A homemade device for simultaneous measurement of pulmonary ventilation and metabolic rate in neonatal rodents.

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Running Head: plethysmography in neonates

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Highlights

- An inexpensive and simple to construct dual chamber plethysmograph sized for neonatal rodents is described.
- The device proved to accurately measure pulmonary ventilation and metabolic rates simultaneously.
- This device will provide an inexpensive alternative approach for investigators that need to assay the ventilatory phenotype of neonatal rodents.

Abstract

Various *in vitro* neonatal rodent models have been developed to study the control of breathing, but translation of the information requires a behavioral assay, which has led to the widespread use of plethysmography to measure breathing in awake neonatal rodents. Best practice requires correcting changes in ventilation to the corresponding change in metabolic rate, which is the main driver of pulmonary ventilation. Obtaining measures of both simultaneously is ideal, though technically difficult. Here we describe a simple, inexpensive home-made dual chamber approach for simultaneous measurement of pulmonary ventilation and metabolic rate. We found that the dual chamber provides values for pulmonary ventilation and metabolic rate that compare favorably with existing approaches.

Key Words: breathing; metabolic rate; neonate; plethysmography; rat

Introduction

In recent decades, a host of cellular, molecular, and genetic approaches have been developed to interrogate the inner workings of the motor and sensory components that underlie the control of breathing. It is often critical to buttress these approaches with a behavioral assay, which has led to the widespread use of plethysmography to measure pulmonary ventilation in awake neonatal rodents. Best practice requires correcting changes in ventilation to the corresponding change in metabolic rate, which is the main driver of pulmonary ventilation. This is especially important when studying the response to hypoxia, as metabolic rate drops precipitously in the hypoxic neonate.

The approaches most often used are flow-through or sealed respirometry, which can be used to measure both pulmonary ventilation and metabolic rate, and head-out plethysmography for measuring the pulmonary ventilation rate. The advantages and disadvantages of each have been widely discussed but can be summarized as follows: In whole body plethysmography, the animal is studied in an enclosed chamber, which allows the animal to be studied awake and unrestrained, and simultaneous measurement of metabolic rate is straightforward. With this method, pressure changes within the chamber are due to both the compression and expansion of alveolar gas as well as changes in volume due to the very small differences in temperature and humidity between gas in the lungs and gas in the chamber, with the latter contributing proportionally more to the total pressure change (Lundblad et al., 2002). This can be problematic in small animals because the pressure changes due to heating and humidification of inhaled gas are very small, making precise measurements of temperature and humidity critical. As a result, some investigators resort to measuring frequency of breathing only, which results in an incomplete behavioral assessment. To circumvent these issues, head out plethysmography was developed. Here, the animal breathes from the atmosphere, and the remainder of the body is enclosed in a chamber. The gas inhaled from the environment expands the pup's thorax leading to airflow from chamber to atmosphere. With proper calibration, the

airflow signal is directly proportional to the change in lung volume. While this method makes it easier to measure lung volume changes, the disadvantages include the neck seal used to isolate the head from the chamber which restrains movement, and the difficulty measuring metabolic rate.

Some clever approaches have been used to address these issues, as explained in the Discussion. Here we describe another approach that allows the simultaneous use of head-out plethysmography and flow-through respirometry. We describe a “dual chamber” design where the animal is placed in a head out chamber, and the entire chamber is then inserted into a larger chamber that is sealed from the environment except for a steady bias flow of gas. Construction of the chamber is described, and validation data are presented. Using a repeat-measures, randomized experimental design, we found that the dual chamber provides values for pulmonary ventilation and metabolic rate that compare favorably with values obtained using more traditional approaches.

Materials and Methods

Animals. All data were obtained from experiments approved by the Institutional Animal Care and Use Committee at The University of Arizona and comply with the ARRIVE guidelines and the National Institutes of Health guide for the care and use of Laboratory animals. A total of 11 male and 11 female animals aged P2-P5 were used, though no sex differences were detected in any of the protocols, so the data are pooled.

