# Sex differences in voluntary activation of the diaphragm

by

Andrew Harry Ramsook

B.P.H.E., B.Ed., The University of Toronto, 2012

M.Sc., The University of British Columbia, 2016

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The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

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the degree of Doctor of Philosophy

in Rehabilitation Sciences

**Examining Committee:**

Dr. Jordan Guenette, Associate Professor, Department of Physical Therapy, UBC
Supervisor

Dr. Michael Koehle, Professor, School of Kinesiology, UBC
Supervisory Committee Member

Dr. Bill Sheel, Professor, School of Kinesiology, UBC
Supervisory Committee Member

Dr. Kristin Cambell, Professor, Department of Physical Therapy, UBC
University Examiner

Dr. Jonathon Little, Associate Professor, School of Health and Exercise Sciences, UBCO
University Examiner

## Abstract

**Purpose:** Three studies were performed to examine how voluntary activation of the diaphragm changes after fatiguing tasks in young, healthy males and females. The change in diaphragm voluntary activation (D-VA) was also compared to the change in contractile function, assessed by the change in transdiaphragmatic twitch pressure ($P_{DI,TW}$).

**Methods:** Study 1 (Chapter 2) investigated the within- and between-session reliability of D-VA using cervical magnetic stimulation (CMS) to evoke twitches. Study 2 (Chapter 3) examined changes in D-VA after high intensity, whole-body exercise between sexes. Study 3 (Chapter 4) examined changes in D-VA after inspiratory pressure threshold loading (IPTL) between sexes.

**Conclusions:** CMS can be used to reliably assess D-VA, measured via intraclass correlation coefficients (ICC) both within- (ICC: 0.76) and between-session (ICC: 0.88). After exercise, D-VA decreased in males and females. The decrease in D-VA was greater in males compared to females (87.4±10.8 *vs* 95.4±4.9%basline, respectively, $p$=0.036). The magnitude of the decrease in D-VA was less than the decrease in $P_{DI,TW}$ (Males: 70.3±12.4, Females: 85.2±16.7%baseline, $p$=0.024). After IPTL, both males and females showed a decrease in D-VA and $P_{DI,TW}$. While males showed a greater decrease in $P_{DI,TW}$ (73.3±12.1 *vs* 87.1±15.0%baseline, respectively, $p$=0.016) compared to females, the decrease in D-VA was similar between sexes (88.2±10.5 *vs* 91.4±7.6%baseline, respectively, $p$=0.432). After IPTL, the decrease in $P_{DI,TW}$ correlated with the total respiratory work performed whereas the decrease in D-VA correlated with time until task failure. The magnitude of the decrease in $P_{DI,TW}$ was greater than the decrease in D-VA after IPTL. Collectively, the results of this thesis suggest that there are sex differences in the change in D-VA after exercise but not IPTL. This thesis provides a foundation for future work to investigate how diaphragm fatigability can affect exercise performance in humans.

## Lay Summary

When breathing muscles fatigue, they lose strength. This loss could be because the muscle is not working effectively or the signal from the brain is not strong enough to make the muscle contract. Females do not fatigue as much as males, but it is unknown if this includes brain-related muscle fatigue. This thesis investigated how the brain contributes to diaphragm fatigue, and if this is different between sexes. In study 1, we determined that measuring diaphragm voluntary activation can reliably test how the brain contributes to diaphragm fatigue. In studies 2 and 3, we measured diaphragm voluntary activation after whole-body exercise and a breathing challenge, respectively. We found that the brain contributes to diaphragm fatigue in males and females but affected males more. However, males and females were affected similarly after the breathing challenge. In conclusion, measuring diaphragm voluntary activation is a reliable technique, and changes in diaphragm voluntary activation are task-specific between sexes.

## Preface

This thesis contains the work of the candidate, Andrew H. Ramsook, under the supervision of Dr. Jordan A. Guenette. Andrew H. Ramsook and Dr. Jordan A. Guenette conceptualized and designed experiments and interpreted data. Data collection and analysis, and document preparation are the work of Andrew H. Ramsook. All data were collected in the Cardiopulmonary Exercise Physiology Laboratory in the Centre for Heart Lung Innovation at St. Paul's Hospital, Vancouver, British Columbia, Canada.

The experiments of *Chapter 2* received ethical approval from Providence Health Care Research Ethics Board (approval number: H17-02680).

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The experiments of *Chapter 3* received ethical approval from Providence Health Care Research Ethics Board (approval number: H19-0725).

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## List of Abbreviations

| | |
|---|---|
| 95%CI | 95% Confidence interval |
| AIC | Akaike information criteria |
| BMI | Body mass index |
| BPNS | Bilateral phrenic nerve stimulation |
| CMAP | Compound muscle action potential |
| CMS | Cervical magnetic stimulation |
| D-VA | Diaphragm voluntary activation |
| ECC | Excitation contraction coupling |
| EMG | Electromyography |
| $EMD_{DI}$ | Crural diaphragm electromyography |
| $EMG_{SCA}$ | Scalenes electromyography |
| $EMG_{SCM}$ | Sternocleidomastoid electromyography |
| $f_b$ | Breathing frequency |
| $FEV_1$ | Forced expired volume in 1 second |
| FRC | Functional residual capacity |
| FVC | Forced vital capacity |
| GET | Gas exchange threshold |
| ICC | Intraclass correlation coefficients |
| IPTL | Inspiratory pressure threshold loading |
| ITT | Interpolated twitch technique |
| MEP | Motor evoked potential |
| MRR | Maximal relaxation rate |
| MVC | Maximal voluntary contraction |
| $P_{DI}$ | Transdiaphragmatic pressure |
| $P_{DI,MAX}$ | Maximal transdiaphragmatic pressure |
| $P_{DI,TW}$ | Transdiaphragmatic twitch pressure |
| $P_{ET}CO_2$ | Partial pressure of end-tidal carbon dioxide |
| $P_{GA}$ | Gastric pressure |
| $PI_{MAX}$ | Maximal inspiratory pressure |
| $P_{MO}$ | Mouth pressure |
| $P_{OES}$ | Oesophageal pressure |
| $PTP_{DI}$ | Pressure-time product of the diaphragm |
| RER | Respiratory exchange ratio |
| $S_pO_2$ | Peripheral oxygen saturation |
| SEM | Standard error of measurement |
| $T_I$ | Inspired time |
| $T_{TOT}$ | Total breath time |
| TLC | Total lung capacity |
| TMS | Transcranial magnetic stimulation |
| TTE | Time to exhaustion |
| TTF | Time to task failure |

| | |
|---|---|
| TTI | Tension-time index |
| $TTI_{DI}$ | Tension-time index of the diaphragm |
| $V_T$ | Tidal volume |
| $\dot{V}CO_2$ | Carbon dioxide production |
| $\dot{V}O_2$ | Oxygen consumption |
| $\dot{V}O_{2MAX}$ | Maximal oxygen uptake |
| $\dot{V}O_{2RM}$ | Oxygen cost of the respiratory muscles |
| $W_b$ | Work of breathing |

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# Chapter 1: Introduction

## 1.1 Background

The respiratory system is regarded as being 'overbuilt' in that it possesses a large capacity to increase minute ventilation ($\dot{V}_E$) to meet the demands of high-intensity aerobic exercise in young, healthy humans (Dempsey, 1986; Dempsey *et al.*, 2020). While this appears to remain true in young, healthy, recreationally active males, there is emerging evidence that the human respiratory system can contribute to exercise limitation in certain healthy populations. Indeed, those living with chronic respiratory disease and some highly trained endurance athletes are examples where the respiratory system is no longer 'overbuilt' to meet the demands of exercise. Many respiratory diseases, including chronic obstructive pulmonary disease and interstitial lung disease, present with unique phenotypes but in most cases the pathology results in changes that reduce the overall capacity of the respiratory system, thereby limiting exercise performance. (Chin *et al.*, 2013; Schaeffer *et al.*, 2017). Alternatively, highly trained athletes impose an incredible physiological, and consequently ventilatory, demand during maximal exercise, which may also limit exercise performance (Johnson *et al.*, 1992; Guenette *et al.*, 2007). Another group that who may experience respiratory limitations to exercise performance are young, healthy females. Historically, there has been a bias favouring male participants in physiology research (Miller, 2014). More recently, there has been a greater emphasis on exploring the influence of biological sex on human physiology, particularly during exercise. Examining the physiological responses to exercise in males and females has led to a greater appreciation for sex differences (and similarities) that were otherwise previously ignored. With respect to the respiratory system, recent research has uncovered a number of differences in morphology and physiology between males and females that may contribute to the respiratory response to exercise (Molgat-Seon *et al.*, 2018b).

### 1.1.1 Sex differences and exercise physiology research

It is important to establish a definition of 'sex' and describe how it differs from 'gender'. Herein, the terms 'sex' and 'gender' will be used in accordance with the definitions provided by the Canadian Institutes of Health Research:

*"Sex refers to a set of biological attributes in humans and animals. It is primarily associated with physical and physiological features including chromosomes, gene expression, hormone levels and function, and reproductive/sexual anatomy. Sex is usually categorized as female or male but there is variation in the biological attributes that comprise sex and how those attributes are expressed. Gender refers to the socially constructed roles, behaviours, expressions and identities of girls, women, boys, men, and gender diverse people. It influences how people perceive themselves and each other, how they act and interact, and the distribution of power and resources in society. Gender identity is not confined to a binary (girl/woman, boy/man) nor is it static; it exists along a continuum and can change over time. There is considerable diversity in how individuals and groups understand, experience and express gender through the roles they take on, the expectations placed on them, relations with others and the complex ways that gender is institutionalized in society."* (Institute of Gender and Health & Canadian Institutes of Health Research, 2020).

Furthermore, given that this thesis primarily concerns itself with sex, the terms 'male' and 'female' will be used to describe the sex of participants (Clayton & Tannenbaum, 2016). This operational definition is important to consider as female participation in sport and research expands. While female participation in sport has grown, there appears to be a lag in the inclusion of female participants in sport and exercise-related research. A study in 2014 examined the sex of participants included in three journals (*The British Journal of Sports Medicine, American Journal of Sports Medicine,* and *Medicine and Science in Sports and Exercise*) and found that females

typically accounted for 35-37% of participants in original research published between January 2011 and August 2013 (Costello *et al.*, 2014). Studying sex differences in all aspects of physiology will provide a better understanding of the human body and how it functions.

## 1.2 Sex differences in the human respiratory system

The main components of the respiratory system are the lungs, airways, rib cage, and respiratory muscles. Between the sexes there are some structural differences that result in several functional consequences. The presence and magnitude of such consequences are best observed when the respiratory system is stressed, such as during whole-body exercise.

### 1.2.1 Sex differences in the structure of the respiratory system

On average, females have smaller lungs compared to their male counterparts (Gutierrez *et al.*, 2004) which holds true even when males and females are matched for height. Curiously, when matched for lung size females also appear to have smaller airways than males (Mead, 1980; Martin *et al.*, 1987; Sheel *et al.*, 2009). Sex differences in airway size are not uniform and appear to vary based on airway generation. Analysis of computed tomography scans in older, former smokers found that differences in airway size can range from 16-31% smaller in females compared to males. The large conducting airways including, but not limited to, the trachea (19.5% smaller in females), left main bronchus (18.1% smaller in females), and right main bronchus (23.8% smaller in females), exhibit a difference in size even when matched for overall lung size in older, healthy ex-smokers (Sheel *et al.*, 2009). These data have recently been supported by a similar study in healthy non-smoking males and females matched for height (Dominelli *et al.*, 2018) and lung size (Christou *et al.*, 2021). The importance of airway luminal area becomes apparent when one considers physical parameters that affect flow, as described by Poiseuille's Law. Specifically, the law states that resistance to airflow is proportional to the radius of the airway to the fourth power.

Therefore, as females are likely to have narrower airways, the resistance for a given flow would be greater. Indeed, there appears to be a strong relationship between the area of the airways and the work of breathing ($W_b$) (Peters *et al.*, 2021). Additionally, there are differences in the morphology of the lungs and rib cage. The male lungs are typically shorter with a wide base, conferring a 'pyramidal' shape, whereas the female lungs are more 'prismatic' in shape, owing to the base and apex of each lung having similar sizes (Torres-Tamayo *et al.*, 2018). Male rib cages tend to be wider, and individual ribs are more horizontally oriented in males compared to their female counterparts (Garcia-Martinez *et al.*, 2016).

### 1.2.2 Sex differences in the function of the respiratory system

At rest, the differences in the structure of the respiratory system between the sexes have virtually no impact on the function of the respiratory system, owing to the balance of the respiratory system's large capacity and the relatively low demand placed upon it at rest. However, during exercise, when the demands placed upon the respiratory system are increased, the functional consequences of the aforementioned sex differences are evident.

It is likely that the increased resistance imposed by smaller airways may explain the higher $W_b$ and oxygen cost of the respiratory muscles ($\dot{V}O_{2RM}$) during exercise, in females compared to males. The $W_b$ is a measure of the physical work performed by the respiratory muscles to achieve a given ventilation (Otis, 1964). As $\dot{V}_E$ approaches ~55 $L \cdot min^{-1}$, the $W_b$ is similar between sexes, however; as $\dot{V}_E$ exceeds ~55 $L \cdot min^{-1}$ the $W_b$ in females is greater than in males for a given $\dot{V}_E$ (Guenette *et al.*, 2007; Dominelli *et al.*, 2015; Molgat-Seon *et al.*, 2017). Furthermore, the sex difference in the $W_b$ is almost exclusively due to a higher resistive rather than viscoelastic component of $W_b$, for a given absolute $\dot{V}_E$ (Guenette *et al.*, 2009). Intuitively, as the $W_b$ increases, so too must the load on the respiratory muscles. Therefore, it stands to reason that the $\dot{V}O_{2RM}$

would be commensurate with the $W_b$. Previous studies have asked participants to voluntarily mimic their ventilatory pattern during exercise while seated at rest, including tidal volume ($V_T$), breathing frequency ($f_b$), and oesophageal pressure ($P_{OES}$), to estimate the $\dot{V}O_{2RM}$ (Aaron et al., 1992). Using this technique, Dominelli *et al.*, found that the $\dot{V}O_{2RM}$ in females was greater than males at both maximal and submaximal intensities (Dominelli *et al.*, 2015). Supporting the association between $W_b$ and $\dot{V}O_{2RM}$, every individual tested exhibited a significant linear correlation between the two outcomes. Moreover, when expressed relative to whole-body maximal oxygen consumption ($\dot{V}O_2$), females dedicated a greater portion of $\dot{V}O_2$ to their respiratory muscles during high-intensity exercise (Dominelli *et al.*, 2015).

