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Respiratory and Metabolic Effects of Active Expiration in Freely Behaving Rats

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ABSTRACT

Aim: Exposure to low oxygen (hypoxia) or high carbon dioxide levels (hypercapnia) leads to a compensatory increase in pulmonary ventilation. Among the motor changes supporting the respiratory responses is the recruitment of abdominal expiratory muscles (ABD), which can enhance expiratory airflow or alter the duration of the expiratory phase. In this study, we assessed the impact of ABD recruitment on metabolic, motor, and ventilatory parameters in unanesthetized, freely behaving animals.

Methods: Sprague–Dawley Holtzman male adult rats ($n=7$) were instrumented to perform simultaneous recordings of pulmonary ventilation, body temperature, diaphragmatic and ABD activities, and O_2 consumption during exposure (20–30 min) to various levels of hypoxia (12%–8% O_2) and hypercapnia (3%–7% CO_2).

Results: Hypoxia or hypercapnia exposure evoked active expiration (AE); however, ABD recruitment did not occur during the entire exposure period, displaying an intermittent profile. The occurrence of AE during hypoxia and hypercapnia conditions was linked to additional increases in tidal volume when compared to periods without ABD activity ($p < 0.05$) and showed no associations with changes in diaphragmatic burst amplitude. Analyses of flow-like patterns suggested that AE during hypoxia recruited expiratory reserve volume during late expiration, while under hypercapnia, it accelerated lung emptying and increased the expiratory flow peak during post-inspiration. AE was also associated with increased oxygen consumption and did not improve air convection requirement.

Conclusion: AE enhances pulmonary ventilation during hypoxia and hypercapnia primarily by increasing tidal volume. However, this motor behavior may also affect other mechanical aspects of the respiratory system to improve alveolar ventilation and gas exchange.

1 | Introduction

Breathing is essential for maintaining oxygen (O_2) and carbon dioxide (CO_2) physiological levels in the arterial blood. The mammalian respiratory motor activity is regulated by a robust yet dynamic neuronal circuitry in the brainstem that coordinates the activity of respiratory muscles controlling airway resistance and thoracic cavity volume [1]. Within this network, a

group of neurons in the ventral surface of the medulla oblongata forms the so-called pre-Bötzinger complex (preBötC), which intrinsically generates oscillations that determine the respiratory rhythm and drive breathing activity [2, 3]. The oscillatory activity of the preBötC is relayed to pontomedullary premotor and motor neurons that control the pattern (timing, shape, amplitude) of the upper airway, thoracic, and abdominal (ABD) muscles [1, 4]. At rest, inspiration initiates with the contractions of

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muscles (e.g., diaphragm) that expand the thoracic cavity and generate the inspiratory inflow. Simultaneously, dilator muscles of the upper airways (e.g., genioglossal and posterior cricoarytenoid muscles) contract to reduce airway resistance and facilitate airflow into the lungs [1]. With the end of the inspiratory phase, the air is drawn out of the lungs due to inspiratory muscle relaxation and the retraction of the chest and lungs by the elastic recoil forces. Despite being generated passively, the expiratory outflow is regulated during the first stage of expiration (also known as post-inspiration) by the contraction of adductor laryngeal muscles (e.g., lateral cricoarytenoid and thyroarytenoid muscles) that narrow the glottis and reduce the expiratory flow velocity [5]. At the end of the expiratory phase (or stage 2 of expiration), no respiratory muscle activity is observed, and expiration ends as the new respiratory cycle begins.

In conditions of elevated metabolic demand or blood gas disturbances, as seen during physical exercise, high altitudes, or in pathological situations promoting hypoxemia and hypercapnia (e.g., sleep-disordered breathing or obstructive lung diseases), the activity of the respiratory network changes to produce compensatory ventilatory responses. In mammals, O_2 - and CO_2 -sensitive cells located in the periphery and central nervous system (also known as peripheral and central chemoreceptors, respectively) detect reductions in O_2 or elevations in CO_2 levels in the arterial blood and trigger responses of increased respiratory frequency and tidal volume [6–8]. These ventilatory adjustments are primarily associated with increased frequency and amplitude of inspiratory muscle contractions driven by the activation of inspiratory premotor and motoneurons in the brainstem and spinal cord, including the preBötC neurons [9]. Expiration can also become an active process during hypoxemia and hypercapnia, with the presence of rhythmic contractions of the abdominal muscles during the second phase of expiration, exhibiting maximal activity immediately before inspiration [10, 11]. This motor response results from the activation of a conditional expiratory oscillator, located rostral to the preBötC, in the lateral parafacial group (pFL), which connects to premotor expiratory neurons [12–14].

Evidence suggests that abdominal muscle contractions during expiration can enhance pulmonary ventilation through different mechanisms. These include accelerating expiratory flow and reducing expiratory time, which facilitates the onset of the next inspiration. Active expiration (AE) can also recruit the expiratory reserve volume to boost tidal volume or promote additional shortening of diaphragmatic muscle fibers during expiration, thus enhancing the muscle tension–contraction relationship and strengthening subsequent inspiration. However, these possibilities can vary according to the species studied [15] or the stimulus applied, especially considering that hypoxia and hypercapnia can promote distinct time-dependent ventilatory changes [6] and metabolic adjustments [16, 17]. Moreover, most studies exploring active expiration induced by hypoxia and hypercapnia conditions used high-intensity stimuli, reduced experimental models (in situ or in vitro), and/or anesthetized animals in which several physiological parameters were artificially maintained steady (e.g., mechanical ventilation, constant body temperature, and intravenous buffer infusion to control blood pH); some reflex mechanisms were eliminated (e.g., vagotomized, paralyzed), and the activity of the CNS was depressed [10, 12, 13, 18–20]. While these

studies provide relevant evidence regarding the mechanisms of AE, their limitations preclude a comprehensive understanding of the functional impact of forced expiration on pulmonary ventilation under conditions of blood gas disturbances.

In the present study, we simultaneously recorded pulmonary ventilation, diaphragmatic and abdominal muscle activities, and metabolic rate in unanesthetized, freely behaving animals exposed to different levels of O_2 and CO_2 to explore the conditions that evoke AE, its correlation with inspiratory motor activity, the influence of AE on ventilatory parameters, and its correlation with the nature (hypoxia vs. hypercapnia) and intensity of the stimulus.

2 | Methods

2.1 | Animals

The experimental protocols and surgical procedures conform to good publishing practice in physiology [21] and the Brazilian National Council for Animal Care (CONCEA) guidelines and were approved by the Ethics Committee on the Use of Animals of São Paulo State University, Araraquara, Brazil (Protocol no. 17/2020). All experiments were performed on adult Sprague–Dawley Holtzman male rats (250–300g) provided by the animal facility of the School of Dentistry of Araraquara (UNESP/FOAR). Animals received food and water *ad libitum* and were housed on a 12h light–12h dark cycle (lights on at 7:00 am) in a temperature ($23^\circ C \pm 1^\circ C$) and humidity ($50\% \pm 10\%$) controlled environment. The experiments were performed during the light phase between 9 am and 5 pm. Following ethical guidelines, the study used the smallest number of animals necessary to ensure statistically valid and unbiased results.

2.2 | Surgical Procedures

Seven days prior to the experiments, the animals were anesthetized with a mixture of ketamine (100 mg/kg; i.p.) and xylazine (10 mg/kg; i.p.). After confirming the anesthetic plane, under aseptic conditions, a pair of fine electrodes made from insulated stainless-steel wires was implanted into the diaphragm (DIA) and abdominal muscles (ABD) to record their electrical activity. To access the midcostal DIA, an incision was made at the right superior–lateral portion of the abdomen, following the ribcage border. On the contralateral side, the oblique ABD muscles were identified, and the electrodes were implanted. The electrode loose ends were tunneled under the skin and attached to an electrical socket on the dorsal region of the animal's neck. During this surgical procedure, temperature monitoring probes (SubCue Datalogger Standard, Alberta, Canada) were delicately inserted into the animal's peritoneal cavity through the same incision performed to access the DIA. The temperature probes were programmed to record the animals' body temperature every 2 min, starting 4 h before the initiation of the experimental protocol until its end. Incisions were sutured at the end of the procedures, and the animals received antibiotics (penicillin; 30 000 IU, i.m.) and anti-inflammatory medication (ketoprofen; 1 mg/kg, s.c.) and were monitored until they regained consciousness.

2.3 | Assessment of Pulmonary Ventilation and Breathing Pattern

Pulmonary ventilation was assessed using whole-body plethysmography, following the method previously described by Seifert et al. [22]. The animals were kept individually in a closed acrylic chamber (4L) connected to a gas mixer (supplied with pure O₂, CO₂, and N₂ tanks) and a vacuum pump, both set at 1.5L/min to maintain equal input and output flows. This configuration allowed uninterrupted respiratory measurements while the chamber was continuously ventilated with humidified normoxic/normocapnic, hypoxic/normocapnic, or normoxic/hypercapnic air mixtures. The plethysmographic chamber was also equipped with sensors monitoring its temperature and humidity during the experiments. The animals' breathing-related pressure oscillations were detected using a highly sensitive pressure transducer (Spirometer, ADInstruments, Bella Vista, Australia) connected to the chamber. These pressure signals were amplified and acquired in a computer using an acquisition system (PowerLab and Labchart 8, ADInstruments, Bella Vista, Australia; sampling rate: 1 KHz). Before starting each experiment, a small animal ventilator (model 683, Harvard Apparatus, USA) was connected to a port of the chamber and used to inject a known air volume (1 mL) at different frequencies (50, 100, 150, and 180rpm) for volume calibration. From the breathing-related pressure signals, we calculated: (i) respiratory frequency (fR; cycles per minute), derived from the time interval between consecutive respiratory peaks; (ii) tidal volume (V_T, mL·kg⁻¹), determined from the cycle amplitude, taking into consideration the calibration volume, animal and chamber's temperature, barometric pressure, and humidity, according to the equations previously described [23, 24], and (iii) minute ventilation (V̇_E, calculated as the product of fR and V_T (mL·kg⁻¹·min⁻¹). We also analyzed the frequency of sighs (events/h), which were identified as augmented breaths (higher than two times baseline V_T) followed by a prolongation of expiratory time [25].