Dual chamber design and dimensions, and EKG vest. Dimensions of the two chambers are shown in Fig. 1A. The dimensions of both chambers are adjusted for larger animals, though the head-out method is difficult in animals older than P12 as they do not tolerate restraint and often break the neck seal. The inner, head-out chamber was made from a 60-cc plastic syringe cut to a length of 70 mm, with an inner diameter (i.d.) of 25 mm and outer diameter (o.d.) of 30 mm. The Luer lock end is used for calibration via injection of known volumes of air. A 3 mm-

diameter hole is drilled into the top and a plastic Luer Lock fitting is epoxied in place and connected to a ¼ inch diameter thick, 4 cm long thick-walled (1 mm) Tygon tube, which is connected to the inflow tube of a Hans-Rudolph pneumotachometer (Model 8430, 3 L/min max flow). Two additional holes, with diameter about 2 mm are drilled into the end of the chamber and used as conduits for the EKG wires and thermocouple. The location of these holes is based on preference. After the wires or thermocouple are passed through, utility wax (Coltene Whaledent utility wax round strips, item # 900-H00817, Florida Dental Supply) is applied as a sealant.

The outer chamber is made from a thick-walled (6 mm), 105 mm-long Plexiglass cylinder with an o.d. of 50 mm and an i.d. of 38 mm. The lid and the bottom of the chamber each have two ports, with one 5 and one 3 mm diameter. The 5 mm holes are threaded to accommodate a 2 mm i.d. nylon hose barb (Masterflex Fittings, item # EW-41517-04) for connecting gas inflow and outflow tubing. The 3 mm holes are for passage of the thermocouple and EKG lead wires. We cut an access slot into the open end of the outer chamber that is 50 mm long and about 12 mm wide (Fig. 1A). This allows the inner, head-out chamber to be inserted easily and accommodates the Tygon tubing that is connected to the pneumotach. After the head-out chamber is inserted, vacuum grease is applied to the inner surface of the lid, and the access slot is covered with a piece of gum rubber (Gum Rubber Sheet Gasket, 1/16" Thick, from Amazon.com). The gum rubber has a hole sized to accommodate the tubing that connects to the pneumotachometer and is 10 mm longer and 20 mm wider than the exposed portion of the access slot. We then use the CO_2 analyzer to check for leaks, and when found they are sealed with vacuum grease.

We used a sealed flow-through respirometer to measure metabolic rate and compared these values with those obtained with dual chamber. The flow-through respirometer is an 85 mm-long Plexiglass cylinder, with an o.d. of 32 mm, an i.d. of 28 mm and a wall thickness of 2 mm. The lid is 27 mm long, with a 50 mm o.d., 35 mm i.d. and wall thickness of 7.5 mm. As with

the larger chamber the lid and bottom have gas inflow and outflow ports fitted with 2 mm i.d. nylon hose barbs, with another 3 mm diameter hole for the thermocouple probe. As with the larger chamber, the inner surface of the lid is coated with vacuum grease before attachment, and leaks around the thermocouple port are sealed with wax.

We also designed a simple vest with 3 electrodes for the measurement of the EKG to obtain heart rate. A photograph of the vest is shown in Fig. 1B. It is made from a piece of canvas sized for 1–2-week-old rat pups, with dimensions 50 x 25 mm. Four strips of Velcro, two with loops on one side and two with hooks on the other, are sewn to the canvas to secure the vest to the animal. The electrodes were made by coiling a 2 cm length of coated stainless-steel wire (0.2 mm, A-M Systems, Inc.), smashing it flat with a hammer, and filing it smooth. The electrodes are then soldered to lead wire for connection to an amplifier (see below) and secured to the canvas with thread. The electrodes are coated with highly conductive electrode gel (signa gel, Parker Laboratories, Inc., Fairfield, New Jersey, USA) before they are attached over the animal's thorax.

The recording equipment used is shown in Fig. 1C. The two ports of the pneumotachometer were attached to the positive and negative ports of a pressure transducer with a range of ± 2 cm H_2O (Validyne DP45-16, Northridge, CA), and amplified with a Validyne Carrier demodulator. The pressure signal, which is proportional to the airflow rate, was sent in parallel to an analog integrator (Grass model 7, Quincy, MA) and an A/D board. The inspiratory component of the airflow signal was rectified and integrated to derive the inspired tidal volume (V_T), with the integrator resetting to zero at the end of each inspiratory cycle. The head-out plethysmograph was calibrated by injecting 0.05, 0.1 and 0.15 ml into the chamber to establish a relation between known volume and the amplitude of the V_T signal derived by the integrator. The digitized airflow signal and inspired V_T signal were displayed on the computer screen (Figs. 2B, 3A and 4A) and stored using Spike II software (Cambridge Electronic Design). The thermocouple probe was attached to a control unit (TCAT-1A Temperature Controller,