As a result of the aforementioned sex differences in respiratory physiology, females are more likely to adopt a ventilatory pattern during exercise that is different from males. For example, to achieve a similar absolute $\dot{V}_E$, females are more likely to assume a more rapid (*i.e.*, higher $f_b$) and shallow (*i.e.*, lower $V_T$) breathing pattern than males (Guenette *et al.*, 2009; Schaeffer *et al.*, 2014). This tachypnoeic breathing pattern may be a means to alleviate the associated elastic $W_b$ at a high lung volume. Following differences in breathing pattern, the respiratory muscle activation strategy between males and females also appears to be different (Mitchell *et al.*, 2018; Molgat-Seon *et al.*, 2018a). Using surface EMG to measure the activation of the sternocleidomastoid ($EMG_{SCM}$) and scalene ($EMG_{SCA}$) muscles, and an oesophageal electrode catheter to measure EMG of the crural diaphragm ($EMG_{DI}$), one can track respiratory muscle activation patterns during exercise. While placing surface electrodes on more superficially located respiratory muscles, such as the sternocleidomastoid, scalene, and intercostal muscles, can easily be achieved by placing electrodes on the skin, the diaphragm is relatively inaccessible for both conventional surface and needle EMG techniques due to its location (Hixon *et al.*, 1969). Using oesophageal catheters allow

surface electrodes to be placed near the diaphragm to measure $EMG_{DI}$ without the associated limitations of surface and needle EMG techniques. Early versions of oesophageal catheters included a single electrode pair (Agostoni *et al.*, 1960; Petit *et al.*, 1960), later iterations of oesophageal catheters have been designed to include multiple pairs of electrodes to account for the changing position of the diaphragm as lung volume changes (Daubenspeck *et al.*, 1989). When measuring respiratory muscle EMG during incremental exercise, Molgat-Seon *et al.* (2018a) observed a greater activation of the scalene muscles at any absolute $\dot{V}_E$ in exercising females compared to males. Females displayed a greater activation of the diaphragm at submaximal absolute $\dot{V}_E$ but this difference was abolished at peak exercise. Finally, $EMG_{SCM}$ was greater in females at a $\dot{V}_E$ above ~40 $L \cdot min^{-1}$ (Molgat-Seon *et al.*, 2018a). When $\dot{V}_E$ was normalized to the maximum $\dot{V}_E$ achieved during exercise, no sex differences in $EMG_{DI}$ were observed, but $EMG_{SCA}$ and $EMG_{SCM}$ were greater in females above 30% of the maximum achieved $\dot{V}_E$ (Molgat-Seon *et al.*, 2018a). When exercising at a constant load equal to 85% of peak work rate, the activation of the respiratory muscles follows a similar pattern, with females activating extra-diaphragmatic (*i.e.*, the scalenes and sternocleidomastoids) inspiratory muscles to a greater degree than males (Mitchell *et al.*, 2018). It is possible that the observed differences in respiratory muscle activation are related to the shape of the rib cage. There is some evidence that, at rest, the rib cage (*i.e.*, intercostal) muscles contribute to the pressure swings during tidal breathing more in females compared to males (Bellemare *et al.*, 2003). This may imply that there is some level of mechanical advantage to generate airflow with intercostal muscles given the structure of the rib cage in females. However, these data were not collected with a concurrent assessment of neural activation and therefore the connection between the morphometry of the rib cage and the activation of extra-diaphragmatic inspiratory muscles remains speculative.

## 1.3 Skeletal muscle fatigue

As it relates to human performance, 'fatigue' can be divided into categories of 'perceived' and 'performance' fatigability (Enoka & Duchateau, 2016). Perceived fatigability is a change in sensation that directly or indirectly affects the performer's ability to execute a task. Some of the most common examples of performance fatigue are symptoms associated with one's psychological state, such as mood, motivation, and expectations (Enoka & Duchateau, 2016). Performance fatigability describes "an objective measure of performance over a period of time" (Enoka & Duchateau, 2016). When discussing skeletal muscle fatigue, most often one is referring to aspects of performance fatigability, such as a decline in force capacity or voluntary activation. Skeletal muscle fatigue is defined as a "*condition in which there is a loss in the capacity for developing force and/or velocity of a muscle, resulting from muscle activity under load and which is reversible by rest*" (1990). Using this definition of skeletal muscle fatigue, this thesis will examine both neural and contractile mechanisms that contribute to the decrease in force generating capacity of the muscle. When skeletal muscle fatigue is due to neural or contractile mechanisms, it has historically been referred to as central or peripheral fatigue, respectively.

### 1.3.1 Neural mechanisms contributing to skeletal muscle fatigue

Neural mechanisms contribute to skeletal muscle fatigue when voluntary drive to that muscle or group of muscles is suboptimal. Throughout the skeletal muscle contraction process, there are a number of sites that can result in a neural fatigue phenotype, including excitatory input to the higher motor centres, excitatory drive to the lower motoneurons, motoneuron excitability, and neuromuscular transmission (Fitts, 1996).

Testing motor cortex excitability, with a combination of EMG and transcranial magnetic stimulation (TMS), provides insight into the excitatory input of higher motor centres. Stimulating

the appropriate area with TMS results in a motor evoked potential (MEP) that is recorded via surface EMG on the corresponding muscle. The size of the MEP, that is the amplitude and area, partially reflects motor cortex excitability (Kent-Braun *et al.*, 2012). By moving the site of stimulation to the corticospinal tracts at the cervicomedullary junction, the corresponding cervicomedullary MEP can differentiate between excitability on the cortical and subcortical levels (Kent-Braun *et al.*, 2012). The role of cortical excitability appears to be most prominent during sustained maximal contractions. However, based on observations that cortical and spinal excitation decrease at similar rates, it is likely that any fatigue-related loss of force is mediated by sources beyond the higher motor centres (McNeil *et al.*, 2009).

While performing fatiguing contractions, the background excitability of the motor neuron pool may change as a result of signals sent via sensory afferents (Kent-Braun *et al.*, 2012). Motor neuron pool excitability is assessed through another EMG recorded response to electrical stimulation of type IA peripheral spindle afferents (*i.e.*, H-reflex). Despite the accepted observation that H-reflex amplitude will decrease with fatiguing contractions, the consequences of a lower H-reflex amplitude with respect to fatigue remains controversial (Kent-Braun *et al.*, 2012).

Changes in the recruitment or discharge of active motor units will influence overall force production. If fewer motor units are recruited, it follows that force will be likewise diminished. While the precise mechanism of motor unit 'dropout' during fatiguing contractions is not known, there are data supporting changes in motor unit recruitment during fatiguing contractions. High threshold motor units can be recruited during sustained and dynamic contractions to replace lower threshold motor units that will no longer fire (Westgaard & de Luca, 1999). Additionally, the motor unit firing rate will also decrease following a fatiguing task (Mettler & Griffin, 2016). A combination of a lower firing rate and a lack of motor units available to be recruited would result

in a loss of force. Fatiguing metabolites may influence neural mechanisms that contribute to skeletal muscle fatigue after both single joint (Kennedy *et al.*, 2014) and whole-body exercise (Amann *et al.*, 2009; Sidhu *et al.*, 2017). It appears that the increased feedback from mechano- and metabo-sensitive group III/IV muscle afferents can inhibit the motor cortex, thus creating an environment where voluntary drive is lower than baseline values (Sidhu *et al.*, 2017).

While techniques like TMS and H-reflex analysis can assess specific components that contribute to fatigability due to neural mechanisms, a large-scale means of measuring neural mechanisms involved in fatigue is the interpolated twitch technique (ITT). Briefly, this method involves delivering two stimuli to the participant, one superimposed during a maximal voluntary contraction, and one control twitch immediately once the muscle is relaxed. The ratio of the amplitude of the superimposed to control twitch is used to determine voluntary activation. A large superimposed twitch indicates that despite attempting a maximal contraction, the muscle is capable of generating more force (Shield & Zhou, 2004). These stimuli can be applied to the motor cortex, the nerve innervating the muscle of interest, or the muscle itself (Kent-Braun *et al.*, 2012). Most healthy well-motivated subjects are capable of near-complete voluntary activation of limb muscles (Merton, 1954) and the diaphragm (Bellemare & Bigland-Ritchie, 1987; McKenzie *et al.*, 1992; Similowski *et al.*, 1996) under resting conditions.

### 1.3.2 Contractile mechanisms contributing to skeletal muscle fatigue

When there is adequate neural drive supporting a muscle contraction yet there is still a deficit in force production compared to rest, fatigue is said to be the result of a failure of the contractile mechanisms of the muscle. Potential sites across the contraction processes that can result in a contractile failure include: a failure in sarcolemma excitability and excitation-contraction coupling (ECC).

Decreases in sarcolemma excitability are observed with skeletal muscle (Fitts, 1996). As the onset of an action potential is based on an absolute threshold, any decrease to the resting potential would reduce the likelihood of action potential propagation, otherwise known as decreased excitability (Kent-Braun *et al.*, 2012).

ECC refers to the series of events that occur in the plasma membrane of skeletal muscle that can lead to calcium release (Calderon *et al.*, 2014). In isolated mouse muscle, ECC-based fatigue appears to occur due to a lack of available calcium ions to form a transient calcium gradient. Furthermore, when caffeine was introduced to the prepared isolated muscle solution, in an effort to facilitate calcium ion channel opening, the previously observed fatigue was reversed (Westerblad & Allen, 1991). The ultimate result of successful ECC is the formation of an actin-myosin cross-bridge. In humans, muscles are composed of primarily three types of fibres. Type I (slow oxidative) fibres rely less on glycolytic means to generate adenosine triphosphate and are considered 'fatigue resistant'. Type IIa (fast oxidative glycolytic) and IIx (fast glycolytic) fibres tend to generate adenosine triphosphate from glycolysis and phosphocreatine stores and are considered very fatigable (Kent-Braun *et al.*, 2012). A key step in cross-bridge formation is the transition from a low- to high-force state. The efficiency of this step can be limited by the accumulation of protons and inorganic phosphate, two key by-products of glycolysis. The likely action for inorganic phosphate is to create an environment that favours the low-force state while an accumulation of protons appears to act through inhibiting the rate constant for high-force cross-bridge formation (Kent-Braun *et al.*, 2012). In essence, during fatiguing contractions, metabolites associated with glycolysis accumulate in the exercising muscle. The metabolic milieu of the muscle then changes to an environment that no longer favours the transition to a high-force state, thereby reducing the force output of the muscle.

### 1.3.3 Fatigability of the diaphragm

The diaphragm is a thin, dome-shaped muscle that separates the abdominal and thoracic cavities. Fibres of the diaphragm insert to two points, the crural diaphragm inserts on the bodies of the lumbar vertebrae while the costal diaphragm inserts along the inner surface of the distal six ribs (Ratnovsky *et al.*, 2008). Upon contraction, the diaphragm flattens, and the thoracic cavity expands. Additionally, the costal diaphragm applies a force that lifts and externally rotates the lower six ribs. The estimated fibre-type distribution of the human diaphragm is roughly even, with slow-twitch fibres comprising 55% of the diaphragm and the balance rather evenly divided between fast-glycolytic and fast-oxidative (24 and 21%, respectively) (Polla *et al.*, 2004). In addition to these fatigue-resistant, slow-twitch fibres, each fibre is surrounded by roughly four-to-six capillaries (Mizuno & Secher, 1989). Despite the number of capillaries surrounding diaphragm muscle fibres being similar to that of peripheral limb muscles in untrained individuals, the relatively smaller size of diaphragm fibres allows for a greater capillary density per area of muscle fibre (Mizuno & Secher, 1989). The greater capillary density and slow-twitch composition of the diaphragm make it highly resistance to exercise-induced fatigue. Despite this, several studies across a breadth of ages and fitness levels offer compelling evidence that the diaphragm is not immune to fatigue (Johnson *et al.*, 1993; Mador *et al.*, 1993; Babcock *et al.*, 1995; Guenette *et al.*, 2010; Archiza *et al.*, 2018; Welch *et al.*, 2018b; Geary *et al.*, 2019; Archiza *et al.*, 2021).

Transdiaphragmatic pressure ($P_{DI}$) is measured via balloon-tipped catheters placed in the stomach and oesophagus and serves as a measure of the force output of the diaphragm. Each balloon measures gastric ($P_{GA}$) and oesophageal pressure ($P_{OES}$), respectively and the absolute difference of the two is the calculated $P_{DI}$. After a fatiguing task, such as high intensity exercise or inspiratory pressure threshold loading (IPTL), decreases in maximal $P_{DI}$ compared to resting

values indicate some level of contractile failure of the diaphragm. While the easiest means to test respiratory muscle strength and subsequent fatigue would be a volitional respiratory manouvre, given these manoeuvres are dependent on participant motivation, volitional tests are not ideal. In order to make objective claims about a change in maximal $P_{DI}$, the phrenic nerves, which innervate the diaphragm, must be maximally stimulated by electrical or magnetic means resulting in an involuntary contraction, or twitch. To determine the presence of contractile failure of the diaphragm, the amplitude of twitches before and after the fatiguing task are compared, and if the post-twitch is lower than the pre-twitch then one can infer the contractile mechanisms of the diaphragm are not performing to their baseline levels and the diaphragm is fatigued (Similowski *et al.*, 1989).

