

# The hyperaemic and metaboreflex response of the diaphragm during fatiguing diaphragmatic work in humans

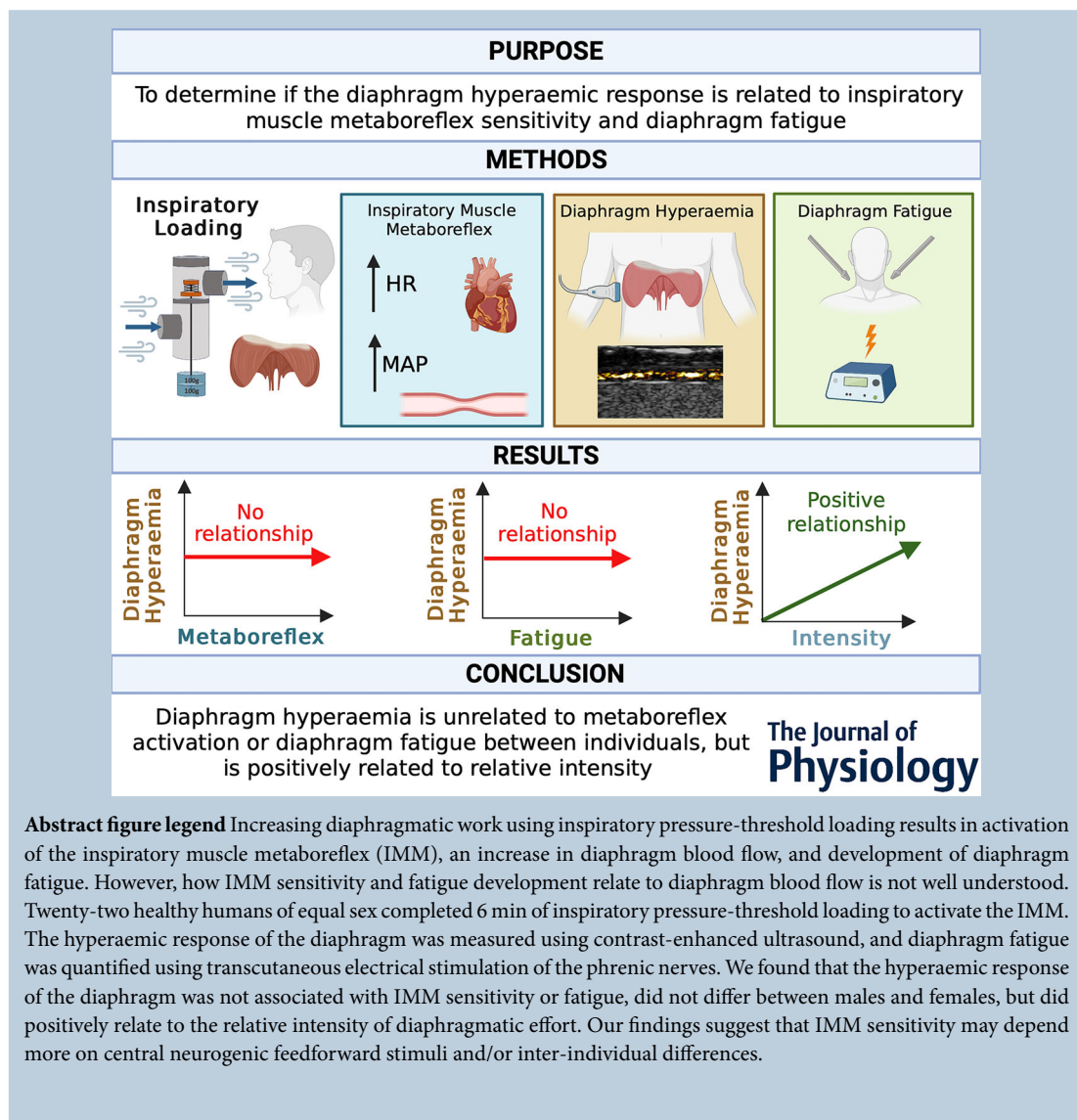
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**Abstract** During inspiratory pressure-threshold loading (ITL), the inspiratory muscle metaboreflex (IMM) increases respiratory muscle blood flow, but its effect on diaphragm blood flow ( $\dot{Q}_{di}$ ) and fatigue in humans, is unclear. Females exhibit an attenuated IMM compared to males during diaphragmatic loading, suggesting less diaphragm blood flow impedance and metaboreflex activation. We hypothesized that diaphragm hyperaemia would relate positively to metaboreflex sensitivity (i.e. mean arterial pressure change;  $\Delta$ MAP) and fatigue development (i.e. transdiaphragmatic twitch pressure change;  $\Delta P_{di,tw}$ ), and be less for females than males. Healthy males and females (11 males and 11 females) completed 6 min of ITL targeting a transdiaphragmatic pressure ( $P_{di}$ ) of 80 cmH<sub>2</sub>O. Contrast-enhanced ultrasound was used to quantify  $\dot{Q}_{di}$  at baseline, 50% and 100% of task completion. The  $\Delta$ MAP during loading and the pre- to post-task  $\Delta P_{di,tw}$ , via bilateral phrenic nerve electrical stimulation, assessed metaboreflex activation and fatigue, respectively. The accumulated pressure-time-product for  $P_{di}$  ( $14,177 \pm 1,497$  cmH<sub>2</sub>O·s; mean  $\pm$  SD) and percentage of maximal  $P_{di}$  ( $\%P_{di,max}$ ,  $51 \pm 8\%$ ) was not different between sexes ( $P > 0.600$ ). At 100% of task completion, IMM activation ( $\Delta$ MAP, +21 mmHg; 95% confidence interval (CI) = 18–24 mmHg;  $P < 0.0001$ ), diaphragm fatigue ( $\Delta P_{di,tw}$ , –18.1 mmHg; 95% CI = –22.2 to –13.9%;  $P < 0.0001$ ) and hyperaemia ( $\Delta \dot{Q}_{di}$ , +15.8 AU s<sup>–1</sup>; 95% CI = 12.5–19.1 AU s<sup>–1</sup>;  $P < 0.0001$ ) did not differ between sexes ( $P \geq 0.352$ ). There was a significant positive relationship between  $\Delta \dot{Q}_{di}$  and  $\%P_{di,max}$  ( $r^2 = 0.211$ ;  $P = 0.0317$ ), but no relationship between  $\Delta \dot{Q}_{di}$  and either  $\Delta$ MAP ( $r^2 = 0.011$ ;  $P = 0.638$ ) or  $\Delta P_{di,tw}$  ( $r^2 = 0.001$ ,  $P = 0.914$ ). Our data demonstrate that diaphragm hyperaemia relates to relative exercise intensity ( $\%P_{di,max}$ ) but is not associated with metaboreflex sensitivity, diaphragm fatigue or sex.

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### Key points

- The inspiratory muscle metaboreflex is activated during fatiguing diaphragmatic loading to redistribute blood flow to working muscles. How diaphragm hyperaemia relates to metaboreflex sensitivity or diaphragm fatigue development is unknown.
- Compared to males, females are reported to have an attenuated inspiratory muscle metaboreflex during both equal absolute and relative intensities, as well as greater diaphragm fatigue resistance during absolute workloads. We investigated whether these sex-based differences result from a lesser hyperaemic response for females compared to males.
- We found that hyperaemia of the diaphragm was positively correlated with fractional transdiaphragmatic pressure but did not relate to the degree of diaphragm fatigue or metaboreflex sensitivity.
- Females and males without differences in diaphragmatic strength did not differ in the magnitude of diaphragm hyperaemia, metaboreflex response, or fatigue development.

**Megan L. Lance** completed her MSc (2024) in the School of Health and Exercise Sciences at UBC Okanagan, under the mentorship of Dr. Glen Foster. She plans to complete a PhD to investigate the regulation of diaphragm blood flow in humans. She received a Stober Undergraduate Research Fellowship to help develop techniques for studying diaphragm blood flow in humans.



## Introduction

To accommodate the diaphragm's blood flow ( $\dot{Q}_{di}$ ) requirements during exercise, there is evidence for the activation of an inspiratory muscle metaboreflex (IMM) to redirect blood flow toward the active respiratory muscles (Dominelli et al., 2017; Musch et al., 1987; St Croix et al., 2000). The IMM is activated during fatiguing respiratory and/or diaphragmatic work, when metabolite build-up stimulates type III/IV afferents within the respiratory muscles to reflexively increase muscle sympathetic nerve activity, heart rate (HR) and mean arterial pressure (MAP) (Sheel et al., 2001; St Croix et al., 2000).

The build-up of metabolites, in turn, is influenced by the generation of high intramuscular pressures relative to maximal diaphragmatic capacity ( $\%P_{di,max}$ ) and prolonged contraction times (i.e. ratio of inspiratory contraction time to full respiratory cycle duration;  $T_I/T_{TOT}$ ). Together, these factors determine the tension-time index of the diaphragm ( $TTI_{di}$ ) (Bellemare et al., 1983), a measure that can characterize the interplay between diaphragmatic workloads and blood flow. Based on invasive measurements in dogs, when the  $TTI_{di}$  exceeds a critical threshold ( $\sim 0.2$ ), there is sufficient mechanical compression of blood vessels that leads to  $\dot{Q}_{di}$  impedance (Bellemare et al., 1983). Additionally, it has been shown in humans that as  $TTI_{di}$  increases past 0.15, there is an inverse relationship between  $TTI_{di}$  and time until task failure, potentially because of greater blood flow limitation (Bellemare & Grassino, 1982; Bird et al., 2025a). As such, several studies in humans have targeted a  $TTI_{di} > 0.2$  to promote impedance of  $\dot{Q}_{di}$ , activate the IMM and induce diaphragm fatigue (Archiza et al., 2021; Geary et al., 2019; St Croix et al., 2000; Welch et al., 2018).

The diaphragm's oxygen demands are met through a rise in both diaphragmatic arteriovenous  $O_2$  difference and  $\dot{Q}_{di}$ , with perfusion continuing to increase even as extraction plateaus (Robertson et al., 1977; Rochester & Bettini, 1976). As such, an increase in diaphragm perfusion appears to play a role in sustaining contractile effort and delaying fatigue. This underscores the potential importance of diaphragm hyperaemia ( $\Delta\dot{Q}_{di}$ ) in supporting recovery and regulating IMM activation, although this relationship remains poorly understood.

The IMM is attenuated for human females compared to human males during isolated fatiguing diaphragmatic work at both equal relative (Welch et al., 2018) and absolute workloads (Geary et al., 2019). Additionally, despite performing at a higher  $\%P_{di,max}$ , females exhibit a similar magnitude of diaphragm fatigue compared to males following an absolute diaphragmatic workload (Geary et al., 2019). Collectively, these findings suggest that females may have less of a detriment to  $O_2$  delivery, less post-exercise hyperaemia and ultimately limited metabolite accumulation and IMM activation during

fatiguing diaphragmatic work. Supporting this, previous research has demonstrated a significant positive relationship between the post-exercise hyperaemia response of the forearm vasculature with increases in MAP during equal relative workloads of handgrip exercise (Taylor et al., 1988).

However, because of the diaphragm's relative inaccessibility and the technological challenges of quantifying  $\dot{Q}_{di}$  in humans, the relationship between the IMM sensitivity and diaphragm fatigue with  $\Delta\dot{Q}_{di}$  is unknown. Furthermore, it is unclear whether  $\Delta\dot{Q}_{di}$  during fatiguing diaphragmatic exercise differs between sexes. Recently, contrast-enhanced ultrasound imaging (CEUS) has been reported as a reliable method for measuring  $\dot{Q}_{di}$  in humans (Bird et al., 2024). Thus, we aimed to determine: (1) whether the sensitivity of the IMM and development of diaphragm fatigue are related to  $\Delta\dot{Q}_{di}$  and (2) whether the attenuated IMM observed in females would lessen the  $\Delta\dot{Q}_{di}$  compared to males. We hypothesized that the  $\Delta\dot{Q}_{di}$  to fatiguing diaphragmic effort would be: (1) positively related to the activation of the IMM and development of diaphragm fatigue and (2) less for females compared to males during a workload-matched loading task.

## Methods

### Ethical approval

Ethical clearance was received from the Clinical Research Ethics Board at the University of British Columbia (H23-0 2158) in accordance with the *Declaration of Helsinki*, except for registration in a database. Written informed consent was obtained from all participants.

### Participants

Healthy and recreationally-active males and females (i.e. moderate physical activity 3–5 days/week) were recruited for the present study. Participants had no history of smoking or vaping, did not self-report any medical history of cardiovascular, respiratory or neurological disease, and were not taking any medication, except for oral contraceptives. Participants were excluded if they had a known or suspected hypersensitivity to perflutren or its components (e.g. polyethylene glycol), an allergy to antihistamines, latex or lidocaine, or were susceptible to anaphylactic reactions. Female participants were excluded if they were pregnant (confirmed by a pregnancy test) and were tested randomly throughout their menstrual cycle.

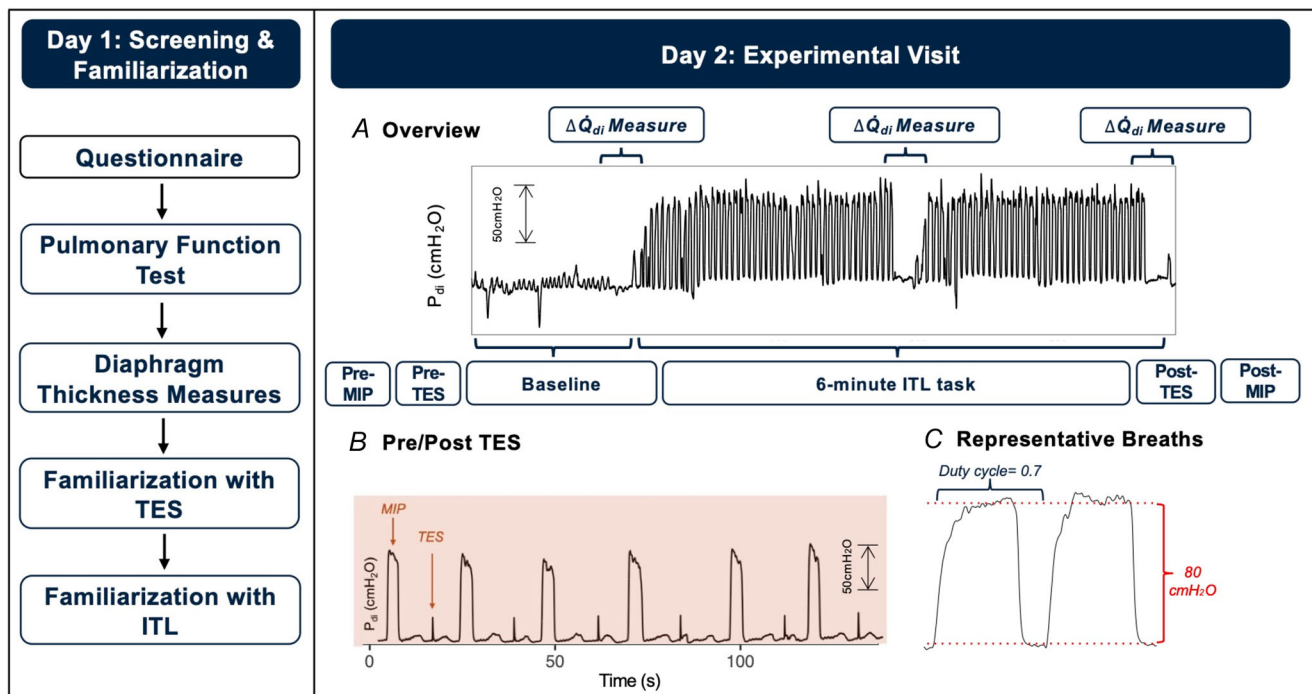
### Experimental overview

A schematic of the study protocol is presented in Fig. 1. Participants completed two testing sessions.

Visit 1 was a screening and familiarization visit, with participants completing the Physical Activity Readiness Questionnaire for Everyone (<https://eparmedx.com/>) form and screening questionnaire to ensure they met the inclusion/exclusion criteria. If the participant was eligible for the study, a pulmonary function test was conducted following the American Thoracic Society and European Respiratory Society's joint guidelines (Quanjer et al., 2012; Stanojevic et al., 2017), and resting blood pressure was determined. Diaphragm measurements were assessed with a 15 MHz transducer (ML6-15; GE Healthcare Technologies, Piscataway, NJ, USA) in the seated position and B-mode cine loops were used to determine the change in diaphragm thickness with quiet tidal breathing and to measure thickening fraction, as previously described (Haaksma et al., 2022). This was followed by instrumentation of two balloon-tipped catheters (47-9005; Cooper Surgical, Trumbull, CT, USA), in which the catheters were inserted nasally and positioned in the oesophagus and stomach to quantify transdiaphragmatic pressure ( $P_{di}$ ) using two differential pressure transducers (DP15; Validyne, Morthridge, CA, USA) as previously described (Milic-Emili et al., 1964). Before balloon insertion, a topical anaesthetic (2% lidocaine hydrochloride; Xylocaine; AstraZeneca, Cambridge, UK) was applied to the participant's nares

and nasopharynx. Participants were familiarized with transcutaneous electrical stimulation (TES) of the phrenic nerves and inspiratory pressure-threshold loading (ITL) to ensure participants could tolerate the stimuli and could perform the breathing task.

The second visit, conducted at least 24 h later, was the experimental visit. Participants refrained from caffeine and alcohol for 12 h and exercise for 24 h beforehand. Participants were instrumented with a 21 gauge antecubital venous catheter for contrast agent infusion and balloon-tipped oesophageal and gastric catheters. Following instrumentation, a contact-activated lancet was used to sample capillary blood from a fingertip, and haemoglobin concentrations were measured using a HemoCue 201+ analyzer (HemoCue AB, Ängelholm Sweden). Participants then completed a series of maximal inspiratory pressure manoeuvres from functional residual capacity to determine their maximal inspiratory muscle volitional strength (MIP) and diaphragmatic volitional strength ( $P_{di,max}$ ). Both manoeuvres were done as a series of separate trials with specific instructions provided. For MIP, participants were instructed to inhale maximally, whereas, for  $P_{di,max}$ , they were asked to inhale from their stomach (i.e. belly breathing). During the completion of three repeatable (<10% variation) manoeuvres for  $P_{di,max}$  and three for MIP, the highest value for each variable was



**Figure 1. Schematic of study protocol**

The study consisted of a screening/familiarization visit (day 1) and an experimental visit (day 2). Panel A shows an overview of the experimental visit, panel B illustrates the transcutaneous electrical stimulation protocol, and panel C shows representative breaths from inspiratory pressure-threshold loading. ITL; inspiratory pressure-threshold loading; MIP; maximal inspiratory pressure;  $P_{di}$ , transdiaphragmatic pressure; TES, transcutaneous electrical stimulation;  $\Delta Q_{di}$ ; diaphragm hyperaemia.

selected from the total pool of six manoeuvres, regardless of which specific test it was recorded.

Transdiaphragmatic twitch pressure ( $P_{di,tw}$ ) in response to TES was recorded before baseline and immediately following ( $\sim 1$  min) the ITL task to assess diaphragm fatigue. Following a 5 min resting baseline, participants performed normocapnic ITL for 6 min. At baseline, 50% and 100% of task completion,  $\dot{Q}_{di}$  measures were acquired for 10 s when the participant performed an end-expiratory apnoea. Another series of MIP and  $P_{di,max}$  manoeuvres was performed immediately following post-exercise TES measures.

### Inspiratory pressure-threshold loading

The ITL apparatus and detailed methodology have been described elsewhere (Eastwood & Hillman, 1995). Briefly, participants inspired against a weighted valve and adding weight increased the inspiratory effort required for inspiratory air flow. Participants were seated in an upright position and breathed through a customized two-way non-rebreathing valve connected to an ITL device and were instructed to breathe from their stomach at the same time as minimizing upper chest movement. Weight was added to a plunger until the participant could reach a  $P_{di}$  of  $+80$  cmH<sub>2</sub>O from their baseline  $P_{di}$  values. Breathing rate ( $f_B$ ) was controlled at 15 breaths  $\text{min}^{-1}$ , with an inspiratory duty cycle (i.e.  $T_I/T_{TOT}$ ) of 0.7 using real-time visual feedback of  $P_{di}$  and the desired square-wave breathing pattern (Fig. 1A and 1C). The task was terminated after 6 min for all participants. Throughout loading, end-tidal partial pressure of carbon dioxide ( $P_{ETCO_2}$ ) was monitored and, if necessary, 100% CO<sub>2</sub> was titrated into the ITL apparatus to maintain normocapnia. Mouth pressures were measured from a port in the mouthpiece connected to a differential pressure transducer (model PA-1; Hans Rudolph, Kansas City, MO, USA).

### Diaphragm electromyography and fatigue measures

Electrical activity (i.e. EMG) of the left costal diaphragm was recorded via adhesive cloth electrodes (10 mm in diameter; ES40076; Cardinal Health, Dublin, OH, USA) arranged in a bipolar configuration over the anterior axillary line of the sixth to eighth intercostal spaces (Glerant et al., 2006) and a ground electrode was placed on the acromion process. Surface EMG signals were amplified ( $100\times$ ) and high-pass filtered (10 Hz) with a pre-amplifier and isolator (NL844 and NL820, respectively; Digitimer, Welwyn Garden City, UK), low-pass filtered (1000 Hz; NL136 module) and sampled at 2000 Hz using an analog-to-digital converter

(Powerlab/16SP ML 880; ADInstruments, Colorado Springs, CO, USA) interfaced with a personal computer.

To quantify diaphragm fatigue, TES of the phrenic nerves was used as previously described (Bellemare & Bigland-Ritchie, 1987; Mador et al., 1996). Briefly, two constant current stimulators (DS7AH; Digitimer) were used to stimulate both phrenic nerves via two manually-placed stimulating electrode pens (cathodes; Compex, Geneva, Switzerland) positioned over the nerves (behind the sternocleidomastoid muscles and parallel to the cricoid cartilage). The anode was a self-adhesive electrode (1700-030; Conmed, Utica, NY, USA) positioned just below the midline of each respective clavicle. Optimal electrode placement was determined on each side separately by adjusting the cathode to search for the site that produced the largest  $P_{di,tw}$  for a given current output. Following placement, each stimulator simultaneously delivered a single supra-maximal electrical stimulus (0.1 ms pulse width) at end-expiration during resting breathing, to evoke a  $P_{di,tw}$ , and the compound muscle action potential (i.e. M-wave) of the left hemi-diaphragm was recorded. The current was incrementally increased by 10 mA until the  $P_{di,tw}$  and M-wave plateaued, observed by no increases in peak-to-peak amplitudes despite an increase in current. To ensure activation of all axons throughout the protocol, an additional 30% of current was added to account for hyperpolarization that can occur during fatiguing exercise (Vagg et al., 1998). Thereafter, a series of six to 10 potentiated twitches (delivery of stimulus to each phrenic nerve simultaneously preceded by  $\sim 5$  s maximal inspiratory effort) and M-waves were recorded before and after the exercise intervention (Fig. 1B). All stimulations were performed at end-expiration by verifying the oesophageal pressure tracing to minimize the influence of lung volumes on  $P_{di,tw}$  (Smith & Bellemare, 1987), and were performed by the same investigator.

### Diaphragm blood flow measures

Contrast-enhanced ultrasound imaging was used to measure  $\dot{Q}_{di}$  as previously described (Bird et al., 2024). Briefly, a constant-rate intravenous infusion (20:1 dilution in saline, 2.5 mL  $\text{min}^{-1}$ ) of lipid-stabilized microbubbles (Definity; Lantheus Medical Inc., Billerica, MA, USA) was administered beginning at the last 2 min of baseline until the end of ITL. During baseline, 50% and 100% of ITL completion, CEUS images were captured at 10 frames per second using a 9L-D transducer and Vivid E9 ultrasound machine in amplitude modulation mode (GE Healthcare Technologies). The imaging settings included a mechanical index of 0.09–0.11, depth of 3–3.5 cm, fundamental frequency of 4 MHz, dynamic range of 57 dB, one focal zone placed at the deep border of the

diaphragm, and no persistence or edge enhancement. Participants were coached into an end-expiratory apnoea near functional residual capacity and CEUS images were acquired after a destruction-replenishment sequence with minimal movement artefact, in which an acoustic flash was used to destroy the microbubbles within the field of view. Ultrasound cine loops were acquired for 10 s following microbubble destruction during an end-expiratory apnoea. Our measurements of  $\dot{Q}_{di}$  serve as an index of diaphragm perfusion derived from microvascular signal intensity within a 2-D region of the muscle (Bird et al., 2024; Wei et al., 1998).