A first-order derivative was applied to the whole-body plethysmography signals to generate an airflow-like signal (dmV/dt), as previously described [26] and illustrated in Figure 1C. The derivative signals were generated using a 201-point window to optimize the signal-to-noise ratio and were used to evaluate the breathing pattern, focusing on the emergence of active expiration. Under resting conditions, when expiration is passive, expiratory flow initiates with a rapid increase that peaks during the first half of expiration, followed by a slow reduction during the second half of expiration. Active expiration was previously described to be associated with the emergence of a second peak during the end of the expiratory phase [18, 27]. Therefore, we assessed the airflow-like signal pattern to identify changes in the inspiratory and expiratory dynamics associated with motor activity changes during exposures to hypoxia or hypercapnia. We also combined analyses of DIA activity and airflow-like signals to identify and quantify the respiratory phase durations. The phases were defined as follows: (1) inspiration (I) – the period from the onset to the peak DIA activity, coinciding with the inspiratory flow-like deflection; (2) post-inspiration (PI) – the period from the onset of expiration to the end of decrementing DIA activity; and (3) expiratory stage 2 (E2) – the late part of the expiratory phase, during which there was no DIA activity. The duration of each respiratory phase was measured in seconds and expressed as a percentage of the total respiratory cycle duration.

2.4 | O₂ Consumption Measurements

Using a flow-through configuration, samples of the inspired and expired gases were drawn through a column of Drierite (Sigma-Aldrich, USA) and then a gas analyzer (ADInstruments, Bella Vista, Australia) to measure the oxygen consumption (V̇O₂, mL·kg⁻¹·min⁻¹) and carbon dioxide production (V̇CO₂, mL·kg⁻¹·min⁻¹), as previously described [28, 29]. The inflow and outflow airs were sampled continuously, and the values of inspired and expired O₂/CO₂ were recorded during all the experiments (PowerLab and LabChart 8, ADInstruments, Bella Vista, Australia; sampling rate: 1 KHz), providing information about the metabolic rate at rest and under hypoxic or hypercapnic conditions. The minimum duration considered for metabolic rate analyses was 10s, which corresponded to 8 to 15 respiratory cycles. Values of V̇O₂ were also used to calculate the air convection requirement (V̇_E/V̇O₂).

2.5 | Recordings of the Diaphragm and Abdominal Muscle Activities in Freely Behaving Animals

The animals were positioned in the plethysmographic chamber to record the electromyographic DIA (DIA_{EMG}) and ABD (ABD_{EMG}) activities, pulmonary ventilation, and metabolic rate under freely behaving conditions. For this purpose, an insulated and shielded cable was connected to the animal's socket and attached to a swivel on the recording chamber lid. From the lid, a cable was then connected to the amplifier (model P511, Grass Technologies, Middleton, USA) for signal amplification (1000×) and band-pass filtering (10–1000Hz). EMG signals were acquired in a computer using an acquisition system (PowerLab and LabChart 8, ADInstruments, Bella Vista, Australia; sampling rate: 2 KHz). The raw signals were integrated (smoothed, 0.05s), and the muscle activity was calculated as the peak amplitude (mV) of the inspiratory and expiratory bursts observed in the integrated DIA_{EMG} (√DIA_{EMG}) and ABD_{EMG} (√ABD_{EMG}), respectively. The AE pattern was defined by the presence of rhythmic ABD_{EMG} activity interposed with DIA_{EMG} activity above tonic levels for at least 5 respiratory cycles [10, 12, 18, 30, 31]. The incidence of active expiration was calculated as the percentage of time when active expiration was present relative to the total time considered for analyses (minimum of 10 min, after excluding artifacts).

2.6 | Experimental Protocol

We performed simultaneous recordings of pulmonary ventilation, body temperature, DIA_{EMG}, and ABD_{EMG}, and O₂ consumption/CO₂ production with a minimum of 7 days after the surgical procedures. Animals were placed in a plethysmograph chamber and allowed to acclimate for at least 30 min before starting the experiments. The signals were recorded under resting conditions (normoxia and normocapnia, 30 min) and during exposure to different levels of hypoxia (12%, 10% and 8% O₂, balanced with N₂, for 20 min/each) or hypercapnia (3%, 5% and 7% CO₂, containing 21% O₂ and balanced with N₂, for 20 min/each). Each gas condition was tested in the same animal on alternate days. That means that the animals were first exposed to three levels of one gas condition (hypoxia or hypercapnia) on the first day and to the other three levels of the second gas condition (hypercapnia or hypoxia) on the following day. The sequence of conditions and stimulus levels was

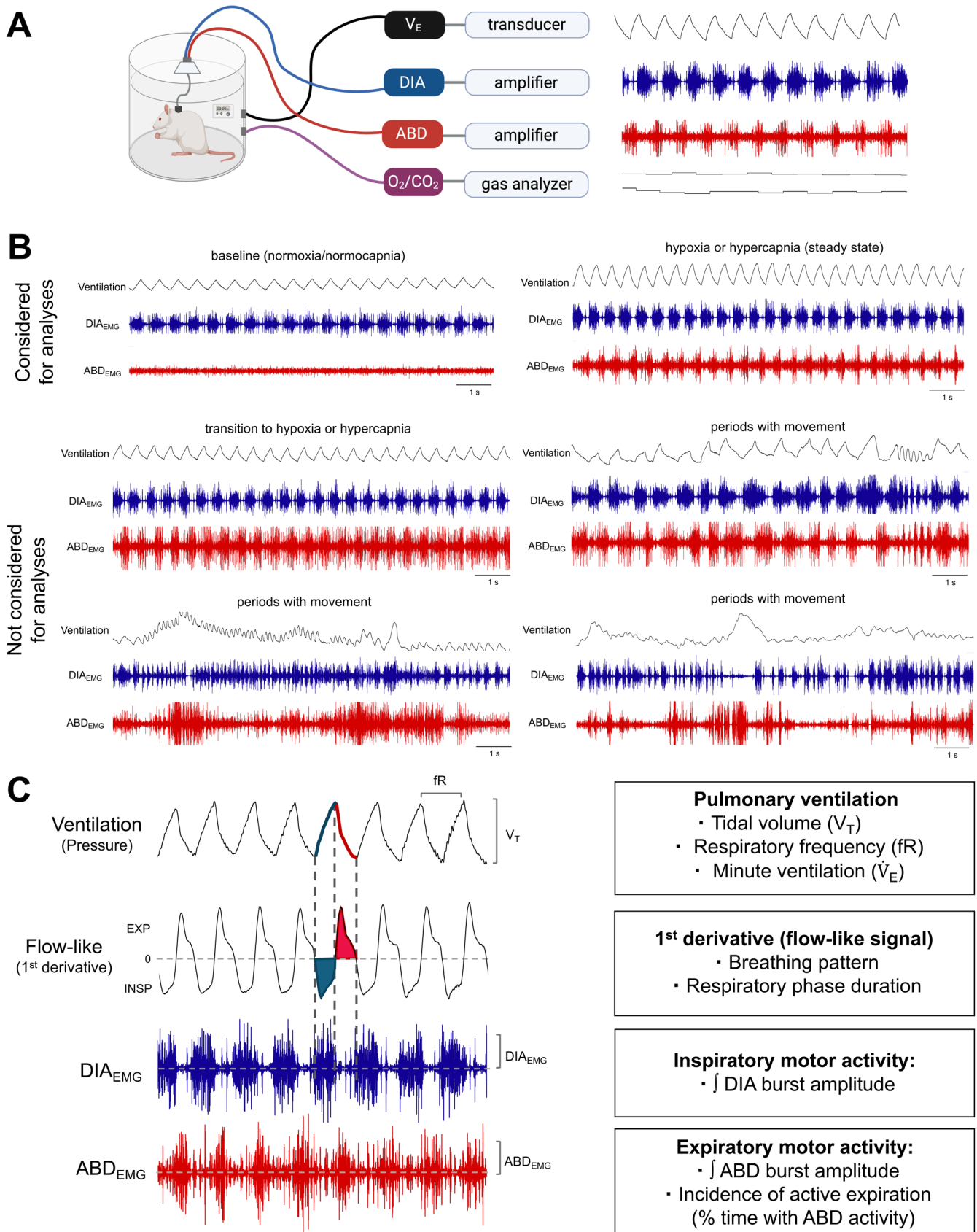


FIGURE 1 | Legend on next page.

applied randomly across animals, respecting an interval of at least one hour between consecutive stimuli. After the experiments, the animals were euthanized with anesthesia overdose (sodium

thiopental, 200mg/kg, i.p.) and the temperature probes were removed from the abdominal cavity for data collection and subsequent analyses (The SubCue Analyzer, Alberta, Canada).

FIGURE 1 | Pulmonary ventilation and EMG recordings in unanesthetized, freely behaving rats. (A) Schematic diagram and representative traces showing the experimental setup and recorded parameters. Pulmonary ventilation (upper black trace), inspiratory (blue trace) and expiratory (red trace) motor activities were measured in unanesthetized, freely behaving rats (see methods for details). Oxygen and carbon dioxide levels inside the chamber (lower black traces, respectively) were also monitored. (B) Examples of traces observed during the experiments. Only steady-state periods during baseline, hypoxia, and hypercapnia. The transition periods from baseline to hypoxia or hypercapnia, as well as periods during which the animal moved, were excluded from the analysis. (C) Methods for data extraction and analyses. From respiratory-related pressure signals (upper trace, black), tidal volume (V_T) and respiratory frequency (fR) were determined and used to calculate minute ventilation ($\dot{V}_E$). The 1st derivative of ventilation traces generated a flow-like signal (second black trace), allowing the analysis of inspiratory (blue-shaded area) and expiratory (red-shaded area) patterns. The electromyograms of the diaphragm muscle (DIA_{EMG} , blue traces) and of the oblique abdominal muscle (ABD_{EMG} , red traces) show bursts occurring during inspiration and in the presence of active expiration, respectively.