Phrenic nerve stimulation techniques originated with electrical bilateral phrenic nerve stimulation (BPNS), developed in the early 1980's (Bellemare & Bigland-Ritchie, 1984). BPNS involved an electrical stimulation delivered by two electrodes behind the sternocleidomastoid muscle. The stimulus intensity was progressively increased until there were no observable increases in compound muscle action potential (CMAP), at which point the stimulus intensity was increased by 20-50% to ensure a maximal stimulation. This particular stimulation technique was extensively used to assess fatigue and overall muscle function of the diaphragm (Bellemare & Bigland-Ritchie, 1987; McKenzie & Gandevia, 1991; McKenzie *et al.*, 1992; Johnson *et al.*, 1993; Babcock *et al.*, 1995). However, several limitations to this technique may prevent its widespread use including: participant discomfort during stimulation, difficulty optimizing the stimulus location, and if needle electrodes are used, a risk to the safety of the participant (Similowski *et al.*, 1989; Wragg *et al.*, 1994). In response, cervical magnetic stimulation (CMS) offers a more

tolerable alternative to electrical stimulation and can similarly activate the phrenic nerves (Wragg *et al.,* 1994).

It is well reported that periods of prolonged respiratory work can result in diaphragm fatigue. Common techniques in research to induce diaphragm fatigue include specific fatiguing tasks, such as IPTL (Welch *et al.*, 2018b; Geary *et al.,* 2019; Boyle *et al.,* 2020; Archiza *et al.*, 2021) and inspiratory resistive breathing tasks (Smith *et al.*, 2016) or high-intensity whole-body exercise (Babcock *et al.*, 1995; Guenette *et al.,* 2010; Archiza *et al.*, 2018; Welch *et al.,* 2018a; Boyle *et al.,* 2020). The majority of the available literature has focused on a change in the $P_{DI}$ to assess the contractile function of the diaphragm; however, some studies have also investigated neural contributions to fatigability (McKenzie *et al.*, 1992; McKenzie *et al.*, 1997).

A loss in the contractile function of the diaphragm is likely influenced heavily by the amount of respiratory work performed by the diaphragm (Johnson *et al.*, 1993; Archiza *et al.*, 2018). Archiza *et al.,* (2018) assessed the role of respiratory muscle work on diaphragm contractile function by having participants perform multiple exercise tests. The first test was a constant load exercise test performed until task failure, while two subsequent tests were completed to 50 and 75% of the total exercise time from the initial test. The authors showed a significant correlation between the decrease in $P_{DI}$ and the cumulative diaphragm force output, assessed via pressure time products, suggesting the amount of work the diaphragm performs influences the magnitude of loss in diaphragm contractile function (Archiza *et al.*, 2018). It is possible that the increase in diaphragm work impairs diaphragm contractile function by impeding blood flow to the diaphragm. In canines, diaphragm blood flow was measured during inspirations at specific tension-time indexes (TTI), a measure of the pressure generated relative to the maximal pressure one could generate over an inspiration (Bellemare *et al.*, 1983). Bellemare *et al.,*(1983) found that when the

TTI exceeded 0.2, diaphragm blood flow began to decrease. Therefore, it is possible that as diaphragm work increases, this increases the TTI thereby impeding blood flow to the diaphragm. This would serve to impair oxygen delivery and the clearance of fatigue inducing metabolites, thus exacerbating fatigue. However, it is important to note that the hyperpnoea of exercise alone does not affect diaphragm function to a similar extent as whole-body exercise. Participants who exhibited a significant loss of contractile function after high intensity exercise did not show evidence of diaphragm fatigue when they replicated the hyperpnoea of exercise while at rest (Babcock *et al.*, 1995). This suggests there may be an interaction between the locomotor and respiratory muscles that contributes to diaphragm fatigability. Previously, Harms *et al* (1997) have shown that manipulating the $W_b$ during exercise affects locomotor muscle blood flow. When the $W_b$ is increased, locomotor muscle blood flow decreases and when the $W_b$ is reduced, locomotor muscle blood flow increases (Harms *et al.*, 1997). Additionally, we have shown that blood flow to the scalene and sternocleidomastoid muscles is lower during exercise than while mimicking the hyperpnoea of exercise at rest (Ramsook *et al.*, 2020). A reduction in available blood flow to the respiratory muscles during exercise could influence diaphragm fatigability.

### 1.4 Sex differences in skeletal muscle fatigue

Generally, females are less likely to develop fatigue when performing intermittent or sustained isometric contractions than males (Hunter *et al.*, 2006; Kent-Braun *et al.*, 2012; Hunter, 2014, 2016). Differences in skeletal muscle fatigability between males and females can likely be attributed to differences in anatomy and physiology; however, specific mechanisms are poorly defined as a result of the historically disproportionate use of males in physiological studies over females (Hunter, 2016). Furthermore, while studies involving isometric tasks have formed the bulk of the current literature, there are fewer available studies in dynamic tasks that investigate a sex

difference and therefore, it is more difficult to assess the influence of sex on fatigue in these dynamic tasks (Hunter, 2014). Sex differences in skeletal muscle physiology, including muscle mass and fibre type distribution, may predispose males to developing fatigue more than their female counterparts. For example, males have greater muscle mass, and a higher proportion of more fatigable, type II muscle fibres. While these muscle characteristics result in the average male being stronger than the average female, so too is male muscle more prone to developing fatigue.

The distribution of fibre types within some muscle group can be different between males and females. Type I (slow twitch) muscle fibres are more fatigue resistant than type II (fast twitch) fibres, owing to the lower rate of metabolite accumulation in type I fibres. In some, but not all skeletal muscles, females have a greater proportion of type I muscle fibres than males (Mannion *et al.*, 1997; Staron *et al.*, 2000; Roepstorff *et al.*, 2006). Muscle mass is often cited as a source of greater fatigability in males. It follows that with, on average, a greater mass of muscle, males will also have, on average, greater absolute muscle strength. Consequently, the metabolic cost of operating more muscle is greater and can exacerbate fatigue. Unfortunately, comparing males and females with similar levels of absolute strength is difficult due to males typically being stronger than females across the majority of muscle groups (Hunter, 2014). That being said, correlations between task failure and absolute strength may provide insight into the role of strength on fatigue. To that end, Hunter and Enoka (2001) designed a study in which males and females performed a fatiguing elbow flexor task at a relative intensity of 20% maximal voluntary contraction (MVC). As expected, the males had greater absolute strength than the females and the females had a significantly longer time to task failure (Hunter & Enoka, 2001). However, when target force was considered as a covariate, the authors discovered that endurance time was inversely related to target force. While target force, and thus maximal force, did not explain the entirety of the findings,

it was enough for the authors to claim that the previously observed sex difference was in fact due to differences in absolute strength (Hunter & Enoka, 2001).

A second potential source of sex differences in fatigue could be blood flow to the working muscles. A reduction in perfusion or local blood flow would exacerbate fatigue by allowing metabolites to accumulate at a faster rate, thereby hastening the impairment in skeletal muscle contractile function (Russ & Kent-Braun, 2003). Greater absolute force generation in males would result in greater intramuscular pressure on the supplying arteries and could promote a more ischemic environment. Additionally, females may experience a greater effect of $\beta_2$-adrenergic receptor mediated vasodilation. Generally, $\beta_2$ adrenergic receptors are more prevalent in type I fibres, which are proportionally greater in females (Roatta & Farina, 2010). Therefore, females may experience better perfusion to exercising muscle due to a greater vasodilatory response through the action of $\beta_2$-adrenergic receptors. In the vastus lateralis, females may experience greater perfusion due to greater capillary density. A histological examination of the vastus lateralis in moderately trained (*i.e.,* $\dot{V}O_{2max}$ 40-65 $mL \cdot kg^{-1} \cdot min^{-1}$) males and females indicates that the number of capillaries surrounding a given muscle fibre is similar between the sexes, but when normalized for muscle fibre cross-sectional area, females have a significantly greater capillary density than males (Roepstorff *et al.*, 2006). However, this is not a universal observation; capillary density in the tibialis anterior is similar between the sexes (Porter *et al.*, 2002). To further examine the role of perfusion on skeletal muscle fatigue, Clark *et al.* (2005) tasked female and male participants to complete an isometric fatiguing task. During a sustained knee extension contraction at 25% MVC, female participants were able to endure the contraction for a significantly greater time than males as expected. On a following visit, the same fatiguing task was performed with the addition of an inflated pressure cuff to occlude blood flow. In the occluded condition, time to task

failure was reduced in both groups to such a degree that no sex difference could be detected (Clark *et al.*, 2005). The results of this study suggest that better perfusion may serve to protect against the development of fatigue in females. Given the lower absolute force generation and greater potential for vasodilation, it stands to reason that females would experience less occlusion of the blood vessels surrounding a particular muscle during dynamic contractions relative to males, thus preserving the contractile properties of the muscle and delaying the onset of fatigue.

Sex differences in any neural mechanisms contributing to fatigability would be linked to synaptic inputs from descending pathways, spinal interneurons, and peripheral afferent feedback (Hunter, 2014). There do not appear to be any differences in baseline voluntary activation between males and females (Yoon *et al.*, 2007), and the influence of sex on changes in voluntary activation is not clear. For example, during isometric contractions, the elbow flexors (Hunter *et al.*, 2006) did not display a sex difference in fatigue due to supraspinal mechanisms, while in the ankle dorsiflexor (Russ & Kent-Braun, 2003) and vastus lateralis muscles (Martin & Rattey, 2007), neural mechanisms, as assessed by central activation ratio and voluntary activation, respectively, appeared to have a greater effect on male participants. Feedback from peripheral afferents may partially explain sex differences in how neural mechanisms contribute to fatigability. Group III/IV afferents are sensitive to fatigue-related metabolites and can increase their discharge rate in response to the accumulation of these metabolites. The increased discharge of group III/IV afferents influences the regulation of central motor drive and thus, voluntary activation (Gandevia, 2001). Given the aforementioned sex differences in muscle physiology, it stands to reason that large muscle groups in males would be more prone to a decline in voluntary activation, the result of a greater activation of group III/IV afferents. A recent study by Yacyshyn *et al.* (2018) used the disparate effects of group III/IV afferents on elbow flexors and extensors to better understand any

potential sex difference in fatigability. A two-minute maximal sustained isometric elbow flexor task led to an inhibition of the elbow extensor motoneurons, but either a facilitation or non-significant change to elbow flexor motoneurons (Yacyshyn *et al.*, 2018). Thus, flexor voluntary activation would, in theory, be unaffected by the task whereas extensor voluntary activation would be inhibited by the increased group III/IV afferent activity. Moreover, the greater presumed group III/IV afferent activity in males would exacerbate any observed fatigue. However, the results did not show any sex difference in fatigability (Yacyshyn *et al.*, 2018). The unexpected results were thought to be due to the unique nature of the elbow flexors and extensors and the type of task that was performed. To date, the aforementioned study appears to be the only one to specifically examine the potential interaction of motoneuron excitability and sex differences in skeletal muscle fatigue. Future considerations for different muscle groups and fatiguing tasks, including dynamic whole-body exercise, should be accounted for to expand the current body of literature.

### 1.4.1 Sex differences in diaphragm fatigability

During high-intensity exercise, the female diaphragm appears to be less fatigable than the male diaphragm (Guenette *et al.*, 2010). Males and females performed a constant-load (90% peak work rate) time-to-exhaustion test, after which diaphragm contractile function was assessed using CMS. It was found that the magnitude of decrease in transdiaphragmatic twitch pressure ($P_{DI,TW}$), used to assess the contractile function of the diaphragm, was greater in males compared to females (Guenette *et al.*, 2010). This sex difference may be partially explained by the higher absolute work rates performed by the males compared to females. This corresponded to a greater absolute $\dot{V}_E$, and $W_b$. In another study, Welch *et al.*, (2018) tasked males and females to perform IPTL until exhaustion. At task failure, both males and females fatigued to similar degrees, but females lasted significantly longer and as a result performed nearly double the amount of cumulative

diaphragmatic work (Welch *et al.*, 2018b). This supports the notion that females are less fatigable since they performed more work for a similar magnitude of fatigue, at least as it relates to the contractile mechanisms that influence fatigability.

With respect to the neural mechanisms that contribute to diaphragm fatigability, there are no studies that have investigated the role of biological sex. There is a case to be made that the inspiratory muscle metaboreflex response during IPTL is attenuated in females compared to males (Welch *et al.*, 2018b; Geary *et al.*, 2019). Using cardiovascular variables as a surrogate to assess the sympathetic activity, studies have found that females tend to have a blunted cardiovascular response when performing IPTL at a given relative intensity (Welch *et al.*, 2018b) and at a given absolute intensity, where the relative intensity was greater in females compared to males (Geary *et al.*, 2019). Given that neural mechanisms contributing to fatigability can be influenced by group III/IV afferents (Taylor *et al.*, 2016), it is possible decreased sympathetic activity during inspiratory work may result in relatively preserved voluntary activation or other measures of the neural influences on diaphragm fatigability.

### 1.5 Summary and rationale

There is a growing appreciation for the study of sex differences in physiology. While there have been significant improvements in understanding the influence of sex on the structure and function of the respiratory system, there remain many unanswered questions. Specifically, as it relates to fatigability, much of the extant literature has focused on changes in $P_{DI,TW}$ to objectively measure a decrement in the contractile function of the diaphragm after tasks; however, the neural mechanisms that contribute to fatigability are relatively under-explored. This may be in part due to a lack of information surrounding the techniques that can assess neural contributions to

diaphragm fatigability. Measuring voluntary activation may be an effective tool to investigate the neural mechanisms that contribute to diaphragm fatigability. However, data formally investigating the utility of this technique may be a barrier to future research. Moreover, the previous studies which have investigated diaphragm voluntary activation (D-VA) have primarily used maximal respiratory manoeuvres that may lack applicability to functional tasks such as exercise or fatiguing tasks like IPTL. Identifying the presence or absence of a neural mechanism contributing to diaphragm fatigability would provide a more comprehensive understanding of diaphragm fatigability. Moreover, exploring potential sex differences in D-VA is pertinent given the known sex differences in respiratory physiology and fatigability.

## 1.6 Purpose

The purpose of this thesis was to investigate how voluntary activation contributes to fatigability of the diaphragm. Moreover, the studies presented in this thesis aimed to explore potential sex differences in D-VA. To address these purposes, three studies were performed. Study 1, presented in *Chapter 2*, determined the within- and between-session reliability of D-VA measurements. Studies 2 and 3, presented in *Chapters 3* and *4*, aimed to explore how different fatiguing tasks affect D-VA in males and females.

## 1.7 Research objectives

Each study was designed to address a specific research question.

1. Can D-VA be reliably measured within- and between-session with twitches evoked by CMS?
2. Does D-VA decrease after a bout of high intensity cycling, and if so, does the decrease in D-VA differ based on sex?

3. Does D-VA decrease after a bout of fatiguing inspiratory work, and if so, does the decrease in D-VA differ based on sex?

These questions were addressed in *chapters 2, 3* and *4*, respectively.

## 1.8 Hypotheses

For the previously outlined objectives our corresponding hypotheses are as follows:

1. CMS evoked twitches can reliably measure D-VA and these measurements will be reliable both between- and within-session.
2. After a bout of high intensity cycling, both males and females will show a decrease in D-VA; however, males will have a greater decrease in D-VA compared to females.
3. After isolated inspiratory work, both males and females will show a decrease in D-VA; however, males will have a greater decrease in D-VA compared to females.

# Chapter 2: Reliability of diaphragm voluntary activation in healthy adults

## 2.1 Introduction

Voluntary activation represents the fraction of evocable neural drive to a muscle, or group of muscles, during a deliberate contraction (Gandevia *et al.*, 1995). The concept of quantifying voluntary activation was developed by Merton through observations that when a muscle twitch, evoked by electrical stimulation, was performed during a voluntary contraction, the amplitude of the superimposed twitch would decrease with increasing tension (Merton, 1954). Without evidence of a superimposed twitch, it was said that activation was maximal, as any additional increase in drive does not result in an increase in force. By expressing these superimposed twitches relative to a twitch of the resting muscle, one is able to quantify voluntary activation (Shield & Zhou, 2004). A decline in maximal voluntary activation after a fatiguing task (*e.g.* exercise), can be interpreted as evidence of neuromuscular mechanisms contributing to fatigue, defined as skeletal muscle fatigue due to mechanisms proximal to the neuromuscular junction (Roussos & Macklem, 1985).

The diaphragm is the primary muscle of inspiration and while the structure and function of the diaphragm suggests a resistance to muscle fatigue, it can still develop fatigue after high-intensity, whole-body exercise (Johnson *et al.*, 1993; Babcock *et al.*, 1995), resistive breathing tasks (Roussos & Macklem, 1977; McKenzie *et al.*, 1997), or inspiratory pressure threshold loading (Welch *et al.*, 2018b). Stimulation of the phrenic nerves elicits an involuntary contraction of the diaphragm and, when combined with a measure of $P_{DI}$, can be used to evaluate diaphragm contractile function.

The contractile component of diaphragm fatigue, evaluated by changes in the amplitude of the $P_{DI,TW}$ evoked after stimulation, has been the primary focus of previous investigations. These investigations have led to a better understanding of the factors limiting exercise performance (Welch *et al.*, 2018a), the inspiratory muscle metaboreflex (Geary *et al.*, 2019), sex differences in respiratory physiology (Guenette *et al.*, 2010; Welch *et al.*, 2018b; Geary *et al.*, 2019), and respiratory muscle function in chronic obstructive pulmonary disease (Mador *et al.*, 2000). In addition to the $P_{DI,TW}$ amplitude, changes to the CMAP can also be associated with skeletal muscle fatigue (Russ & Kent-Braun, 2003); however, evidence of this in the diaphragm is scarce. In contrast  diaphragm fatigued due to changes in contractile function, the neural mechanisms contributing to diaphragm fatigue has received limited attention. By measuring changes in D-VA using either electrical (Bellemare & Bigland-Ritchie, 1984) or magnetic (Similowski *et al.*, 1996) stimulation, in addition to $P_{DI,TW}$ amplitude, one can more comprehensively examine fatigability of the diaphragm. Quantification of D-VA is performed by comparing the amplitude of a twitch evoked during a voluntary contraction, referred to as a superimposed twitch, to a control twitch evoked while the muscle is at rest (Shield & Zhou, 2004). Furthermore, by asking participants to perform multiple voluntary contractions at various relative intensities, one can examine the relationship between the amplitude of the superimposed twitch and the voluntary force generated (Bellemare & Bigland-Ritchie, 1984).

Previous studies have confirmed that well-motivated participants are capable of near-maximal D-VA at rest (Gandevia & McKenzie, 1985; McKenzie *et al.*, 1992); however, to our knowledge, there have been no formal reports on the test-retest reliability of assessing D-VA using CMS. Determining the reliability of CMS-derived measures of D-VA is necessary for future longitudinal and cross-sectional studies examining diaphragmatic function (Allen *et al.*, 1995). In

addition, there is growing concern regarding the lack of replication between different research groups in human physiology research (Wagner, 2017). Thus, by formally examining the test-retest reliability of D-VA using CMS, we hope to provide a foundation for future studies to examine neural contributions to diaphragmatic fatigue following different interventions (*e.g*. exercise and inspiratory muscle loading) across the spectrum of health and individuals with chronic cardiorespiratory conditions. Accordingly, the purpose of this study was to investigate both the within-session and between-day reliability of D-VA in healthy adults. We also sought to examine the relationship between superimposed twitch and voluntary force ($P_{DI}$) of the diaphragm with magnetic evoked stimulations. We hypothesized that both within-session and between-day measures of D-VA would exhibit good reliability based on previously reported reliability for voluntary activation in other limb muscles (Nuzzo *et al.*, 2019).

## 2.2 Methods

### 2.2.1 Participants.

Healthy young males and females were included if they had spirometry within normal limits (Tan *et al.*, 2011) and reported no cardiopulmonary disorders. Women were tested randomly throughout their menstrual cycle. All experimental procedures received ethical approval from the Providence Health Care Research Institute and the University of British Columbia Clinical Research Ethics Board (H17-02680).

### 2.2.2 Experimental Overview.

After providing written informed consent, participants completed pulmonary function testing for screening purposes. Participants were then instrumented with a dual-balloon oesophageal electrode catheter used to assess $EMG_{DI}$ along with $P_{OES}$ and $P_{GA}$ pressure (Guangzhou Yinghui Medical Equipment Co. Ltd., Guangzhou, China). $P_{DI}$ was calculated as the difference between

$P_{GA}$ and $P_{OES}$. Figure 2-1 summarizes the order of the primary experimental procedures performed in this study. All participants were thoroughly familiarized with the manoeuvers on each testing day. The familiarization period included multiple trials with breaks in between and visual feedback along with instruction from the laboratory technician. After these familiarization periods, participants were allowed to rest for 5-10 minutes before proceeding with the actual measurement. First, a recruitment curve was developed for each participant under resting conditions by performing a minimum of three unpotentiated twitches at increasing stimulator output intensity to determine if the phrenic nerves were maximally stimulated. After the $P_{DI,TW}$ recruitment curve was established, participants then completed a block of five interpolated twitches (consisting of a stimulation delivered during a maximal inspiratory manoeuvre and a second stimulation when the participant returned to functional residual capacity [FRC]) to assess baseline diaphragm contractile function and D-VA. After resting for a minimum of 10 minutes, participants underwent a twitch occlusion protocol by delivering superimposed twitches during inspiratory manoeuvres at 25, 50, 75, and 100% of the $P_{DI}$ achieved during maximal inspiratory pressure manoeuvres performed at FRC, in randomized order, to assess the relationship between voluntary $P_{DI}$ generation and superimposed twitch amplitude. Finally, a second block of five interpolated twitches, identical to the first, was completed after resting for a minimum of 10 minutes to provide a second, within-session measure of D-VA and diaphragm contractile function. A second visit performed $\geq$ 48 hours after visit 1 was completed to test the between-day reliability of each technique. Visit 2 was identical to visit 1 apart from the pulmonary function tests, which were not performed during visit 2.

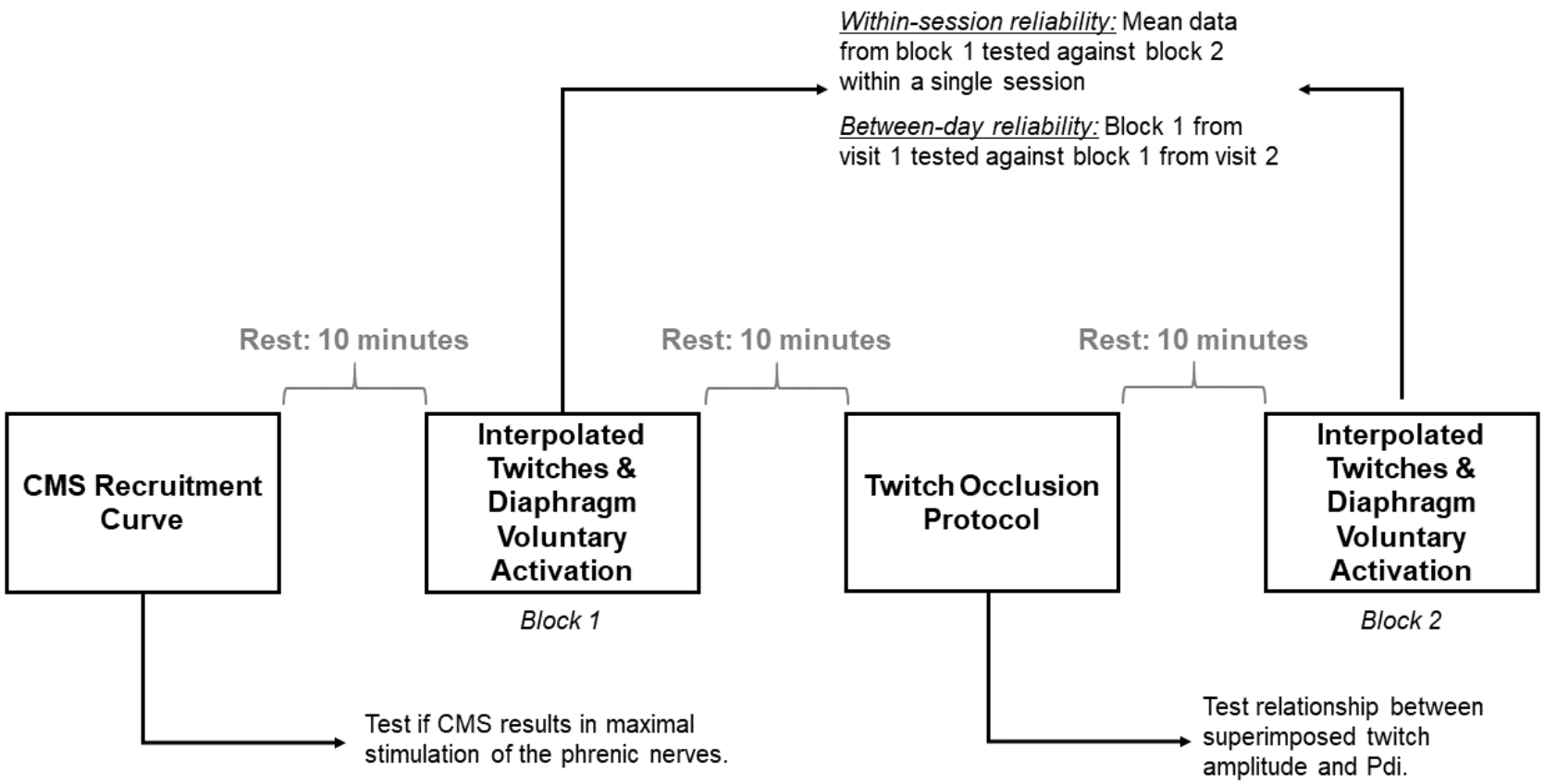


**Figure 2-1. Experimental overview for experiment of Chapter 2.**

### 2.2.3 Pulmonary Function.

Spirometry, plethysmography, and maximal inspiratory pressures were assessed according to standard recommendations (American Thoracic Society/European Respiratory Society, 2002; Miller *et al.*, 2005) using a commercially available cardiopulmonary testing system (Vmax 229d with Autobox 6,200 DL; SensorMedics; CA, USA) with values being expressed as percentages of predicted (Wilson *et al.*, 1984; Gutierrez *et al.*, 2004; Tan *et al.*, 2011).

### 2.2.4 Respiratory Pressures.

The catheter used in this study was equipped with two balloons for measuring $P_{OES}$ and $P_{GA}$. Each balloon was connected to a calibrated differential pressure transducer (model DP15-34; Validyne Engineering; CA, USA). The catheter was completely inserted such that both balloons were in the stomach of the participant. The catheter was then slowly withdrawn in 1 cm increments while the participant performed inspiratory sniffs. Once a negative deflection in the $P_{OES}$ was observed on a sniff, the catheter was removed a further 7 cm (*i.e.*, the length of the balloon) to ensure that the entire oesophageal balloon was within the oesophagus. Optimal $P_{OES}$ balloon position was confirmed using the occlusion technique (Baydur *et al.*, 1982). If the occlusion test suggested suboptimal balloon positioning, then the catheter was moved in 1cm increments until the occlusion test indicated the balloon was in the appropriate location. $P_{DI}$ was calculated as the difference between $P_{GA}$ and $P_{OES}$. Data were converted from analogue to digital (Power Lab 16/35, ADInstruments, Colorado Spring, CO, USA) and collected using LabChart software (LabChart v7.3.7 pro, ADInstruments; Colorado Springs, CO, USA). Participants were able to view data as they were being collected as a form of visual feedback.

### 2.2.5 Electromyography.

The catheter system used for measuring $P_{OES}$ and $P_{GA}$ also included nine 1 cm electrodes, which formed five recording pairs separated by 3.2 cm to measure crural diaphragm EMG. Data were collected with the same hardware and software as the respiratory pressures. Oesophageal catheter systems have been used previously to record CMAP from the diaphragm and was chosen instead of surface electrodes to minimize the influence of extra-diaphragmatic muscles in measuring the diaphragm CMAP (Luo *et al.*, 1998). All EMG data were collected through a bio-amplifier (model RA-8; Yinghui Medical Technology, Guangzhou, China) and sampled at 10 kHz (PowerLab 16SP, ADInstruments; CO, USA) and digitally band-pass filtered (20-500 Hz). The EMG pair that provided the largest peak-to-peak amplitude for any given manoeuvre was used for subsequent EMG analysis (see: CMAP Analysis).

### 2.2.6 Cervical Magnetic Stimulation.

A single twitch was delivered through a 90 mm circular coil connected to a 2 tesla magnetic stimulator (Magstim $200^2$, Magstim; Whitland, UK). For all stimulations, participants were seated with their neck slightly flexed and delivered near FRC initially based on a hand signal from the participant. Lung volume was confirmed by monitoring the $P_{OES}$ to be within ± 1.5 $cmH_2O$ of baseline levels. Baseline $P_{OES}$ was determined from a period (1-2 min) of quiet resting breathing prior to any stimulations. The optimal stimulation point was determined prior to testing by holding the coil horizontally at the spinous process of the seventh cervical vertebra ($C_7$) and applying a single stimulus at 70% stimulator output (Welch *et al.*, 2017). The amplitude of the $P_{DI,TW}$ and amplitude of the CMAP were recorded for each stimulus. The coil was then moved in increments of ~1 cm both above and below $C_7$ and repeated until the greatest $P_{DI,TW}$ and CMAP` amplitudes

were recorded. The stimulation point was then marked in ink on the participant's neck and used for all subsequent stimulations. Twitches were discarded during all procedures if a) the stimulation was not performed at FRC; b) the stimulation occurred during oesophageal peristalsis; c) cardiac artefact was superimposed over the CMAP; or d) there was noticeable $EMG_{DI}$, suggesting the diaphragm had not relaxed (Laghi *et al.*, 1996).

### 2.2.7 CMS Recruitment Curve.

A recruitment curve was developed for each participant to determine if CMS was capable of maximally stimulating the phrenic nerves. Each recruitment curve consisted of a minimum of three unpotentiated twitches at increasing stimulator output (60, 70, 80, 90, 95, and 100% stimulator output) separated by a minimum of 30 seconds to avoid twitch potentiation as done previously (Rafferty *et al.*, 1999; Taylor & Romer, 2009; Guenette *et al.*, 2010; Welch *et al.*, 2017). Maximal stimulation of the phrenic nerves were determined by a plateau in the amplitude of the $P_{DI,TW}$ and CMAP. Evidence of a plateau was first evaluated by visual inspection and by objectively determining if the difference in $P_{DI,TW}$ and CMAP between maximal and submaximal stimulator output intensities was less than the individual coefficient of variation of $P_{DI,TW}$ and CMAP, respectively (Welch *et al.*, 2018b).

### 2.2.8 Interpolated Twitches and Diaphragm Voluntary Activation.

Two blocks of interpolated twitches were performed on each session. One block before and one a minimum of 10 minutes after the twitch occlusion protocol. The interpolated twitch manoeuvre involved the stimulation of the phrenic nerves twice at 100% stimulator output, one during a maximal inspiratory effort (superimposed twitch) and one following the maximal inspiratory effort

performed against a semi-occluded breathing apparatus (control twitch) (Similowski *et al.*, 1996). Each block included a minimum of five twitch manoeuvres. The first two twitch manoeuvres were discarded and the first three acceptable twitches were used for the analysis (Welch *et al.*, 2017). The maximal inspiratory manoeuvres were initiated at "the end of a normal breath out" (*i.e.* FRC) and were sustained for 2-4 seconds, with any effort lasting less than 1.5 seconds being excluded from the analysis (Laveneziana *et al.*, 2019a). Participants were also coached in diaphragmatic breathing and instructed to maintain a similar breathing pattern when performing each maximal inspiratory manoeuvre. Participants were given visual feedback of $P_{OES}$, $P_{GA}$ and $P_{DI}$ in real time, and verbal encouragement during each manoeuvre (Laporta & Grassino, 1985) to ensure appropriate technique and effort. A superimposed twitch was delivered once the participant displayed a steady $P_{DI}$ trace after a minimum of 1 second. A control twitch was delivered once the participant returned to FRC following a single breath after the maximal inspiratory effort. An example of a raw trace of a single interpolated twitch manoeuvre, including superimposed and control twitches, is provided in Figure 2-2. Peak-to-peak CMAP amplitude was assessed to ensure the quality of the delivered stimulation. Any twitch with a submaximal CMAP amplitude, judged by being lower than the coefficient of variation subtracted from the amplitude of the largest observed CMAP amplitude, was discarded. $P_{DI,TW}$ data were assessed for amplitude, contraction time, half-relaxation time, and maximal relaxation rate according to established guidelines (American Thoracic Society/European Respiratory Society, 2002). Briefly, contraction time was calculated as the time from the delivery of the stimulus until peak pressure; half-relaxation time was calculated as the time for $P_{DI}$ to return to 50% peak $P_{DI,TW}$ amplitude; and maximal relaxation rate was calculated as the first derivative of pressure with respect to time over the first half of the $P_{DI}$ relaxation curve, and normalized to peak $P_{DI,TW}$ amplitude. D-VA was estimated using the

interpolated twitch technique, which was simultaneously obtained during the interpolated twitch manoeuvres. Voluntary activation was calculated as the ratio of the superimposed $P_{DI,TW}$ to control $P_{DI,TW}$ using the following equation:

Voluntary Activation (%) = [1-(superimposed twitch/control twitch)] x 100% (Merton, 1954)

Within-session reliability of D-VA was assessed on each day comparing the first block of interpolated twitches against the second block. Between-day reliability of D-VA was evaluated by comparing interpolated twitches from block 1 on day 1 against block 1 on day 2.

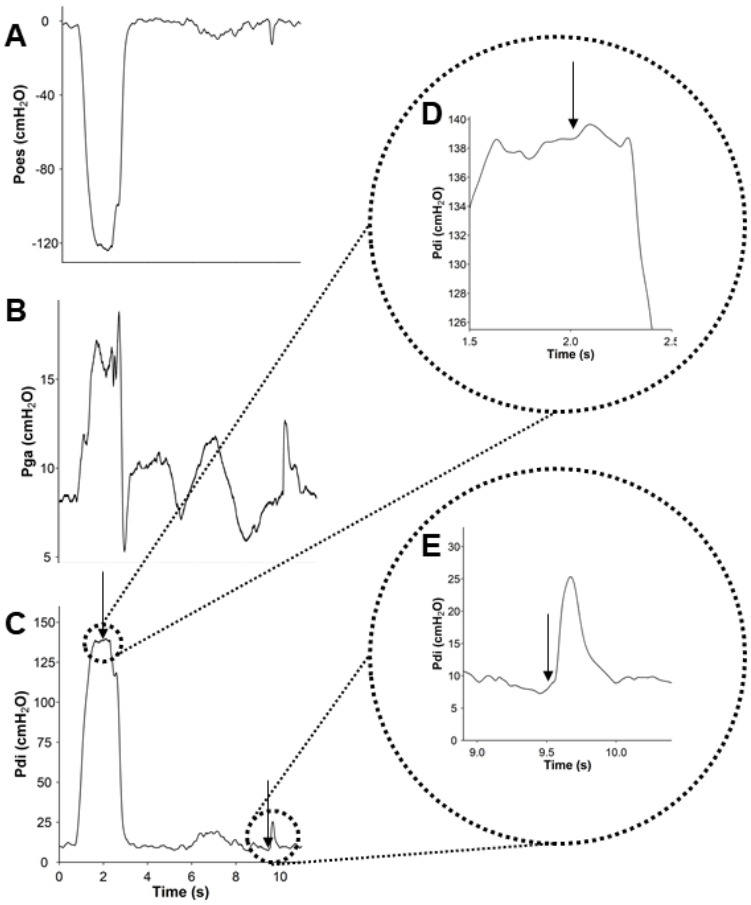


**Figure 2-2. Raw data from a single interpolated twitch manoeuvre.**
Data presented are $P_{OES}$ (panel **A**), $P_{GA}$ (panel **B**) and $P_{DI}$ (panel **C**). Panel **D** expands on the superimposed twitch from the interpolated twitch manoeuvre and panel **E** expands on the resting, control twitch from the interpolated twitch manoeuvre. Arrows represent time at which the phrenic nerves were stimulated. *Abbreviations:* $P_{OES}$, oesophageal pressure; $P_{GA}$, gastric pressure; $P_{DI}$, transdiaphragmatic pressure.

### 2.2.9 Twitch Occlusion Protocol.

The twitch occlusion protocol was used to assess the relationship between superimposed twitch amplitude and voluntary $P_{DI}$. Participants completed three inspiratory efforts targeting 25, 50, 75, and 100% of their maximal $P_{DI}$ ($P_{DI,MAX}$) generated during an inspiratory effort from FRC, in randomized order. $P_{DI,MAX}$ was determined as the largest mean $P_{DI}$ sustained for 1 second during the maximal inspiratory efforts performed without CMS of the phrenic nerves. Participants were

instructed to maintain the target pressure for 2-4 seconds during the twitch occlusion protocol and any manoeuvre lasting less than 1.5 seconds was excluded. A stimulus at 100% of stimulator output was delivered to elicit a superimposed twitch during each inspiratory effort. Superimposed twitch amplitude was determined as the difference between peak $P_{DI}$ and $P_{DI}$ at the moment of stimulation. Voluntary $P_{DI}$ was taken as the average $P_{DI}$ 1 second prior to stimulation. Participants were given visual feedback of their $P_{OES}$, $P_{GA}$, $P_{DI}$, and mouth pressure in real time and were coached in maintaining a stable $P_{DI}$ for as long as possible. Visual feedback consisted of a visible band on the data acquisition software that was set at the target $P_{DI}$ ± 3 $cmH_2O$. All participants practiced maintaining their target $P_{DI}$ for 2-4 seconds, without stimulations before proceeding to inspiratory efforts with stimulations. The 75 and 100% trials were followed by 10 minutes of rest to allow the participant to recover from any potential diaphragm fatigue the trial may have induced. If either superimposed or control twitch CMAP peak-to-peak amplitude was found to be submaximal, defined as different from the largest CMAP by greater than the coefficient of variation of the measurement, the manoeuvre was not considered for analysis.

### 2.2.10 CMAP Analysis.

A custom script developed in MATLAB (R2018b, MathWorks, MA, USA) processed raw EMG data to analyze each CMAP. Raw EMG were processed offline with a fourth order Butterworth filter (cut-off frequency: 20-500 Hz). Filtered EMG were then rectified to calculate peak-to-peak amplitude, along with onset and offset. Peak-to-peak amplitude was calculated as the sum of the two rectified EMG peaks. A baseline threshold was calculated as the mean of the rectified EMG signal 40 ms before the stimulation ± 2 standard deviations. The onset was defined as the first point before the first peak that was greater than this threshold and the offset was defined as the

first point after the second peak to fall below the threshold. CMAP duration was calculated as the difference between offset and onset. All EMG data were visually inspected and verified.

### 2.2.11 Statistical Analyses.

A repeated measures ANOVA with Tukey's post-hoc test was performed to determine if the phrenic nerves were maximally stimulated with CMS and if recruitment curves were different between days. Maximal stimulation was examined by comparing $P_{DI,TW}$ and CMAP peak-to-peak amplitude at submaximal stimulator output intensities against maximal (100%) stimulator output intensity. Intraclass correlation coefficients (ICC) for D-VA, $P_{DI,TW}$ parameters (half-relaxation time, contraction time, twitch amplitude, and maximal relaxation rate), and CMAP parameters (peak-to-peak amplitude, onset, and duration) between days were calculated via two-way mixed effects models to provide a relative index of test-retest reliability. Interpretation of ICC values were defined as 'poor', 'moderate', 'good', or 'excellent' if the values were < 0.5, 0.5-0.75, 0.75-0.9, or > 0.9, respectively (Koo & Li, 2016). To provide an index of test-retest reliability the standard error of measurement (SEM) was also calculated (Hopkins, 2000). To assess the relationship between superimposed twitch and voluntary $P_{DI}$, we fit the data to three different, mixed effects regression models that contain a random intercept and random slope to account for within subject clustering of data due to repeated measurements taken from the same participant and to allow for random variation in the slopes for each participant. Each model represented a different type of relationship (linear, quadratic, and cubic) between superimposed twitch amplitude and $P_{DI}$. All three models were then evaluated against each other to determine which model yielded the best fit. The model with the lowest Akaike information criteria (AIC) score was chosen as the best fitting model. Group data are presented as mean ± standard deviation. ICC values are reported

as ICC; 95% Confidence Interval (95% CI). A *p* value < 0.05 was considered statistically significant.

## 2.3 Results

### 2.3.1 Participants and CMS Recruitment curve.

A total of 20 participants were enrolled in this study. When individual recruitment curves were inspected, a plateau in both $P_{DI,TW}$ and CMAP were observed in 13/20 participants (Figure 2-1). The 7 participants that were not maximally activated at 100% stimulator output were excluded from analysis as measuring D-VA requires a supramaximal stimulus to be valid. Participant characteristics are presented in Table 2-1. All participants had normal spirometry when expressed as a percent of normative values (Tan *et al.*, 2011). Inspiratory muscle strength, as assessed by a maximal inspiratory pressure manoeuvre was slightly above predicted, but within normal limits (Wilson *et al.*, 1984). There was no difference in the recruitment curve between days in $P_{DI,TW}$ ($p = 0.69$) or CMAP ($p = 0.71$).

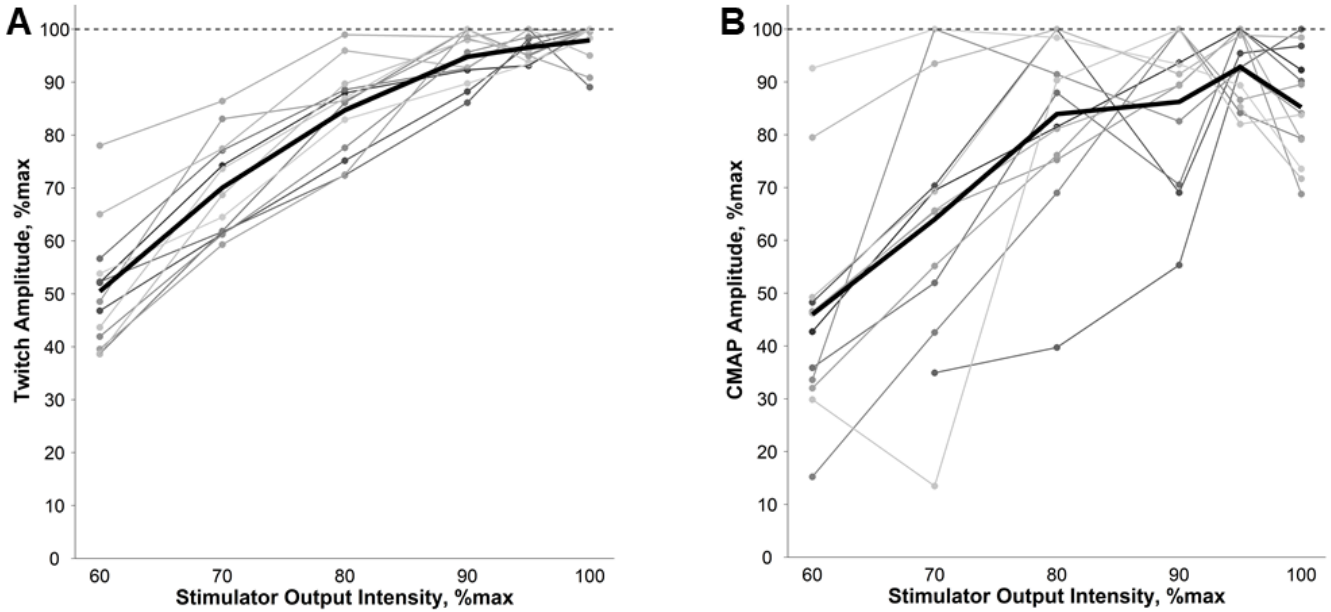


**Figure 2-3. $P_{DI,TW}$ (A) and CMAP amplitude (B) responses to increasing stimulator output intensity**
*Abbreviation*: $P_{DI,TW}$ transdiaphragmatic twitch pressure, CMAP, compound muscle action potential.

**Table 2-1. Participant information.**

| | |
|---|---|
| Age, years | 25 ± 3 |
| Sex, M : F | 4 : 9 |
| Height, cm | 168 ± 11 |
| Mass, kg | 65 ± 14 |
| Body mass index, $kg \cdot m^{-2}$ | 23 ± 4 |
| *Pulmonary Function* | |
| FVC, L (%predicted) | 4.6 ± 1.3 (102 ± 16) |
| $FEV_1$, L (%predicted) | 3.6 ± 0.8 (95 ± 12) |
| $FEV_1$/FVC (%predicted) | 80 ± 8 (94 ± 9) |
| TLC, L (%predicted) | 5.7 ± 1.3 (94 ± 10) |
| $PI_{MAX}$, $cmH_2O$ (%predicted) | -97 ± 30 (111 ± 22) |

Data are presented as mean ± SD. Abbreviations: FVC, forced vital capacity; $FEV_1$, forced expired volume in 1 second; TLC, total lung capacity; $PI_{MAX}$, maximal inspiratory pressure

### 2.3.2 Interpolated Twitches and Diaphragm Voluntary Activation.

Potentiated $P_{DI,TW}$ amplitude (*i.e.*, the resting twitch from the interpolated twitch manoeuvre) did not significantly change after the twitch occlusion protocol, during either visit 1 (pre: 36.0 ± 8.9 vs. post: 33.5 ± 9.1 $cmH_2O$, p = 0.11) or visit 2 (pre: 35.6 ± 10.1 vs. post: 34.4 ± 7.8 $cmH_2O$, p = 0.89) nor did CMAP peak-to-peak amplitude (Visit 1 – pre: 0.50 ± 0.28 vs. post: 0.51 ± 0.31 mV, p = 0.96; Visit 2 – pre: 0.44 ± 0.34 vs. post: 0.46 ± 0.24 mV, p = 0.45). Within-session SEM values are found in Table 2-2 and Table 2-3 for visits 1 and 2, respectively. Within-session D-VA displayed ‘good’ reliability during visit 1 (ICC: 0.76; 95%CI: 0.21-0.93) and visit 2 (ICC: 0.90; 95%CI: 0.67-0.90). Table 2-4 shows between-day comparisons. No differences were observed in the pressure characteristics of twitches between visits, and they displayed ‘good’ between-day reliability based on the ICC values. CMAP properties from the diaphragm were ‘moderately’ reproducible between visits. D-VA was not different between visits 1 and 2 (91% ± 6 vs. 92% ±

5, p = 0.68). Maximal D-VA was found to have ‘good’ reliability between visit 1 and visit 2 (ICC: 0.88; 95%CI: 0.67-0.95). The between-day standard error of measurement for diaphragm-VA was 2.7. The average difference between resting end-expiratory $P_{OES}$ and $P_{OES}$ at the onset of the control stimulations on visits 1 and 2 were 0.1 ± 0.5 and -0.06 ± 0.5 $cmH_2O$, respectively, indicating that stimulations were performed close to FRC.

**Table 2-2. Visit 1 within-session reliability of diaphragm-voluntary activation and twitch parameters.**

| | Block 1 | Block 2 | ICC (95%CI) | SEM | P value |
|---|---|---|---|---|---|
| Voluntary activation, % | 91 ± 6 | 92 ± 6 | 0.76 (0.21-0.93) | 3.9 | 0.54 |
| Contraction time, milliseconds | 126.9 ± 9.0 | 125.7 ± 8.7 | 0.88 (0.61 – 0.96) | 4.1 | 0.58 |
| Twitch amplitude, $cmH_2O$ | 36.0 ± 8.9 | 33.5 ± 9.1 | 0.89 (0.65-0.97) | 3.9 | 0.11 |
| Half-relaxation time, milliseconds | 74.4 ± 8.2 | 75.0 ± 14.4 | 0.83 (0.44 – 0.95) | 6.6 | 0.87 |
| $MRR/P_{DI}$, $cmH_2O \cdot second^{-1}/cmH_2O$ | 0.019 ± 0.0024 | 0.019 ± 0.0029 | 0.53 (0.10 – 0.86 | 0.0022 | 0.29 |
| $EMG_{DI}$ – Peak to peak amplitude, mV | 0.50 ± 0.28 | 0.51 ± 0.31 | 0.86 (0.54 – 0.96) | 0.15 | 0.96 |
| $EMG_{DI}$ – Onset, milliseconds | 7.4 ± 1.8 | 7.6 ± 1.8 | 0.42 (0.18-0.82) | 1.8 | 0.80 |
| $EMG_{DI}$ – Duration, milliseconds | 20.4 ± 6.6 | 19.7 ± 5.5 | 0.42 (0.19 – 0.82) | 5.4 | 0.78 |

Data are presented as mean ± SD. The MRR has been normalized to the twitch amplitude for comparison purposes. P values obtained from paired *t-tests* or Wilcoxon-signed rank tests if not normally distributed between block 1 and block 2. *Abbreviations:* SEM, standard error of measurement; MRR, maximum relaxation rate; $P_{DI}$, transdiaphragmatic pressure; $EMG_{DI}$, crural diaphragm electromyography.

**Table 2-3. Visit 2 within-session reliability of diaphragm voluntary activation and twitch parameters.**

| | Block 1 | Block 2 | ICC (95%CI) | SEM | P value |
|---|---|---|---|---|---|
| Voluntary activation, % | 92 ± 5 | 94 ± 5 | 0.90 (0.67-0.97) | 2.2 | 0.17 |
| Contraction time, milliseconds | 126.7 ± 8.2 | 126.9 ± 8.2 | 0.86 (0.54-0.96) | 4.1 | 0.41 |
| Twitch amplitude, $cmH_2O$ | 35.6 ± 10.1 | 34.4 ± 7.8 | 0.88 (0.61 – 0.91) | 4.3 | 0.89 |
| Half-relaxation time, milliseconds | 73.0 ± 9.7 | 85.2 ± 3.14 | 0.30 (0.13 – 0.79) | 12.0 | 0.17 |
| $MRR/P_{DI}$, $cmH_2O \cdot second^{-1}/cmH_2O$ | 0.019 ± 0.0037 | 0.019 ± 0.0023 | 0.84 (0.54 – 0.96) | 0.0016 | 0.34 |
| $EMG_{DI}$ – Peak to peak amplitude, mV | 0.44 ± 0.34 | 0.46 ± 0.24 | 0.90 (0.68 – 0.97) | 0.13 | 0.45 |
| $EMG_{DI}$ – Onset, milliseconds | 7.2 ± 1.5 | 7.0 ± 1.2 | 0.42 (0.19 – 0.73) | 1.3 | 0.71 |
| $EMG_{DI}$ – Duration, milliseconds | 24.2 ± 5.0 | 21.8 ± 6.5 | 0.57 (0.41-0.81) | 4.6 | 0.09 |

Data are presented as mean ± SD. The MRR has been normalized to the twitch amplitude for comparison purposes. P values obtained from paired *t-tests* or Wilcoxon-signed rank tests if not normally distributed between block 1 and block 2. *Abbreviations:* SEM, standard error of measurement; MRR, maximum relaxation rate; $P_{DI}$, transdiaphragmatic pressure; $EMG_{DI}$, crural diaphragm electromyography.

**Table 2-4. Between-day reliability.**

| | ICC (95% CI) | SEM | P value |
|---|---|---|---|
| Voluntary activation, % | 0.88 (0.67 – 0.95) | 2.7 | 0.68 |
| Contraction time, milliseconds | 0.80 (0.45-0.92) | 5.2 | 0.93 |
| Twitch amplitude, $cmH_2O$ | 0.87 (0.65-0.95) | 4.8 | 0.95 |
| Half-relaxation time, milliseconds | 0.70 (0.19-0.89) | 6.6 | 0.64 |
| MRR/$P_{DI}$, $cmH_2O \cdot second^{-1}/cmH_2O$ | 0.70 (0.19-0.89) | 0.0020 | 0.58 |
| $EMG_{DI}$ – Peak to peak amplitude, mV | 0.93 (0.82-0.97) | 0.11 | 0.64 |
| $EMG_{DI}$ – Onset, milliseconds | 0.33 (-0.08-0.71) | 1.6 | 0.83 |
| $EMG_{DI}$ – Duration, milliseconds | 0.43 (-0.54-0.79) | 5.0 | 0.11 |

Between-day reliability was evaluated by comparing data from block 1 on visit 1 (mean data in Table 2-2) against block 1 on visit 2 (mean data in Table 2-3). The MRR has been normalized to the twitch amplitude for comparison purposes. P values obtained via paired *t-tests* or Wilcoxon-signed rank tests if not normally distributed between visit 1 and visit 2. *Abbreviations:* ICC, intraclass correlation coefficient; 95%CI, 95% confidence interval; SEM, standard error of measurement; MRR, maximum relaxation rate; $P_{DI}$, transdiaphragmatic pressure; $EMG_{DI}$, crural diaphragm electromyography.

### 2.3.3 Twitch Occlusion Protocol.

When asked to target 25, 50, 75, and 100% of their $P_{DI,MAX}$, D-VA was 21% ± 19, 51% ± 20, 74% ± 14, and 92% ± 8 during visit 1, and 24% ± 17, 51% ± 20, 74% ± 10, and 94% ± 6 during visit 2. A linear, quadratic, and cubic model were each used to evaluate the relationship between superimposed twitch amplitude and voluntary $P_{DI}$ generation. While all three models resulted in significant correlations between superimposed twitch and voluntary $P_{DI}$ (all $p < 0.001$), the quadratic model had the best fit based on the AIC score being lowest (1449.8) (Figure 2-4) compared to the linear (AIC: 1490.8) and cubic model (AIC: 1453.4).