Physitemp, Clifton, NJ) that maintained chamber temperature at 33 ± 0.15 °C (mean ± SD) by controlling a heat lamp. This ambient temperature corresponds to normal nesting conditions for neonatal rats (Mortola, 1984). For measurement of metabolic rate, the gas analyzer pulled air through the flow-through respirometer at a rate of 100 ml/min, and 150 ml/min for the larger dual chamber. The effluent gas exiting the chamber passed through a Dri-Rite canister and then to O_2 and CO_2 analyzers (iWorx GA 200). The output from the CO_2 and O_2 sensors was digitized using the Spike II A/D board and software as above (Figure 1C). Temperature was monitored and maintained as described above.

Validation of the dual chamber for measurement of metabolic rate and *in vivo* plethysmography. In six animals (3M, 3F), we examined the response of the dual chamber to a change in gas concentration. In normal use, room air is pulled through the chamber at a constant flow rate by the gas analyzer's pump. To examine ventilatory and metabolic responses to test gases using the dual chamber, we used a calibrated Matheson rotameter set to deliver a certified test gas consisting of 12% O_2, 5% CO_2, balance N_2. The gas inflow rate was set to 155 ml/min, and the gas analyzer was set to pull gas from the chamber at a rate of 150 ml/min. Thus, the rate of incoming gas was just above the rate at which gas was removed from the chamber. We estimated the time needed for chamber gas concentration to match test gas concentration by computing the time constant for O_2 and CO_2 (Fig. 2A). We continued to apply the test gas for 5 min to measure the steady state response to hypoxic hypercapnia, with measurements made approximately 4 min after the test gas was introduced into the chamber. We also considered the possibility that gas flow into or out of the chamber disturbs the pups and changes breathing rate and/or depth. Accordingly, in another group of six pups (3M, 3F) we examined the response to compressed air, using the same inflow and outflow rates as used with hypoxic-hypercapnia.

The accuracy of resting, steady state ventilatory and metabolic rate measurements in the dual chamber was assessed in a separate group of 10 pups (5M, 5F). Metabolic rate

obtained in the dual chamber was compared to values measured with a flow-through respirometer sized for small animals. Validation of the dual chamber for measuring pulmonary ventilation was done by comparing values obtained with the dual chamber to those obtained with standard head out plethysmography. We also examined the influence of the EKG vest on measures of pulmonary ventilation and metabolic rate in the dual chamber. Thus, each of the 10 pups was studied in each of five conditions, with the order of presentation randomized: flow-through respirometer alone; head-out chamber alone; head-out chamber plus an EKG vest; dual chamber; dual chamber plus the EKG vest. Measurements were made for 30 min in each condition to achieve a steady state, and the animals were given a 30 min recovery between measurements. Key comparisons include inspired V_T, breath frequency, their product (pulmonary ventilation rate, $\dot{V}_I$), oxygen consumption ($\dot{V}O_2$) CO2 production ($\dot{V}CO_2$), the respiratory exchange ratio (RER, $\dot{V}CO_2/\dot{V}O_2$), and heart rate.

Statistics. To compare the different methods for measuring ventilation and metabolism we used a one-way, repeated measures analysis of variance (ANOVA). If the ANOVA was significant, paired contrasts were analyzed with the Bonferroni *post hoc* procedure. To mitigate effects of differences in body weight, and for ease of comparison with published values, $\dot{V}_I$, $\dot{V}O_2$ and $\dot{V}CO_2$ are expressed as ml/min/kg body weight^{-1}. Paired t--tests were used to examine the influence of adding compressed gas to the chamber, and for comparing baseline values with the values obtained in hypoxic hypercapnia. All values in the text are expressed as the mean ± 1 SD. For all tests, statistical significance was defined as a P-value of ≤ 0.05. We used Prism software (GraphPad Software, San Diego, CA) for all statistical analyses.

Results

The time constant to convert the chamber gas concentration from the room air value to the steady state value obtained with an inflow of test gas containing 12% O_2, 5% CO_2, balance N_2 in six pups averaged 54 ± 5.3 sec for CO_2 and 41 ± 5.5 sec for O_2, similar to the representative values shown in Fig. 2A. Fig. 2B is an exemplary recording showing that the addition of compressed air into the chamber (at arrow in Fig. 2B) had no impact on either V_T, frequency or heart rate. This was true in all six pups, with V_T averaging 93 ± 16 μL at baseline and 86 ± 18 μL with the addition of compressed air. Frequency averaged 105 ± 28 breaths/min at baseline and 105 ± 11 breaths/min with the addition of compressed air, $\dot{V}_I$ averaged 958 ± 261 ml/min/kg at baseline and 906 ± 221 ml/min/kg with compressed air, and heart rate averaged 363 ± 49 beats/min at baseline and 327 ± 33 beats/min with compressed air.

