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A low cost, simplified, and scaleable pneumotachograph and face mask for neonatal mouse respiratory measurements

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Abstract

Introduction—Neonatal respiratory disorders are a leading cause of perinatal mortality due to complications resulting from premature births and prenatal exposure to drugs of abuse, but optimal treatments for these symptoms are still unclear due to a variety of confounds and risk factors. Mouse models present an opportunity to study the underlying mechanisms and efficacy of potential treatments of these conditions with controlled variables. However, measuring respiration in newborn mice is difficult and commercial components are expensive and often require modification, creating a barrier and limiting our understanding of the short and long-term effects of birth complications on respiratory function.

Methods—Here, we present an inexpensive and simple flow through pneumotachograph and face mask design that can be easily scaled for parallel, high-throughput assays measuring respiration in neonatal mouse pups. The final apparatus consists of three main parts: a water-jacketed chamber, an integrated support tray for the pup, and a pneumotachograph consisting of a two side-arm air channel that is attached to a pressure transducer.

Results—The pneumotach showed a linear response and clean, steady respiratory traces in which apneas and sighs were clearly visible. Administration of caffeine in P0.5 CD1 wildtype neonates resulted in an increase in tidal volume, minute ventilation, and minute ventilation normalized to oxygen consumption as well as a decrease in periodic instability.

Discussion—The described methods offer a relatively simple and inexpensive approach to constructing a pneumotachograph for non-invasive measurements of neonatal mouse respiration,

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enhancing accessibility and enabling the high-throughput and parallel characterizations of neonatal respiratory disorders and potential pharmacological therapies.

Keywords

respiration; breathing; newborns; neonate; mouse; pneumotach; plethysmography; Newborn Abstinence Syndrome; Sudden Infant Death Syndrome

1 Introduction

Several techniques exist to measure respiration in neonatal mice, but each has its limitations¹. One technique which has proven very successful for studies in enabling the measurement of respiratory parameters in both wild-type mice treated with a drug, as well as mutant animals with respiratory deficits²⁻⁶ is head-out plethysmography consisting of a pneumotachograph and face mask, which overcomes the limitations of low signal-to-noise ratios, limited data collection windows, and the lack of absolute tidal volume measurements of other techniques. However, current commercial pneumotach systems are 1) designed for adult mouse measurements with less sensitivity for neonate applications; 2) prohibitively expensive for multiple units; and 3) must be yet further modified or adapted for use in neonate measurements.

Our goal was to lower the barrier of entry for these types of studies by reducing costs and making available expanded, explicit instructions that build off of prior work⁷. Thus, we sought to build upon existing head-out pneumatochography methods^{2,7,8} to generate an inexpensive and simple design for producing a face mask and pneumotach combination with minimal dead space for highly sensitive measurements specifically in un-anesthetized newborn mice. Our low-cost strategy and straightforward construction (using inexpensive, simple tools that are widely available for <\$150 USD) enables the production of multiple pneumotach based respiratory measurement stations for parallel high throughput studies to accommodate study of respiratory deficits and screening of potential therapeutic drugs for prenatal drug exposure, premature birth deficits, and congenital respiratory defects that may be found within international mouse mutagenesis efforts⁹. To validate the applicability of our system, we treated wild type P0.5 mouse pups with saline (control) and caffeine, a respiratory stimulant and established therapy for neonatal apneas. We found that administration of caffeine resulted in respiratory phenotypes that mimicked those seen in previous studies, demonstrating the potential application of our approach and setting the stage for parallel high-throughput studies of neonatal respiratory dysfunction and potential treatments.

2 Methods

2.1 Mice

Timed pregnant CD1 DAMs were monitored for parturition. All respiratory measurements were made between 7 am and 4 pm the day of birth. All experimental animals were treated in compliance with the United States Department of Health and Human Services and the Baylor College of Medicine IACUC guidelines.

2.2 Pneumotach Design and Construction

In head-out plethysmography, a head-out mask attached to the snout is connected to a pneumotach consisting of a single central channel connected to two side-arm channels for pressure measurements⁶⁻⁸. Air is drawn through the pneumotach central channel and into the mask by an attached vacuum pump through a gas flow-through port in the face mask resulting in a constant turnover of the gas in the facemask and the ability to regularly sample expired gasses. As the air flows through the central channel, pressure that is proportional to the flow rate is created along the channel walls. During inhalation and exhalation, the flow rate in the central channel is increased and decreased proportionately to the lung volume displacement. This change in flow as a function of lung volume also results in a change in the central channel pressure, which can be measured at the side arm channel ports⁷. This method of measurement provides an accurate representation of tidal volume and enables longer continuous studies over various gas compositions and treatments while normalizing breathing to metabolic rate with the use of in-line gas analyzers²⁻⁴.