2.7 | Data and Statistical Analyses

Analyses were conducted offline using LabChart 8 (ADInstruments, Bella Vista, Australia). As pulmonary ventilation, muscle activity, and metabolic rate were recorded simultaneously, the physiological data were extracted within the same time periods (Figure 1A,C), allowing for correlations between responses. Only periods of quiet breathing and after the stabilization of the gas conditions in the chamber (steady state, 3–5 min after the initiation of exposure) were considered for analyses (Figure 1B). Transitions from normoxia/normocapnia to hypoxia or hypercapnia and periods containing artifacts due to animal movements, exploratory behaviors, and grooming were excluded from the analyses (Figure 1B). All parameters were quantified in their original units and grouped according to the gas condition and intensity. Changes induced by hypoxia or hypercapnia were compared to the corresponding baseline parameters prior to each stimulus. Methods for data extraction and analyses are summarized in Figure 1C.

The results were presented as mean $\pm$ SD. The normal distribution of the data was verified using the Shapiro–Wilk normality test. Changes in ventilation, muscle activity, and metabolic rate during hypoxia or hypercapnia were analyzed with one-way ANOVA for repeated measures followed by Tukey's post-test. Correlations between air convection requirement ($\dot{V}_E/\dot{V}O_2$) and active expiration incidence, as well as between $\dot{V}O_2$ and active expiration incidence, were examined using simple linear regressions. Respiratory phase durations during baseline, hypoxic, and hypercapnic conditions were compared using two-way ANOVA with Tukey's post-test. Statistical analyses and graphic operations were conducted with GraphPad Prism (GraphPad Software, USA, Version 8). Differences were considered significant when $p < 0.05$.

3 | Results

3.1 | Active Expiration and Its Functional Impact During Hypoxia Exposure in Unanesthetized Rats

In our experimental conditions, acute exposure to all hypoxic levels (12%, 10%, and 8% O_2) increased pulmonary ventilation and respiratory motor activity and reduced metabolic rate (Figures 2 and 3), as previously documented [16, 32]. As for the presence of AE, the most intense hypoxic stimulus (8% O_2) elicited ABD contractions in all animals tested ($n = 7$), while 10% and 12% O_2 were able to evoke AE in 6 and 5 (out of 7) rats, respectively (Figure 2A). However, the AE pattern during acute

hypoxia was not an all-or-nothing event, as reported in other studies using anesthetized or reduced preparations [10, 13, 33]. In fact, we noted that the unanesthetized animals exhibited periods with the presence and absence of AE throughout the period of exposure, with no clear transition pattern. Interestingly, the incidence of AE (i.e., % time the animal presented AE during exposure) was similar across hypoxia levels (Figure 2B).

To assess the impact of AE on ventilation during hypoxia exposure, we compared, within the same animal, the changes in ventilatory parameters, diaphragmatic activity, and metabolic rate in moments when the ABD expiratory recruitment was present or absent (i.e., no AE) (Figure 2C–E). In all intensities of hypoxia tested, the emergence of AE promoted a further increase in $\dot{V}_E$ compared to the periods when the ABD muscle was not recruited (12% O_2 : 2865 ± 327 vs. 2479 ± 622 mL·kg⁻¹·min⁻¹, $p = 0.037$, Figure 2C₁; 10% O_2 : 2958 ± 590 vs. 2403 ± 638 mL·kg⁻¹·min⁻¹, $p < 0.001$, Figure 2D₁; 8% O_2 : 2812 ± 495 vs. 2388 ± 542 mL·kg⁻¹·min⁻¹, $p = 0.011$, Figure 2E₁). These additional increases in $\dot{V}_E$ were due to higher V_T responses in the presence of AE (12% O_2 : 24.5 ± 3 vs. 18.9 ± 2.8 mL·kg⁻¹, $p = 0.009$, Figure 2C₂; 10% O_2 : 27.2 ± 4 vs. 21.3 ± 4 mL·kg⁻¹, $p = 0.018$, Figure 2D₂; 8% O_2 : 27.4 ± 5 vs. 24.9 ± 6 mL·kg⁻¹, $p = 0.005$, Figure 2E₂). The fR was not different from baseline during exposure to 10% and 8% O_2 (Figure 2D₃, E₃). In contrast, the milder hypoxic stimulus (12% O_2) elevated the fR (no AE vs. baseline, 130.2 ± 18 vs. 97.7 ± 7 cpm, $p = 0.014$, Figure 2C₃) – a response that was attenuated in the presence of AE (116.4 ± 11 vs. 130.2 ± 18 cpm, $p = 0.04$, Figure 2C₃), but remained above baseline (AE vs. baseline, 116.4 ± 11 vs. 97.7 ± 7 cpm, $p = 0.026$, Figure 2C₃). Hypoxia exposure also strengthened DIA contractions in all levels tested (12% O_2 : no AE vs. baseline, 8.8 ± 2.6 vs. 7.2 ± 2.1 mV, $p = 0.012$; AE vs. baseline, 8.9 ± 2.5 vs. 7.2 ± 2.1 mV, $p = 0.003$, Figure 2C₅; 10% O_2 : no AE vs. baseline, 8.7 ± 2.3 vs. 7.1 ± 2.3 mV, $p = 0.001$; AE vs. baseline, 8.5 ± 2.6 vs. 7.1 ± 2.3 mV, $p = 0.008$, Figure 2D₅; 8% O_2 : no AE vs. baseline, 8.8 ± 2.5 vs. 7.1 ± 2.2 mV, $p = 0.009$; AE vs. baseline, 9.2 ± 2.5 vs. 7.1 ± 2.2 mV, $p < 0.001$, Figure 2E₅). The amplitude of DIA_{EMG} bursts was not altered by the presence of AE (12% O_2 : no AE vs. AE, 8.8 ± 2.6 vs. 8.9 ± 2.5 mV, $p = 0.902$, Figure 2C₅; 10% O_2 : no AE vs. AE, 8.7 ± 2.3 vs. 8.5 ± 2.6 mV, $p = 0.524$, Figure 2D₅; 8% O_2 : no AE vs. AE, 8.8 ± 2.5 vs. 9.2 ± 2.5 mV, $p = 0.296$, Figure 2E₅).

Our analysis of $\dot{V}O_2$ revealed that the hypoxia-induced reduction in the metabolism was smaller during the periods of ABD muscle recruitment (no AE vs. AE—12% O_2 : 9.6 ± 2.4 vs. 7.3 ± 2.5 mL·kg⁻¹·min⁻¹, $p = 0.009$, Figure 3A; 10% O_2 : 11.4 ± 4.3 vs. 9.0 ± 3.9 mL·kg⁻¹·min⁻¹, $p = 0.016$, Figure 3B; 8% O_2 : 11.7 ± 2.7

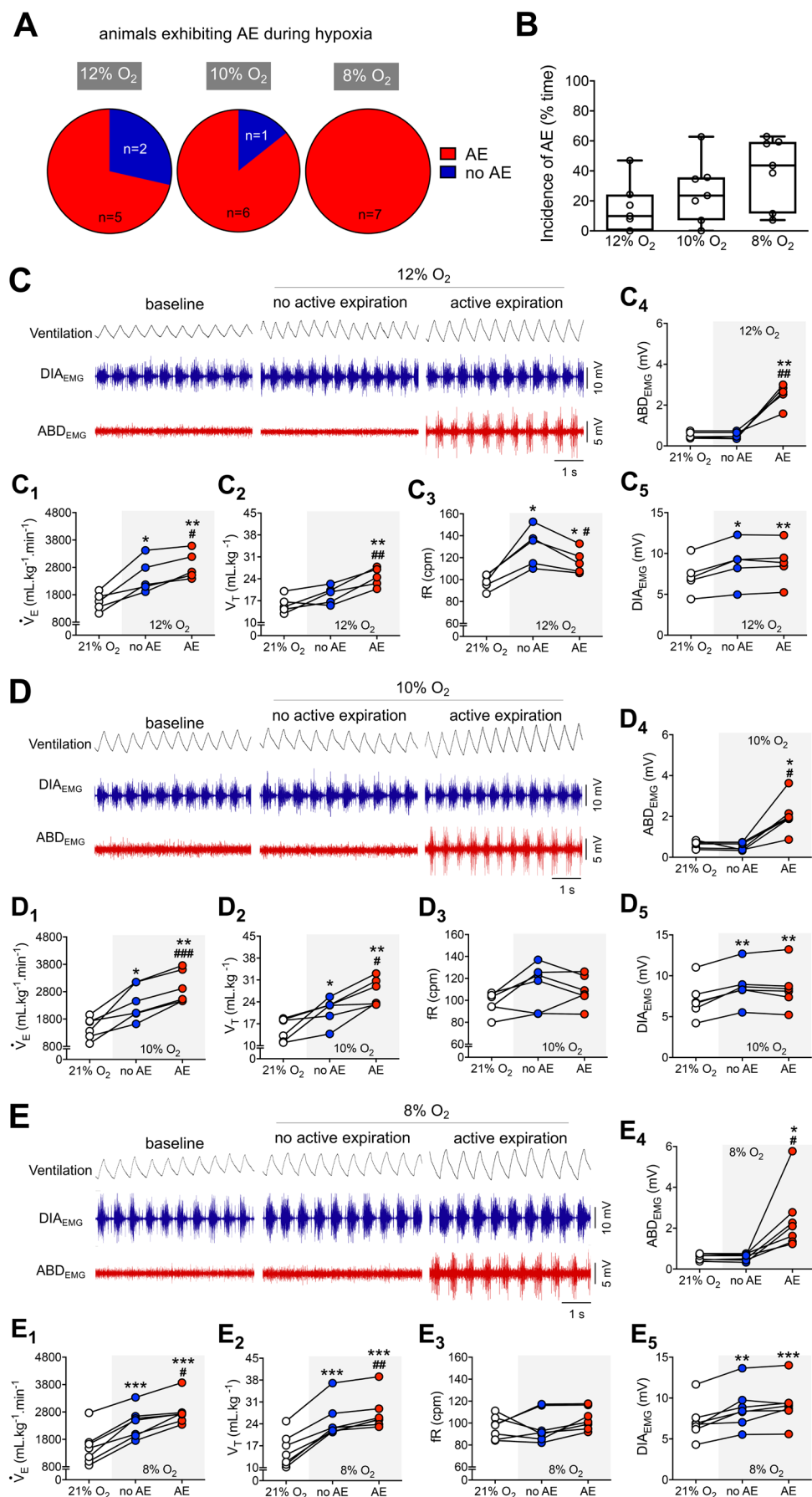


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FIGURE 2 | Functional characterization of active expiration during different hypoxia levels. (A) Pie graphs depicting the proportion of rats presenting (red) or not presenting (blue) active expiration during exposure to different levels of hypoxia (Left, 12% O₂; middle, 10% O₂; and right, 8% O₂). (B) Boxplots (line represents the mean) showing the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) during exposure to different levels of hypoxia (Left, 12% O₂; middle, 10% O₂; and right, 8% O₂). (C–E) Representative traces of pulmonary ventilation (Ventilation, upper traces, black) and electromyograms of the diaphragm (DIA_{EMG}, middle traces, blue) and oblique abdominal muscles (ABD_{EMG}, lower traces, red) during baseline (21% O₂) and hypoxia conditions (C, 12% O₂; D, 10% O₂; and E, 8% O₂). Traces also show periods during hypoxia exposure without active expiration (i.e., absence of ABD rhythmic muscle activity) and with active expiration (i.e., rhythmic ABD muscle activity). C₁–C₅, D₁–D₅, and E₁–E₅. Graphs showing individual values of minute ventilation ($\dot{V}_E$; C₁, D₁, and E₁), tidal volume (V_T ; C₂, D₂ and E₂), respiratory frequency (fR; C₃, D₃, and E₃), DIA_{EMG} (C₄, D₄, and E₄), and ABD_{EMG} (C₅, D₅, and E₅) amplitudes, analyzed during baseline periods (baseline, white symbols) and hypoxia periods without (no AE, blue symbols) and with active expiration (AE, red symbols). C₁–C₅, 12% O₂; D₁–D₅, 10% O₂; and E₁–E₅, 8% O₂. For all experiments $n = 5-7$. * $p < 0.05$ vs. 21% O₂, ** $p < 0.01$ vs. 21% O₂, *** $p < 0.001$ vs. 21% O₂, # $p < 0.05$ vs. no AE, ## $p < 0.01$ vs. no AE, ### $p < 0.001$ vs. no AE (repeated measures one-way ANOVA).

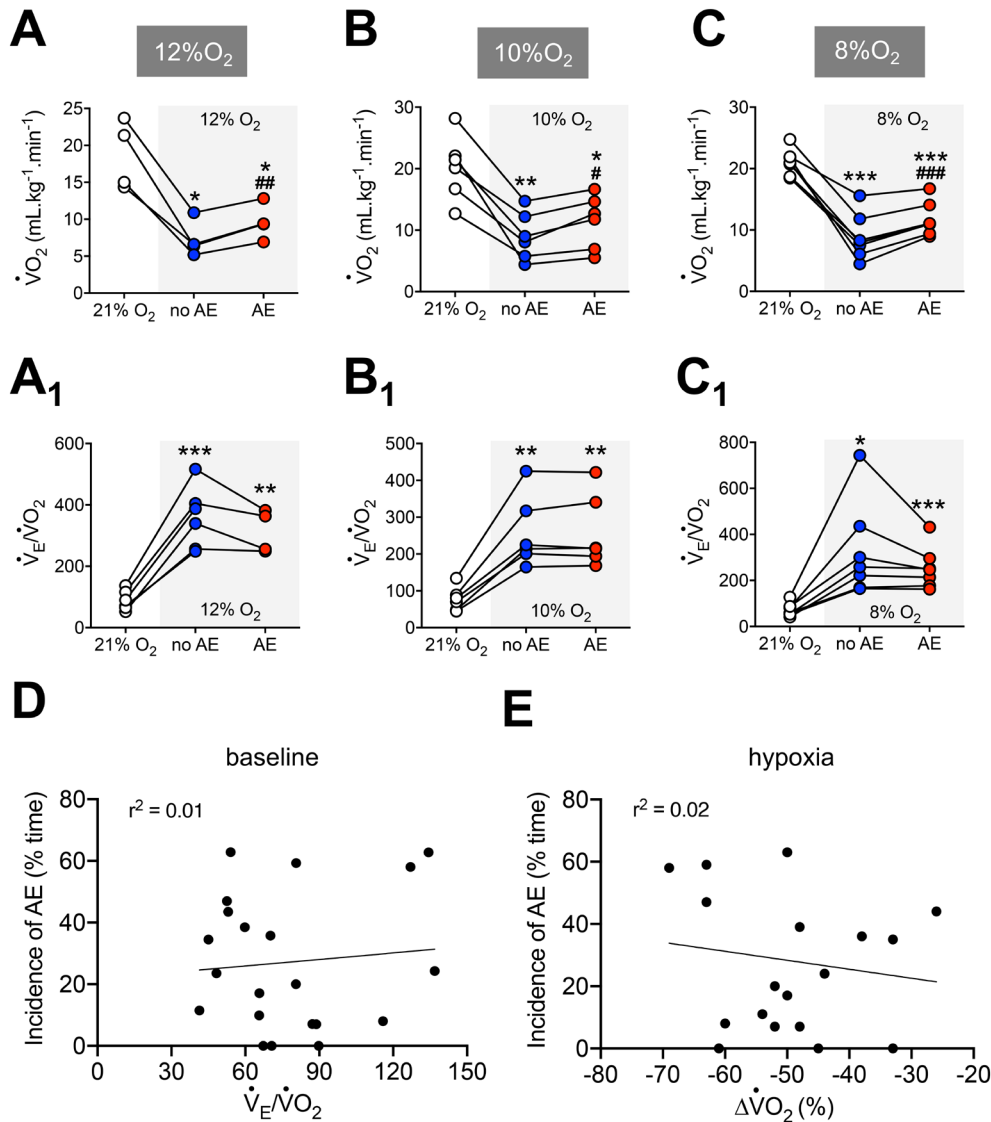


FIGURE 3 | Correlation between active expiration and metabolic measurements during different hypoxia levels. (A–C) Graphs showing individual values of oxygen consumption ($\dot{V}O_2$) during baseline (baseline, white symbols) and hypoxia conditions without (no AE, blue symbols) and with active expiration (AE, red symbols). A, 12% O₂; B, 10% O₂; and C, 8% O₂. A₁, B₁, and C₁. Individual values of air convection requirement ($\dot{V}_E/\dot{V}O_2$) during baseline (baseline, white symbols) and hypoxia conditions without (no AE, blue symbols) and with active expiration (AE, red symbols). A₁, 12% O₂; B₁, 10% O₂; and C₁, 8% O₂. (D) Correlation (linear regression) between the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) and respiratory equivalent ($\dot{V}_E/\dot{V}O_2$) during hypoxia. (E) Correlation (linear regression) between the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) and changes in oxygen consumption ($\dot{V}O_2$) during hypoxia. For all experiments $n = 5-7$. * $p < 0.05$ vs. 21% O₂, ** $p < 0.01$ vs. 21% O₂, *** $p < 0.001$ vs. 21% O₂, # $p < 0.05$ vs. no AE, ## $p < 0.01$ vs. no AE, ### $p < 0.001$ vs. no AE (repeated measures one-way ANOVA).

vs. $8.8 \pm 3.7 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p < 0.001$, Figure 3C), indicating that the occurrence of AE was associated with an additional energy expenditure. As a result, $\dot{V}_E/\dot{V}O_2$, which increased during all hypoxic conditions ($p < 0.05$), remained similar in the absence or presence of AE (no AE vs. AE—12% O_2 : 358.9 ± 100.7 vs. 312.7 ± 70.1 , $p = 0.339$, Figure 3A₁; 10% O_2 : 257.9 ± 96.4 vs. 259.4 ± 99.26 , $p = 0.949$, Figure 3B₁; 8% O_2 : 327.4 ± 205.4 vs. 254.1 ± 90.8 , $p = 0.294$, Figure 3C₁). The incidence of AE did not correlate with baseline $\dot{V}_E/\dot{V}O_2$ ($r^2 = 0.01$, Figure 3D) or the magnitude of $\dot{V}O_2$ reductions during hypoxia ($\Delta\dot{V}O_2$, $r^2 = 0.02$, Figure 3E), indicating that neither resting nor hypoxia-induced metabolic responses affected significantly the expression of this motor behavior.

All levels of hypoxia also increased the frequency of sighs (baseline: 16.7 ± 3.1 events/h vs. 12% O_2 : 79.3 ± 29.7 events/h, $p = 0.005$; 10% O_2 : 80.1 ± 20.1 events/h, $p = 0.001$; 8% O_2 : 65.0 ± 31.3 , $p = 0.032$, Figure 6A,C). The sigh events were frequently followed by a brisk and decrementing ABD burst (Figure 6B). Under hypoxia conditions, when AE was present, we observed a brief period of attenuation of ABD bursts during the post-sigh period (Figure 6B). Based on evidence showing that vagal afferents from lung stretch receptors appear to influence the emergence of AE [10], we evaluated whether the AE incidence during hypoxia would be influenced by the sigh expression. However, no correlation was found between the incidence of AE and sighs ($r^2 = 0.025$, Figure 6D).

3.2 | Active Expiration and Its Functional Effects During Hypercapnia in Unanesthetized Rats

In conditions of increased inspired CO_2 levels (3%, 5%, and 7% O_2), the animals exhibited increased pulmonary ventilation and respiratory motor activity, with no changes in metabolic rate (Figures 4 and 5), as previously reported [17]. Similarly to hypoxia, only the most intense hypercapnic stimulus (7% CO_2) promoted AE in 100% ($n = 7$) of the animals, while 5% and 3% CO_2 evoked AE in only 6 and 5 (out of 7) rats, respectively (Figure 4A). Likewise, the AE pattern during acute hypercapnia was not an all-or-nothing event, as described in studies using anesthetized or reduced preparations [10, 13], and its incidence was not different across hypercapnic levels ($p > 0.05$, Figure 4B).