Following the apnoea, participants performed an inspiratory capacity manoeuvre to determine end-expiratory lung volume (EELV) at the time of CEUS measurements by subtracting inspiratory capacity from total lung capacity. The skin was marked at the probe site for consistent placement between measurements. Vascular conductance of the diaphragm ( $VC_{di}$ ) was calculated as  $\dot{Q}_{di}/MAP$ . All CEUS examinations were conducted by the same investigator.

### Cardiovascular and respiratory measures

All cardiovascular and respiratory variables were acquired using an analogue-to-digital converter (ML880 PowerLab 16/35; ADInstruments, Colorado Springs, CO, USA) at 200 Hz and monitored using LabChart data acquisition, version 8.1.30 (ADInstruments). Non-invasive measures of beat-by-beat blood pressure by finger pulse photoplethysmography (Finometer PRO; Finapres Medical Systems, Amsterdam, The Netherlands), HR by lead II electrocardiogram (FE132; ADInstruments) and oxyhaemoglobin saturation by pulse oximetry (7500FO; Nonin Medical, Plymouth, MN, USA) were continuously recorded. Participants breathed through a mouthpiece attached to a bacteriological filter and the inspiratory pressure-threshold loader while wearing a nose clamp. Respired end-tidal oxygen ( $P_{ETO_2}$ ) and  $P_{ETCO_2}$  gas pressures were sampled on the expiratory side of the experimental setup by a gas analyser (ML206; ADInstruments). Inspiratory and expiratory flow were measured separately using two pneumotachometers (models 3813 and 4813; Hans Rudolph, Shawnee, KS, USA) connected to differential pressure amplifiers (model PA-1; Hans Rudolph). The expiratory pneumotachometer was attached to a pneumotach heater control (3850A series; Hans Rudolph) set to 37°C, whereas the inspired pneumotachometer was left at ambient temperature. Inspiratory measures were only recorded during inspiratory capacity manoeuvres and baseline measures because a pneumotach was not connected to the inspiratory pressure-threshold loader.

### Data analysis

Cardiorespiratory data were averaged on a breath-by-breath basis and binned into baseline (final 2 min), 50% and 100% task completion (final minute each). The  $P_{di,tw}$  was determined as the change in pressure from stimulus onset to peak pressure. The first two potentiated twitches were excluded due to incomplete potentiation, as indicated by rising  $P_{di,tw}$  amplitudes. Twitches not occurring at end-expiration, identified via  $P_{oes}$ , were also excluded. M-wave characteristics were analysed from the diaphragm EMG recordings, and included duration, latency, amplitude, and area. The amplitude and area were normalized to the largest M-wave recorded at baseline and expressed as a percentage.

The accumulated force output (i.e. pressure-time product) of the diaphragm ( $PTP_{di}$ ) and oesophagus ( $PTP_{oes}$ ) were calculated by integrating  $P_{di}$  and  $P_{oes}$  over the periods of inspiration for the entire duration of the ITL protocol, respectively. The  $TTI_{di}$  was calculated as the product of  $P_{di}/P_{di,max}$  and  $T_I/T_{TOT}$ , in which  $P_{di,max}$  was determined from functional residual capacity.

Ultrasound cine loops were analysed via freely available software (narnar app; narnar, Lake Oswego, OR, USA) to measure video intensity on a frame-by-frame basis within a region of interest (ROI). Time and acoustic intensity data were determined for ROIs within the diaphragm and model fitted to the following wash-in curve (Bird et al., 2024):

$$y = A(1 - e^{-\beta t})$$

where  $y$  is the video intensity at the pulsing interval,  $A$  is the plateau video intensity reflecting microbubble content, an index for microvascular blood volume ( $MBV_{di}$ ) and  $\beta$  is reflecting microbubble flux rate, an index for microvascular flux rate ( $MFR_{di}$ ). The multiplication of  $MBV_{di}$  and  $MFR_{di}$  is proportionate to  $\dot{Q}_{di}$ . This analysis technique has been used extensively to characterize relative microvascular perfusion (Rim et al., 2001; Weber et al., 2006; Wei et al., 1998).

### Statistical analysis

Statistical analyses were performed using R, version 4.3. (R Foundation for Statistical Computing, Vienna, Austria). Normality was confirmed from visual inspection of quantile–quantile normality plots and the histogram of residuals. Data are presented as the mean  $\pm$  SD, whereas statistical contrasts are presented as mean differences  $\pm$  95% confidence interval (CI).  $P < 0.05$  was considered statistically significant. Participant anthropometric parameters, pulmonary function data and basic diaphragm structure were compared between sexes using an independent samples Student's  $t$  test. Changes in cardiorespiratory measures and  $\dot{Q}_{di}$  were

compared using a linear mixed-effect model with time (baseline, 50% and 100% of ITL) and sex as fixed factors, and participant as a random factor (random intercept model). For cardiorespiratory data, the minute before each corresponding CEUS measurement (i.e. before 50% and 100% loading) was averaged and used for comparison. When significant effects were identified, Tukey's honestly significant difference (HSD) was used to identify pairwise differences.

A plateau of  $P_{di,tw}$  and M-wave amplitude was determined using a linear mixed-effect model with stimulator intensity (% of max) and sex as fixed factors and participant as a random factor, where 100% is the stimulator intensity required to record the highest peak-to-peak amplitude during the diaphragm muscle recruitment protocol. The change in  $P_{di,tw}$  from baseline to post-ITL was compared between sexes using a two-way independent samples Student's *t* test.

Associations between cardiorespiratory, diaphragm fatigue and  $\dot{Q}_{di}$  measures were assessed using linear models and Pearson correlation coefficients for between-participant comparisons (i.e. comparing baseline to 100% task completion across individuals). To assess within-subject relationships, accounting for repeated measurements from the same individual, a repeated measures correlation (Bland & Altman, 1995) was performed, permitting comparisons between baseline, 50% and 100% of task completion within individuals. This approach accounts for inter-individual variability and isolates the common within-participant association across the sample.

## Results

### Participant characteristics

In total, 24 participants (12 females and 12 males) were recruited for this study. Out of the 24 participants, one female and one male were unable to complete the ITL task during the familiarization visit, bringing the final sample size of this investigation to 22 (11 males and 11 females). Because of technical issues, TES data are missing for one male participant,  $\dot{Q}_{di}$  measurements at 50% of task completion are missing from three participants (two females and one male) and M-wave recordings are missing from four participants (two females and two males). Participant demographics, pulmonary function, diaphragm parameters and haemoglobin data are presented in Table 1. Male participants had a greater height, mass, lung volume, diffusion capacity and haemoglobin concentration compared to females ( $P < 0.0001$ ). When expressed as a percent of predicted values based on age, sex, height and ethnicity in accordance with the Global Lung Function Initiative (<https://gli-calculator.ersnet.org/>), pulmonary function,

lung volumes and diffusion capacity measurements were not different ( $P \geq 0.316$ ) between sexes. Males had a significantly greater MIP ( $P = 0.00609$ ) and end-inspiration diaphragm thickness during tidal breathing ( $P = 0.0315$ ) compared to females, but  $P_{di,max}$  was not different between sexes ( $P = 0.713$ ).

### Diaphragm blood flow

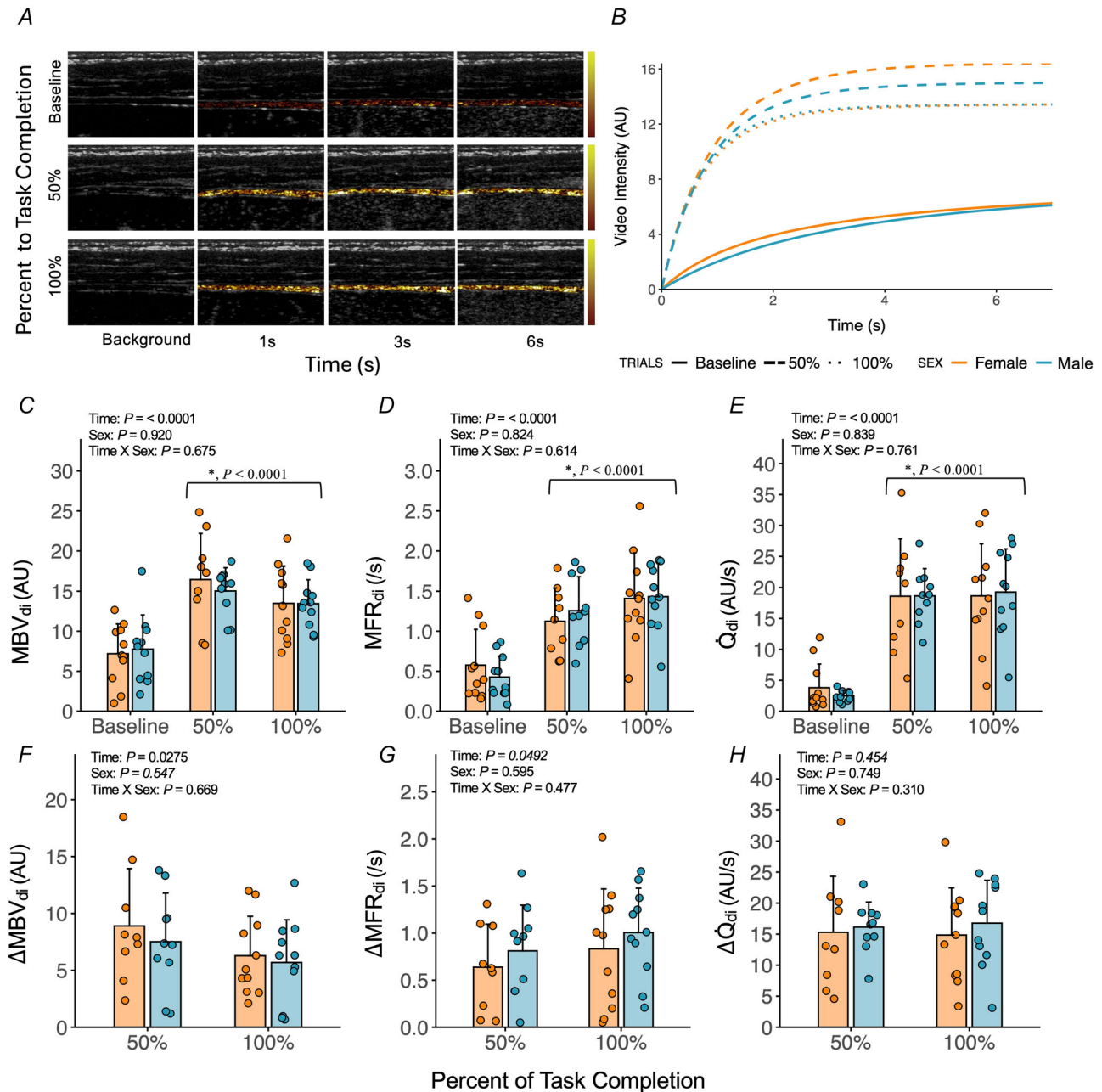
Diaphragm blood flow data are presented in Fig. 2. There was a main effect of time ( $P < 0.0001$ ) but not sex ( $P \geq 0.824$ ) for  $MBV_{di}$ ,  $MFR_{di}$  and  $\dot{Q}_{di}$  throughout ITL. There was a significant increase of  $MBV_{di}$  (+6.0 AU; 95% CI = 3.9–8.0 AU;  $P < 0.0001$ ),  $MFR_{di}$  (+0.9  $s^{-1}$ ; 95% CI = 0.7–1.2  $s^{-1}$ ;  $P < 0.0001$ ) and  $\dot{Q}_{di}$  (+15.8 AU  $s^{-1}$ ; 95% CI = 12.5–19.1 AU  $s^{-1}$ ;  $P < 0.0001$ ) from baseline to 100% of task completion, with similar increases from baseline to 50% of task completion ( $P \geq 0.0689$ ) (Fig. 2, C, D and E).

