (24, 25) may explain why housing had such dramatic effects on this response. That the hypoxic response pattern for testosterone was similar suggests that similar mechanisms may be involved.

Effect of Housing on the Anapyrexic Response

Core T<sub>b</sub> is regulated by the hypothalamic structures, including the preoptic area, the anterior hypothalamus, and the PVN (10). During hypoxia, decrease in T<sub>b</sub> (anapyrexia) is part of the integrated strategy aiming to reduce V<sub>O2</sub> at a time when O<sub>2</sub> availability is reduced. Our laboratory previously showed that this degree and duration of hypoxic challenge can induce anapyrexia (14, 20), but, because neither NMS nor chronic CORT administration affected this response, the reduced anapyrexic response of rats housed in triads was unexpected. During hypoxia, thermoregulation is influenced by hormones, as estradiol injection in median preoptic area of female rats maintains T<sub>b</sub> constant during cold exposure (49). Moreover, our laboratory has previously showed that estradiol supplementation (alone or in combination with progesterone) prevents anapyrexia in rat pups (34). Consequently, the elevated estradiol level measured in rats housed in triads likely explains the housing-related differences in anapyrexic responses.
Conclusions

The current experiments provide experimental evidence for the existence and persistence of long-lasting effects of stressors on respiratory control development. Exposure to stress during early life has long-lasting consequences on respiratory control development (9, 30, 39). While the first 2 wk are currently viewed as a critical period for respiratory control development (1), elimination of the deleterious effects of NMS by triad housing indicates that this system remains substantially malleable well beyond this period. The present data do not indicate why housing had such an effect, but knowing that social interactions and play promote “optimal” central nervous system development in young rats (15, 48), we propose that such interactions were higher in pups housed in triads than in pairs. The overall effects of housing on respiratory regulation reported here may be viewed as marginal, but it is noteworthy that, in humans, the HVR of patients with sleep-disordered breathing is 29% greater than in healthy subjects (41). Considering that the HVR attenuation resulting from triad housing in NMS rats is well within that range, the physiological benefits from this form of enrichment may be significant. Based on this result and the reduced basal HPA axis function, triad housing may seem preferable for juvenile rats; however, a broader range of housing conditions needs to be tested to determine which approach is optimal for this species. Results of such study would be useful in the development of animal care guidelines. Nevertheless, these observations raise important questions about what should be considered as “normal” respiratory parameters in this species, keeping in mind that paired housing is a very common, but not uniform, animal care practice.

From a more applied perspective, results from the present study are consistent with clinical work showing that, in infants, breastfeeding and kangaroo care, which allow proximity and interactions between the infant and the mother, are forms of uniform, animal care practice.

ACKNOWLEDGMENTS

We express our most sincere appreciation to Dr. Nathalie Laflamme, consultant in statistics, and for the technical assistance of Melanie Pelletier and Sylvie Viger, as well as the precious help and advice of Kenia Bicego.

GRANTS

This work was supported by the Canadian Institute of Health Research (R. Kinkead and V. Joseph), the Canada Research Chair in Respiratory Neurobiology (R. Kinkead), and a doctoral scholarship by the Fonds de la Recherche en Santé du Québec (S. Fournier).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES