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The CO_2-stimulus duration and steady-state time-point used for data extraction alters the cerebrovascular reactivity outcome measure.

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New Findings

- What is the central question of this study?

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CO_2-stimulus duration and steady-state time-point alters CVR

Cerebrovascular reactivity (CVR) is a common functional test to assess brain health. Impaired CVR has been associated with all-cause cardiovascular mortality. This study investigated whether the duration of the CO_2-stimulus and the time-point used for data extraction would alter the CVR outcome measure.

- What is the main finding and its importance?

This study demonstrated CVR measures calculated from 1- and 2-minute CO_2-stimulus durations were significantly higher than CVR calculated from a 4-minute CO_2-stimulus. CVR calculated from the first 2-minutes of the CO_2-stimulus were significantly higher than CVR calculated from the final minute if the duration was ≥4-minutes. This study highlights the need for consistent methodological approaches.

Abstract

Cerebrovascular reactivity to carbon dioxide (CVR) is a common functional test to assess brain vascular health, though conflicting age and fitness effects have been reported. Studies have used different CO_2-stimulus durations to induce CVR and extracted data from different time-points for analysis. Therefore, this study examined whether these differences alter CVR, and explain conflicting findings. Eighteen healthy volunteers (24±5 years) inhaled four CO_2-stimulus durations (1, 2, 4 and 5-min) of 5% CO_2 (in air) via the open-circuit Douglas bag method, in a randomised order. CVR data were derived from transcranial Doppler (TCD) measures of middle cerebral artery blood velocity (MCAv), with concurrent ventilatory sensitivity to the CO_2 stimulus (VE-CO_2). Repeated measures ANOVAs compared CVR and VE-CO_2 measures between stimulus durations and steady-state time-points. An effect of stimulus duration was observed (p=0.002, η^2=0.140), with 1-min (p=0.010) and 2-min (p<0.001) differing from 4-min, and 2-min differing from 5-min (p=0.019) durations. VE-CO_2 sensitivity increased ~3-fold from 1-min to 4- and 5-min durations (p<0.001, η^2=0.485). CVR calculated from different steady-state time-points within each stimulus duration were different

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(p<0.001, η^2=0.454); specifically, for 4-min (p=0.001) and 5-min (p<0.001), but not 2-min stimulus durations (p=0.273). These findings demonstrate that methodological differences alter the CVR measure.

1. Introduction

Effective regulation of brain blood flow is vital for optimal brain function both momentarily and across the lifespan. Resting cerebral blood flow (CBF) declines by approximately 50% across healthy adulthood (Buijs *et al.* 1998; Scheel *et al.* 2000; Stoquart-ElSankari *et al*., 2007; Ainslie *et al.* 2008), and this coincides with a decline in the most potent regulatory mechanism of CBF - its responsiveness to changes in arterial carbon dioxide pressure (PCO_2; termed cerebrovascular responsiveness to carbon dioxide or cerebrovascular reactivity (CVR)). This response demonstrates how well pH balance is maintained and is a crucial homeostatic function to ensure biological processes in the brain operate optimally. CBF is reduced and CVR impaired in clinical diseases such as atrial fibrillation (Junejo *et al.* 2019), stroke (Markus *et al.* 2001) and dementia (den Abeelen *et al.* 2014). Furthermore, reduced CVR is associated with all-cause cardiovascular mortality (Portegies *et al.* 2014). Thus, CVR has become a common functional test to assess brain vascular health.

It is well recognized that regular exercise has a positive effect on brain function (Voss *et al.* 2011) and CVR appears to be a sensitive measure that can be used to determine this. Previous studies have shown that a greater CVR is associated with higher aerobic fitness (Bailey *et al.* 2013; Barnes *et al.* 2013), with CVR improving following 12 weeks of exercise training (Murrell *et al.* 2013). However, conflicting observations have been reported. For example, a group of Masters athletes with a life-long history of aerobic exercise training demonstrated a blunted CVR (Thomas *et al.* 2013), as compared to

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age-matched sedentary adults. One possible explanation for such inconsistent findings is that methodological inconsistencies are present throughout the scientific literature in this area. For example, the open-circuit inhalation of CO_2 has been administered for a stimulus duration of 1.5 (Vernieri *et al.* 2009), 3 (Murrell *et al.* 2013), 4 (Guiney *et al.* 2015; Kastrup *et al.* 2001) or 5 minutes (Lavallee *et al.* 2009), as well as with different CO_2-stimulus concentrations (5% or 7%; see Fierstra *et al.* 2013 for full review). Further, CVR has been derived from varying durations of the overall stimulus (e.g., 15, 30 or 60 seconds of data). In addition, there is variation in how long participants have been breathing CO_2 before data are extracted for calculation (i.e., how the time-point of the steady-state period is defined) as well as an unclear methodological approach to determine how to identify the steady-state period (e.g. 90 seconds into the stimulus duration versus the end of the stimulus duration, please see Vernieri *et al.* 2009; List *et al.* 2015; Murrell *et al.,* 2013).

1.1. **Study Aims and Hypotheses**

To maximise the utility of CVR as a measure of brain vascular health it is important to identify whether methodological nuances affect the CVR outcome measure. Therefore, the overall aim of this study was to compare different methods of calculating the CVR outcome measure within the same individuals using the open-circuit technique where individuals inhaled a fixed fractional concentration of 5% CO_2 from a pre-mixed Douglas bag. This open-circuit approach is the most commonly used technique because it uses low specification, relatively inexpensive equipment, and therefore is used in clinical settings to determine health status and disease risk (e.g., den Abeelen *et al.* 2014; Portegies *et al.* 2014). Nevertheless, the nature of this open-circuit approach introduces an influence from the ventilatory chemoreflexes (Fierstra et al. 2013), therefore the time-course effect of elevated PCO_2 on the ventilatory response ($VE\text{-}CO_2$) was also examined to determine how this may

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influence the CVR measure. The study had two main aims for examining variations in data collection and analysis: 1) Mean CVR outcome measures and VE-CO_2 values were compared by calculating the final 30 seconds of four different stimulus durations of breathing 5% CO_2 (1, 2, 4 and 5 minutes), and 2) Mean CVR calculations derived from different methods of data extraction (including the time-point of the steady-state period used to determine data extraction and the duration of data extraction) within the same stimulus duration were compared. We hypothesized that: 1) the stimulus duration of 1, 2, 4 or 5 minutes would not give the same calculated CVR outcome, and 2) the time-point of data extraction would alter the CVR outcome (i.e., the CVR calculated by extracting data from earlier in the stimulus duration would be different from those calculated from data extracted at the end of the stimulus duration for durations longer than 2 minutes, possibly due to changes in VE- CO_2).

2. Materials and Methods

2.1. Ethical Approval

Ethical approval was obtained for all experimental protocols and procedures by the University of Birmingham Ethics Committee and conformed to the Declaration of Helsinki, except for registration in a database (project code: ERN_14-0555). All testing took place in the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham. Prior to participation, a detailed verbal and written explanation of the study was provided and written informed consent to participate was obtained.

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2.2. Study Design and Protocol

Eighteen healthy volunteers (8 male and 10 female; mean age 24 ±5 years) participated in the study and were included in the analysis. Participants were required to make two visits. During the first visit participants provided informed consent and completed a general health questionnaire to check that they met the inclusion/ exclusion criteria. Participants were not taking any medication and had no history of cardiovascular, cerebrovascular or respiratory disease. Following successful screening participants then completed a familiarisation session of the open-circuit gas challenge protocol. They were asked to lie in supine position, relax and breathe as naturally as possible. First, participants lay supine for ~20 minutes while they were instrumented with equipment (detailed below). Once instrumented, baseline data were collected for 5 minutes. They then breathed 1 minute of a 5% CO_2 (in air) stimulus, followed by 5 minutes of room air, followed by 5 minutes of the 5% CO_2 stimulus. After satisfactory completion of familiarisation trials (i.e., no adverse reactions to breathing the CO_2 stimulus were experienced by the participant and adequate Doppler signals were identified by the researcher), participants were invited back for the second visit that was a full experimental testing session. For this second visit, four different stimulus durations of the same 5% CO_2 stimulus were administered (1, 2, 4 and 5 minutes) and each included a 5-minute baseline recovery period (Figure 1A). These stimulus durations were performed in a randomised order between participants to minimize any order effect. For both the familiarisation and experimental visits, participants were asked to avoid vigorous exercise and alcohol 24 hours prior to study participation, caffeine for 12 hours and heavy meals for 4 hours.

2.3. Measures and Equipment

Beat-by-beat middle cerebral blood velocity (MCAv) and blood pressure (BP) along with breath-by-breath respiratory rate and volume and end-tidal CO_2 partial pressure were continuously measured during the gas challenge protocol. MCAv was assessed using transcranial Doppler (TCD) (Multi Dop

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X, DWL, Compumedics Ltd Germany) with a 2-MHz probe placed over each temporal window to measure bilateral MCAv. Probes were prepared with ultrasound gel and held in place with a headset. Search and identification procedures were performed in accordance with established guidelines (Willie *et al.* 2011).

BP was measured using a finger cuff placed on the middle finger of the left hand (Portapres, Finapres, Medical System BV, Netherlands). Respiratory rate and volume were measured using a heated pneumotachograph (3813 Series, Hans Rudolph Inc, Kansas, USA) attached to a facemask, while fractional changes in inspired and expired O_2 and CO_2 were measured via a sample line attached to the facemask and a fast responding gas analyser (ML206, ADInstruments Ltd, New Zealand). Measures were recorded at 1k Hz via an analogue-to-digital converter (Powerlab, ADInstruments) and displayed in real time and stored for offline analysis using commercially available software (LabChart v7.3.5, ADInstruments). Calibration of equipment was performed before each testing session.

2.4. **Data Analysis**

The researcher was blinded to the order in which participants received the different stimulus durations when calculating resting CBF and CVR measures. The analysis was split into two parts. For aim 1, data from the last 30 seconds of each of the four CO_2 stimulus durations (1, 2, 4 and 5 minutes) and 60 seconds of baseline data before each stimulus duration were extracted (Figure 1B). For aim 2, data were extracted from two different steady-state time-points (i.e., after 60 seconds of stimulus duration and at the end of stimulus duration; Figure 1C), for two different durations (60 and 30 seconds) from three stimulus durations (2, 4 and 5 minutes). The 1-minute stimulus duration was not included in this analysis as there was not enough time for MCAv to reach steady-state (i.e., plateau). Finally, **3-**

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minute stimulus duration CVR **measures** were calculated from the final 30 seconds of data 3 minutes into the stimulus duration for both the 4- and 5-minute stimulus durations.

Mean right and left MCAv and CVR measures were compared for hemispatial effects. Mean baseline MCAv was calculated preceding each gas stimulus and analysed separately to see if they were different. Mean absolute and relative changes (percentage increase from baseline to hypercapnia) in MCAv and CVR measures were calculated. Ventilation sensitivity was also calculated and compared across stimulus durations. The following equations were used to calculate absolute and relative CVR (Equation 3.1 and 3.2), and VE-CO_2 (Equation 3.3).

Absolute CVR:

$$\frac{\text{hypercapnic (5\% } CO_2\text{) MCAv} - \text{resting MCAv}}{\text{hypercapnic (5\% } CO_2\text{) } P_{ET}CO_2 - \text{resting } P_{ET}CO_2}$$

Equation 3.1

Relative CVR:

$$\frac{((\text{hypercapnic (5\% } CO_2\text{) MCAv} - \text{resting MCAv}) / \text{resting MCAv}) * 100}{\text{hypercapnic (5\% } CO_2\text{) } P_{ET}CO_2 - \text{resting } P_{ET}CO_2}$$

Equation 3.2

VE-CO_2:

$$\frac{\text{hypercapnic (5\% } CO_2\text{) VE} - \text{resting VE}}{\text{hypercapnic (5\% } CO_2\text{) } P_{ET}CO_2 - \text{resting } P_{ET}CO_2}$$

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Equation 3.3

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After preliminary analysis and for completeness, a further analysis was performed on the 4- and 5-minute stimulus durations to determine whether CVR calculated 3-minutes into the stimulus duration differed to CVR values calculated from the 2-minute stimulus duration.

2.5. **Statistical Analysis**

Statistical analysis was performed in SPSS software (IBM SPSS version 22.0, Chicago, IL). Measures were compared using repeated analysis of variance (ANOVAs) with the main factors being stimulus duration (1, 2, 4 and 5 minutes), steady-state time-point (1 and 2), and duration of data extraction (60 and 30 seconds). Data are presented as means and standard deviations. Statistical significance was set at $p = 0.050$ and η^2 is used as the effect size throughout. Bonferroni post-hoc comparisons were performed to identify which stimulus durations were significantly different. For aim 2, paired t-tests were used to examine effects of time-point for each of the different stimulus durations separately.

3. Results

3.1. **Baseline Measures**

There was no significant effect for mean MCAv between baseline measures ($F(3,48) = 1.73$, $p = 0.17$, $\eta^2 = 0.098$), nor was there any effect between hemispheres ($F(1,16) = 0.41$, $p = 0.53$, $\eta^2 = 0.025$).

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3.2. Aim 1: Does the CO_2 Stimulus Duration Alter the Cerebrovascular Reactivity to Carbon Dioxide Measure?

3.2.1. Cerebrovascular Reactivity to Carbon Dioxide

There was a significant main effect of stimulus duration for both relative and absolute CVR measures (relative: $F(3,48) = 3.49$, $p = 0.037$, $\eta^2 = 0.161$; absolute: $F(3,48) = 3.10$, $p = 0.048$, $\eta^2 = 0.162$) where CVR calculated from the 2-minute stimulus duration was higher than CVR calculated from the 4-minute stimulus duration (Figure 2A and B, upper panel). Neither stimulus duration displayed a significant effect for hemisphere (relative: $F(1,16) = 1.99$, $p = 0.18$, $\eta^2 = 0.111$; absolute: $F(1,16) = 1.86$, $p = 0.190$, $\eta^2 = 0.104$). Therefore, analyses were performed on average CVR measures of bilateral MCAv (averaged right and left side). Relative and absolute CVR measures and VE-CO_2 values are presented in Table 1 with significant main effect *p* values in bold. Post-hoc differences are shown in Figures 2A and 2B.

Table 1. Mean and standard deviation (SD) for average (right and left MCAv) relative and absolute CVR measures and VE-CO_2 values for each stimulus duration. Repeated-measures ANOVAs revealed significant differences between stimulus durations.

		CO_2 stimulus duration					
		1 minute	**2 minute**	**4 minute**	**5 minute**	***p***	***η^2***
Relative CVR	mean	2.99	3.20	2.52	2.64	**0.002**	0.140
(% change in MCAv / mm Hg change in $P_{ET}CO_2$)	SD	1.07	1.38	1.04	1.07		
Absolute CVR	mean	1.97	2.14	1.72	1.76	**0.036**	0.153
(cm/s/mm Hg)	SD	0.71	0.99	0.77	0.72		
VE-CO_2	mean	0.32	0.70	0.90	0.95	**0.000**	0.485
(L/min/mm Hg)	SD	0.22	0.25	0.36	0.43		

Abbreviations: CO_2, carbon dioxide; CVR, cerebrovascular reactivity; MCAv, middle cerebral artery blood velocity; $P_{ET}CO_2$, end-tidal carbon dioxide; VE-CO_2, ventilation sensitivity to CO_2.

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3.2.2. Within-Individual Variability

We observed visual differences in the beat-to-beat MCAv-response profile between durations. For example, a steady-state profile typified the 4- and 5-minute tests, whereas the 1-minute test tended to peak in the final seconds of the stimulus duration. Further, there was variation within individuals across the four stimulus durations for measures of CVR; CoV ranging from 7 – 46% between individuals (Figure 2A and 2B, lower panel).

3.2.3. Ventilatory Sensitivity to Carbon Dioxide

Ventilatory sensitivity to CO_2 (VE-CO_2; average from the 30-second time window selected, see Section 3.3.3 for calculation) increased ~3-fold from the 1 minute to the 4- and 5-minute stimulus durations ($p < 0.001, \eta^2 = 0.485$) (Table 1; Figure 2C, upper panel).

3.3. Aim 2: Does the Time-Point of Steady-State or the Duration of Data Extraction Alter the Cerebrovascular Reactivity to Carbon Dioxide Measure?

The purpose of this next analysis was to investigate whether the time-point of steady-state used for data extraction (time-point 1 or 2; Figure 1C) and the duration of data extracted (30 or 60 s) altered the CVR outcome measure. Time-point of steady-state and duration of data extraction were examined for the 2-, 4- and 5-minute CO_2 stimulus for both CVR and VE-CO_2 measures. One important note is that for the 2-minute stimulus, because of the shorter length, data are extracted from almost identical time-points regardless of the approach used (i.e., data are extracted following 1 minute of the stimulus up to the end of the

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stimulus for time-point 1, and data are extracted 1 minute preceding the end of the stimulus backwards to 1 minute into the stimulus for time-point 2 and would be almost identical since the stimulus duration was exactly 2-minutes). **Mean MCAv traces and mean $P_{ET}CO_2$ traces for all four stimulus durations** (1, 2, 4 and 5 minutes) for one participant are shown in Figure 5.

Cerebrovascular Reactivity to Carbon Dioxide

There was a significant effect of stimulus duration: $F(2,68) = 4.78$, $p = 0.020$, $\eta^2 = 0.123$, where CVR calculated from the 2-minute stimulus duration was higher than CVR calculated from the 4-minute stimulus duration (similar to aim 1). There was also a significant main effect of steady-state time-point: $F(1,34) = 28.24$, $p < 0.001$, $\eta^2 = 0.454$, where CVR calculated from time-point 1 was higher than CVR calculated from time-point 2. The effect of data extraction duration was not significant: $F(1,34) = 1.12$, $p = 0.300$, $\eta^2 = 0.032$ (Figure 3). Therefore, further analysis was performed for average CVR measures of bilateral MCAv (averaged right and left side) calculated from 60 seconds of extracted data.

There was a significant main effect of time-point: $F(1,17) = 14.373$, $p = 0.001$, $\eta^2 = 0.458$, and a trend towards stimulus duration: $F(2,34) = 28.24$, $p = 0.064$, $\eta^2 = 0.149$. Further, a significant time-point by stimulus duration interaction was observed ($p = 0.008$, $\eta^2 = 0.232$). Paired t-tests were used to examine effects of time-point for each of the different stimulus durations separately (Figure 3A). As expected, given the similar time-points for the 2-minute stimulus duration, CVR measures were not significantly different ($p = 0.273$). However, there was a significant effect of time-point for the 4-minute ($p = 0.001$) and 5-minute ($p < 0.001$) stimulus durations, where CVR calculated from time-point 1 was higher than CVR calculated from time-point 2. In summary, this analysis shows that the

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time-point of steady-state used to determine data extraction does alter the CVR measure (Figure 4A and Table 2).

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3.3.1. Ventilatory Sensitivity to Carbon Dioxide

There was a significant effect of time-point: $F(1,17) = 54.76$, $p < 0.001$, $\eta^2 = 0.763$, where VE-CO_2 sensitivity calculated from time-point 2 was higher than VE-CO_2 sensitivity calculated from time-point 1. Effects of extract duration and stimulus duration were not significant ($F(1,17) = 2.52$, $p = 0.131$, $\eta^2 = 0.129$ and $F(2,16) = 0.746$, $p = 0.490$, $\eta^2 = 0.085$ respectively) (Figure 3). However, there were significant interactions between time-point and stimulus duration: $F(2,34) = 15.90$, $p < 0.001$, $\eta^2 = 0.483$ and time-point and data extraction duration: $F(2,34) = 14.61$, $p = 0.001$, $\eta^2 = 0.462$. Therefore, two separate ANOVAs were performed for each method of data extraction, 60 seconds of data extracted from time-point 1 and time-point 2 (Table 3; Figure 4B).

When data were extracted from time-point 2, VE-CO_2 sensitivity increased from the 2 minute to the 4 and 5 minute test durations (similar to in aim 1). In contrast, when data were extracted from time-point 1 there was less variability within the VE-CO_2 sensitivity measure between the stimulus durations (i.e., data is extracted from approximately the same time-point despite the stimulus duration).

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Table 2. Mean and standard deviation (SD) for average (right and left MCAv) relative CVR measures, calculated using data extracted from different time-points (time-point 1 and 2) from 3 stimulus durations (2, 4 and 5 minutes). Separate within subject ANOVAs were performed for each steady-state time-point (with stimulus duration and duration of data extraction as factors) and each stimulus duration (with steady-state time-point and duration of data extraction as factors).

CVR (% change in MCAv / mm Hg change in PETCO2)		**CO_2 stimulus duration**				
Time-point of data extraction		2 minute	4 minute	5 minute	*p*	η^2
Time-point 1	mean	3.26	2.81	3.02		
	SD	1.27	0.70	0.80	0.221	0.085
Time-point 2	mean	3.22	2.50	2.62		
	SD	1.24	0.92	0.97	**0.024***	0.213

Abbreviations: CVR, cerebrovascular reactivity (CVR); MCAv, middle cerebral artery blood velocity.

Table 3. Mean and standard deviations (SD) for VE-CO_2 values calculated using different methods of data extraction (steady-state time-point and data extraction duration) from 3 stimulus durations (2, 4 and 5 minutes). Separate within subject ANOVAs were performed for each steady-state time-point (with stimulus duration and duration of data extraction as factors) and each stimulus duration (with steady-state time-point and duration of data extraction as factors).

VE-CO_2 (L/min/mm Hg)		**CO_2 stimulus duration**				
Method of data extraction		2 minute	4 minute	5 minute	*p*	η^2
Time-point 1	mean	0.64	0.60	0.54		
	SD	0.21	0.23	0.23	0.299	0.069
Time-point 2	mean	0.63	0.89	0.91		
	SD	0.20	0.31	0.40	**0.009****	0.243

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Abbreviations: CO_2, carbon dioxide; VE-CO_2, ventilation sensitivity to CO_2.

3.3.2. **Three Minute Stimulus Duration for the** Cerebrovascular Reactivity to Carbon Dioxide **Measure**

In addition, CVR measures were calculated from the final 30 seconds of data 3 minutes into the stimulus duration for both the 4 and 5 minute stimulus durations. Paired t-tests revealed no differences between relative CVR measures calculated from the mean right MCAv ($t = 0.474$; $p = 0.643$), a trend for a difference between relative CVR measures calculated from the left MCAv ($t = 1.965$; $p = 0.070$) and no differences for measures calculated from average (right and left) MCAv ($t = 1.686$; $p = 0.504$) when comparing between the 4 minute and 5 minute stimulus durations. Further, a repeated-measures ANOVA comparing average CVR measures obtained from 3 minutes into the 4- and 5-minute stimulus duration with the CVR measures obtained from the 2-minute stimulus duration, were also not significantly different ($p = 0.184$, $\eta^2 = 0.121$).

4. Discussion

The purpose of this study was to compare CVR measures and VE-CO_2 values calculated from: 1) different CO_2 stimulus durations, and 2) different durations of data extraction and different time-point locations of steady-state for data extraction. The main findings were: 1) Within-participant variation increased when the stimulus duration was longer than 2 minutes, likely due to increased effects of the ventilatory response; and 2) CVR outcome measures calculated after 60 seconds of stimulus (time-point 1) were higher than measures obtained at the end of the stimulus duration (time-point 2). In addition, there was less variability in the CVR outcome measure when it was derived from 60 seconds after stimulus onset (time-point 1), indicating a more reliable point to determine CVR. Collectively, these findings demonstrate that methodological differences of stimulus duration and time-point used

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to determine the steady-state, do indeed alter the CVR outcome measure and VE-CO_2 values. Based on these findings, CVR outcome measures calculated 60 seconds after stimulus onset (time-point 1) with a stimulus duration of between 2 and 3 minutes appear to be less affected by methodological error or individual variability and therefore more reliable for both within (i.e., repeated measures) and between (cross-sectional) study comparison. Given that CVR is a common method of assessing brain health and has been applied in both healthy and clinical populations (e.g. Murrell *et al*, 2013, den Abeelen *et al.* 2014, Portegies *et al.* 2014), it is important that any variance caused by different methodologies is understood so that this measure can be more effectively and consistently used in research and clinical settings.

4.1. Aim 1: Does the CO_2 Stimulus Duration Alter the Cerebrovascular Reactivity to Carbon Dioxide Measure?

In aim 1, we found a significant effect of the CO_2 stimulus duration on the CVR outcome measure, as well as marked variability within the same individuals across the different stimulations (see Figure 2A and 2B). On average, the CVR measure increased between the 1-minute and the 2-minute stimulus duration, and was lowest when taken from the 4- and 5-minute stimulus durations. These differences are likely driven by the change in VE-CO_2 that increased approximately 3-fold as the stimulus duration increased (Figure 2C). Indeed, the difference in VE-CO_2 values between 1 and 2 minutes were significant, becoming progressively less different as the stimulus duration increased to 4 and 5 minutes. The VE-CO_2 values reported in this study support those previously shown in the literature (Lucas *et al.* 2011, using 4-min of 7% CO_2; Murrell *et al.* 2013, using 3-min of 5% CO_2); Brothers *et al.* 2014, using 5-min stepped-changes in CO_2). Thus, indicating that rather than a measurement error, these different values reflect a change in the physiological processes responding to the CO_2 stimulus.

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In some individuals, after 2-minutes of CO_2 stimulus, MCAv had only just reached steady-state, or may still be increasing to reach steady-state (Regan *et al.* 2013), and explains why the calculated CVR measures from the 2-minute CO_2 stimulus were higher in this study. Consequently, open-circuit, steady state CVR measures calculated by extracting data from a 2-minuite period may be less affected by increases in ventilation triggered by the chemoreceptors due to elevated $PaCO_2$, particularly for responders who are slow to reach steady-state or when 60 seconds of data are extracted. However, measures taken during this period may lead to a higher calculated CVR value. To address this in the current study, CVR measures from the final 30 seconds of data that were obtained 3 minutes into the stimulus duration for both the 4-and 5-minute CO_2 stimuli were compared with the CVR measure calculated using the 2-minute CO_2 stimulus. This comparison revealed no significant difference between these CVR measures. Given this, perhaps a 2- or 3-minute stimulus duration is the most suitable to use to obtain an accurate CVR measure, since they are least affected by intra-individual variation in vascular and respiratory responses, including the time to reach steady-state. Further, a shorter duration will also not unnecessarily overstress participants.

4.2. Aim 2: Does the Time-Point of Steady-State or the Duration of Data Extraction Alter the Cerebrovascular Reactivity to Carbon Dioxide Measure?

For aim 2, we found significant effects of steady-state time-point on CVR measures, though only when calculated from the 4- and 5-minute CO_2 stimulus. The lack in variability from the 2-minute CO_2 stimulus was expected since the data are extracted from effectively the same point for both steady-state time-point approaches (as detailed above). In contrast, there was no effect of data extraction duration for each stimulus duration on the CVR measure (30 vs. 60 seconds; $p = 0.300$).

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The duration of data extraction (60 or 30 seconds) does not seem to affect CVR measures based on this dataset. However, a consistent approach should be used that avoids any interference resulting from the on-kinetics of the stimulus and the gradual increase to steady-state that occurs within the first 2 minutes. Extracting a relative small proportion of the dataset is the commonly used approach in research centres using Doppler ultrasound to calculate the CVR measure. In contrast, research centres using magnetic resonance imaging (MRI) to measure CVR will extract data from the entire time course as well as the preceding baseline to calculate the CVR measure using a linear regression. Further, in protocols using several different levels of hypercapnia, data may be extracted from the entire protocol (Driver *et al.* 2016), rather than a relatively small segment. These approaches that utilise more of the dataset may avoid the possible issues with ventilatory response sensitivity observed herein, because all the data where this effect may be more pronounced (and may occur at different time-points between individuals) is being included to calculate the response. This leads to question whether CVR outcome measures (in addition to resting CBF measures) are different between different imaging modalities (i.e., TCD and MRI), a question beyond the scope of this study though warranting further investigation.

4.3. **Limitations and Methodology Considerations**

Limitations within this study include that it is difficult to test natural variation that may appear in the same individual across time. This is because it is impossible to test exact reliability between stimulus durations, as we cannot give a participant the different stimulus durations at the same time (i.e., the 2-minute stimulus duration cannot be given at the same time as the 4- or 5-minute stimulus duration). This study came as close as possible in achieving this by comparing four different stimulus durations within an hour whilst allowing adequate recoveries between them. Further, between participants, the stimulus durations were administered in a randomised order to minimize potential order effects. Further research is needed to compare these measures between days. Intra-individual variation in CO_2

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responses may be explained by genetic factors as well as sex, fitness level and other circumstances (Secher *et al.* 2015), indicating that the CVR measure is far more complicated than often presumed. Nevertheless, the CVR outcome measure is often collected from a single clinical visit, where the individual has not had time for their physiological measures to reach a resting baseline, and then used to predict current health status and disease risk.

Another limitation with this study and with TCD as a method is that we are unable to consider possible effects of vessel diameter change in response to CO_2, and how these may contribute to changes in blood velocity, which is currently debated in the literature (e.g., Brothers and Zhang, 2016; Hoiland and Ainslie, 2016). However, our findings can direct us towards an approach that avoids as much variability as possible in CVR measures and lead us to consider other factors. Further, within-individual variability will likely lead to conflicting reports of CVR when other methods are used; including MRI, isotropic methods and volumetric flow.

A familiarization trial was performed with each participant to ensure they were comfortable with the 5% CO_2 gas and experienced no adverse effects. This trial also ensured that sufficient MCA signals could be obtained that could be replicated for the second visit. Mean MCAv values were also compared between the four baselines. On visual inspection, there is notable variability between the baseline velocities with some participants showing more variability than others (i.e., CoV ranging from 1 – 8%). This may be a natural variability that occurs across time in response to many environmental influences and warrants further investigation, particularly if day-to-day variation within the same individual may also influence the CVR measure. In contrast, there is less variability between the baseline mean $P_{ET}CO_2$ values (i.e., CoV ranging from 0 – 5%; mean CoV = 2.4%).

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4.4. Perspectives and Future Directions

To maximise the utility of CVR as a measure of brain vascular health it is important to identify where methodological nuances affect CVR outcome measures. In considering where inconsistencies between studies alter the measure we can determine their collective interpretation of how CVR depicts brain vascular health in various populations. It may then be necessary to move towards a standardised, optimised method to measure CVR. This is imperative to identify given that study findings are often compared and some studies have been used to predict risk of cardiovascular mortality (Markus *et al.* 2001; Portegies *et al.* 2014).

Given the methodological considerations discussed when considering intra-individual variation, future research may aim to establish where the peak CVR is likely to occur and the extent to which individual responses range from the lowest to highest CVR measure, in addition to further investigation of kinetic CVR responses. Our findings demonstrated that some individual's CVR measures vary considerably depending on the stimulus duration used, whereas others would remain relatively stable (as shown in Figure 2A and 2C, lower panel). Recently, Carter and colleagues have introduced an alternative approach to assess CVR via CO_2 administration, by quantifying the shear stress-induced vasodilatory response of the intracranial artery (Carter *et al*. 2016). The findings of the present study would support targeting this initial response, since there was less inter-subject variability with the shorter stimulus durations. We could also consider implementing the ventilation response into the calculation in some way. For example, determining the point where CVR is at its peak and the ventilation response is at its lowest. Perhaps this approach would give a more accurate

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representation of CVR measures that is less influenced by within-subject variability and the ventilatory response.

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In addition to the open-circuit technique, re-breathing and stepped end-tidal clamping are other approaches that are used to measure CVR with transcranial Doppler (TCD). Investigating differences with these approaches was beyond the scope of this study, but it is likely that different approaches introduce more CVR variability, with potentially greater variations than that observed here for this within-technique comparison. For example, changes in the arteriovenous gradient of PCO_2 will change the interaction effect between vascular and ventilatory sensitivities (Reid and Leigh 1967; Xie *et al.* 2006; Fan *et al.* 2010; Ainslie and Duffin 2011), which is an obvious difference between the rebreathing and open-circuit techniques that may alter the CVR outcome measure, as reviewed elsewhere (Ogoh *et al.,* 2008; Skow *et al*. 2013; Boulet *et al*. 2016; Mackay *et al.* 2016). Such differences in the physiological response has obvious implications for the interpretation of the outcome CVR measure within and between cohorts of interest. Further, magnetic resonance imaging (MRI) also provides a measure of CVR. Though in contrast to TCD, reactivity measures are derived from changes in the blood-oxygen-level dependent (BOLD) signal (Thomas *et al.* 2013; Bhogal *et al.* 2016; Zhou *et al.* 2015). As well as variations within one methodological approach, the choice of measurement technique differs across studies (TCD or MRI). While challenging, a full comparison of all the availability approaches is needed to clarify differences between all these techniques and which one (or combination) will best quantify brain vascular health and reliably predict clinical risk.

We also recommend future research investigating sex differences between these measures with a larger sample size; although we did not observe significant sex differences in our small sample except for the 4-minute absolute CVR measure where females had higher CVR.

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4.5 Conclusion

The present study showed that the stimulus duration does alter the CVR measure, as does the method of data extraction (i.e., choice of steady-state time-point); though these effects are different between stimulus durations. Given these findings, whilst also considering that slow responders may take longer to reach steady-state, we recommend using a 3-minute stimulus duration where data is extracted from the end of the stimulus duration (taking a 30-60 second average). Our findings strongly indicate that a more consistent approach in collecting data and calculating the CVR measure is required. To achieve this, a better understanding of what is indeed the best method of calculating this response is also required. Gold-standard approaches are needed so that findings between studies can be compared and clinical use of CVR measures are more robust.

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Additional information

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Disclosure/Conflict of interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Author Contribution statement

CVB, KM, RAIL & SJEL contributed to the conception or design of the work. CVB, KM, RAIL, ACW & SJEL contributed to the acquisition, analysis, or interpretation of data for the work. CVB, KM, RAIL, ACW & SJEL contributed to drafting of the work or revising it critically for important intellectual content. All authors approved the final version of the manuscript. All authors agree to be accountable for all aspects of the work in ensuring 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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Figure 1. Methodology illustration. A is a schematic of the gas challenge protocol. Shaded boxes show the stimulus durations (1, 2, 4 and 5 minutes of 5% CO_2; random order of administration between participants). White boxes show baseline periods where participants were breathing room air. B shows a representative CO_2 trace of gas delivery to a participant taken from LabChart. Shows where for aim 1 30 seconds of data were extracted from the end of four different CO_2-stimulus durations (1, 2, 4 and 5 minutes). C shows percentage CO_2 trace from LabChart showing where data were extracted from two different steady-state time-points. Time-point 1: after 60 seconds of stimulus duration and Time-point 2: end of stimulus duration, for two different durations (60 and 30 seconds).

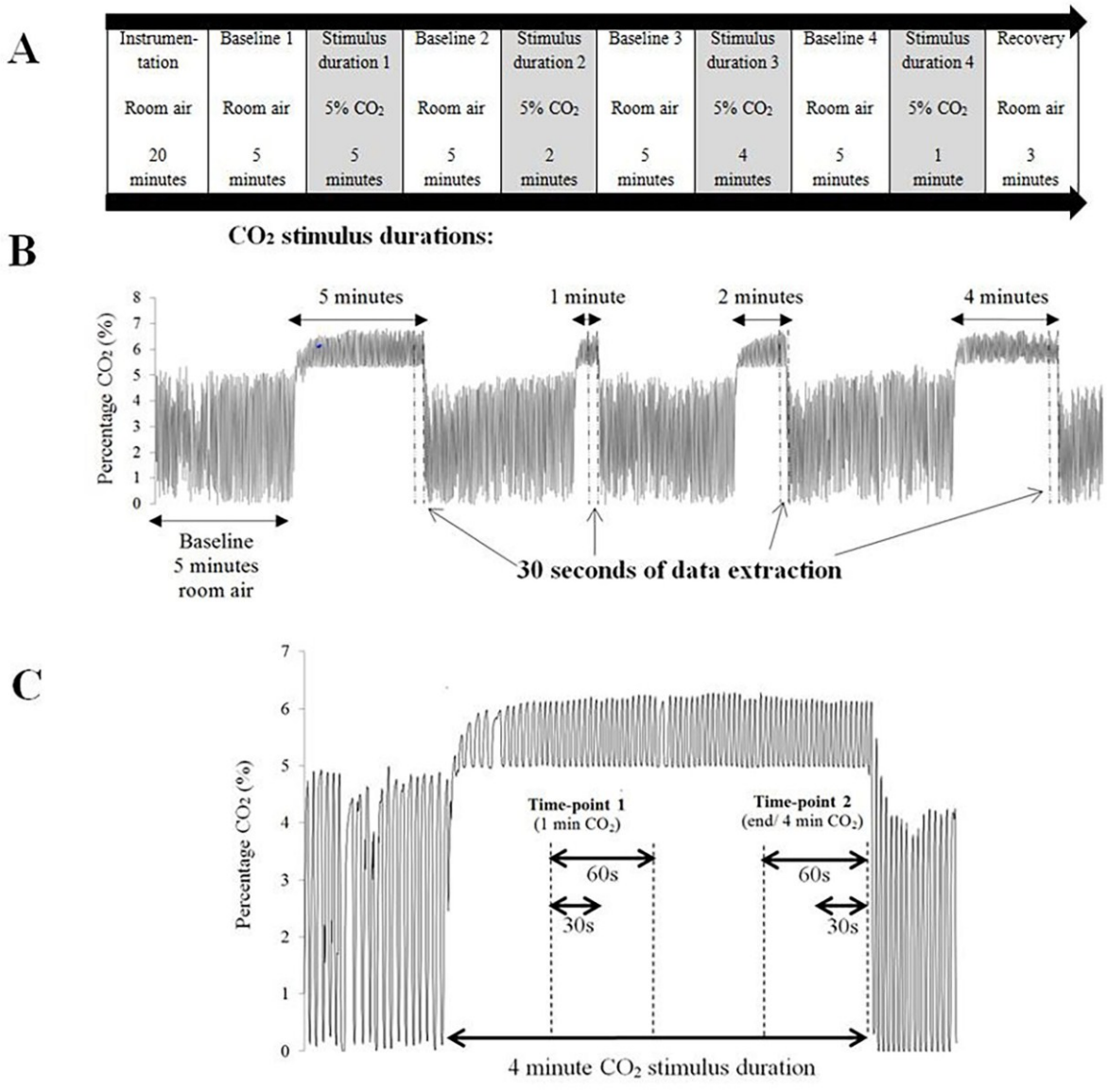

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Figure 2. Upper panel shows mean (± SD) relative CVR (A), absolute CVR (B) and VE-CO_2 (C) calculated for each stimulus duration. Significant post-hoc effects and trends towards significance of stimulus duration are shown in bold font. Lower panel shows within-individual variability in relative CVR (A), CVR (B) and VE-CO_2 (C) for each participant between CO_2-stimulus durations. Within-individual covariance ranged from 7-46% for CVR and 13-77% for VE-CO_2. Each symbol represents one participant. Abbreviations: CO_2, carbon dioxide; CVR, cerebrovascular reactivity; MCAv, middle cerebral artery blood velocity; $P_{ET}CO_2$, end-tidal carbon dioxide; VE-CO_2, ventilation sensitivity to CO_2.

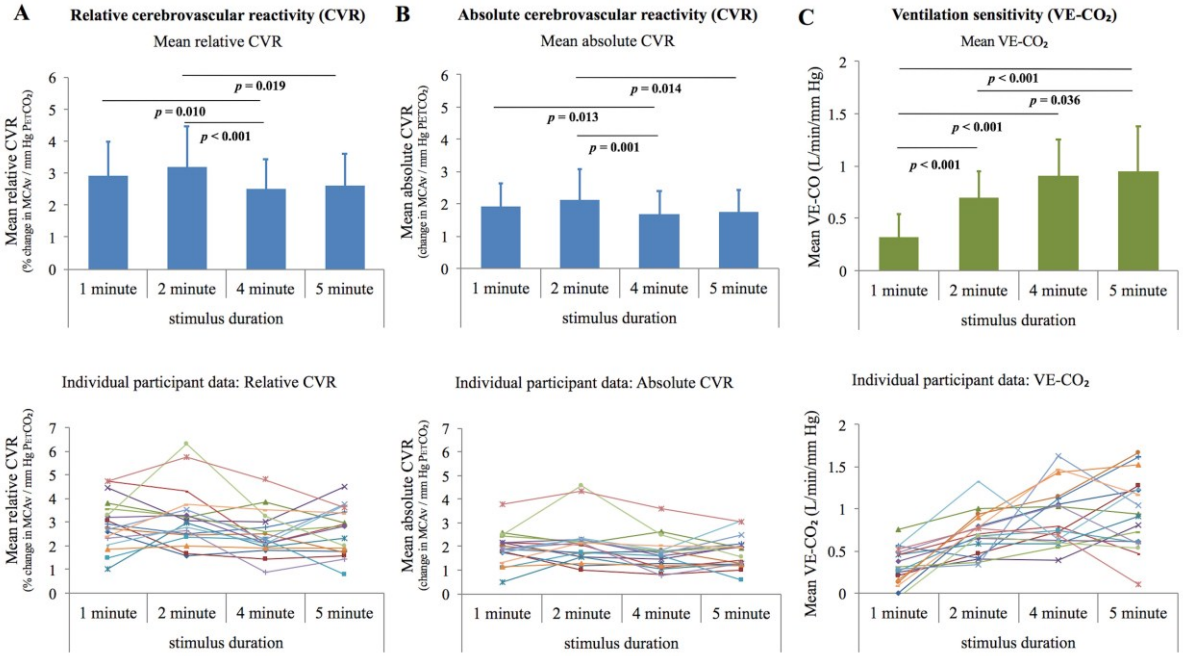

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Figure 3. Mean ± SD relative CVR measures (averaged right and left MCAv) (A) and VE-CO_2 values (B). Graphs show measures calculated for two different durations (30 and 60 seconds) from three stimulus durations (2, 4 and 5 minutes). For CVR and VE-CO_2, there were no significant differences between measures calculated from a 30 and 60 second duration of data extraction. Abbreviations: CO_2, carbon dioxide; CVR, cerebrovascular reactivity; MCAv, middle cerebral artery blood velocity; $P_{ET}CO_2$, end-tidal carbon dioxide, VE-CO_2, ventilation sensitivity to CO_2.

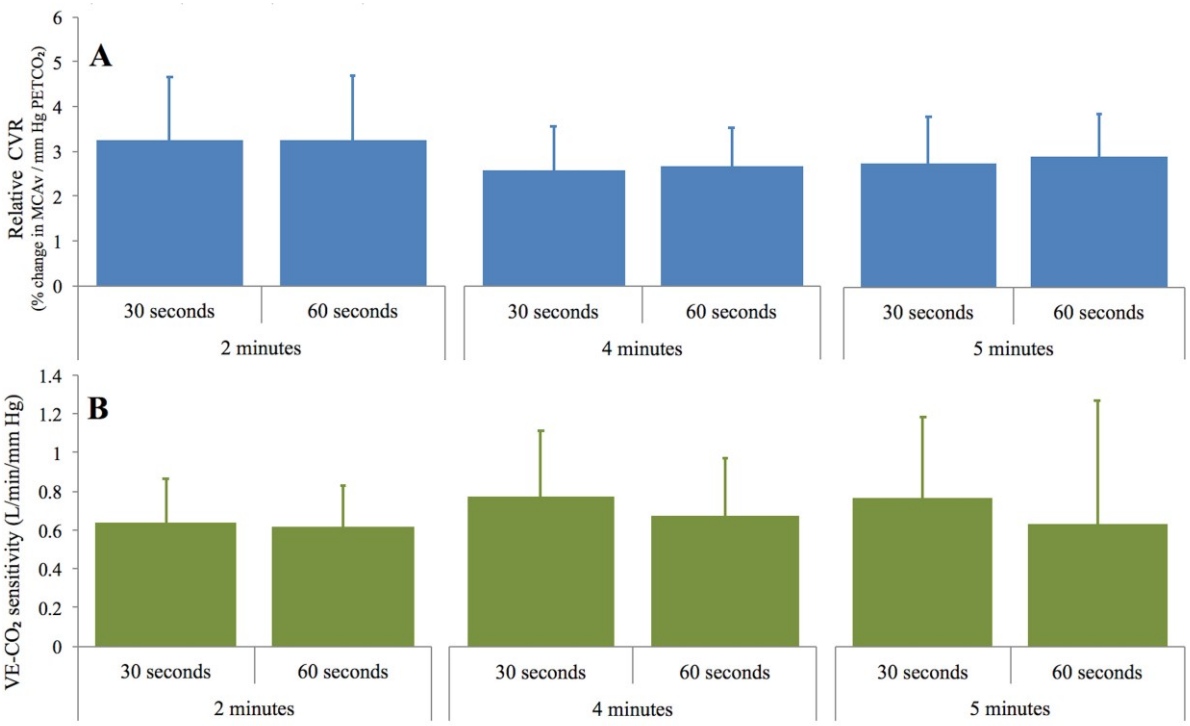

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Figure 4. Mean ± SD relative CVR measures (averaged right and left MCAv) (A) and VE-CO_2 values (B). Graphs show measures calculated from two steady-state time-points from three stimulus durations (2, 4 and 5 minutes). For CVR, post hoc comparisons revealed a significant effect of time-point for the 4-minute and 5-minute stimulus durations. For VE-CO_2, post hoc comparisons revealed a significant effect of time-point for the 2- minute, 4-minute and 5-minute stimulus durations. Abbreviations: CO_2, carbon dioxide; CVR, cerebrovascular reactivity; MCAv, middle cerebral artery blood velocity; $P_{ET}CO_2$, end-tidal carbon dioxide, VE-CO_2, ventilation sensitivity to CO_2.