There was a main effect of time ( $P < 0.0001$ ) but not sex ( $P \geq 0.547$ ) for the  $\Delta MBV_{di}$  and  $\Delta MFR_{di}$ . The  $\Delta MBV_{di}$  was significantly lower by 1.9 AU (95% CI = 0.1–3.6 AU) at 100% of task completion compared to 50% ( $P = 0.0351$ ) (Fig. 2F). The  $\Delta MFR_{di}$  was significantly higher by 0.2  $s^{-1}$  (95% CI = 0.0–0.4  $s^{-1}$ ) at 100% of task completion compared to 50% ( $P = 0.0492$ ) (Fig. 2G). There was no effect of time ( $P = 0.454$ ) or sex ( $P = 0.749$ ) on the  $\Delta \dot{Q}_{di}$  at 100% of task completion compared to 50% (Fig. 2H). There was a main effect of time ( $P < 0.0001$ ) but not sex ( $P = 0.793$ ) on  $VC_{di}$  (Table 2) in which it increased from baseline to 50% (+0.14 AU  $s^{-1}$  mmHg $^{-1}$ ; 95% CI = 0.11–0.17 AU  $s^{-1}$  mmHg $^{-1}$ ) and 100% (+0.14 AU  $s^{-1}$  mmHg $^{-1}$ ; 95% CI = 0.11–0.17 AU  $s^{-1}$  mmHg $^{-1}$ ) of task completion ( $P < 0.0001$ ).

There was a main effect of time ( $P < 0.0001$ ) and sex ( $P = 0.00962$ ), and no interaction ( $P = 0.480$ ) on the EELV as a percentage of total lung capacity (Table 2), in which it was significantly lower at 50% (–8.7%; 95% CI = –12.6% to 4.8%) and 100% (–8.4%; 95% CI = –12.3% to –4.5%) of task completion compared to baseline ( $P < 0.0001$ ) and it was higher for females by 8% (95% CI = –2.0% to 15.0%) compared to males ( $P = 0.0123$ ). There was no effect of time ( $P = 0.0705$ ) or sex ( $P = 0.955$ ) on the length of apnoea required to obtain the CEUS measures. The apnoea length at baseline, 50% and 100% of task completion were 14.4 s (95% CI = 12.9–15.9 s), 16.0 s (95% CI = 14.4–17.5 s) and 15.3 s (95% CI = 13.8–16.9 s), respectively.

### Cardiovascular response

The cardiovascular responses to ITL at baseline, 50%, and 100% of task completion are shown in Fig. 3 and Table 2. There was a main effect of time ( $P < 0.0001$ ) but not sex ( $P = 0.849$ ) for HR (Fig. 3A). Heart rate increased



**Figure 2. Contrast-enhanced ultrasound measurements of diaphragm blood flow ( $\dot{Q}_{di}$ )**

A, background-subtracted, colour-coded contrast-enhanced ultrasound images of an individual male participant at baseline, as well as 50%, and 100% of task completion. B, destruction-replenishment curves from average time-intensity data within the diaphragm at baseline and 50%, and 100% of task completion, and between males and females.  $N = 22$  (11 females: 11 males) for all measures except for the 50% timepoint, in which  $N = 19$  (9 females: 10 males). Absolute values of microbubble content of the diaphragm (MBV<sub>di</sub>) (C), microbubble flux of the diaphragm (MFR<sub>di</sub>) (D) and  $\dot{Q}_{di}$  (E), and absolute changes from baseline in  $\Delta$ MBV<sub>di</sub> (F),  $\Delta$ MFR<sub>di</sub> (G) and  $\Delta$  $\dot{Q}_{di}$  (H). Absolute values (C), (D) and (E) were compared using a linear mixed-effect model with time (baseline, 50% and 100% of inspiratory loading) and sex as fixed factors, and participant as a random factor (random intercept model). Changes from baseline (F), (G) and (H) were compared using a linear mixed-effect model with time (50%, and 100% of inspiratory loading) and sex as fixed factors, and participant as a random factor (random intercept model). When significant effects were identified, Tukey's HSD was used to identify pairwise differences. \*Significantly different compared to baseline.

**Table 1. Participant demographics, pulmonary function, diaphragm ultrasound and haematology.**

Variable	Male (n = 11)	Female (n = 11)	Total (n = 22)	P
<b>Demographic characteristics</b>				
Age (years)	28 ± 6	26 ± 4	27 ± 5	0.329
Height (cm)	181 ± 5	165 ± 6	173 ± 10	<0.0001
Mass (kg)	78 ± 5	63 ± 7	71 ± 10	<0.0001
BMI (kg m <sup>-2</sup> )	23.8 ± 1.9	23.3 ± 2.6	23.6 ± 5.1	0.606
<b>Pulmonary function</b>				
<b>Spirometry</b>				
FVC (L)	6.2 ± 0.9	4.3 ± 0.5	5.2 ± 1.2	<0.0001
FVC (% predicted)	110 ± 15	113 ± 12	111 ± 13	0.690
FEV <sub>1</sub> (L)	4.7 ± 0.6	3.4 ± 0.4	4.1 ± 0.8	<0.0001
FEV <sub>1</sub> (% predicted)	101 ± 10	105 ± 14	103 ± 12	0.479
FEV <sub>1</sub> /FVC (%)	76 ± 4	80 ± 8	79 ± 6	0.142
FEV <sub>1</sub> /FVC (% predicted)	92 ± 5	93 ± 8	92 ± 7	0.923
<b>Lung volumes</b>				
TLC (L)	7.6 ± 1.1	5.7 ± 0.6	6.7 ± 1.3	<0.0001
TLC (% predicted)	104 ± 18	110 ± 10	107 ± 15	0.344
FRC (L)	3.6 ± 0.6	2.8 ± 0.4	3.2 ± 0.7	<0.00354
FRC (% predicted)	103 ± 13	105 ± 14	104 ± 12	0.726
RV (L)	1.8 ± 0.5	1.4 ± 0.5	1.6 ± 0.5	0.106
RV (% predicted)	106 ± 28	92 ± 37	99 ± 33	0.316
<b>Diffusing capacity</b>				
D <sub>L</sub> CO (mL min <sup>-1</sup> mmHg <sup>-1</sup> )	39.4 ± 5.7	26.6 ± 5.6	33.0 ± 8.5	<0.0001
D <sub>L</sub> CO (% predicted)	107 ± 16	106 ± 17	106 ± 9	0.959
D <sub>L</sub> CO/V <sub>A</sub> (mL min <sup>-1</sup> mmHg <sup>-1</sup> L <sup>-1</sup> )	5.1 ± 0.6	5.3 ± 0.8	5.2 ± 0.7	0.517
D <sub>L</sub> CO/V <sub>A</sub> (% predicted)	99 ± 13	109 ± 15	104 ± 14	0.143
<b>Inspiratory muscle strength</b>				
MIP <sub>FRC</sub> (cmH <sub>2</sub> O)	155 ± 31	120 ± 21	137 ± 31	0.00609
MIP <sub>FRC</sub> (% predicted)	139 ± 29	128 ± 22	133 ± 26	0.331
P <sub>di,max</sub> (cmH <sub>2</sub> O)	162 ± 24	165 ± 18	164 ± 21	0.713
<b>Diaphragm ultrasound</b>				
Diaphragm depth (mm)	18.5 ± 2.7	16.7 ± 3.2	17.6 ± 3.0	0.176
End-expiration thickness (mm)	1.5 ± 0.3	1.2 ± 0.3	1.4 ± 0.3	0.0553
End-inspiration thickness (mm)	2.8 ± 0.6	2.3 ± 0.4	2.5 ± 0.6	0.0315
ΔTidal thickness (mm)	1.3 ± 0.7	1.0 ± 0.4	1.1 ± 0.6	0.223
Tidal thickening fraction (%)	88.8 ± 52.5	83.3 ± 42.3	87.2 ± 45.6	0.696
<b>Haematology</b>				
Haemoglobin concentration (g L <sup>-1</sup> )	144.6 ± 8.2	132.3 ± 7.4	138.5 ± 9.9	0.00131

Note: Data are presented as the mean ± SD. Values between sexes were compared using independent samples Student's *t* test.

Abbreviations: BMI, body mass index; D<sub>L</sub>CO, diffusion capacity of the lung for carbon monoxide transfer; D<sub>L</sub>CO/V<sub>A</sub>, D<sub>L</sub>CO corrected for alveolar volume; FEV<sub>1</sub>, forced expired volume in one second; FRC, functional residual capacity; FVC, forced vital capacity; MIP<sub>FRC</sub>, maximal inspiratory pressure at functional residual capacity; P, probability for a difference between sexes based on an independent samples Student's *t* test; P<sub>di,max</sub>, maximal transdiaphragmatic pressure at functional residual capacity; RV, residual volume; TLC, total lung capacity.

by 22 beats min<sup>-1</sup> (95% CI = 18–26 beats min<sup>-1</sup>) from baseline to 50% (*P* < 0.0001) and 25 beats min<sup>-1</sup> (95% CI = 21–30 beats min<sup>-1</sup>) from baseline to 100% (*P* < 0.0001) of task completion (Fig. 3A). The ΔHR was significantly greater at 100% task completion compared to 50% task completion (3.5 beats min<sup>-1</sup>; 95% CI = 1.5–5.5 beats min<sup>-1</sup>, *P* = 0.001) and was not different between sexes (*P* = 0.324) (Fig. 3B).

There was a main effect of time (*P* < 0.0001) but not sex (*P* = 0.832) for MAP (Fig. 3C). Mean arterial pressure increased by 19 mmHg (95% CI = 17–29 mmHg) at 50% (*P* < 0.0001) and 21 mmHg (95% CI = 18–24 mmHg) at 100% (*P* < 0.0001) task completion (Fig. 3C). The ΔMAP was not significantly different at 100% of task completion compared to 50% of task completion (1.5 mmHg; 95% CI = -0.8 to 3.8 mmHg, *P* = 0.196) (Fig. 3D). The ΔMAP

Table 2. Respiratory and cardiovascular response to inspiratory pressure-threshold loading