As observed in hypoxia, the increases in $\dot{V}_E$ during exposure to all levels of hypercapnia were higher during periods with AE compared to those with no AE (3% CO_2 : 2337 ± 596 vs. $1997 \pm 455 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p = 0.026$, Figure 4C₁; 5% CO_2 : 2955 ± 758 vs. $2334 \pm 695 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p = 0.049$, Figure 4D₁; 7% CO_2 : 4247 ± 775 vs. $3483 \pm 931 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p = 0.020$, Figure 4E₁). These increases in $\dot{V}_E$ associated with AE occurred due to augmented V_T responses (3% CO_2 : 21.1 ± 3.5 vs. $18.6 \pm 3.2 \text{ mL} \cdot \text{kg}^{-1}$, $p = 0.011$, Figure 4C₂; 5% CO_2 : 25.7 ± 5.3 vs. $21.0 \pm 6.1 \text{ mL} \cdot \text{kg}^{-1}$, $p = 0.001$, Figure 4D₂; 7% CO_2 : 33.0 ± 5.5 vs. $26.1 \pm 4.8 \text{ mL} \cdot \text{kg}^{-1}$, $p = 0.004$, Figure 4E₂). In contrast, the increase in fR during hypercapnia was not significantly affected by the presence of AE (3% CO_2 : no AE vs. baseline, 106.8 ± 9.4 vs. $81.6 \pm 7.3 \text{ cpm}$, $p = 0.009$; AE vs. baseline, 109.4 ± 9.4 vs. $81.6 \pm 7.3 \text{ cpm}$, $p = 0.012$, Figure 4C₃; 5% CO_2 : no AE vs. baseline, 111.8 ± 12.7 vs. $89.9 \pm 8.2 \text{ cpm}$, $p = 0.008$; AE vs. baseline, 113.9 ± 20.5 vs. $89.9 \pm 8.2 \text{ cpm}$, $p = 0.059$, Figure 4D₃; 7% CO_2 : no

AE vs. baseline, 131.8 ± 13.2 vs. $103.3 \pm 5.3 \text{ cpm}$, $p = 0.004$; AE vs. baseline, 128.8 ± 11.0 vs. $103.3 \pm 5.3 \text{ cpm}$, $p = 0.008$, Figure 4E₃). Furthermore, 5% and 7% CO_2 stimuli, but not 3%, evoked significant increases in DIA_{EMG} burst amplitude (3% CO_2 : no AE vs. baseline, 7.4 ± 0.9 vs. $6.6 \pm 1.4 \text{ mV}$, $p = 0.293$; AE vs. baseline, 7.9 ± 2.0 vs. $6.6 \pm 1.4 \text{ mV}$, $p = 0.099$, Figure 4C₅; 5% CO_2 : no AE vs. baseline, 7.2 ± 1.6 vs. $6.2 \pm 1.3 \text{ mV}$, $p = 0.033$; AE vs. baseline, 8.0 ± 1.5 vs. $6.2 \pm 1.3 \text{ mV}$, $p = 0.011$, Figure 4D₅; 7% CO_2 : no AE vs. baseline, 8.0 ± 1.5 vs. $6.8 \pm 1.0 \text{ mV}$, $p = 0.029$; AE vs. baseline, 7.9 ± 1.7 vs. $6.8 \pm 1.0 \text{ mV}$, $p = 0.105$, Figure 4E₅). The emergence of AE did not modify the hypercapnia-induced changes in the DIA_{EMG} activity (3% CO_2 : no AE vs. AE, 7.4 ± 0.9 vs. $7.9 \pm 2.0 \text{ mV}$, $p = 0.783$, Figure 4C₅; 5% CO_2 : no AE vs. AE, 7.2 ± 1.6 vs. $8.0 \pm 1.5 \text{ mV}$, $p = 0.261$, Figure 4D₅; 7% CO_2 : no AE vs. AE, 8.0 ± 1.5 vs. $7.9 \pm 1.7 \text{ mV}$, $p = 0.932$, Figure 4E₅).

Concerning the metabolic rate, the $\dot{V}O_2$ of the animals exposed to hypercapnia remained unchanged compared to baseline values when AE was absent (Figure 5A–C). On the other hand, $\dot{V}O_2$ was higher in the presence of AE compared to periods without AE (3% CO_2 : 19.3 ± 5.5 vs. $16.1 \pm 5.3 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p = 0.005$, Figure 5A; 5% CO_2 : 19.3 ± 9.8 vs. $15.6 \pm 9.7 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p = 0.015$, Figure 5B; 7% CO_2 : 19.0 ± 5.7 vs. $15.24 \pm 5.3 \text{ mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, $p = 0.015$, Figure 5C). Therefore, as observed under hypoxia conditions, the increases in $\dot{V}_E$ associated with AE during hypercapnia (Figure 4C₁–E₁) occurred at the expense of additional O_2 consumption. As a result, $\dot{V}_E/\dot{V}O_2$ was not improved during the presence of AE (3% CO_2 : no AE vs. AE, 126.4 ± 30.4 vs. 123.5 ± 21.8 , $p = 0.898$, Figure 5A₁; 5% CO_2 : no AE vs. AE, 161.8 ± 66.6 vs. 153.8 ± 53.6 , $p = 0.899$, Figure 5B₁; 7% CO_2 : no AE vs. AE, 345.3 ± 158.5 vs. 311.2 ± 121.3 , $p = 0.333$, Figure 5C₁). The incidence of AE during hypercapnia did not correlate with baseline $\dot{V}_E/\dot{V}O_2$ ($r^2 = 0.021$, Figure 5D) or the magnitude of changes in $\Delta\dot{V}O_2$ ($r^2 = 0.09$, Figure 5E). Finally, hypercapnia did not change the occurrence of sighs (Figure 6E,F); therefore, no association between AE and sigh frequency were noted in this condition.

3.3 | Effects of Active Expiration on Inspiratory and Expiratory Dynamics and Phase Durations

The airflow-like signals generated by the first-order derivative of whole-body plethysmography signals (dmV/dt) enabled us to estimate the changes in inspiratory and expiratory timing and efforts in animals exposed to hypoxia and hypercapnia and link these changes to the emergence of AE. Using DIA_{EMG} activity as a reference, we divided the respiratory cycle into the following phases: (i) inspiration (I), from the onset to the peak of DIA_{EMG} bursts; (ii) post-inspiration (PI), coinciding with the decrease in DIA_{EMG} activity after its peak; and (iii) stage 2 of expiration (E2), a period marked by the absence of DIA_{EMG} activity. In all animals showing AE under different levels of hypoxia or hypercapnia, we observed that maximal ABD_{EMG} activity occurred during the E2 phase (Figure 7A,C). However, the effect on expiratory efforts varied between gas conditions. Under hypoxia (all levels tested), we noted that both inspiratory and expiratory flow-like peaks increased, suggesting enhanced respiratory efforts during both phases (Figure 7B). In the presence of AE (Figure 7B), an additional peak in the expiratory phase during E2 was noted (absent under baseline conditions or without AE),

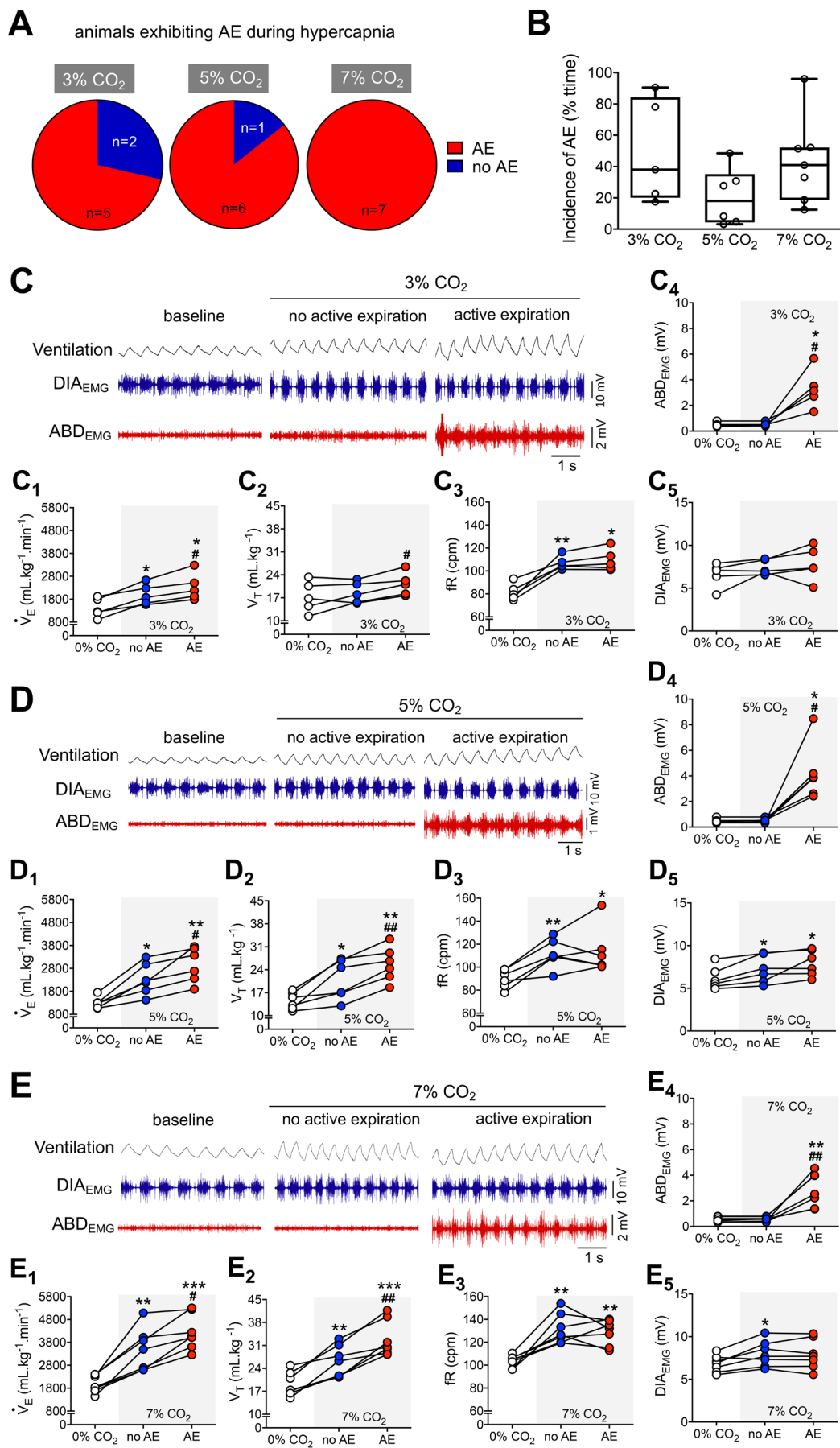


FIGURE 4 | Legend on next page.