To compare the dual chamber for measuring metabolic rate and pulmonary ventilation with some selected existing approaches we studied 10 pups at the following ages: P2, N = 2; P3, N = 3, P4, N = 3 and P5, N = 2. Body weight ranged from 8.5-12.9 g and averaged 10.9 ± 1.2 g. A representative recording from an animal studied in the dual chamber and wearing the EKG vest is shown in Fig. 3A. The traces, from top down, include the EKG, inspired V_T, airflow, and the concentrations of O_2 and CO_2 in the chamber effluent. Note the stability of breathing and heart rate during this epoch, as well as the quality of the airflow tracing. Figure 3B contains all data used to assess the concordance between the dual chamber and more traditional approaches for measuring pulmonary ventilation and metabolic rate. $\dot{V}_I$ averaged 1445 ± 207 ml/min/kg in the head out chamber and was roughly 20 % higher in the dual chamber (1801 ± 207 ml/min/kg) ($P<0.01$, 3Ba). Variability in the dual chamber was also higher than that obtained with the standard head-out approach (coefficient of variation (CV) 14 and 11.5 %, respectively). The higher $\dot{V}_I$ in the dual chamber compared to the head-out plethysmograph was due to higher V_T (Fig. 3Bb), as frequency did not differ between any of the trials (Fig. 3Bc). V_T averaged 0.084

± 0.01 ml/breath in the head-out chamber and 0.11 ± 0.02 ml/breath in the dual chamber (P<0.01, Fig. 3Bb). Attaching the EKG vest during either head-out or dual-chamber plethysmography had no significant influence on V_T or $\dot{V}_I$ (Fig. 3Ba).

$\dot{V}O_2$ (Fig. 3Bd) averaged 47 ± 6.5 ml/min/kg in the flow-through respirometer, 40 ± 10 in the dual chamber (P<0.05, flow-through respirometer vs. dual chamber), and 36 ± 6.4 ml/min/kg when the dual chamber was combined with the EKG vest (P<0.01, flow-through respirometer vs. dual chamber + vest). $\dot{V}CO_2$ measured in the flow-through respirometer chamber (Fig. 3Be) averaged 37 ± 5 ml/min/kg, which was not different than that measured in the dual chamber (32 ± 7 ml/min/kg). $\dot{V}CO_2$ measured in the dual chamber while pups wore the EKG vest averaged 30 ± 5.5 ml/min/kg (P<0.001 compared to the flow-through respirometer chamber, Fig. 3Be). RER averaged 0.77 ± 0.03 in the flow-through respirometer, 0.81 ± 0.06 in the dual chamber and 0.81 ± 0.06 when the dual chamber was combined with the EKG vest (P=NS, Fig. 3Bf). The gas convection requirement for O_2 ($\dot{V}_I$ / $\dot{V}O_2$) averaged, respectively, 36 ± 5 and 42 ± 6 in the dual chamber and in the dual chamber with the pup wearing the EKG vest (Fig. 3Bg, P<0.05). The $\dot{V}_I$ / $\dot{V}CO_2$ ratio averaged 44 ± 5 and 52 ± 7 in the dual chamber and the dual chamber plus vest (Fig. 3Bh, P<0.05).

We analyzed heart rate in each pup under each of three conditions; resting quietly on a paper towel; in the head-out plethysmograph; and in the dual chamber plethysmograph. Heart rate averaged 354 ± 29 on the paper towel, 368 ± 49 with the pup in the head-out plethysmograph, and 366 ± 24 b/min with the pup in the dual chamber (P=NS, Fig. 3Bi).

The baseline and steady state ventilatory and metabolic rate response to the addition of compressed gas containing 12% O_2, 5% CO_2, balance N_2 is shown in a representative animal in Fig. 4A. Chemoreceptor stimulation increased both frequency (120-to-168 breaths/min) and tidal volume (98-to-185 µl/breath) in this animal. The data for all six pups show that frequency

averaged 105 ± 28 breaths/min at baseline, and 114 ± 22 breaths/min during hypoxic hypercapnia (Fig. 4B, P=0.2786); V_T averaged 82 ± 22 µl/breath at baseline and 143 ± 52 µl/breath in hypoxic hypercapnia (Fig. 4C, P=0.0093); $\dot{V}_I$ averaged 1,253 ± 224 ml/min/kg at baseline and 2360 ± 722 ml/min/kg in hypoxic hypercapnia (Fig. 4D, P=0.016); and the ventilatory equivalent for CO_2 ($\dot{V}_I/\dot{V}CO_2$) averaged 27 ± 6.3 at baseline, and rose to 48 ± 21 in hypoxic hypercapnia (Fig. 4E; P=0.0343).

Discussion

We compared measurements of pulmonary ventilation, metabolic rate, and heart rate obtained with a homemade dual chamber plethysmograph to values measured using head-out plethysmography and unrestrained flow-through respirometry in neonatal rodents. To the best of our knowledge, this approach has not been used previously, and we are unaware of other direct comparisons of different methods for measuring ventilation and metabolism in neonatal rodents. We compared measurements of pulmonary ventilation made with the dual chamber with those made with standard head-out plethysmography in each of 10 pups. There was no difference in breathing frequency measured in the head-out versus dual chamber configuration, though values for V_T and $\dot{V}_I$ were significantly higher in the dual chamber. On average, $\dot{V}_I$ was 20% higher in the dual chamber than in the head-out chamber (1801±393 vs 1445±207 ml/min/kg), and the data were more variable (coefficient of variation 22 vs. 14%). However, the difference was consistent, with 9 of the 10 pups having a higher value in the dual chamber than in the head-out chamber. The V_T measured in the dual chamber was higher than that measured in the head out plethysmograph alone. This finding was not anticipated, and at this time we have no reasonable explanation for the discrepancy. We also found that both $\dot{V}O_2$ and $\dot{V}CO_2$ were lower in the dual chamber than in the flow-through respirometer in 8 of 10 pups. The RER

measured in the dual chamber was the same as that measured with the flow-through respirometer, indicating that the difference in $\dot{V}O_2$ and $\dot{V}CO_2$ between the methods were of similar magnitude.

Average values for ventilation and metabolism measured in neonatal rodents within the same age range that we studied, but using several different methods, have been reported. The values obtained with the different approaches are quite similar, especially when considering the inherent variability in these technically demanding measurements. (Mortola et al., 1989) used the manometric measurement in a closed chamber with a CO_2 absorber to measure $\dot{V}O_2$, and in separate experiments measured ventilation with head-out plethysmography. Our values for $\dot{V}_I$ measured with head-out plethysmography were slightly higher than values reported by Mortola et al (1445 vs. 1264 ml/min/kg). Likewise, our values for $\dot{V}O_2$ were 47 and 40 ml/min/kg in the flow-through respirometer and dual chamber, respectively, whereas Mortola et al reported a value of 34.5 ml/min/kg using a sealed respirometer. (Bavis et al., 2010) used a head-out plethysmograph similar to that used by (Matsuoka and Mortola, 1995) to measure pulmonary ventilation, but measured $\dot{V}O_2$ separately using a flow-through respirometer. Their reported values for $\dot{V}_I$ (roughly 1400 ml/min/kg) and $\dot{V}O_2$ (46 ml/min/kg) were also well within the range reported here. Using a commercial system, (Niane et al., 2009) report average values for $\dot{V}_I$ and $\dot{V}O_2$ of 1790 and 54 ml/min/kg, respectively, in 4-day old rat pups.

The most similar approach to the dual chamber described here is one designed by (Matsuoka and Mortola, 1995), and subsequently incorporated into commercial systems. Their approach was to place the animal in a rear chamber, with the head entering a front chamber through a hole cut in a thick layer of Parafilm. The Parafilm separated the animal's head from the body. The rear chamber was for measurement of breathing pattern with head-out