The design of our apparatus used to monitor the breathing of the mouse pups consisted of three main components: a water-jacketed chamber for body temperature stabilization as mouse neonates do not thermoregulate, particularly when separated from the litter¹⁰, a removable tray that supports the mouse pup and forms one-half of the face mask, and a pneumotachograph body that contains the other half of the face mask and ports for pressure measurements, calibration and gas flow-through for downstream sampling of expiratory gasses (Fig. 1A,F and Fig. 2). The measurements given here are appropriate for P0.5-P1.5 mouse neonates (animals between 1-2g in weight) but can be scaled up or down as necessary based on the work of Mortola and Noworaj⁷.

2.2.1 Chamber construction—The main chamber consisted of an outer shell and an inner shell to create a flow-through water jacket for temperature stabilization (Fig. 1B). The outer shell was constructed using a 47.63mm (1(x0215E)) piece of 25.40mm (1") ID, 31.75mm (1¼") OD acrylic tube, a 3.175mm (1/8") × 50.80mm (2") × 50.80mm (2") acrylic sheet and a 25.40mm (1") × 12.70mm (1/2") × 12.70mm (1/2") piece of acrylic bar stock. One open face of the tube was solvent welded to the acrylic sheet using Weld-On #4 and #16 acrylic solvents to ensure a watertight seal. The sheet was routed to be flush with the tube. A 25.40mm (1") × 12.70mm (1/2") face of the acrylic block was then solvent welded on to the back of the acrylic sheet. Two intersecting 5.105mm (.201") holes and one 3.175mm (1/8") hole were drilled through this block to provide a way to mount the chamber onto a rail connected to a magnetic base. The 5.105mm holes were tapped with a ¼-20 UNC tap so that 6.35mm (¼") machine screws could be used to lock the apparatus to the mounting rod. The inner shell was made from a 42.86mm (1 1/16") piece of 19.05mm (¾") ID, 25.40mm (1") OD acrylic tube, which was welded onto a 3.175mm (1/8") thick acrylic sheet with Weld-On #4 and Weld-On #16 acrylic solvents and routed to be flush with the tube. After this, the outer wall of the inner shell was milled down 1.59mm (1/16") starting 3.175mm (1/8") back from the open end and going all the way to the closed end. This is ideally done using a lathe but can also be accomplished with a CNC mill or even just a Dremel tool. This provides space for water to circulate around the chamber. The inner shell was then inserted into the outer shell until the open ends were flush and the pieces were solvent welded together. Two

3.175mm ($\frac{1}{8}$ ") channels were then drilled through the outer shell to be inlet and outlet ports for temperature controlled water. One port was on top of the chamber near the open face while the other was on the bottom near the capped end. Polycarbonate luer fittings were also solvent welded onto these ports using #16 acrylic solvent to facilitate connecting of the circulating water bath.

2.2.2 Pup Tray Construction—The pup was placed in a tray that slides into the water-jacketed chamber. This tray was made of a 41.28mm (1(x0215D)") long piece of 12.70mm ($\frac{1}{2}$ ") ID, 19.05mm ($\frac{3}{4}$ ") OD acrylic tube of which the top half was removed from the rear 34.93mm (1(x0215C)") of the tube (Fig. 1D). The edges of this tube were well sanded with 400 and 1200 grit sandpaper to ensure a smooth edge.