FIGURE 4 | Functional characterization of active expiration during different hypercapnia levels. (A) Pie graphs depicting the proportion of rats presenting (red) or not presenting (blue) active expiration during exposure to different levels of hypercapnia (Left, 3% CO₂; middle, 5% CO₂; and right, 7% CO₂). (B) Boxplots (line represents the median) showing the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) during exposure to different levels of hypercapnia (Left, 3% CO₂; middle, 5% CO₂; and right, 7% CO₂). (C–E) Representative traces of pulmonary ventilation (Ventilation, upper traces, black) and electromyograms of the diaphragm (DIA_{EMG}, middle traces, blue) and oblique abdominal muscles (ABD_{EMG}, lower traces, red) during baseline (0% CO₂) and during hypercapnia (C, 3% CO₂; D, 5% CO₂; and E, 7% CO₂). Traces also show periods without active expiration (i.e., absence of ABD rhythmic muscle activity) and with active expiration (i.e., rhythmic ABD muscle activity) during hypercapnia exposure. C₁–C₅, D₁–D₅, and E₁–E₅. Graphs showing individual values of minute ventilation ($\dot{V}_E$; C₁, D₁, and E₁), tidal volume (V_T ; C₂, D₂ and E₂), respiratory frequency (fR; C₃, D₃, and E₃), DIA_{EMG} (C₄, D₄, and E₄), and ABD_{EMG} (C₅, D₅, and E₅) amplitudes, analyzed during baseline (baseline, white symbols) and hypercapnia conditions without (no AE, blue symbols) and with active expiration (AE, red symbols). C₁–C₅, 3% CO₂; D₁–D₅, 5% CO₂; and E₁–E₅, 7% CO₂. For all experiments $n = 5-7$. * $p < 0.05$ vs. 0% CO₂, ** $p < 0.01$ vs. 0% CO₂, *** $p < 0.001$ vs. 0% CO₂, # $p < 0.05$ vs. no AE, ## $p < 0.01$ vs. no AE (repeated measures one-way ANOVA).

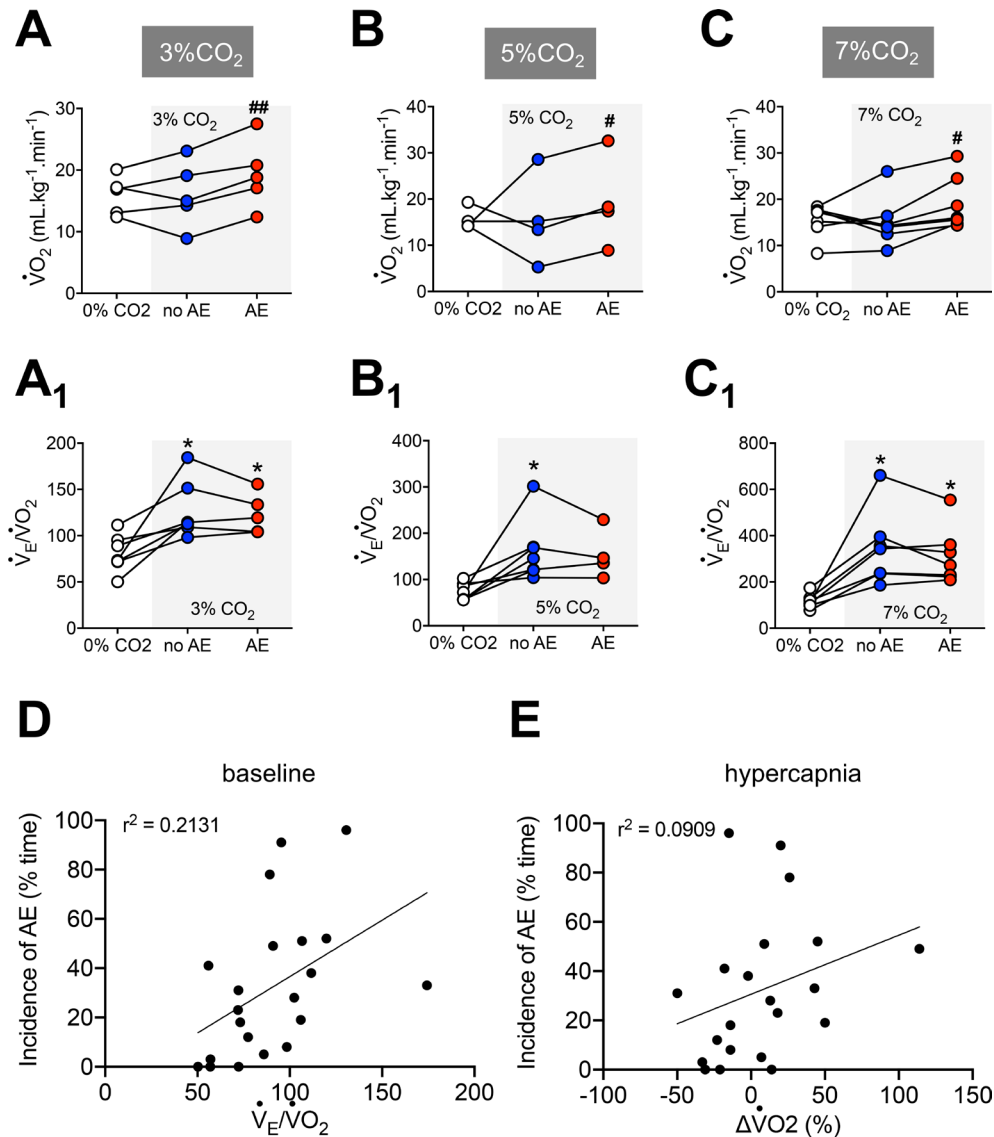


FIGURE 5 | Correlation between active expiration and metabolic measurements during different hypercapnia levels. (A–C) Graphs showing individual values of oxygen consumption ($\dot{V}O_2$) during baseline (baseline, white symbols) and hypercapnia conditions without (no AE, blue symbols) and with active expiration (AE, red symbols). A, 3% CO₂; B, 5% CO₂; and C, 7% CO₂. A₁, B₁, and C₁. Individual values of respiratory equivalent ($\dot{V}_E/\dot{V}O_2$) during baseline (baseline, white symbols) and hypercapnia conditions without (no AE, blue symbols) and with active expiration (AE, red symbols). A₁, 3% CO₂; B₁, 5% CO₂; and C₁, 7% CO₂. (D) Correlation (linear regression) between the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) and air convection requirement ($\dot{V}_E/\dot{V}O_2$) during hypercapnia. (E) Correlation (linear regression) between the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) and changes in oxygen consumption ($\dot{V}O_2$) during hypercapnia. For all experiments $n = 5-7$. * $p < 0.05$ vs. 0% CO₂, # $p < 0.05$ vs. no AE, ## $p < 0.01$ vs. no AE (repeated measures one-way ANOVA).