plethysmography, and the front chamber was used to measure $\dot{V}O_2$ with a flow-through method, as used here. In these studies, the average $\dot{V}O_2$ in 2-8-day-old rat pups ranged from 32-46 ml/min/kg depending on age, which compares favorably to the average of 40 ml/min/kg that we obtained using the dual chamber in pups of approximately the same age. We initially tried the chamber described by Matsuoka and Mortola, but we had difficulty keeping the pup from breaking the Parafilm seal with head movement. Perhaps the thick seal used by Matsuoka and Mortola, which requiries up to four layers of Parafilm, covers more of the face and head leading to irritation and squirming. However, we acknowledge that any method that requires a flexible neck seal is inherently prone to leaks, and vigilance is required to ensure that leaks are detected and corrected with vacuum grease or other sealants. We also noted that the increased movement of the animal when fitted with the thick seal creates pressure artifacts as the forward and backward movement of the seal alters chamber volume, an artifact that has been noted previously (Flandre et al., 2003). The neck seal that we use is made from a thin, single layer of latex cut from a glove and this approach is well tolerated by most neonatal rat pups.

(Avraam et al., 2015) used another clever approach to measure ventilation and metabolism in mouse pups, that involves sealing a face mask to the snout, and placing the pup in an enclosed chamber but with the pup's mask externalized through a gasket in the wall of the chamber. The externalized mask is connected to a bias flow system, with a pneumotach detecting changes in airflow and a gas analyzer measuring the exhaled O_2 and CO_2 concentrations. This approach yielded values well within the range reported by others and us, with an average $\dot{V}_I$ of 1012 ml/min/kg, and an average $\dot{V}O_2$ of 43 ml/min/kg. Though this approach (Avraam et al., 2015) has many advantages, it does add dead space and likely changes the configuration of the upper airway. Moreover, because the mask is attached to a fixed gasket the animal must remain still to avoid breaking the seal, and head movement is not possible. On the other hand, this method and others that use a facemask allow faster changes

in test gas concentrations owing to the small size of the mask compared to the relatively large chamber volume required with our method.

Though there are many ways to obtain ventilatory and metabolic rate measures in neonatal rodents, we are unaware of other quantitative, randomized, repeat-measures comparisons of some of the more commonly used approaches. Although we report some differences in absolute measures of $\dot{V}_I$ and $\dot{V}O_2$ between the methods tested, in our view, the systematic nature of the differences provides confidence that the dual chamber can be used to assess differences between treatment groups, or between interventions within a treatment group. We do not yet understand the reason for the lower $\dot{V}O_2$ in the dual chamber compared to that measured in a traditional, flow-through respirometer. It could simply be the result of the larger volume of the dual chamber, which reduces the resolution of measures of effluent O_2 and CO_2 concentrations (Bavis et al., 2010; Lighton, 2017). Or it could simply reflect reduced movement due to the restraint of the head-out plethysmograph. Support for the latter comes from the observation that metabolic rate was lower still when the dual chamber also included the additional restraint offered by the EKG vest (Figs. 3d and 3e). Other possibilities include isolation from the environment in the dual chamber, bringing the metabolic rate closer to its true resting value, or perhaps inducing sleep, which we did not document. An important caveat is that the slightly higher $\dot{V}_I$ and lower $\dot{V}O_2$ in the dual chamber compared to corresponding values obtained with traditional head out plethysmography and the respirometer results in a higher $\dot{V}_I/\dot{V}O_2$ ratio; the ramifications of this potential error should be considered when designing experiments that will be carried out using the dual chamber. We also assessed the response to hypoxic hypercapnia as a functional test of the dual chamber in six neonatal rats (3 Males, 3 Females). $\dot{V}_I$ increased from 1,253 ml/kg/min at baseline to 2,360 ml/kg/min in hypoxic hypercapnia, which is similar to ventilatory responses obtained with the same level of hypoxic

hypercapnia in earlier studies (Abu-Shaweesh et al., 1997; Huang et al., 2010; Saetta and Mortola, 1987).

A key motivation for designing and evaluating this device was to provide a low-cost option for investigators that do not want to invest in a commercial plethysmography system. We estimate that assembling our system from scratch could be done for about 18,000 USD, or much less if some equipment is already available, and/or used equipment is obtained. In contrast, we received a quote of 68,000 USD for a commercial, “turnkey” system that purports to provide accurate values for ventilation and metabolism in neonatal rodents. In conclusion, the dual chamber approach described here adds an inexpensive option for the simultaneous measurement of pulmonary ventilation and metabolic rate in neonatal rodents.

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Additional information.

Competing interests. There are no competing or conflicts of interest.

Author contributions. Christine Hoyer-Kimura designed the chamber, Brennan Boyd collected and analyzed all the data, Lila Wollman consulted on data interpretation and edited the manuscript, and Ralph Fregosi designed the experiments, made the graphs and figures, conducted the statistical analyses, and wrote the manuscript.