Variables	Percent to task completion				P	Sex	X		
	Baseline		100%						
	Males	Females	Males	Females					
<b>Respiratory</b>									
$P_{di}$ (% max)	6.3 ± 2.3	5.8 ± 2.3	48.0 ± 8.0 <sup>†</sup>	49.7 ± 5.8 <sup>†</sup>	50.9 ± 8.0 <sup>†</sup>	51.0 ± 9.2 <sup>†</sup>	<0.0001	0.867	0.694
$P_{M,peak}$ (% max)	0.7 ± 0.3	0.6 ± 0.2	18.7 ± 3.3 <sup>†</sup>	25.5 ± 7.7 <sup>**†</sup>	18.8 ± 6.6 <sup>†</sup>	24.6 ± 7.1 <sup>†</sup>	<0.0001	0.0135	0.00214
PTP <sub>di</sub> (cmH <sub>2</sub> O-s/breath)	14 ± 10	9 ± 4	169 ± 23 <sup>†</sup>	165 ± 21 <sup>†</sup>	182 ± 12 <sup>†</sup>	167 ± 34 <sup>†</sup>	<0.0001	0.198	0.506
PTP <sub>di</sub> /PTP <sub>oes</sub>	1.6 ± 0.4	1.5 ± 0.5	2.2 ± 0.3 <sup>†</sup>	2.2 ± 0.6 <sup>†</sup>	2.4 ± 0.3 <sup>†</sup>	2.4 ± 0.7 <sup>†</sup>	<0.0001	0.837	0.768
$P_{oes,tw}$ (cmH <sub>2</sub> O)	-3.2 ± 1.8	-2.4 ± 2.4	-	-	-3.6 ± 2.1 <sup>†</sup>	-3.3 ± 2.2 <sup>†</sup>	0.0342	0.512	0.345
$T_I/T_{TOT}$	0.39 ± 0.08	0.35 ± 0.07	0.68 ± 0.04 <sup>†</sup>	0.69 ± 0.06 <sup>†</sup>	0.70 ± 0.03 <sup>†</sup>	0.69 ± 0.05 <sup>†</sup>	<0.0001	0.336	0.203
TTI <sub>di</sub>	0.05 ± 0.02	0.05 ± 0.02	0.33 ± 0.06 <sup>†</sup>	0.34 ± 0.07 <sup>†</sup>	0.36 ± 0.06 <sup>†</sup>	0.34 ± 0.07 <sup>†</sup>	<0.0001	0.825	0.373
$P_{ETCO_2}$ (mmHg)	38 ± 3	36 ± 4	36 ± 3 <sup>†</sup>	35 ± 4 <sup>†</sup>	37 ± 2	37 ± 3	0.00758	0.549	0.0694
$f_b$ (breaths min <sup>-1</sup> )	11 ± 5	12 ± 4	15 ± 0 <sup>†</sup>	15 ± 0 <sup>†</sup>	15 ± 0 <sup>†</sup>	15 ± 0 <sup>†</sup>	<0.0001	0.593	0.886
$\dot{V}_E$ (L min <sup>-1</sup> )	12 ± 4	9 ± 3	34 ± 5 <sup>†</sup>	25 ± 7 <sup>†</sup>	31 ± 5 <sup>†</sup>	23 ± 6 <sup>†</sup>	<0.0001	<0.001	0.0337
$V_T$ (L)	1.0 ± 0.5	0.8 ± 0.2	2.3 ± 0.3 <sup>†</sup>	1.7 ± 0.5 <sup>†</sup>	2.1 ± 0.3 <sup>†</sup>	1.6 ± 0.4 <sup>†</sup>	<0.0001	<0.001	0.252
$\bar{P}_M$ (cmH <sub>2</sub> O)	-0.07 ± 0.2	-0.6 ± 0.2	-20.3 ± 2.3 <sup>†</sup>	-19.0 ± 4.7 <sup>†</sup>	-20.1 ± 2.4 <sup>†</sup>	-18.3 ± 4.1 <sup>†</sup>	<0.0001	0.258	0.363
$\bar{P}_{oes}$ (cmH <sub>2</sub> O)	-7.7 ± 1.6	-8.3 ± 2.5	-27.2 ± 3.2 <sup>†</sup>	-28.0 ± 5.4 <sup>†</sup>	-27.4 ± 3.2 <sup>†</sup>	-26.4 ± 4.2 <sup>†</sup>	<0.0001	0.884	0.388
$\bar{P}_{di}$ (cmH <sub>2</sub> O)	20.1 ± 3.0	22.2 ± 2.7	80.1 ± 5.9 <sup>†</sup>	78.3 ± 7.1 <sup>†</sup>	84.4 ± 5.6 <sup>†</sup>	77.8 ± 10.9 <sup>†</sup>	<0.0001	0.289	0.018
EELV (%TLC)	55.4 ± 7.0	62.5 ± 6.6	46.4 ± 12.7 <sup>†</sup>	54.1 ± 5.9 <sup>†</sup>	45.2 ± 8.5 <sup>†</sup>	55.9 ± 8.8 <sup>†</sup>	<0.0001	0.00962	0.480
Apnea length (s)	14.5 ± 2.9	14.4 ± 2.4	16.4 ± 1.6	15.5 ± 2.3	16.6 ± 4.1	14.1 ± 2.2	0.0705	0.955	0.729
<b>Cardiovascular</b>									
$S_{pO_2}$ (%)	96 ± 1	97 ± 1	98 ± 0 <sup>†</sup>	98 ± 0 <sup>†</sup>	98 ± 1 <sup>†</sup>	98 ± 1 <sup>†</sup>	<0.0001	0.208	0.0929
HR (beats min <sup>-1</sup> )	68 ± 6	71 ± 12	92 ± 8 <sup>†</sup>	91 ± 12 <sup>†</sup>	95 ± 10 <sup>†</sup>	95 ± 13 <sup>†</sup>	<0.0001	0.849	0.395
MAP (mmHg)	87 ± 7	85 ± 6	104 ± 11 <sup>†</sup>	107 ± 7 <sup>†</sup>	107 ± 10 <sup>†</sup>	108 ± 10 <sup>†</sup>	<0.0001	0.832	0.120
SBP (mmHg)	120 ± 5	115 ± 7	140 ± 9 <sup>†</sup>	142 ± 10 <sup>†</sup>	144 ± 9 <sup>†</sup>	143 ± 11 <sup>†</sup>	<0.0001	0.636	0.0941
DBP (mmHg)	70 ± 9	70 ± 7	87 ± 13 <sup>†</sup>	90 ± 6 <sup>†</sup>	89 ± 12 <sup>†</sup>	91 ± 9 <sup>†</sup>	<0.0001	0.634	0.361
$VC_{di}$ (AU s <sup>-1</sup> mmHg <sup>-1</sup> )	0.03 ± 0.01	0.05 ± 0.05	0.18 ± 0.06 <sup>†</sup>	0.17 ± 0.09 <sup>†</sup>	0.20 ± 0.08 <sup>†</sup>	0.17 ± 0.08 <sup>†</sup>	<0.0001	0.793	0.238

Note: Data are presented as the mean ± SD. Absolute values for all variables were compared using a linear mixed-effects model with time (baseline, 50%, and 100% of inspiratory loading) and sex as fixed factors, and participant as a random factor (random intercept model). When significant effects were identified, Tukey's HSD was used to identify pairwise differences.  $N = 22$  (11 males, 11 females) for all comparisons except for the 50% timepoint, in which  $N = 19$  (9 females; 10 males) for EELV and apnea length.

Abbreviations: DBP, diastolic blood pressure; EELV, end-expiratory lung volume;  $f_b$ , breathing frequency; HR, heart rate; MAP, mean arterial pressure;  $P_{di}$ , trans-diaphragmatic pressure;  $\bar{P}_{di}$ , mean transdiaphragmatic pressure;  $P_{oes,tw}$ , oesophageal pressure at time of transcutaneous electrical stimulation of the phrenic nerves; PTP<sub>di</sub>, pressure-time-product of the diaphragm; PTP<sub>di</sub>/PTP<sub>oes</sub>, pressure-time-product of the diaphragm to the pressure-time-product of the oesophagus;  $P_{M,peak}$ , peak mouth pressure;  $T_I/T_{TOT}$ , inspiratory time to total time of a breath; TLC, total lung capacity; TTI<sub>di</sub>, tension-time index of the diaphragm;  $P_{ETCO_2}$ , partial pressure of end-tidal carbon dioxide;  $\bar{P}_{oes}$ , mean inspiratory oesophageal pressure;  $\bar{P}_M$ , mean inspiratory mouth pressure;  $\bar{P}_{M, peak}$ , peak inspiratory mouth pressure; SBP, systolic blood pressure;  $S_{pO_2}$ , arterial oxyhaemoglobin saturation;  $VC_{di}$ , vascular conductance of the diaphragm;  $V_E$ , minute ventilation;  $V_T$ , tidal volume; X, interaction effect of time and sex.

\*  $P < 0.0001$ .

\*\*  $P = 0.0175$  compared to males.

†  $P < 0.0001$ .

‡  $P = 0.00899$  compared to baseline.

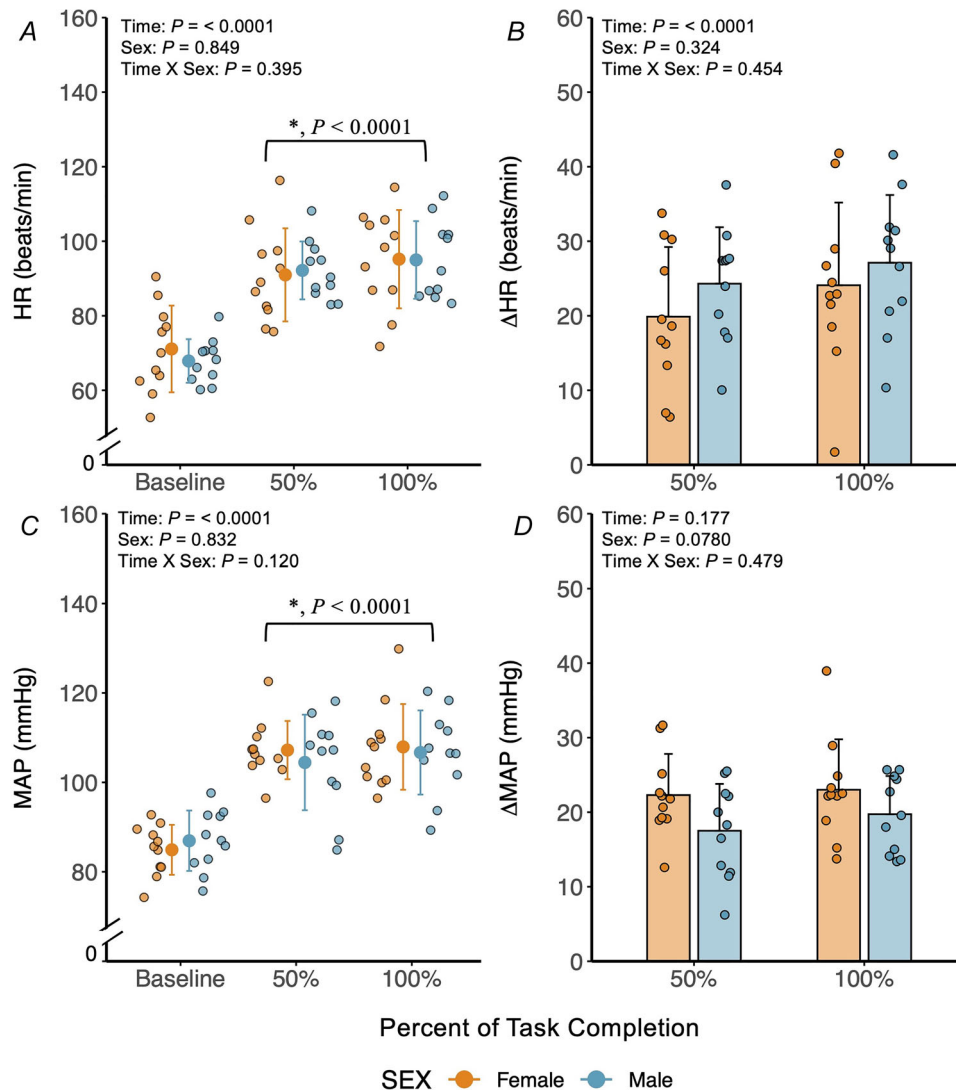
was not significantly different between males and females ( $P = 0.0780$ ).

### Diaphragm neuromuscular function

A plateau of the  $P_{di,tw}$  and the M-wave amplitude of the left diaphragm was obtained with TES of the phrenic nerves (Fig. 4). When compared with 100% of stimulator output needed to record maximal responses, there was no significant difference for either  $P_{di,tw}$  or the M-wave

amplitude of the left diaphragm at 80%, 90% or 130% of the stimulator intensity ( $P > 0.568$ ) (Fig. 4). Supra-maximal stimulation required a current of  $183 \pm 56$  mA for males and  $150 \pm 15$  mA for females and was not significantly different between sexes ( $P = 0.106$ ).

Baseline potentiated twitches were not different between males ( $39.3 \pm 11.8$  cmH<sub>2</sub>O) and females ( $41.2 \pm 11.7$  cmH<sub>2</sub>O,  $P = 0.266$ ). There was a main effect of time ( $P < 0.0001$ ) but not sex ( $P = 0.354$ ) for  $P_{di,tw}$  (Fig. 5A). Transdiaphragmatic twitch pressure decreased



### Figure 3. Cardiovascular responses to inspiratory pressure-threshold loading

The absolute heart rate (HR) (A) and mean arterial pressure (MAP) (C) during baseline, 50% and 100% of task completion. The absolute differences of HR (B) and MAP (D) from baseline to 50% and 100% of task completion during inspiratory pressure-threshold loading. Absolute values (A) and (C) were compared using a linear mixed-effect model with time (baseline, 50% and 100% of inspiratory loading) and sex as fixed factors, and participant as a random factor (random intercept model). Changes from baseline (B) and (D) were compared using a linear mixed-effect model with time (50% and 100% of inspiratory loading) and sex as fixed factors, and participant as a random factor (random intercept model). When significant effects were identified, Tukey's HSD was used to identify pairwise differences.  $N = 22$  (11 females, 11 males) for all measures. \*Significantly different compared to baseline.

by  $-7.2$  cmH<sub>2</sub>O (95% CI =  $-9.7$  to  $-5.1$  cmH<sub>2</sub>O) from baseline to post-ITL ( $P < 0.0001$ ). There was a significant reduction in  $P_{di,tw}$  ( $-18.1\%$ ; 95% CI =  $-22.2\%$  to  $-13.9\%$ ;  $P < 0.0001$ ) that was not significantly different between sexes ( $P = 0.352$ ) (Fig. 5B). The average coefficient of variation between subsequent stimuli for baseline potentiated twitches was  $5.4 \pm 2.6\%$  for males and  $5.3 \pm 2.7\%$  for females. To facilitate comparisons to other studies (Guenette et al., 2009), fatigue of the diaphragm was assumed if there was a  $\geq 11\%$  reduction of  $P_{di,tw}$  relative to baseline levels, as determined from the 95% CI of the coefficient of variation of baseline twitches. Based on this definition, two females and four males did not develop significant diaphragm fatigue. However, all participants had a reduction in  $P_{di,tw}$ , and were included in all analyses. There was a decrease in the oesophageal pressure at the time of stimulation from baseline to post-ITL from  $-2.8$  cmH<sub>2</sub>O (95% CI =  $-4.0$  to  $-1.6$  cmH<sub>2</sub>O) to  $-3.4$  cmH<sub>2</sub>O (95% CI =  $-4.6$  to  $-2.2$  cmH<sub>2</sub>O) ( $P = 0.0351$ ).