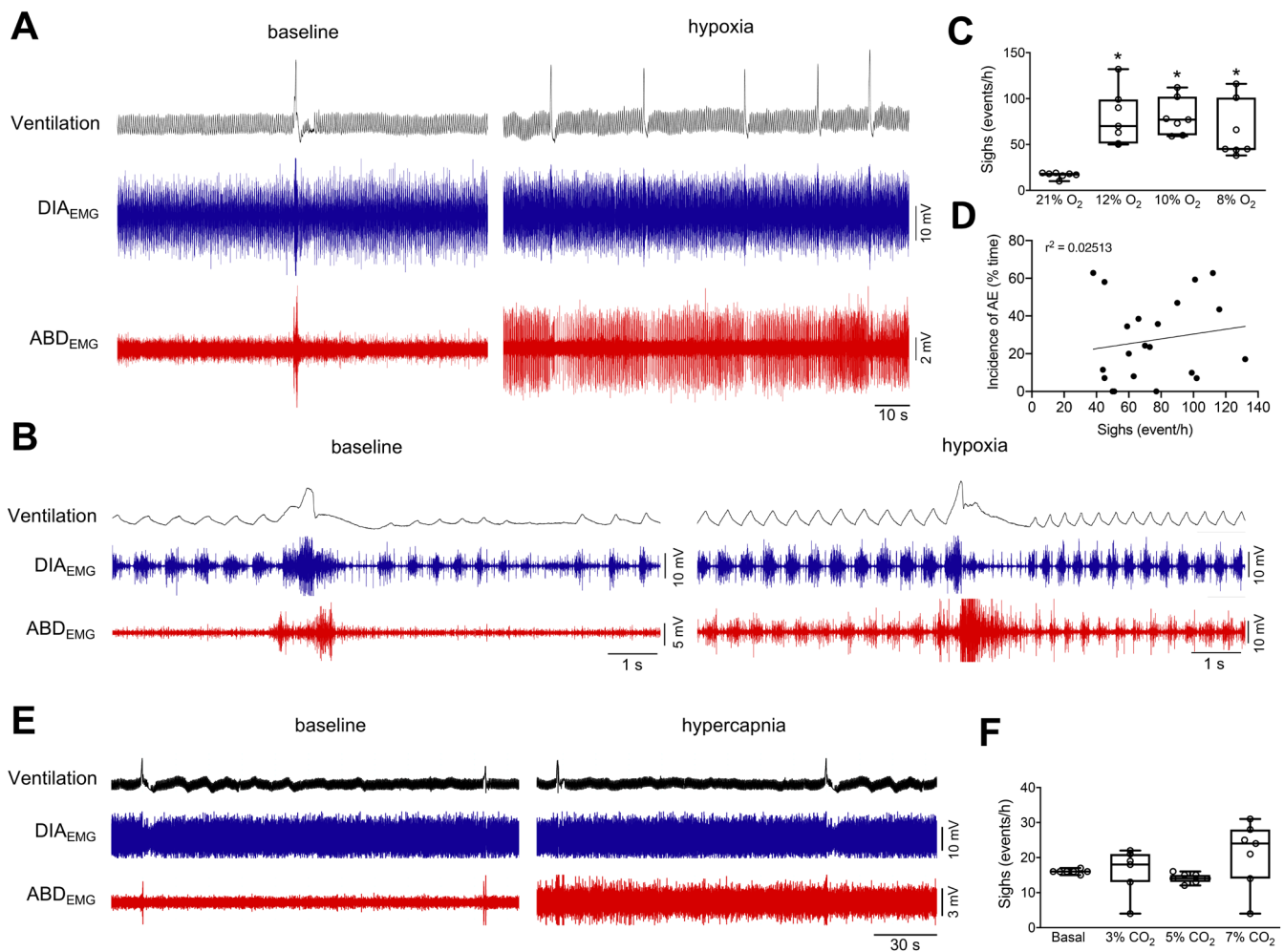


FIGURE 6 | Correlation between active expiration and sighs during hypoxia and hypercapnia. (A) Representative traces of pulmonary ventilation (Ventilation, upper traces, black) and electromyograms of the diaphragm (DIA_{EMG}, middle traces, blue) and of the oblique abdominal muscles (ABD_{EMG}, lower traces, red) during baseline (left) and hypoxia conditions (right). (B) Expanded traces of Ventilation, DIA_{EMG}, and ABD_{EMG} showing inspiratory and expiratory motor activity patterns during sighs during baseline (left) and hypoxia (right) conditions. (C) Occurrence of sighs (events/h) during baseline (21% O₂) and different hypoxia levels (12%, 10%, and 8% O₂). (D) Correlation (linear regression) between the incidence of active expiration (% time the animal exhibited ABD muscle recruitment) and sigh occurrence (events/h). (E) Representative traces of pulmonary ventilation (Ventilation, upper traces, black) and electromyograms of the diaphragm (DIA_{EMG}, middle traces, blue) and of the oblique abdominal muscles (ABD_{EMG}, lower traces, red) during baseline (left) and hypercapnia conditions (right). (F) Occurrence of sighs (events/h) during baseline (0% CO₂) and different hypercapnia levels (3%, 5%, and 7% CO₂). **p* < 0.05 vs. 21% O₂ (repeated measures one-way ANOVA).

along with a further increase in the inspiratory flow-like peak. During hypercapnic conditions (all levels tested), the inspiratory and expiratory flow-like peaks were higher than baseline, indicating increased respiratory efforts similar to those observed under hypoxia (Figure 7D). However, with AE present, the expiratory flow-like pattern remained unchanged, while significant increases were verified in both the inspiratory and expiratory peaks (Figure 7D). Consequently, these analyses suggest that although ABD_{EMG} reaches its maximal activity during the E2 phase, the influence of AE on airflow and pulmonary mechanics may differ between hypoxia and hypercapnia.

We observed that, similar to the expiratory flow pattern, respiratory phase times changed differently during hypoxia and hypercapnia exposure. The durations of the respiratory phases were measured during exposure to 8% O₂ or 7% CO₂, as all animals exhibited AE expiration under these conditions.

However, similar findings were noted at other levels of hypoxia and hypercapnia tested (data not shown). During hypoxic conditions (*n* = 7), when AE was absent, the respiratory phase durations were similar to baseline (no AE vs. baseline—INSP: 42.9 ± 2.7 vs. 44.6 ± 3.5%; PI: 36.3 ± 2.0 vs. 34.7 ± 2.5%; E2: 20.7 ± 1.3 vs. 20.6 ± 2.4%; *p* > 0.05; Figure 8A). With the emergence of AE during hypoxia, the PI phase duration lengthened (43.9% ± 6.1%, *p* = 0.0430 and 0.0191 compared to baseline and hypoxia—no AE, respectively; Figure 8A) while the E2 phase became shorter (17.1% ± 3.0%, *p* = 0.0015 and *p* = 0.0581 compared to baseline and hypoxia—no AE, respectively; Figure 8A). The I phase remained unchanged during AE (39.1% ± 3.1%, *p* > 0.05 compared to baseline and hypoxia—no AE, Figure 8A). Under hypercapnic conditions without AE (*n* = 7), the duration of the PI phase markedly reduced (no AE vs. baseline: 10.3 ± 3.6 vs. 33.0% ± 7.3%, *p* = 0.0003), the E2 phase significantly increased (no AE vs. baseline: 41.1 ± 3.6 vs. 22.4% ± 4.8%, *p* < 0.0001),

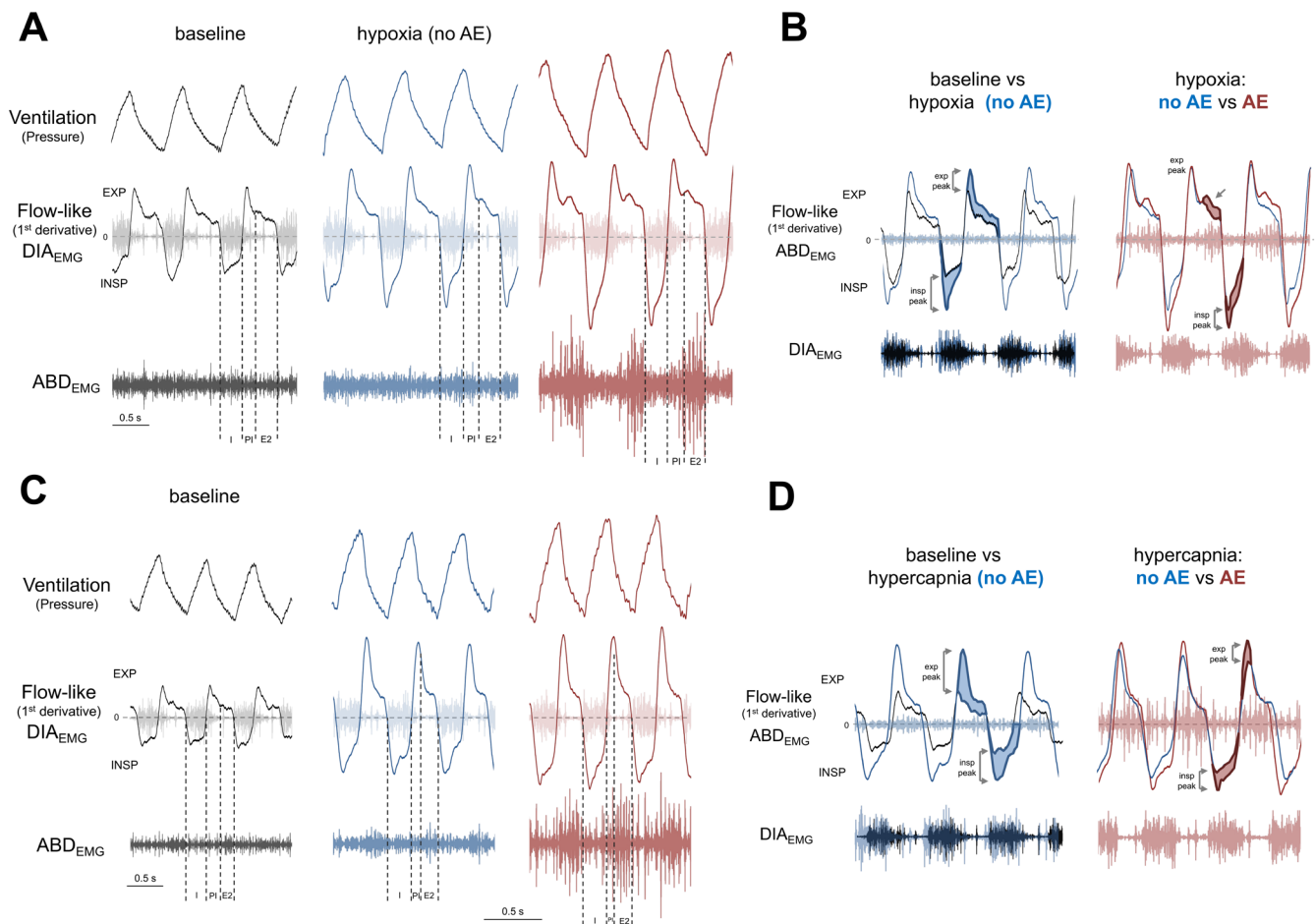


FIGURE 7 | Active expiration and inspiratory/expiratory dynamics during hypoxia and hypercapnia. (A and C) Examples of ventilation traces (upper traces), superimposed traces of flow and diaphragm electromyogram (flow-like signal and DIA_{EMG}, middle traces), and abdominal electromyogram (ABD_{EMG}, lower traces) during baseline (black) and during hypoxia (A) or hypercapnia (C) periods without (blue) and with active expiration (red). Vertical dotted lines indicate respiratory phases: I (inspiration), PI (post-inspiration), E2 (expiratory stage 2). (B and D) Flow-derived analyses of the inspiratory and expiratory patterns during baseline (black traces) and during hypoxia (B) or hypercapnia (D) periods without (blue traces) and with active expiration (red traces). Inspiratory (insp) and expiratory (exp) peaks are indicated by shaded areas in the flow-like traces.

and the I phase did not change compared to baseline (no AE vs. baseline: 48.6 ± 1.0 vs. $45.0 \pm 4.1\%$, $p = 0.1630$; Figure 8B). This pattern of reduced PI, prolonged E2, and unchanged I phase durations was not modified by the presence of AE (I: $47.4 \pm 1.6\%$, $p > 0.05$ compared to baseline and hypercapnia—no AE; PI: $12.6 \pm 1.2\%$, $p = 0.0003$ and 0.1776 $p > 0.05$ compared to baseline and hypercapnia—no AE, respectively; E2: $12.6 \pm 1.2\%$, $p < 0.0001$ compared to baseline and hypercapnia—no AE; Figure 8B).

4 | Discussion

In mammals, AE is recognized as a respiratory behavior that enhances pulmonary ventilation in situations of blood gas disturbances. A substantial body of evidence in the literature describes the conditions that lead to ABD expiratory recruitment and the corresponding central mechanisms that underpin the emergence of the AE pattern [10, 12–15, 18–20, 30, 31, 34–47]. Studies performed in humans have characterized the pattern of ABD activation during hypoxia and hypercapnia exposure [48–50]. Experimentally, most of the studies were conducted

on anesthetized, vagotomized, mechanically ventilated rats or reduced (in situ) rodent preparations, which provided detailed and relevant information. These studies offered important insights into the potential effects of AE on breathing parameters that would improve pulmonary ventilation. However, limitations in these studies, such as the depressant effects of anesthesia, absence of afferent information, tracheostomy, and clamped metabolic parameters, hindered a comprehensive interpretation of how ABD recruitment during expiration would affect ventilation and blood gas homeostasis. In our study, we combined measurements of pulmonary ventilation, DIA_{EMG} and ABD_{EMG} activities, and metabolic rate in unanesthetized, freely behaving animals to obtain detailed information and advance our comprehension of the ventilatory effects of AE on breathing under conditions of O₂ deficiency or CO₂ excess. Our data show that AE evoked during hypoxia and hypercapnia improves minute ventilation mainly by increasing tidal volume without affecting the inspiratory motor activity. However, due to its metabolic cost, AE does not improve air convection requirement, suggesting that this motor behavior may have other effects on the respiratory system rather than causing hyperventilation.