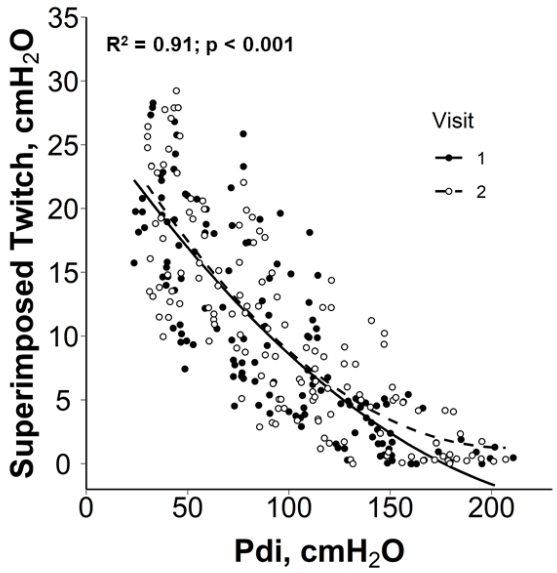


**Figure 2-4. Quadratic model of the relationship between superimposed twitch amplitude and $P_{DI}$.**
Filled circles are data from visit 1 and open circles are data from visit 2. Solid line represents the group model for visit 1 and the dashed line represents the group model for visit 2. $R^2$ and p values represent strength and significance of correlation, respectively, for both visits pooled together. *Abbreviation:* $P_{DI}$, transdiaphragmatic pressure

## 2.4 Discussion

### 2.4.1 Major Findings.

The primary aim of this study was to determine the reliability of CMS-evoked stimulations of the diaphragm to assess D-VA in healthy adults. Our findings suggest that measuring diaphragm-VA with CMS is a reliable technique both between and within days. Overall, this study expands on previous work examining D-VA in humans (McKenzie *et al.*, 1992; Similowski *et al.*, 1996), and the reliability of D-VA suggests that it can be used to assess the effect of an intervention on D-VA over multiple sessions.

### 2.4.2 Diaphragm Voluntary Activation.

When a muscle is maximally stimulated during a submaximal voluntary contraction, the inactive motor units are recruited and generate a twitch response. The magnitude of this superimposed twitch diminishes as voluntary activation increases (Merton, 1954). When activation is maximal, there is no observable superimposed twitch response as all available motor units are already firing. A decline in voluntary activation after fatiguing contractions is indicative of fatigue due to neural mechanisms as opposed to contractile mechanisms (Shield & Zhou, 2004).

We observed slightly less diaphragm-VA (91% ± 6) as a group compared with previously published values of 95% ± 1.5 by McKenzie et al. (1992). One of our participants had very low diaphragm-VA values (< 80%) during both visits (Figure 2-5), which partially contributed to this finding. Despite this, this participant still displayed similar reliability to the group (SEM for visits 1 and 2 were 1.8 and 4.8, respectively). Moreover, their maximal inspiratory pressure was within the normal predicted values. Although all participants were familiarized with performing maximal

inspiratory manoeuvres, we cannot rule out the possibility that this participant was consistently performing similar submaximal efforts. Our lower D-VA values may also be due to differences in methodology. For example, McKenzie et al. (1992), used bilateral electrical stimulation of the phrenic nerves in four male participants to assess D-VA. The advantage to using electrical stimulation is the added confidence that the delivered stimulus will result in maximal activation of the phrenic nerves. The drawback to electrical stimulation is the potential cutaneous pain for the participant (Wragg *et al.*, 1994). Moreover, co-activation of neck muscles can make it difficult to maintain the stimulations at the optimal site. We attempted to control for potential submaximal stimulations by only including manoeuvres that had CMAPs that were similar in amplitude to the greatest CMAP recorded during each session.

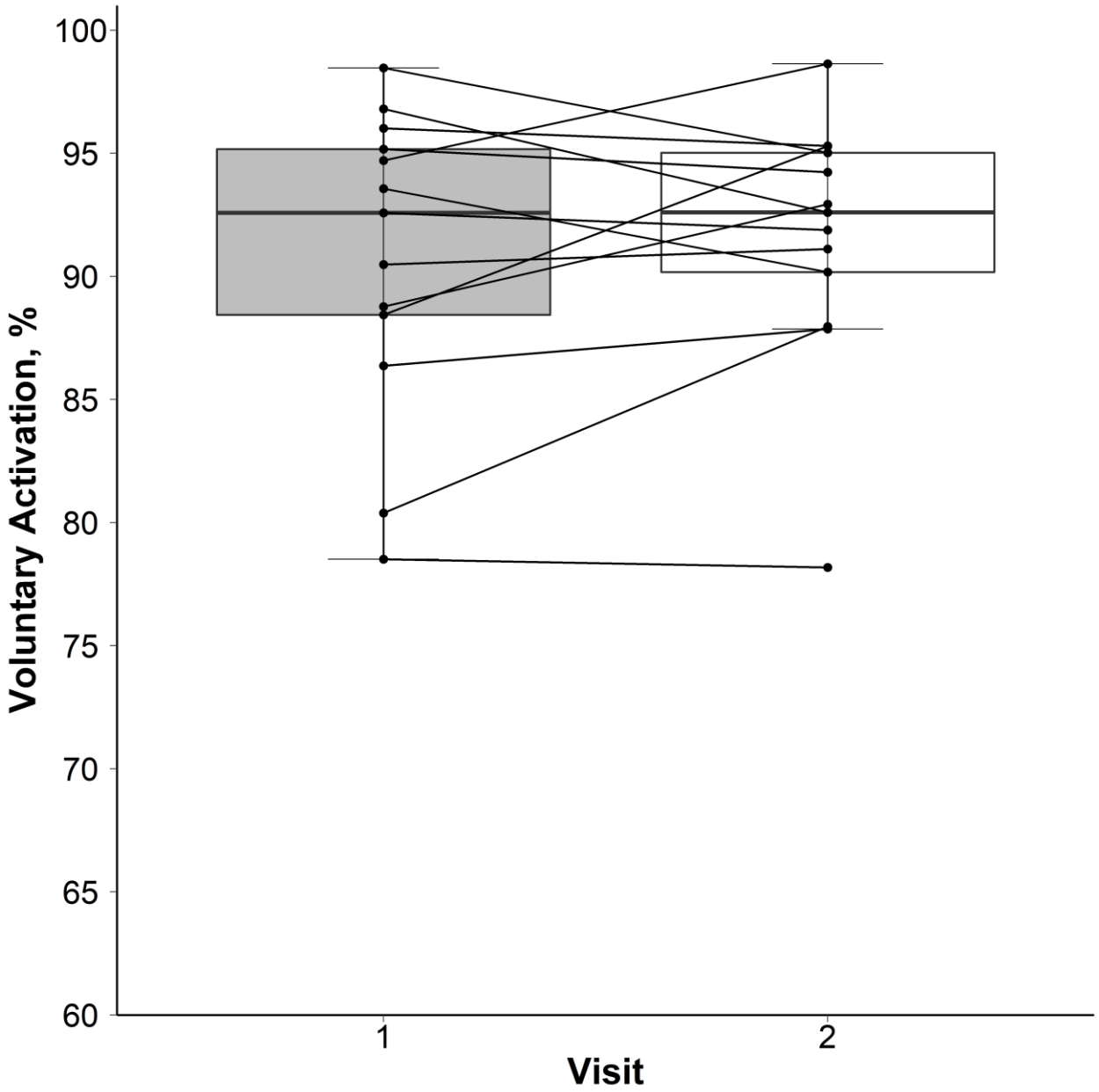


**Figure 2-5. Box and whisker plot showing maximal diaphragm-VA between visits**
Points represent individual values. Upper and lower bands of box represent interquartile range. Middle line within box represents median. Error bars represent 95% confidence intervals.

Another study that examined the use of CMS-evoked twitches to measure D-VA also observed that most participants (6/7) were capable of maximal D-VA, as evidenced by the absence of a superimposed twitch during maximal voluntary inspiratory manoeuvres (Similowski *et al.*,

1996). In contrast, no participants in our study reached 100% diaphragm-VA for all trials. All participants were thoroughly familiarized to both the maximal inspiratory pressure manoeuvres and CMS used to measure D-VA, and participants were able to replicate similar levels of D-VA on their second visit. Therefore, it is unlikely that D-VA levels less than 100% are due to poor participant technique in the current study.

We also explored the relationship between CMS evoked superimposed twitches and $P_{DI}$ generation with the twitch occlusion protocol. Using the twitch occlusion protocol to determine how the superimposed twitch amplitude falls in relation to increasing voluntary force or pressure is important for attempting to predict maximal force. Such predictions could be useful in certain clinical populations where maximal voluntary efforts are difficult to obtain. A linear relationship is then fit to the submaximal efforts and the point at which the line crosses the x-axis (*i.e.*, the amplitude of the superimposed twitch is 0) would be the individual's estimated maximal voluntary force. However, for such predictions to be valid, the relationship between force and superimposed twitch must be linear or the maximal force could be over or underestimated. We have confirmed earlier findings that the relationship between superimposed twitch amplitude and $P_{DI}$ is not linear (Bellemare & Bigland-Ritchie, 1984). In both our data and the original findings of Bellemare and Bigland-Ritchie (1984), a linear model fit to submaximal data would underestimate true maximal $P_{DI}$. These findings are in contrast to the results of Similowski et al. (1996), who also used CMS to measure D-VA and determined the fall in superimposed twitch amplitude was linear with increasing $P_{DI}$ (Similowski *et al.*, 1996), although they did not attempt to fit other (*i.e.*, quadratic or cubic) models to their data as done in the present study. Similowski et al. (1996), posit that coactivation of neck muscles observed at high intensity contractions could have contributed to the deviation from linearity observed by Bellemare and Bigland-Ritchie (1984). In our study, we

instructed participants to perform their inspiratory efforts primarily with their diaphragm. Before engaging in any inspiratory efforts, participants were coached by placing one hand on their abdomen and one on their ribs while breathing. Participants were told to breathe while feeling the hand on their abdomen moving in and out while the hand on the ribs remained relatively stationary. Moreover, the participants were instructed to keep their shoulders down and relaxed as best they could throughout the manoeuvre to avoid using their neck muscles. Another possible factor that may have influenced our twitch occlusion protocol data could be the effect of potentiation. Twitch potentiation, or a temporary increase in twitch tension after a muscular contraction, has been shown to occur after contractions as low as 33% of maximal diaphragm voluntary contraction (Mador *et al.*, 1994). Our twitch occlusion protocol was performed in a randomized order, which may have helped to mask this effect for the group; however, given that the effect of potentiation could last for more than 8 minutes (Mador *et al.*, 1994), it is possible that even a single effort could have influenced subsequent trials. Despite incongruous results between Similowski et al (1996) and our findings, it appears that measures of maximal D-VA are best made from maximal efforts. Given the results of the current study and the findings of Bellemare and Bigland-Ritchie (1984), it does not seem appropriate to estimate voluntary activation from submaximal efforts in the diaphragm, at least in young, healthy participants.

To our knowledge, this is the first study to formally investigate the reliability of D-VA measurements made with CMS-evoked twitches. We found the reliability of diaphragm-VA to be good between days. Our ICC values are within the range of measurements in other upper and lower limb muscles (0.60 - 0.96) as recently summarised (Nuzzo *et al.*, 2019). Furthermore, we have provided within-session measures of reliability using SEM for both $P_{DI,TW}$ and D-VA, two key

measurement techniques that can be used to assess fatigability of the diaphragm. These findings may be used to inform future studies in sample size calculations.

While the results of our study suggest that D-VA measurements can be reliable between and within days in young, healthy men and women, future studies are needed to examine the reliability and utility of D-VA in other populations, such as those with respiratory disease, inspiratory muscle weakness, and healthy aging, among others. Improving our understanding of D-VA may provide new insight into the mechanisms of dyspnoea and exercise intolerance across the spectrum of health and chronic disease. For example, we recently demonstrated a link between diaphragm fatigue due to a loss of contractile function and both exercise performance and the multi-dimensional components of exertional dyspnoea (Boyle *et al.*, 2020). Understanding neural contributions to diaphragm fatigue of the diaphragm using D-VA may provide additional insight into these relationships. Studying sex differences is another potential application of D-VA measures. For example, there is evidence of increased diaphragm fatigue resistance in women compared to men during both exercise (Guenette *et al.*, 2010) and inspiratory pressure threshold loading (Welch *et al.*, 2018b; Geary *et al.*, 2019). Whether there are sex-based differences in the neural mechanisms that contribute to diaphragm fatigue remains unknown.

### 2.4.3 Methodological Considerations.

Factors that can affect the reliability of voluntary activation measurements have been covered in greater detail elsewhere (Nuzzo *et al.*, 2019). The interpolated twitch technique is dependent on maximal stimulations. The potential for submaximal phrenic nerve stimulation is an established limitation of the CMS technique (Similowski *et al.*, 1996). Similowski *et al.* (1996) used CMS to examine D-VA and showed peak-to-peak amplitudes of the diaphragm CMAP to be similar

between CMS at maximal stimulation intensity compared to values produced by maximal bilateral electrical stimulation, even in participants where a plateau in the CMAP amplitude was not observed. The similarities between electrical and CMS evoked CMAP amplitude led these authors to conclude that CMS resulted in a consistent maximal stimulation. We attempted to minimize the effect of submaximal phrenic nerve stimulation by only including twitches that were near the maximal peak-to-peak amplitude observed for an individual on a given visit. This means the delivered stimulus resulted in a similar electrical response at the level of the muscle for every twitch included in the analysis. We also made an effort to ensure each stimulation was delivered at a similar lung volume by monitoring $P_{OES}$. Changes in lung volume affect the length-tension relationship of the diaphragm and could, in turn, influence $P_{DI,TW}$ amplitude. We must acknowledge though that our threshold for excluding twitches based on end-expiratory $P_{OES}$ (± 1.5 $cmH_2O$) does not guarantee perfectly consistent lung volumes between stimulations.