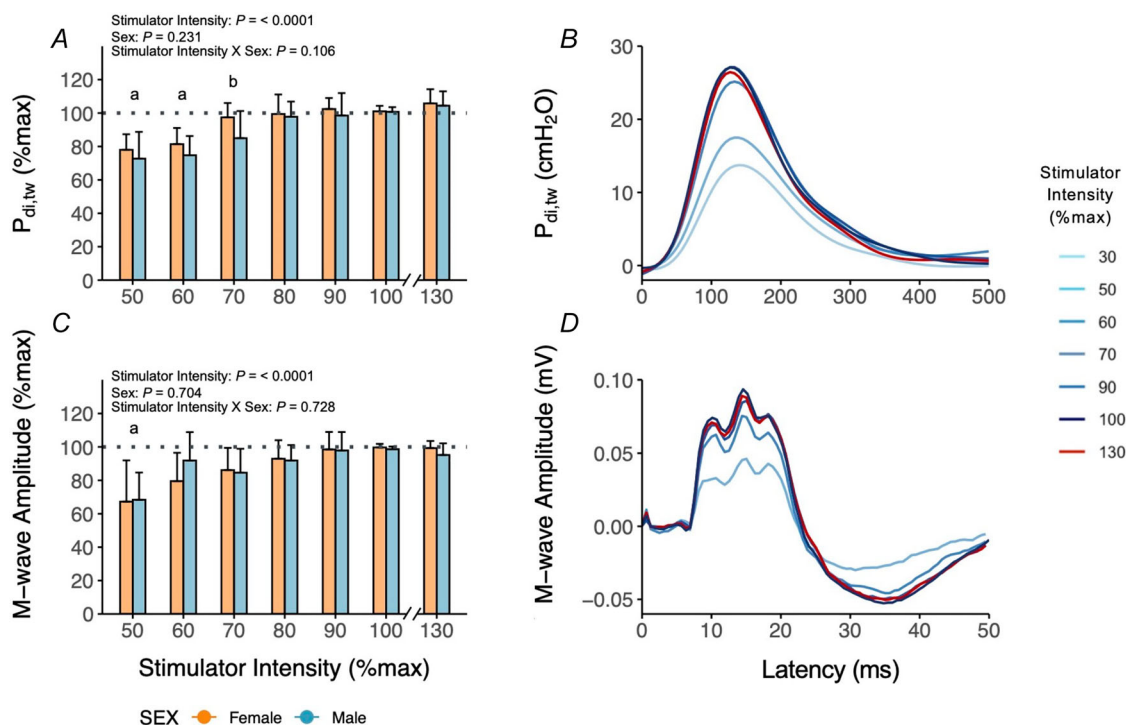
There was no main effect of time ( $P = 0.934$ ) for MIP between baseline ( $109$  cmH<sub>2</sub>O; 95% CI =  $95$ – $123$  cmH<sub>2</sub>O)

and post-ITL ( $106$  cmH<sub>2</sub>O; 95% CI =  $94$ – $122$  cmH<sub>2</sub>O). There was a main effect of sex ( $P = 0.001$ ), in which males had a significantly greater MIP compared to females ( $+41$  cmH<sub>2</sub>O; 95% CI =  $17$ – $66$  cmH<sub>2</sub>O,  $P = 0.00141$ ). There was no main effect of time ( $P = 0.0839$ ) or sex ( $P = 0.725$ ) for  $P_{di,max}$  during baseline ( $154$  cmH<sub>2</sub>O; 95% CI =  $141$ – $166$  cmH<sub>2</sub>O) and post-ITL ( $147$  cmH<sub>2</sub>O; 95% CI =  $134$ – $160$  cmH<sub>2</sub>O).

There was no effect of time ( $P \geq 0.171$ ) or sex ( $P \geq 0.331$ ) for the left diaphragm M-wave amplitude, area, duration, or latency. Following ITL, the left M-wave normalized amplitude, normalized area, duration, and latency were  $-2.3$  (95% CI =  $-14.3\%$  to  $9.7\%$ ),  $+3.2\%$  (95% CI =  $-11.5\%$  to  $+17.8\%$ ),  $+0.9$  ms (95% CI =  $-1.3$  to  $+3.1$  ms) and  $+0.2$  ms (95% CI =  $-0.1$  to  $+0.5$  ms) relative to baseline, respectively.

### Diaphragmatic work and respiratory variables

Respiratory variables are provided in Table 2. Throughout ITL, the accumulated  $PTP_{di}$  was not different



**Figure 4. Diaphragm recruitment**

The transdiaphragmatic twitch pressure ( $P_{di,tw}$ ) (A),  $N = 21$  (11 females, 10 males) and compound muscle action potential (M-wave) amplitude of the left diaphragm (C),  $N = 18$  (9 females, 9 males), during bilateral transcutaneous electrical stimulation of the phrenic nerves across increasing stimulator current intensities. Values are expressed as a percentage of the maximal  $P_{di,tw}$  or M-wave amplitude achieved during the recruitment process. A linear mixed-effects model was used to compare peak-to-peak amplitudes, with stimulator intensity (% of maximal) and sex as fixed factors and participant as a random factor. When significant effects were identified, Tukey's HSD was used to identify pairwise differences. Representative  $P_{di,tw}$  (B) and M-wave (D) tracings from a female participant. <sup>a</sup> $P < 0.0001$ , <sup>b</sup> $P = 0.0007$ , compared to 100% stimulator current used to evoke maximal  $P_{di,tw}$  and M-wave amplitude.

between males ( $14,351 \pm 919$  cmH<sub>2</sub>O·s) and females ( $14,003 \pm 1,947$  cmH<sub>2</sub>O·s;  $P = 0.600$ ). Males and females performed the task at a percentage of  $P_{di,max}$  (males:  $51\% \pm 8\%$ , females:  $51\% \pm 9\%$ ,  $P = 0.870$ ) that was not different, whereas females performed ITL at a higher percentage of their MIP (males:  $19\% \pm 7\%$ , females:  $26\% \pm 8\%$ ,  $P = 0.013$ ). There were no significant differences in  $f_B$  between sexes ( $P = 0.593$ ), whereas males had a greater minute ventilation and tidal volume compared to females ( $P < 0.0005$ ). The diaphragmatic contribution to total inspiratory muscle force output (i.e.  $PTP_{di}/PTP_{oes}$ ; males:  $2.4 \pm 0.3$ , females:  $2.4 \pm 0.7$ ) and the  $TTI_{di}$  (males:  $0.36 \pm 0.06$ ; females:  $0.34 \pm 0.07$ ) was not significantly different between sexes ( $P > 0.825$ ). There was no effect of time between 50% and 100% of task completion for the percentage of  $P_{di,max}$ , peak mouth pressure,  $PTP_{di}/PTP_{oes}$  and  $TTI_{di}$  ( $P \geq 0.203$ ).

### Diaphragm hyperaemia and its relationship to metaboreflex sensitivity, fatigue and pressure-generation

Within individuals, there were significant relationships between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$  ( $r = 0.857$ ,  $P < 0.0001$ ),  $\Delta HR$  ( $r = 0.857$ ,  $P < 0.0001$ ),  $\Delta P_{di,tw}$  ( $r = 0.7714$ ,  $P = 0.002$ ),  $\%P_{di,max}$  ( $r = 0.898$ ,  $P < 0.0001$ ),  $TTI$  ( $r = 0.913$ ,  $P < 0.0001$ ) and  $\Delta PTP_{di}$  per breath ( $r = 0.902$ ,  $P < 0.0001$ ). Between individuals, the  $\Delta\dot{Q}_{di}$  was not associated with  $\Delta MAP$  (Fig. 6A) or  $\Delta HR$  ( $r^2 = 0.007$ ;  $P = 0.706$ ). There was also no association between

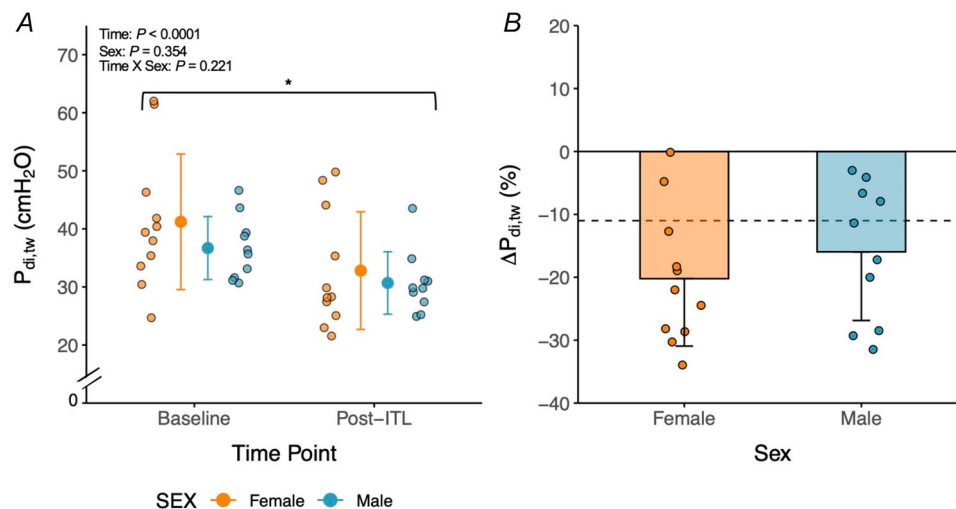
$\Delta\dot{Q}_{di}$  and  $\Delta P_{di,tw}$  (Fig. 6B) between individuals. There was a significant positive relationship between  $\Delta\dot{Q}_{di}$  and  $\%P_{di,max}$  (Fig. 6C) and  $\Delta PTP_{di}$  per breath ( $r^2 = 0.184$ ,  $P = 0.466$ ) but not  $TTI_{di}$  (Fig. 6D), or between  $\Delta MAP$  and  $\%P_{di,max}$  ( $r^2 = 0.005$ ;  $P = 0.758$ ).  $VC_{di}$  and  $\%P_{di,max}$  were significantly related ( $r^2 = 0.215$ ;  $P = 0.030$ ), but there was no relationship between  $\Delta P_{di,tw}$  and  $\%P_{di,max}$  ( $r^2 = 0.01$ ;  $P = 0.710$ ) or  $TTI_{di}$  ( $r^2 = 0.0001$ ,  $P = 0.961$ ) between individuals.

## Discussion

### Main findings

The purpose of the present study was to determine: (1) whether the sensitivity of the IMM and diaphragm fatigue development relates to the diaphragm's hyperaemic response and (2) whether the attenuated IMM response and diaphragm fatigue resistance previously reported for females (Geary et al., 2019; Smith et al., 2016; Welch et al., 2018) would lessen the hyperaemic response of the diaphragm compared to males.