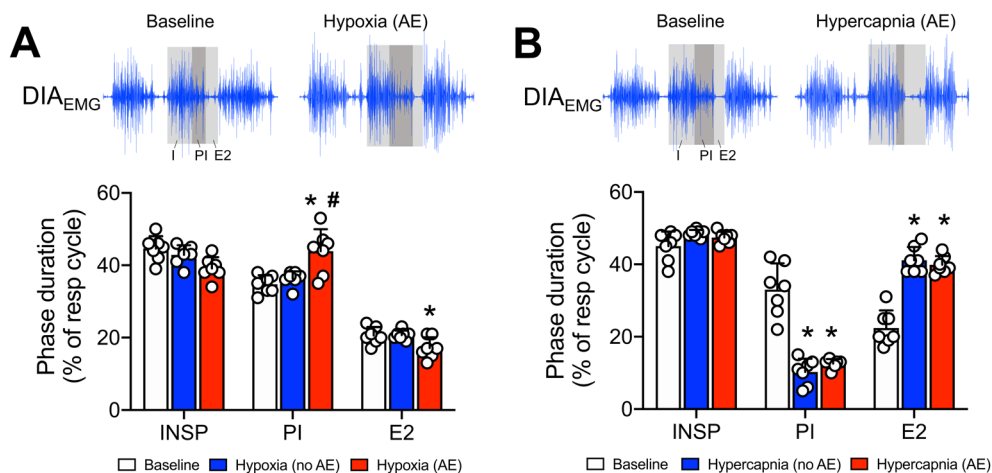


FIGURE 8 | Respiratory phase durations and active expiration during hypoxia and hypercapnia. Average values of the duration of the inspiratory (I), post-inspiratory (PI), and late expiratory (E2) phases in unanesthetized animals ($n=7$) during hypoxia (panel A) or hypercapnia (panel B) exposure without (no AE) and with active expiration (AE). Values are expressed as a percentage of the total cycle duration. The top traces illustrate representative recordings of the diaphragm electromyogram (DIA_{EMG}), which were used to define the respiratory phases (see the Methods section for more information). *Different from baseline conditions (normoxia/normocapnia); #Different from no AE conditions; $p < 0.05$ (repeated measures two-way ANOVA).

The ventilatory responses to hypoxia and hypercapnia are often associated with an increase in respiratory frequency and diaphragmatic activation caused by the stimulation of inspiratory pre-motor and motoneurons, including the inspiratory rhythm-generating neurons in the preBötC [8]. This enhanced inspiratory motor output accelerates the breathing rate and increases the depth of inspiration, as seen in our animals exposed to hypoxia and hypercapnia during periods without abdominal recruitment. Regarding the hypoxic ventilatory response, evidence indicates that it shows a biphasic pattern, with a large initial increase in ventilation, followed by a secondary depression if the hypoxic stimulus persists for several minutes (as in our experiment protocol), mainly due to a reduction in respiratory frequency [6, 51]. Because our evaluations started 3–5 min after the initiation of the hypoxia exposure, the animals were likely during the secondary phase of the hypoxic ventilatory response, which explains why we did not find relevant changes in respiratory frequency, except at 12% O_2 . We did not consider the initial minutes of hypoxia exposure for analysis because the O_2 levels in the chamber were equilibrating and the animals were frequently moving, which hindered accurate evaluations of EMG activity, ventilation, and O_2 consumption.

In both hypoxia and hypercapnia situations, regardless of the intensity of the stimulus, the presence of AE amplified pulmonary ventilation due to additional increases in V_T . These observations support similar observations previously obtained in other experimental conditions [18, 36, 45]. However, unlike other studies, we noted that AE did not promote relevant changes in respiratory frequency, except at 12% O_2 . Evidence obtained from in situ preparations suggested an association between AE and increased respiratory frequency in response to the activation of O_2 peripheral chemoreceptors in the carotid bodies [33]. In contrast, other studies performed in vagotomized and anesthetized animals or reduced preparations showed that AE evoked by hypercapnia or direct activation/disinhibition of expiratory-generating neurons in the pFL leads to expiratory phase prolongation and a reduction in respiratory frequency [13, 18, 19, 36].

These observations, combined with our results, indicate that AE elicited by hypoxia and hypercapnia primarily affects the lung volume in an unanesthetized animal containing all peripheral afferents and an intact central nervous system. However, we do not exclude that AE may influence respiratory frequency. In another study performed also in unanesthetized conditions, it was reported that AE can be recruited during sleep to promote stability in respiratory frequency when breathing irregularities arise [45]. We speculate that the effects of AE on respiratory rhythm may depend on the experimental condition or the animal's state (i.e., sleep or vigilance), which could affect the excitability of the respiratory network.

The changes in V_T during periods with AE did not correlate with increases in DIA_{EMG} burst amplitude, suggesting a direct effect of AE on lung volume. The highest ABD activity during hypoxia or hypercapnia occurred in the second stage of expiration (E2). This timing aligns with the firing patterns of expiratory oscillator neurons in the pFL and expiratory motoneurons in the spinal cord [13, 33, 52]. ABD activation during the E2 phase is thought to enhance expiratory flow and recruit expiratory reserve volume, thereby amplifying the subsequent inspiratory volume [15]. Consistent with this possibility, our analyses of flow-like signals during hypoxia demonstrated that an increased expiratory effort during E2 was associated with an increased inspiratory peak. During hypercapnia, in contrast, the presence of AE was associated with the amplification of expiratory efforts during the first part of expiration (post-inspiration) rather than the E2 phase. These observations suggest that the effect of AE on lung dynamics differs between hypoxia and hypercapnia, even though abdominal muscles are similarly activated during the same phase. We hypothesize that AE increases expiratory flow and recruits expiratory reserve volume during the E2 phase under hypoxia exposure, while during hypercapnia, it accelerates lung emptying and augments expiratory flow during the post-inspiratory phase. This possibility aligns with our analyses showing prolonged DIA_{EMG} post-inspiratory (breaking) activity and reduced E2 phase duration during hypoxia, and reduced

DIA_{EMG} post-inspiratory and prolonged E2 phase duration during hypercapnia. Moreover, these distinct effects of AE on expiratory flow may occur if these gas conditions differentially modulate upper airway resistance during expiration, restricting expiratory flow during hypoxia and facilitating it under hypercapnia. This hypothesis parallels previous reports showing that post-inspiratory laryngeal adductor muscle activity increases during the activation of carotid-body O₂ chemoreceptors [9, 39], and that the activity of dilator muscles in the pharynx and larynx increases during hypercapnia [13, 52]. However, these possibilities require further investigation.

A relevant novel finding in our study is the observation that the increase in $\dot{V}_T$ caused by AE does not improve air convection requirement ($\dot{V}_E/\dot{V}O_2$). Although this finding may not be surprising—since one might expect an increase in metabolic rate to support muscle contractions—it suggests that the rise in $\dot{V}_E$ induced by AE is proportional to the energy expenditure required for recruiting abdominal muscles, thus not leading to significant improvements in pulmonary ventilation. This interpretation, however, requires additional and detailed experiments to be fully elucidated, including measurements of arterial blood gas during periods with and without AE and analyses of O₂ extraction ratio. Nevertheless, these findings indicate that the functional significance of AE may not be restricted to alveolar ventilation and may also contribute to other aspects, such as controlling upper airway pressure or distribution of ventilation, which ultimately facilitates gas diffusion, or improving respiratory stability [45, 53]. This scenario of various effects of AE on the respiratory system combined with its metabolic cost may explain why this respiratory behavior shows a fragmented and non-uniform expression in unanesthetized animals under hypoxia and hypercapnia. Evidence shows that the intensity of ABD recruitment can be influenced by the animal's vigilance state or posture [30, 45, 54, 55]. Although we believe that the former did not interfere significantly with our analyses due to the short-term nature of our exposure (20–30 min) and the fact that acute hypoxia/hypercapnia exposure evokes arousal responses [56], the latter is a possibility that was not controlled in our experiments. Previous studies have documented an inconsistent pattern of ABD activation during hypercapnic conditions and sleep [30, 57]. Our current study supports these earlier observations and reports the same fragmented AE pattern under hypoxia exposure.

In conclusion, our study provides detailed insights into the occurrence and functional impacts of AE on pulmonary ventilation, respiratory motor activity, and metabolism in unanesthetized rats exposed to several levels of hypoxia and hypercapnia. Our data indicate that AE occurs periodically during situations of reduced O₂ or excessive CO₂ availability and enhances expiratory flow and tidal volume to boost lung ventilation independently of increases in diaphragmatic activity. During hypoxia, AE appears to recruit the expiratory reserve volume during the E2 phase, while during hypercapnia, AE augments the expiratory flow peak during the post-inspiratory phase to accelerate lung emptying. However, the onset of AE incurs an additional metabolic cost and does not improve air convection requirement, suggesting that this motor behavior may influence other aspects of the respiratory system that can improve alveolar ventilation and gas exchange. Understanding the physiological role of AE during hypoxia and

hypercapnia is crucial, given the prevalence of conditions involving blood gas and metabolic disturbances, such as chronic obstructive pulmonary disease, sleep apnea, or high-altitude acclimatization. Future research exploring the association between impaired AE recruitment and respiratory diseases opens avenues for therapeutic modulation of AE in these contexts.

Author Contributions

Isabela P. Leirão: conceptualization, formal analysis, investigation, methodology, project administration, validation, visualization, writing – original draft, writing – review and editing and revision. **Pedro L. Katayama:** formal analysis, writing – original draft, writing – review and editing and revision. **Daniel B. Zoccal:** conceptualization, data curation, formal analysis, funding acquisition, methodology, project administration, resources, supervision, validation, writing – review and editing and revision. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work to ensure that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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