2.2.3 Pneumotachograph Construction and Assembly—To construct the pneumotachograph and face mask, a 25.40mm (1") $\times$ 25.40mm (1") $\times$ 12.70mm ($\frac{1}{2}$ ") acrylic block was used to form the pneumotachograph, and a 6.35mm ($\frac{1}{4}$ ") piece of 6.35mm ($\frac{1}{4}$ ") ID, 12.70mm ($\frac{1}{2}$ ") OD acrylic tube was used to form the face mask (Fig. 1C). Also two small thick neodymium magnets (6.35mm ($\frac{1}{4}$ ") diameter, 1.59mm ($\frac{1}{16}$ ") thick), a 17.46mm (.69") $\times$ 12.70mm ($\frac{1}{2}$ ") piece of 1.59mm ($\frac{1}{16}$ ") thick super-soft adhesive backed silicone rubber and a 17.46mm (.69") $\times$ 6.35mm ($\frac{1}{4}$ ") $\times$ 12.70mm ($\frac{1}{2}$ ") acrylic piece were used to build a bell housing for near square wave gas switching (Fig. 1E). To build the pneumotachograph, a .79mm ($\frac{1}{32}$ ") central channel was drilled 4.76mm ($\frac{3}{16}$ ") up from a 12.70mm ($\frac{1}{2}$ ") side on one of the 25.40mm (1") $\times$ 12.70mm ($\frac{1}{2}$ ") faces of the acrylic block. This face can be referred to as the front and the 12.70mm ($\frac{1}{2}$ ") side measured from is the bottom. Two .79mm ($\frac{1}{32}$ ") side arms were drilled on the bottom to intersect the first channel. The side arms were 8mm apart and the first placed about 8.7mm from the front edge. Also, two .79mm ($\frac{1}{32}$ ") diagonal channels were drilled both starting in the center of the front face 1.59mm ($\frac{1}{16}$ ") above the first channel and terminating on the center of either side. These were drilled to create a port for calibration injections and a port to attach to a vacuum pump and rotameter for gas flow through control. The acrylic tube piece was then solvent welded to the front around the airflow channels to act as a face mask for the pup. Opposite the face mask, a 6.35mm ($\frac{1}{4}$ ") hole was drilled 12.70mm ($\frac{1}{2}$ ") from the top and 1.59mm ($\frac{1}{16}$ ") deep, into which one of the magnets was fixed with super glue. Also, a 5.105mm (.201") hole was drilled 4.76mm ($\frac{3}{16}$ ") from the top and 12.70mm ($\frac{1}{2}$ ") deep and tapped with a $\frac{1}{4}$ -20 UNC tap for mounting. One more 5.105mm (.201") hole was drilled and tapped into the top, 9.53mm ($\frac{3}{8}$ ") from the back edge to intersect the mounting hole, and a 6.35mm ($\frac{1}{4}$ ") through hole was drilled into the side to intersect the first mounting hole. To facilitate the connection of tubes, blunt-ended 22 gauge needles were press fitted into the two air channels on the bottom face and the two air channels on the side face thus allowing tubes with luer connections to be easily attached. For the bell housing to enable gas inflow switching, within the small acrylic piece, a 1.59mm ($\frac{1}{16}$ ") air channel was drilled into a 17.46mm (.69") $\times$ 12.70mm ($\frac{1}{2}$ ") face, 6.35mm ($\frac{1}{4}$ ") from a 12.70mm ($\frac{1}{2}$ ") edge. On the same face, a 6.35mm ($\frac{1}{4}$ ") hole positioned 12.70mm ($\frac{1}{2}$ ") from the 12.70mm ($\frac{1}{2}$ ") edge was drilled 1.59mm ($\frac{1}{16}$ ") deep into which the second magnet was affixed. Also, a 1.59mm ($\frac{1}{16}$ ") deep rectangle was milled out of this face. The rectangle was 6.35mm ($\frac{1}{4}$ ") wide and 7.94mm ($\frac{5}{16}$ ") tall in order to surround the 1.59mm ($\frac{1}{16}$ ") air channel and extend all the way

to the bottom edge of the acrylic piece. On the opposite face, a polycarbonate luer fitting was solvent welded over the 1.59mm ($\frac{1}{16}$ ") hole for a gas connection. This small piece is intended to magnetically attach to the pneumotachograph to provide a bell housing for near square-wave gas switching. Finally, the rubber was cut to match the face with the milled rectangle and attached with the adhesive side to the milled face.

When assembled, the face mask and pneumotach have a total volume of 0.22878 mL. This is a significant reduction of the amount of dead space when compared to the reported 3-4mL face mask only volume using a cut syringe for P8 juvenile measurements^{2,4}. An exploded view of the set-up can be seen in Fig. 1F.

If building pneumotachographs with bulk raw materials, these chambers can be built for about \$3.68 apiece. More realistically, with the available order sizes of the raw materials, the parts for 10 of these chambers will cost \$114.82 or \$119.22 for 15 chambers (about \$8 each). All acrylic machining can be done with a Dremel rotary tool attached to a Dremel work station and cross vise for a one time cost less than \$100 USD. Tooling, man hours, and materials for 15 chambers is still less than a single commercial pneumotachograph (ex: Harvard Apparatus Pneumotachometer, \$404 USD) which must be yet further modified to include a facemask and a separate water jacketed chamber must be built or ordered (ex: Glass water jacket from Vitriforms, \$200 USD).