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FIGURE LEGENDS.

Fig. 1. Design of the dual chamber. *Panel A*. The "chamber-in-chamber" design is comprised of a standard, homemade head-out plethysmograph for measuring breathing frequency and airflow, with the plethysmograph inserted into the larger outer chamber. The lid is applied, and gum rubber is used to seal the access slot. Air is pulled through the outer chamber by the O_2 and CO_2 analyzer for open circuit measurement of metabolic rate. The design and use of the dual chamber plethysmograph are described in detail in *Methods*. *Panel B*. The vest with homemade electrodes for measuring heart rate (see *Methods*). *Panel C.* Schematic diagram of the dual chamber interfaced with necessary equipment for recording pulmonary ventilation, metabolic rate, and heart rate. The system is described in detail in *Methods*.

Fig. 2. Performance of the dual chamber. *Panel A*. Changes in CO_2 and O_2 concentrations as gas is introduced into the dual chamber, configured for normal use, including an animal in the chamber (9 g, P4 female rat pup). For this experiment we used a calibrated Matheson rotameter set to deliver gas at a rate of 155 ml/min. We set the flow rate of the gas analyzer at 150 ml/min. The test gas was certified to contain 12% O_2, 5% CO_2, balance N_2. Thus, the rate of incoming gas was just above the rate at which gas was removed from the chamber. As shown on the figure, the time constant for a change to roughly 63% of the steady state value was 54.8 and 46.6 sec for CO_2 and O_2, respectively. *Panel B*. We were concerned that the animals may sense the addition of compressed gas into the chamber, leading to increased vigilance and changes in $\dot{V}_I$ and/or heart rate. Accordingly, in 6 pups we recorded airflow and the EKG before (shown to the left of the downward arrow in panel B), and after the addition of compressed air. As shown in this recording, there are no discernible effects of gas inflow on breathing or heart rate. The average data for the 6 pups is given in Results.

Fig. 3. **Representative recording and summary of method comparison data**. *Panel A*. From top down, the EKG, the time integral of inspired airflow, the peak height of which is

equivalent to inspired tidal volume; airflow signal from the pneumotach (inspiration upwards), and the CO_2 and O_2 concentrations (in percent) in the chamber effluent gas. *Panel B*. Ten neonatal rats aged P1-P5 were studied under each of several conditions: in the head-out chamber alone; in the dual chamber; in the head-out chamber but wearing the EKG vest; in the dual chamber but wearing the EKG vest. *Panels* Ba, Bb and Bc show $\dot{V}_I$ Vt and frequency in each condition; *Panels* Bd, Be and Bf show $\dot{V}O_2$, $\dot{V}CO_2$ and the RER, and *Panels* Bg, Bh and Bi show $\dot{V}_I/\dot{V}O_2$, $\dot{V}_I/\dot{V}CO_2$ and heart rate. Data in *Panels* Ba-Bf and Bi were analyzed with one-way ANOVA and Bonferroni-corrected post-hoc comparisons. Data in *Panels* Bg and Bh were analyzed with an unpaired t-test. A detailed description of the statistical results is provided in *Methods* and *Results*. *, P<0.05; **, P<0.01; ***, P<0.001.

Fig. 4. **Representative recording and summary of the ventilatory response to chemoreceptor stimulation obtained with the dual chamber**. *Panel A.* Use of the dual chamber to measure the ventilatory response to chemoreceptor stimulation. The breath cycles shown during hypoxic hypercapnia were obtained in the steady state, at approximately 4 min after the test gas was introduced into the chamber. From top down, the traces show the concentrations of CO_2 and O_2 in the chamber effluent, inspired tidal volume, and pulmonary airflow, with inspired flow represented by downward deflections. *Panels B-E* show pulmonary function data from each of the six animals that were exposed to inspired gas consisting of 12% O_2, 5 % CO_2, balance N_2 (“Hypoxic Hypercapnia”). For each animal, the average data computed under baseline conditions (BL, filled circles) and hypoxic hypercapnia (H/H, unfilled circles) are shown. Note that frequency did not change significantly, though V_T, $\dot{V}_I$ and $\dot{V}_I/\dot{V}CO_2$ all increased significantly. *, P<0.05 for BL vs. H/H; **, P<0.01 for BL vs. H/H.