We found that the  $\Delta\dot{Q}_{di}$  response was positively associated with the  $\%P_{di,max}$  during ITL. In this study, the absence of a relationship between  $\Delta\dot{Q}_{di}$  with the  $\Delta MAP$  or  $\Delta P_{di,tw}$ , suggests that the IMM sensitivity at a given workload may depend on other factors such as individual variability of the metaboreflex sensitivity or central neurogenic feedforward stimuli (explained later). We observed no sex-based differences for  $\Delta\dot{Q}_{di}$ ,



### Figure 5. Diaphragm fatigue

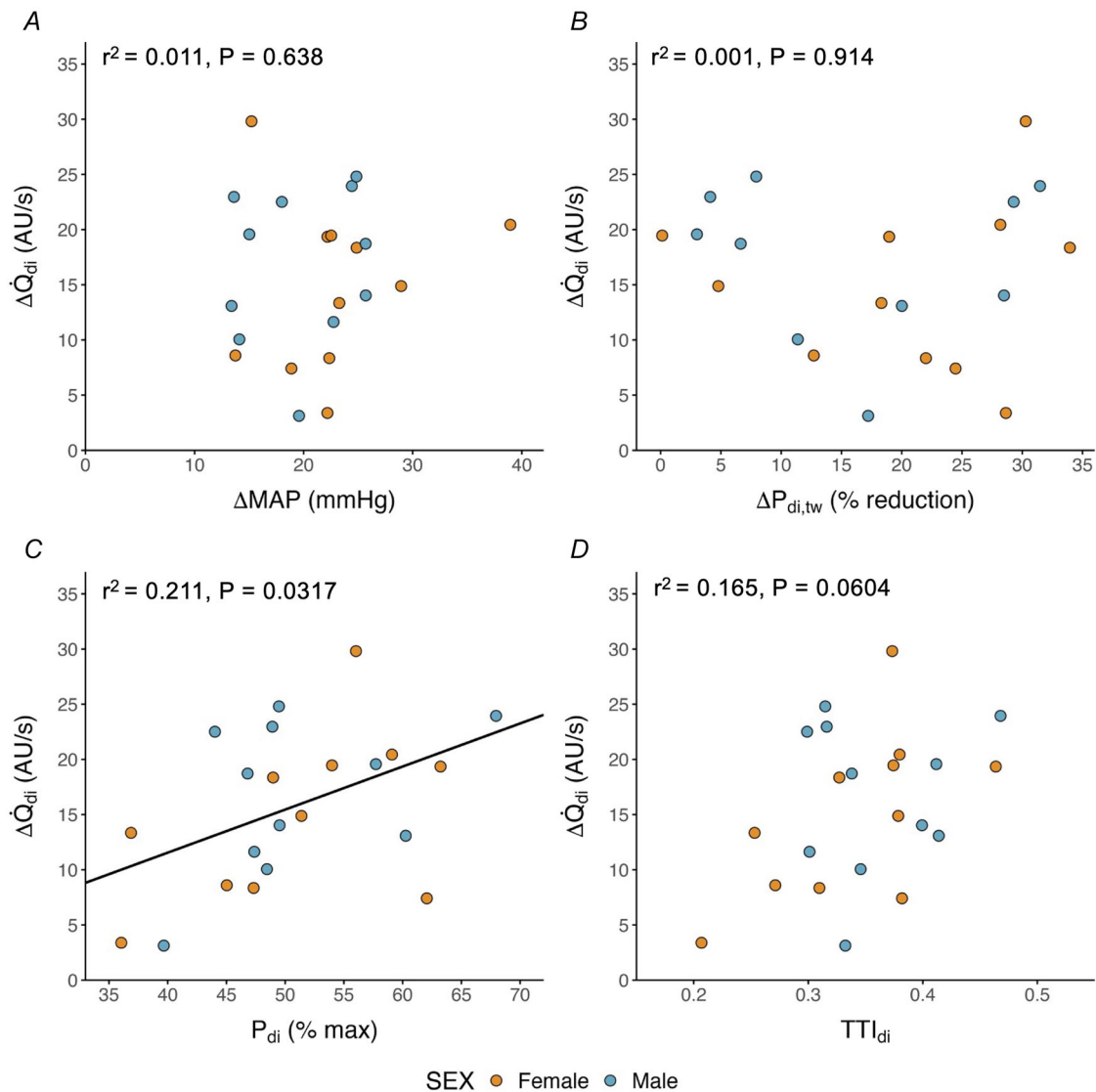
The absolute diaphragmatic twitch pressure ( $P_{di,tw}$ ) at baseline and immediately after 6 min of ITL (A) compared using a linear mixed-effects model with time and sex as fixed factors and participant as a random factor. The change in  $P_{di,tw}$  from baseline to post-ITL (B) was compared between sexes using an independent samples Student's  $t$  test. The dashed line represents the threshold for fatigue development based on  $1.96 \times$  the coefficient of variation between consecutive stimuli of the baseline potentiated twitches. When significant effects were identified, Tukey's HSD was used to identify pairwise differences.  $N = 21$  (11 females, 10 males) for all measures. The average coefficient of variation between subsequent stimuli was  $5.4 \pm 2.6\%$ . \* $P < 0.0001$  compared to baseline.

IMM sensitivities or diaphragm fatigue, probably because the females and males recruited for this study did not have different diaphragm strengths. Our findings indicate that diaphragm hyperaemia is related to the relative diaphragmatic workload during ITL.

Taken together, we found that at an absolute workload, IMM sensitivity and diaphragm fatigue are not major determinants of diaphragm hyperaemia. Additionally, no sex-based differences for IMM sensitivity and fatigue development were observed when diaphragmatic strength was not different between males and females, which coincided with no sex differences for diaphragm hyperaemia.

### Diaphragm hyperaemia

We used CEUS to record  $\dot{Q}_{di}$  during fatiguing diaphragmatic work to examine its association with IMM activation in humans. Throughout ITL, there was a significant increase in  $\dot{Q}_{di}$  from baseline to 100% of task completion (Fig. 2E). There was no significant difference for  $\Delta\dot{Q}_{di}$  at 50% or 100% of task completion, suggesting that a similar metabolic demand of the diaphragm was met with a similar hyperaemic response (Manohar, 1990). However, the  $\Delta MBV_{di}$  was significantly lower at 100% of task completion, whereas the  $\Delta MFR_{di}$  was significantly higher at 100% of task completion, compared to 50%,



**Figure 6. The relationship of diaphragm hyperaemia with metaboreflex sensitivity, diaphragm fatigue, and diaphragm pressure generation**

Relationships, using linear models and Pearson correlation coefficients, between changes from baseline to 100% of task completion in diaphragm blood flow ( $\Delta\dot{Q}_{di}$ ) and mean arterial pressure ( $\Delta$ MAP) (A), transdiaphragmatic twitch pressure ( $\Delta P_{di,tw}$ ) (B), percent of maximal transdiaphragmatic pressure ( $\%P_{di,max}$ ) (C) and tension-time index ( $TTI_{di}$ ) (D).  $N = 22$  (11 females, 11 males) for (A), (C) and (D), and  $N = 21$  (11 females, 10 males) for (B).

potentially reflecting a progressive increase of blood flow impedance throughout the protocol, necessitating a compensatory increase of perfusion pressures (Boushel, 2010).

As the  $TTI_{di}$  was above 0.3 in this study,  $\dot{Q}_{di}$  measures are probably more reflective of post-contraction hyperaemia in comparison to the study by Bird et al. (2024), which had a relatively low  $TTI_{di}$  of 0.2 and no IMM response. We showed a significant positive relationship between  $\dot{Q}_{di}$  and  $\%P_{di,max}$  (Fig. 6C), suggesting that the degree of muscle ischaemia and subsequent hyperaemia relates to the relative exercise intensity. A similar relationship was observed between  $VC_{di}$  and  $\%P_{di,max}$ ; this parallel suggests that our observations are primarily vaso-genic in nature, reflecting an increased metabolic vasodilatory drive rather than being explained by changes in perfusion pressure (Sheel et al., 2018).

Within our study, there was a large variation in the  $\Delta\dot{Q}_{di}$  between individuals, similar to the observations made by Bird et al. (2024) over the range of 10%–50%  $P_{di,max}$ . The variability is probably a result of inter-individual differences that affect  $\dot{Q}_{di}$  responses, including the diaphragm's mechanical efficiency, cardiac output, training status, sex and skeletal muscle fibre type (Bird et al., 2024; Gliemann et al., 2019; Robertson et al., 1977). Such variability could influence our findings by potentially obscuring the relationship between  $\dot{Q}_{di}$  and both IMM sensitivity and fatigue because individuals with greater mechanical efficiency would require smaller increases of  $\dot{Q}_{di}$  and MAP (Venturelli et al., 2021).

### Diaphragm hyperaemia and metaboreflex sensitivity

There was no relationship between the  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$  (Fig. 6A), which, in contrast to our hypothesis, suggests that the degree of IMM activation does not dictate the extent of diaphragm hyperaemia. The absence of a correlation between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$  in the present study contrasts previous research conducted using the forearm vasculature that showed that the degree of post-exercise hyperaemia of the forearm was positively related to the prevailing MAP during increasing intensities of sub-maximal, rhythmic exercise (Taylor et al., 1988). However, this discrepancy may be due to different experimental approaches. In the present study, participants targeted a single absolute workload, and responses were compared across participants, whereas, in the study by Taylor et al. (1988), the metaboreflex and hyperaemic responses were assessed across five different workloads using a within-individual design. Consequently, inter-individual differences for metaboreflex activation could explain the lack of relationship between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$  in the present study. In our study, when accounting for individual differences using a repeated measures correlation, a

significant association was observed between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$ , suggesting that within-participant variability plays an important role in determining the association between IMM activation and hyperaemia.

The variability of  $\Delta MAP$  observed in this study may be explained by factors such as physical fitness (Kaufman & Hayes, 2002), and genetics (Notay et al., 2018). For example, previous studies have shown that single-nucleotide polymorphisms in receptors found in skeletal muscle group III/IV afferents play an important role in determining the MAP response to exercise (Notay et al., 2018). Additionally, inter-individual differences for the perception of effort could affect autonomic control and cardiovascular regulation because the perceived rate of exertion can evoke cardiovascular adjustments independent of afferent input (Williamson, 2010).

The absence of a relationship between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$  in the present study may also be explained by influences on skeletal muscle hyperaemia independent of sympathetic drive. For example, vasodilatory substances released by contracting muscles (e.g. potassium, adenosine) and the endothelium (e.g. nitric oxide, prostaglandins) contribute to local blood flow regulation within the microcirculation (Joyner & Casey, 2015). Regarding the diaphragm, Supinski et al. (1990) observed a significant increase in  $\dot{Q}_{di}$  during norepinephrine infusion to the fatiguing diaphragm, suggesting that the diaphragm is protected from direct vasoconstrictor effects through functional sympatholysis (Supinski et al., 1990). Therefore, the diaphragm's ability to autoregulate its blood flow (Hussain et al., 1988) may explain the absence of a relationship between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$  in our study.

### Diaphragm hyperaemia and diaphragm fatigue

In the present study, there was no relationship between  $\Delta\dot{Q}_{di}$  and  $\Delta P_{di,tw}$  (Fig. 6B). As such, we must reject the hypothesis that the extent of diaphragm fatigue would determine the hyperaemic response (Bellemare & Grassino, 1982). Additionally, we found no significant relationship between  $\Delta P_{di,tw}$  and  $TTI_{di}$  or  $\%P_{di,max}$ . The relationship between intramuscular pressure, tissue oxygenation and fatigue development has previously been studied by Kramer et al. (2005) in the multifidus muscle. Although they hypothesized that higher intramuscular pressure would result in tissue hypoxia, which in turn would increase muscular fatigue, they found: (1) high-intramuscular pressure was not always associated with tissue hypoxia and (2) tissue hypoxia was not always associated with muscle fatigue. Thus, they concluded that inter-individual variability in the physiological responses to sustained muscular contractions explained their results.

There is conflicting literature regarding the role that  $\dot{Q}_{di}$  plays in fatigue development. Aubier et al. (1985)

showed that hypocalcaemia reduced diaphragmatic force in mechanically ventilated dogs, even when  $\dot{Q}_{di}$  and blood pressure were held constant, suggesting that the fatigue observed was independent of  $\dot{Q}_{di}$ . Pope et al. (1989) further supported this by showing that fatigue occurred despite sustained  $\dot{Q}_{di}$  and phrenic venous oxygen tensions  $>30$  mmHg, indicating oxygen delivery was not limiting. However, their use of 100% oxygen and mechanical ventilation may have masked blood flow and perfusion limitations, known to be reduced under these conditions (Horn et al., 2022). Lastly, the post-contraction hyperaemia during relaxation probably compensates for any  $\dot{Q}_{di}$  occlusion during contractions, ensuring that total  $\dot{Q}_{di}$  and oxygen delivery are maintained (Buchler et al., 1985).