2.3 Experimental Design

P0.5 respiration was measured in the custom built head-out mask-pneumotachograph system described above. For calibration and experiments, room air was drawn through the mask-pneumotachograph and O₂ and CO₂ gas analyzers (AEI) by a vacuum pump (AEI) attached to the gas flow-through port on the mask. The rate of gas flow-through was continuously controlled via a rotameter. In our experiments, we used a flow rate of 12.5 mL/min. For calibration prior to an experiment, the face mask was sealed with a piece of nitrile rubber (cut from a glove, Fig. 2B). Gas composition was calibrated by drawing defined gas mixtures from the gas flow-through port that were fed through the bell housing attached by a magnet to the end of the pneumotachograph (Fig. 1E). During gas composition calibration, ventilation was also calibrated as a series of 20 μ L pipetman injections via a second port into the empty facemask at a rate of 3Hz. Pipette injections of increasing volume showed a linear response from 20 μ L up to 200 μ L (Fig. 3).

For experimental assays following calibration, a small opening was made in the nitrile rubber to fit the snout (nose and mouth) of a P0.5 mouse. The mouse was taken out of its home cage, weighed, and then a circle of Impregnum F, Polyether Impression material^{2,11} was applied to its face (Fig. 2D). The pup was then affixed to the face mask with the snout protruding through the circular opening and held in place until the Impregnum material had solidified (~1 minute) (Fig. 2E). The mouse rested on a platform attached to the face mask that fit inside a temperature controlled chamber (36°C) to maintain the mouse pup at 34-35°C (Fig. 2F).

Pneumotachograph pressure changes were measured at the side arms by a Validyne DP45 differential pressure transducer and CD15 carrier demodulator and recorded with

LabChartPro in real time. The pneumotachograph trace was integrated to produce a respiratory waveform. Waveforms were analyzed offline using LabChartPro and custom written MATLAB code to determine respiratory rate (V_f), tidal volume (V_T), minute ventilation (V_E) and pattern analysis. O_2 consumption was determined by comparing the gas composition between calibration in the empty chamber without the mouse pup and live breathing using an AEI oxygen sensor and analyzer. Chamber temperature was constantly monitored using a ThermoWorks MicroThermo 2 and probe and was recorded with LabChartPro in real time.

After attachment to the face mask, mice were allowed to acclimate for 10 minutes in room air. Data was recorded for another 20 minutes under room air conditions. Neonates were then injected interscapularly with caffeine dissolved in saline at a concentration of 20 mg/kg or an equivalent volume of saline. Data was recorded for 30 minutes post-injection.

2.4 Data analysis

Respiratory waveforms were collected when the neonate was at rest and readings were free from movement artifacts. There was usually some amount of physical movement upon initial placement of the pup into the face mask (generally 1-5 minutes). Pups were thus allowed to acclimate for the first 10 minutes after placement, during which no data was used. After acclimation, limited physical movement was seen in the pups (obvious as a large deviation of the whole waveform from zero as well as camera monitoring), and was excluded from data analysis. A minimum of 1 minute cumulative data compiled from traces at least 10 seconds long from the last 10 minutes of a given experimental condition were analyzed. Apart from integration, no filtering, smoothing or other manipulations were applied to the pressure waveform. Tidal volume (V_T) was determined by comparing peak (mV) height to calibration injections (mV/ μ l) after we demonstrated that the construction showed a linear response to volume injections (Fig. 3A).

Poincaré plots and sigh and apnea measurements were determined using 2 minutes of movement-free traces from each breathing condition. Poincaré plots are generated by plotting either the breathing period or amplitude versus that of the subsequent breath in a continuous series of success breaths. The Peak Analysis package in LabChart was used to measure the intervals between and the amplitude of each peak. The data were exported to Excel to determine the number of apneas and sighs, length of apneas, and coefficients of variation and the primary data was plotted using MATLAB. Sighs were defined as a breath with an amplitude at least twice as large as the average breath. Apneas were defined as an interbreath interval (IBI) that was longer than 1.5 seconds. The coefficient of variation (CV) of the IBI and amplitude was also calculated from the same 2-minute trace compilation of each breathing condition (standard error IBI or amplitude / mean IBI or amplitude).