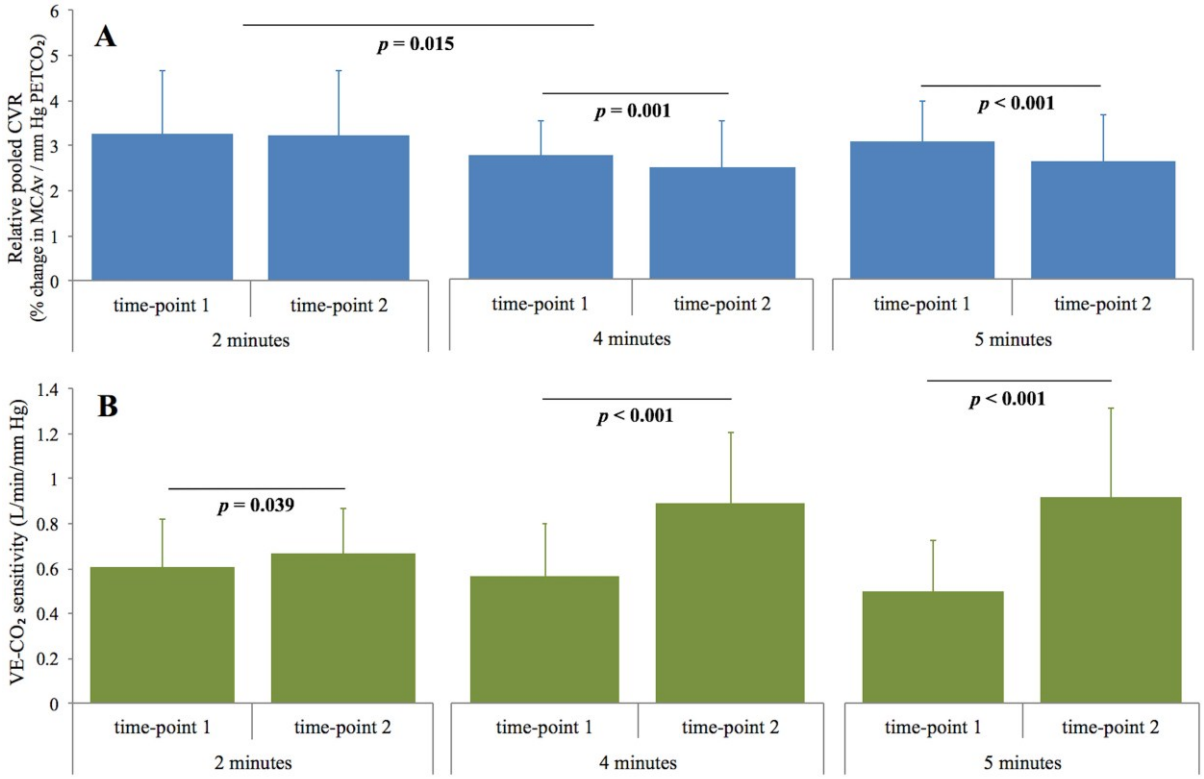

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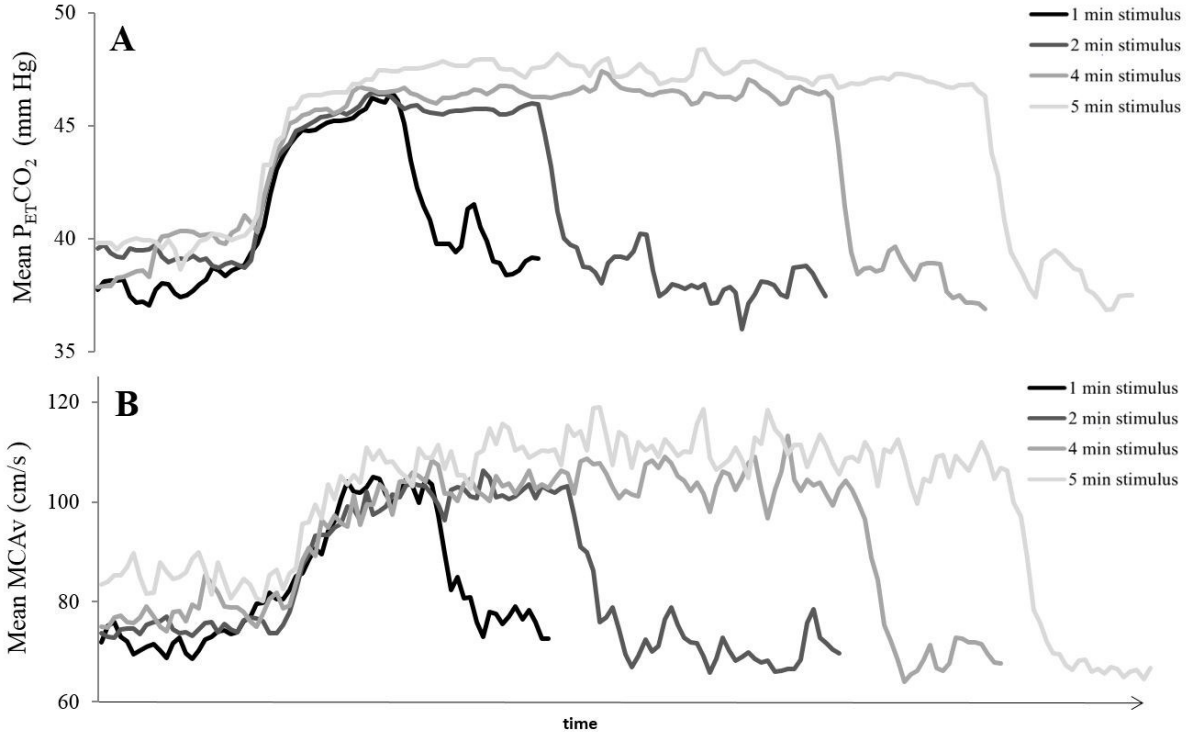
Figure 5. Mean $P_{ET}CO_2$ (A) and mean MCAv (B) for all four stimulus durations (1, 2, 4 and 5 minutes) for one participant.



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