Conversely, Supinski et al. (1988) demonstrated, in mechanically ventilated dogs, that hyperperfusion of the diaphragm can partially reverse fatigue, suggesting that  $\dot{Q}_{di}$  may be a determinant of fatigue resistance. Later work by Supinski et al. (1993), using an *in situ* canine diaphragm model, showed that administration of a vasodilator failed to increase  $\dot{Q}_{di}$  or alter fatigue progression, suggesting that the diaphragm may exhaust its vasodilatory reserve as fatigue develops. Supporting the idea that maintaining perfusion may mitigate fatigue, Gayan-Ramirez & Decramer (2002) showed, in rats, that intermittent spontaneous breathing during mechanical ventilation, allowing periodic increases of diaphragm perfusion, helped preserve force output. Similarly, Bark & Scharf (1986) provide evidence that  $\dot{Q}_{di}$  can limit force production because they observed  $\dot{Q}_{di}$  impedance as  $P_{di}$  increased during pacing-induced fatigue in dogs. Their study also highlighted that the relationship between  $\dot{Q}_{di}$  and  $TTI_{di}$  was not fixed but varied depending on the combination of  $T_I/T_{TOT}$  and stimulation frequency. These findings underscore the complexity of interpreting  $\dot{Q}_{di}$  during fatiguing contractions and highlight the need for techniques that can detect within-participant, within-region changes in perfusion to better understand its role in fatigue development.

### Sex-based differences

Males and females in this study were subjected to a similar amount of fatiguing diaphragmatic work, in which there were no significant sex-based differences for  $PTP_{di}$ ,  $TTI_{di}$  or  $\%P_{di,max}$  during ITL. With ITL, the increase of  $\dot{Q}_{di}$  and  $VC_{di}$  for males and females was not different (Fig. 2H). Although we rejected the hypothesis that females would present with less of an increase of  $\dot{Q}_{di}$ , as a result of an attenuated diaphragm hyperaemic response, we anticipated that diaphragm strength would be greater for males than females. Consequently, males and females probably experienced a similar degree of mechanical compression of vasculature within the

diaphragm, intramuscular occlusion of blood flow and diaphragm hyperaemia when  $\dot{Q}_{di}$  measurements were acquired (Bellemare et al., 1983; Bird et al., 2024). The absence of sex-based differences for  $\dot{Q}_{di}$  may also reflect similarities in diaphragm vascular control between sexes. Although systemic vascular control is often influenced by hormonal differences (Boese et al., 2017), previous studies in rats have reported no sex-based differences for diaphragm blood flow (Smith et al., 2017) or VC (Horn et al., 2024), which is consistent with our findings.

Additionally, the activation of the IMM, measured by a significant increase in  $\Delta MAP$  and  $\Delta HR$ , was not significantly different between males and females (Fig. 3). The absence of sex-based differences for IMM activation is probably the result of a similar extent of the mechanical constraint of  $\dot{Q}_{di}$ , which would result in a similar extent of metabolite build-up and activation of type III/IV afferents (Bellemare et al., 1983; St Croix et al., 2000). These findings are consistent with the findings of Archiza et al. (2021), who showed similar MAP and HR responses between sexes when diaphragm strength-matched males and females targeted an absolute  $P_{di}$  of 92 cmH<sub>2</sub>O for 5 min. Similar findings were also observed for the elbow flexor muscles, in which females exhibited reduced MAP and HR responses to fatiguing exercise only if their maximal strength was lower than males (Hunter & Enoka, 2001), but not if the sexes were strength-matched (Hunter et al., 2004). These results suggest that absolute strength, rather than sex *per se*, may be a key determinant of the pressor response during exercise. Previous work has demonstrated that individuals with greater absolute strength exhibit a higher MAP response, despite exercising at a similar relative intensity (30% of MVC) (Parmar et al., 2018). This is possibly because of the greater production of metabolites, increased occlusion of feed arteries and a more pronounced compromise in oxygen delivery, leading to a stronger pressor reflex. However, it remains incompletely understood whether sex-based differences in other modulators of the IMM, such as arterial baroreflex sensitivity, central integration of afferent signals and sympathetic transduction, also influence the IMM response (Fu & Ogoh, 2019).

A strength of the present study was the utilization of TES of the phrenic nerves to avoid the significant limitation associated with cervical magnetic stimulation, in which supramaximal stimulation is not reached in all participants, resulting in the exclusion of participant data (Angus et al., 2023; Mador et al., 1996). Following ITL, the magnitude of diaphragm fatigue, as shown by a reduction of  $P_{di,tw}$ , was not different between the sexes (Fig. 5). The reductions of  $P_{di,tw}$  that we observed occurred without a change to the M-wave, suggesting that fatigue development was probably related to disruption of excitation-contraction coupling rather than the transmission of action potentials (Barkhaus et al., 2024).

The lack of sex-based differences for the magnitude of diaphragm fatigue (reduction of  $P_{di,tw}$ ) is consistent with other studies, regardless of whether diaphragm strength was matched (Archiza et al., 2021) or unmatched (Geary et al., 2019). However, when males have greater diaphragmatic strength than females, Geary et al. (2019) concluded that females were fatigue resistant, because females performed ITL at a higher  $\%P_{di,max}$ , but had a similar reduction of  $P_{di,tw}$  compared to males. Similarly, others concluded that females were diaphragm fatigue resistant because males and females had a similar reduction of  $P_{di,tw}$ , but females took longer to reach task failure, suggesting sex-based differences for the rate of diaphragm fatigue rather than its magnitude (Welch et al., 2018). Although there was no significant reduction of MIP or  $P_{di,max}$  from baseline to post-ITL for males or females, suggesting central fatigue probably would not occur (Bellemare & Bigland-Ritchie, 1984), additional measures comparing a superimposed twitch during a MIP to a control twitch would provide further insight (Ramscook et al., 2022).

### Methodological considerations

First, CEUS measurements are taken during an apnoea, which precludes continuous assessment of dynamic blood flow regulation during inspiratory loading. As such, our data provide insight into the hyperaemic response rather than real-time flow regulation during contractions. Additionally, although our protocol probably imposed transient flow restriction ( $TTI > 0.3$ ) (Bellemare et al., 1983), we cannot distinguish whether the observed increases of  $\dot{Q}_{di}$  reflect elevated metabolic demand, a release of mechanical compression, or the decrease of  $O_2$  requirements occurring 10–30 s after stopping muscle contractions (Armstrong & Laughlin, 1983). Second, diaphragm fibre length affects  $\dot{Q}_{di}$ , in that  $\dot{Q}_{di}$  decreases with increased fibre length because of stretching of intramuscular vessels, increasing vascular resistance (Supinski et al., 1986). Because EELV was significantly lower at 50% and 100% of task completion compared to baseline ( $\sim 800$  mL), the resultant increase of fibre length may have attenuated  $\dot{Q}_{di}$  and led to an overall underestimation of  $\Delta\dot{Q}_{di}$ . Additionally, females in the present study had a significantly higher EELV during CEUS measurements compared to males, which may have led to an overestimation of  $\Delta\dot{Q}_{di}$  in females. Third, because CEUS-derived measures of  $\dot{Q}_{di}$  are dependent on the contrast concentration, several factors limit the ability for CEUS to measure absolute blood volume: (1) indexing to an arterial blood pool sample may be confounded by haematocrit differences between microvascular and systemic circulation (Pries & Secomb, 2005); (2) it is difficult to quantify how many microvessels contain

microbubbles, as the low microbubbles to red blood cell ratio (McClatchey et al., 2020) and uneven distribution of red blood cells throughout the microvasculature (Poole et al., 2020) could result in microvessels not containing a single microbubble; and (3) as blood flow and microvascular haematocrit increase (from  $\sim 15\%$  to  $\sim 45\%$ ), the fractional volume accessible to microbubbles may decline, confounding interpretation of signal intensity (Poole et al., 2013). Further research is required to better understand microbubble distribution within the microvascular circulation and their interactions with red blood cells and vessel junctions. Additionally, validation studies of CEUS for assessing muscle perfusion during exercise are needed, employing rigorous and standardized methodology. However, CEUS remains a valuable tool for estimating blood flow because it has been shown to reflect global blood flow (Rim et al., 2001; Wei et al., 1998) and correlate with skeletal muscle capillarization (Weber et al., 2006).

Additional considerations of our study design include our initial expectation that diaphragm strength would be greater for males than in females. This assumption was based on prior data from Geary et al. (2019) and general population data that report males typically have greater respiratory muscle strength (ATS/ERS, 2002). The absolute target for  $P_{di}$  is also worth considering because six participants (two females and four males) did not develop sufficient diaphragm fatigue. Although a higher target may have induced diaphragm fatigue for all participants, it would also increase participant withdrawal rates because more individuals may not have been able to complete the task, as demonstrated by two participants being unable to reach the target in this study.

There are several methodological considerations for the lack of a relationship between  $\Delta\dot{Q}_{di}$  and  $\Delta MAP$ . First, activating the metaboreflex through muscle ischaemia is problematic for investigating the blood flow component of the IMM because the mechanical impedance to blood flow within the diaphragm produced to induce the reflex simultaneously disrupts the increase in diaphragm blood flow (Boushel, 2010). Second, although we did our best to isolate diaphragmatic work by targeting  $P_{di}$ , activation of accessory respiratory muscles, and/or mechano-reflex and central neurogenic feedforward stimuli, could contribute to the IMM response and influence blood flow distribution amongst the respiratory muscles (Dominelli et al., 2017).

Finally, we did not control for the menstrual cycle because previous studies have shown endogenous fluctuations in oestrogen throughout the menstrual cycle phases do not influence the increases of HR or MAP following metaboreflex activation within the upper and lower limbs (Hartwich et al., 2013; Lee et al., 2023; Parmar et al., 2018). However, because females possess a greater expression of vasodilatory  $\beta_2$ -adrenergic receptors that

have an increased sensitivity attributed to oestrogen, it has been shown that the IMM is heightened for post-menopausal females compared to young females (Joyner et al., 2016; Leahy et al., 2023). Although recent work in animals has provided critical insight into the effects of sex and ageing on  $\dot{Q}_{di}$  (Horn et al., 2024, 2025), our current understanding of diaphragm perfusion remains informed almost exclusively by animal studies.

## Conclusions

This was the first study in humans to examine the relationship between  $\dot{Q}_{di}$ , IMM and diaphragm fatigue development. We found a significant positive relationship between exercise intensity and the diaphragm's hyperaemic response. However, there was no relationship between the sensitivity of the IMM, the extent of diaphragm fatigue and the  $\dot{Q}_{di}$  response within our sample. Our results suggest that the sensitivity of the IMM may depend more on central neurogenic feed-forward stimuli and/or inter-individual differences for the metaboreflex sensitivity. Additionally, the absence of a relationship between  $\dot{Q}_{di}$  and fatigue suggests that oxygen extraction remained sufficient, and the diaphragm was not perfusion-limited under these conditions. Lastly, there were no sex-based differences for  $\Delta\dot{Q}_{di}$ , probably as a result of no differences in diaphragmatic strength between males and females and/or absence of sex-based differences for vascular control. These findings emphasize that  $\dot{Q}_{di}$  correlates with exercise intensity, is unaffected by sex, and is not a main determinant of metaboreflex sensitivity of the respiratory muscles during inspiratory pressure-threshold loading at a single absolute workload. Although CEUS is a valuable tool for assessing relative changes in diaphragm perfusion, further methodological validation studies are needed to support its application and interpretation during dynamic aerobic exercise (Bird et al., 2025b; Horn & Poole, 2025). Accordingly, the conclusions of the present study should be interpreted within the context of its design constraints and methodological limitations.

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## Additional information

### Data availability statement

The de-identified numerical datasets used and/or analysed during the present study are available from the corresponding author upon reasonable request from a qualified researcher.

### Competing interests

The authors declare that they have no competing interests.

### Author contributions

M.L.L., C.D.B., P.B.D., C.J.M. and G.E.F. contributed to the conception and design of the study. M.L.L., C.D.B., P.A.W.,

M.M.C., S.F.T. and G.E.F. were involved in data acquisition. M.L.L. and G.E.F. prepared figures and tables. The manuscript was drafted by M.L.L. and G.E.F., with all authors involved in revising the manuscript before submission. All authors edited and approved the final version of the manuscript submitted for publication. All authors have approved the final version of the manuscript and agreed to be accountable for all aspects of the work. All persons designated as authors qualify for authorship and all those who qualify for authorship are listed.

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### Keywords

contrast-enhanced ultrasound imaging, diaphragm blood flow, inspiratory muscle metaboreflex, muscle fatigue, phrenic nerve stimulation

### Supporting information

Additional supporting information can be found online in the Supporting Information section at the end of the HTML view of the article. Supporting information files available:

### Peer Review History