2.5 Statistics

Results (V_f , V_T , V_E , V_{O_2} , V_E/V_{O_2} , number of sighs, number and length of apneas, and CVs of IBI and amplitude) were compared between all cohorts using a linear mixed-effects regression model with injection type (caffeine and saline) and time point (pre or post injection) as fixed effects and animal ID as a random effect with MATLAB. The p-values

reported correspond to the statistical significance of the difference between caffeine and saline post-injection vs. pre-injection. A p-value of <0.05 was used to indicate statistical significance. Standard error of the mean is shown on all charts.

3 Results

Our pneumotach design provided steady, regular respiratory traces in which both apneas and sighs were clearly visible and detectable (Fig. 4). In order to demonstrate the applicability of our experimental design and construction, P0.5 CD1 mouse neonates were treated with caffeine, a respiratory stimulant and common neonatal therapy used for premature apneas. Caffeine has been shown to increase overall ventilation (V_E) and reduce the number of apneas in a hypoxic exposure rat pup model¹².

After caffeine administration, P0.5 neonates ($n=11$) showed significant increases in V_T ($p=0.01$), V_E ($p=0.0006$), and V_E/V_{O_2} ($p=0.009$) as compared to pre-injection conditions and saline controls ($n=11$) (Fig. 5). In both conditions, neonates showed increased V_f ($p=0.002$) and V_{O_2} ($p=0.0002$) after injection with no difference between caffeine and saline injections. There was a trend towards a reduced number of apneas after caffeine administration ($p=0.08$) compared to an increase in saline controls and no difference in number of sighs or average apnea length (Fig. 6). Poincaré analysis of interbreath interval and amplitude showed that caffeine administration resulted in a reduction in periodic instability as indicated by the coefficient of variation ($p=0.04$) (Fig. 7) and a small but statistically insignificant reduction in volume instability ($p=0.18$) in neonates treated with caffeine as compared to saline controls (Fig. 8).

There were no differences in pre-injection baseline respiratory parameters between those treated with saline and those treated with caffeine.

4 Discussion

We present a precision-constructed side-arm pneumotach and face mask for measuring respiratory parameters in un-anesthetized P0.5 mouse neonates. This design on a well established approach provides an accurate and reliable measure of breathing frequency, tidal volume, oxygen consumption, and pattern parameters in newborn mouse pups and has a significant reduction in the pneumotach and face mask dead space (>2.7 mL) as compared to that reported in previous studies⁴. Apparatus dead space should be minimized to avoid rebreathing of expired air. It may be possible to further reduce the dead space in our apparatus, as the 0.228 mL volume still represents a substantial volume compared to the ~ 10 μ L tidal volume of a P0.5 mouse. A second limitation of head-out plethysmography is potential stress induced by restraint in the face mask. To minimize this confound, all our measurements were taken at least 20 minutes after the animal was placed into the face mask during movement free periods.

The construction measurements given are appropriate for animals between 1-2g in weight but can be scaled up for older/heavier animals if needed, using the parameters published by Mortola and Noworaj⁷. Importantly, the whole system can be easily manufactured with a simple and inexpensive set of tools with minimal material costs to produce an accurate and

sensitive pneumotachograph system suited for neonate studies, in contrast to expensive commercial components designed for adult mouse studies that require further adaptation. This method can also be used to quantify the effects of gas challenges (i.e. low O₂ or high CO₂) using an additional bell housing apparatus that can be placed over the channel through which air is drawn into the face mask. We expect that this system can be easily combined with EEG leads and pulse oximetry to simultaneously measure cardiovascular parameters to further inform upon neonatal phenotypes, as in other studies¹³.

The results of this study demonstrate the practicality of the pneumotach device design, using a common and validated neonatal treatment with known respiratory effects. Our results are confirmed by previous studies showing that caffeine administration increases overall ventilation and reduces apneas in a hypoxic exposure rat pup model¹². We see a similar phenotype in P0.5 neonates upon caffeine administration as compared to saline controls, with increased V_T, V_E, V_E/V_{O2}, and decreased periodic instability. While one previous study in P8-9 mouse pups did not see significant changes in ventilation after caffeine administration, this may be due to a difference in caffeine sensitivity in younger pups, the higher concentration used in this study, or differences in strain³.

We believe that this design approach to neonate pneumotachography will provide a valuable alternative to expensive commercial components that must be further adapted for studying the mechanisms behind neonatal respiratory dysfunction and efficacy of potential treatments. The inexpensive cost and ease of construction lowers several barriers to enable high-throughput parallel screening of potential therapeutic drugs, the screening of genetic mouse models toward defining disease mechanisms, and functional circuit mapping to define druggable neural correlates for a range of congenital respiratory disorders.

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Abbreviations

V_f	respiratory rate (breaths/min)
V_T	tidal volume (mL/breath/g)
V_E	minute ventilation (respiratory rate $\times$ tidal volume, mL/min/g)
V_{O_2}	oxygen consumption (mL/min/g)
V_E/V_{O_2}	minute ventilation normalized to oxygen consumption

Figure 1. Construction and schematic of the water-jacketed chamber, face mask and pneumotach design

Shown is an overall drawing of all of the parts (A) as well as specific schematics and photos for the outer and inner shell of the main chamber (B), the pneumotachograph and face mask (C), the holding tray for the neonatal mouse pups (D), and the pneumotach attachment for near square-wave gas switching (E). An exploded schematic of the components can be seen in (F).

Figure 2. Photos of the system and set-up for measuring mouse neonate respiration

Shown is a photo of the constructed set-up in the lab (A) with labels for tubing and connections. First, a piece of nitrile rubber (here, cut from a piece a glove) is used to seal the face mask for calibration (B). Following a series of 20 μ l pipetman injections, a small circular opening was made in the nitrile rubber with a scalpel (C). A layer of Impregnum F, Polyether Impression material was applied to the face of a pup (D) and the pup was then affixed to the face mask with the snout protruding through the circular opening and held in place until the Impregnum material had solidified (E). The pup tray was then inserted into the heated water chamber to keep the pup at a neutral temperature (F).

Figure 3. Linear signal obtained from increasing volume injections into the pneumotachograph
A) Representative trace showing raw amplitudes from injections of increasing volumes of 20 µL starting at 20 µL up to 200 µL. B) Quantification of peak amplitudes from the raw trace. A linear relationship can be seen between peak amplitude of the trace and volume of the injection.

Figure 4. Representative traces from two P0.5 neonatal pups before and after caffeine and saline injections

With this technique, reliable and accurate respiratory traces are obtained from which absolute breathing frequency and volume can be derived. Both raw (red) and integrated (green) traces are shown in each condition. Before injection (**A, B** and **E, F**), baseline breathing traces are seen with a small level of periodic and volume instability. After caffeine administration (**C, D**), increases in V_f and V_T and a reduction in periodic instability can be seen. After saline administration (**G, H**), an increase in V_f is also seen, but is accompanied by a decrease in V_T and an increase in periodic instability.

Figure 5. Administration of caffeine increases respiratory parameters in P0.5 wildtype mouse neonates as compared to pre-injection conditions and saline controls

Upon caffeine administration, P0.5 neonates (n=11) showed increased V_T (B), $\dot{V}_E$ (C), and $\dot{V}_E/\dot{V}_{O_2}$ (E) as compared to pre-injection conditions and saline controls (n=11). Under both conditions (saline and caffeine), V_f (A) and $\dot{V}_{O_2}$ (D) increased after injection compared to pre-injection baseline parameters. *p<0.05, **p<0.01, ***p<0.001.

Figure 6. Administration of caffeine in P0.5 neonates reduces periodic instability as compared to pre-injection conditions and saline controls

After caffeine administration, P0.5 neonates (n=11) showed a small but statistically insignificant reduction in apneas (B) with no change in number of sighs (A) or average apnea length (C). P0.5 neonates showed a reduced periodic instability after caffeine administration as compared to pre-injection conditions and saline controls (n=11, $p=0.04$) with a small but insignificant decrease in volume instability ($p=0.18$). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 7. Representative Poincaré plots of interbreath interval from P0.5 neonatal pups treated with caffeine or saline

The interbreath interval (IBI) is plotted as a function of the previous IBI. Animals treated with caffeine (**A, B**) showed tighter clustering and a smaller coefficient of IBI variation after caffeine administration (right) than before (left). In contrast, animals treated with saline (**C, D**) showed higher levels of periodic instability after saline injection (right) than before (left).

Figure 8. Representative Poincaré plots of breath amplitude from P0.5 neonatal pups treated with caffeine or saline

Amplitude is plotted as a function of the previous breath's amplitude. Apart from small changes in amplitude, no significant changes in volume instability can be seen in either caffeine (**A, B**) or saline (**C, D**) conditions between pre-injection (left) and post-injection (right) time points.