Additionally, with respect to CMS-evoked twitches to measure D-VA, we must acknowledge that CMS does not exclusively stimulate the phrenic nerves (Similowski *et al.*, 1989). The site of stimulation was located near the level of $C_7$ in all participants. While this represented the greatest non-potentiated $P_{DI,TW}$ amplitude and CMAP peak-to-peak amplitude, it also undoubtedly stimulated nerves that innervate the shoulder and neck. Activation of extra-diaphragmatic inspiratory muscles could affect the amplitude of the superimposed and control twitches and could therefore influence the interpretation of D-VA. In the biceps brachii, antagonist activation can result in small responses that influence the overall torque response at lower but not maximal or near maximal activation (Awiszus *et al.*, 1997).

Another limitation is that the positioning of the catheter may have influenced our CMAP values. Specifically, the catheter used in the present study was positioned in order to optimize $P_{OES}$ and $P_{GA}$ values rather than optimizing the $EMG_{DI}$ signal, which would be required to adequately measure the reliability of the CMAP (Luo *et al.*, 1998). We chose to do so as the respiratory pressures were more important for the current study, and the multiple electrode pairs of the oesophageal catheter still allowed us to record diaphragm CMAPs from all stimulations. This established limitation of combined $EMG_{DI}$ and dual balloon catheters is a result of the fixed distance between the electrodes and balloons and can be avoided by using two separate catheters, one for $EMG_{DI}$ and another for respiratory pressures.

Finally, we did not control for menstrual cycle phase or use of oral contraceptives in our female participants. There is evidence that the natural fluctuations in circulating hormones associated with the menstrual cycle can influence voluntary activation of the knee flexor muscles (Ansdell *et al.*, 2019b). It is possible that the effect of menstrual cycle phase may extend to D-VA; however, this remains speculative and requires further investigation. Regardless, controlling for menstrual cycle phase and use of oral contraceptives would most likely only serve to strengthen our reliability data by minimizing potential variability within and between our female participants.

## 2.5 Conclusions

The results of this study suggest that CMS-evoked twitches can reliably measure diaphragm-VA in young, healthy men and women. Measuring D-VA in addition to evaluating changes in the contractile (*i.e.*, $P_{DI,TW}$) and electrical (*i.e.*, CMAP) properties of the diaphragm can offer a wider view into the causes and consequences of diaphragm fatigue. Future work is required to assess the

reliability of D-VA measurements in older adults and in patients that have compromised respiratory muscle function.

# Chapter 3: Sex differences in diaphragm voluntary activation after exercise

## 3.1 Introduction

Skeletal muscle fatigue has been defined as a "condition in which there is a loss in the capacity for developing force and/or velocity of a muscle, resulting from muscle activity under load and which is reversible by rest" (1990). In a broader context, the concept of fatigue includes two core attributes: performance fatigability, a reduction in an objective measure of force; and perceived fatigability, changes in the sensations that affect the performer (Enoka & Duchateau, 2016). Measures of performance fatigability eschew accompanying adjectives such as central or peripheral fatigue, which are intended to isolate a source of fatigue, and instead favour focusing on the mechanisms that limit performance (Enoka & Duchateau, 2016). The human diaphragm, the primary muscle of inspiration, exhibits a loss in the capacity to generate pressure after whole-body exercise at high intensities (Johnson *et al.*, 1993). This loss in pressure generation is a sign that the contractile mechanisms of the muscle are not functioning to a similar level after exercise compared to the resting state. However, it is also possible for neural mechanisms to contribute to fatigability (Gandevia, 2001).

Sex differences in fatigability are well reported over a breadth of muscle groups for tasks performed at similar relative intensities (Hunter, 2016). During submaximal dynamic contractions at a moderate velocity, females tend to be less fatigable than males (Yoon *et al.*, 2015), but this sex difference is reduced when performing dynamic contractions at maximal velocity (Senefeld *et al.*, 2013). With respect to the diaphragm, females appear to be more resistant to reductions in diaphragmatic contractile function after high intensity constant work rate exercise (Guenette *et al.*, 2010; Welch *et al.*, 2018a). It is worth nothing that the primary focus in previous studies of

diaphragm fatigability has been on changes in contractile function. The contribution of neural mechanisms to fatigability of the diaphragm has received less attention.

The neural contribution to performance fatigability can be estimated by quantifying the voluntary activation of a muscle (Shield & Zhou, 2004). D-VA has been estimated by comparing the amplitude of an evoked twitch during a maximal voluntary contraction to the amplitude of a control twitch when the muscle is at rest (Bellemare & Bigland-Ritchie, 1984; Similowski *et al.*, 1996; Ramsook *et al.*, 2021). Research has shown that after sustained maximal inspiratory efforts (Luu *et al.*, 2020) and expulsive manoeuvres (McKenzie *et al.*, 1992), the voluntary activation of the diaphragm can decrease in male participants, suggesting that neural mechanisms contribute to some level of performance fatigability of the diaphragm. Importantly, the aforementioned studies used tasks that specifically targeted the diaphragm through increased respiratory work, as opposed to whole body exercise, which requires locomotor and respiratory muscles to compete for a finite cardiac output. Fatigability is task specific, and it is possible that the diaphragm may respond differently to whole body exercise compared to isolated inspiratory work. Moreover, neither study included female participants, thus it remains unknown if females respond similarly to their male counterparts. Expanding on previous work, we sought to determine whether D-VA would decrease after whole-body exercise, similar to the changes in the contractile function of the diaphragm assessed by $P_{DI,TW}$. Moreover, we aimed to explore if a sex difference was present in the change of D-VA after exercise. We hypothesized that *i*) D-VA would contribute to diaphragm fatigability in males and females, and *ii*) males would exhibit a greater loss in D-VA after exercise compared to males.

## 3.2 Methods

### 3.2.1 Ethical approval

All participants provided written, informed consent prior to beginning the study. All experimental procedures were approved by The University of British Columbia and Providence Health Care Research Ethics Board (UBC-PHC REB Number: H19 – 00725). The study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database.

### 3.2.2 Participants

A sample size calculation using G*Power (3.1.9.6) determined a total sample of 16 participants (8 males and 8 females) was required to detect a difference in D-VA between sexes, assuming a medium effect size of 0.4, $\alpha = 0.05$, power = 0.80, a correlation among repeated measures ($\rho$) = 0.5, and a non-sphericity correction ($\varepsilon$) = 0.70. We estimated a medium effect size because existing literature comparing D-VA between males and females after exercise were lacking. We recruited participants with no smoking history and no symptoms of cardiovascular or respiratory disease. Females were tested randomly throughout their menstrual cycle and were permitted to use oral contraceptives or intrauterine devices. While there is an effect of menstrual cycle phase on baseline knee extensor voluntary activation, there does not appear to be an effect of menstrual cycle phase on the change in voluntary activation after exercise (Ansdell *et al.*, 2019b). Moreover, neither menstrual cycle phase (McNulty *et al.*, 2020) nor oral contraceptive use (Elliott-Sale *et al.*, 2020) have a meaningful impact on exercise performance.

### 3.2.3 Experimental design

Each participant reported to the laboratory on two occasions separated by a minimum of 48 hours to allow for recovery between visits. Participants avoided strenuous exercise for 24 hours prior to each session and refrained from caffeine and alcohol on the day of testing. The first session

involved anthropometric and pulmonary function measurements followed by an incremental cycling test to determine peak work rate and gas exchange threshold (GET). The second session included a time-to-exhaustion (TTE) cycling test at a constant work rate equal to 60% of the difference between their GET and peak work rate determined on visit 1 (Lansley *et al.*, 2011). Diaphragm fatigue measurements, including $P_{DI,TW}$ and D-VA, were measured prior to exercise, within 5 minutes of completing exercise, and at 15 and 30 minutes after exercise. Respiratory muscle EMG, dyspnoea, and cardiorespiratory variables were measured throughout the TTE test.

### 3.2.4 Pulmonary function tests

Spirometry and plethysmography were measured using a commercially available testing system (Vmax 229d with Autobox 6,200 DL; SensorMedics, Yorba Linda, CA, USA) and respiratory muscle strength was measured through a semi-occluded mouthpiece connected to a calibrated pressure transducer (model DP15-34; Validyne Engineering, CA, USA) in accordance with standard recommendations (Miller *et al.*, 2005; Wanger *et al.*, 2005; Laveneziana *et al.*, 2019b). Pulmonary function and respiratory muscle strength data are expressed as absolute values and as %predicted (Black & Hyatt, 1969; Gutierrez *et al.*, 2004; Tan *et al.*, 2011).

### 3.2.5 Cycle exercise tests

Cycle exercise was performed on an electronically braked upright ergometer (VIAsprint 200P; Ergoline, Bitz, Germany). Metabolic and cardiorespiratory parameters were assessed using a commercially available metabolic cart (TrueOne 2400, Parvo Medics, Sandy, UT, USA), customized to output the raw expired flow signal. Heart rate was recorded continuously during exercise via a heart rate monitor (Polar H10, Polar Electro, Kempele, Finland). Inspired flow was collected by a separate pneumotachometer (Series 3813; Hans Rudolph, Shawnee, KS, USA) connected to an amplifier (PA-1 Series 1110, Hans Rudolph, Shawnee, KS, USA). At rest and

every two-minutes during exercise, participants performed dynamic inspiratory capacity manoeuvres. To assess perceptions of dyspnoea and leg discomfort, participants were asked to rate their overall sensation of "breathing discomfort" and "leg discomfort" using a 0-10 modified category-ratio scale (Borg, 1982). The scale was anchored such that '0' represented no breathing/leg discomfort at all, and '10' represented the most intense breathing/leg discomfort the participant had ever experienced or could imagine experiencing.

The incremental cycling test on visit 1 began with a 6-minute rest period followed by a 1-minute warm-up of unloaded pedalling. Exercise started at 25 W and increased in a stepwise fashion every 2-minutes until volitional exhaustion or if cycling cadence fell below 60 rpm despite verbal encouragement. Maximal work rate was defined as the highest work rate sustained for 30 seconds. The TTE test on visit 2 also began with a 6-minute rest period and was followed by a 1-minute warmup at 50% of the exercising load. Exercise load was determined by the work rate at the individual participant's GET plus 60% of the difference between the work rate at GET and peak work rate from visit 1 (Lansley *et al.*, 2011). GET was estimated using the V-slope method (Beaver *et al.*, 1986). The TTE test ended when the participant reached volitional exhaustion or if pedal cadence fell below 60 rpm. Participants were notified when cadence was approaching 60 rpm and asked if they could keep their cadence above 60 rpm, otherwise no verbal encouragement was given during the TTE test.

### 3.2.6 Respiratory pressures and the work of breathing

An oesophageal balloon catheter (no. 47-9005; Cooper Surgical, Trumbull, CT, USA) filled with 1 mL of air measured $P_{OES}$. Appropriate placement of the oesophageal catheter was confirmed via the occlusion test (Baydur *et al.*, 1982). A second combined electrode balloon catheter (Guangzhou Yinhgui Medical Equipment Co. Ltd., Guangzhou, China) was filled with 1.2 mL of air and used

to measure $P_{GA}$ and $EMG_{DI}$. Placement of the electrode balloon catheter was based on the optimization of the $EMG_{DI}$ signal as previously described (Luo *et al.*, 2008). Both catheters were connected to calibrated differential pressure transducers (model DP15-34; Validyne Engineering, CA, USA). $P_{DI}$ was calculated as the difference between $P_{GA}$ and $P_{OES}$. The work of breathing ($W_b$) was calculated at baseline and every two minutes of exercise by creating ensemble averaged $P_{OES}$-tidal volume loops and then calculating the area within the $P_{OES}$-tidal volume loop plus the area that was outside of the loop but within a right angle triangle with points at end-inspiratory and end-expiratory lung volume, which represents a portion of the elastic $W_b$ (Dominelli & Sheel, 2012). $W_b$ values were then multiplied by breathing frequency to represent a unit of power (*i.e.*, $J \cdot min^{-1}$).

### 3.2.7 Inspiratory muscle electromyography

$EMG_{DI}$ signals were collected with a bio-amplifier and amplified 1000x (model RA-8; Yinghui Medical Technology, Guangzhou, China). The electrode catheter included nine electrodes separated by 3.2 cm, forming five recording electrode pairs to measure $EMG_{DI}$. $EMG_{SCM}$ and $EMG_{SCA}$ were measured by placing bipolar surface electrodes over the muscle belly and collected with a wireless surface EMG system (MyoSystem 1400A; Noraxon, Scottsdale, AZ, USA). $EMG_{SCM}$ electrodes were placed midway between the mastoid process and medial clavicle while $EMG_{SCA}$ electrodes were placed at the level of the cricoid process within the posterior triangle of the neck. To quantify EMG during exercise, EMG signals were first band pass filtered (20 – 500 Hz) and then converted to root mean square using a 0.1 second moving average window. EMG data were selected manually on a breath-by-breath basis to avoid cardiac artefact and are expressed as a percentage of the highest EMG value obtained for each respective muscle during an inspiratory capacity manoeuvre, performed either at rest or during exercise.

### 3.2.8 Diaphragm neuromuscular function

The phrenic nerves were stimulated using cervical magnetic stimulation (CMS). Each stimulus was delivered through a 90-mm circular coil attached to a 2-tesla magnetic stimulator (Magstim 200$^2$; Magstim, Whitland, UK). To keep lung volume similar during CMS, all stimuli were triggered near functional residual capacity based on a hand signal from the participant when they were "at the end of a normal breath out," which was later confirmed by checking the end-expiratory $P_{OES}$ signal. The optimal stimulation site was found by delivering stimuli at 70% stimulator output intensity starting at the seventh cervical vertebra and moving up and down in ~1 cm increments until the greatest $P_{DI,TW}$ amplitude was recorded. That point was marked in ink on the participant's neck and used for all subsequent stimulations. To test if CMS resulted in a maximal stimulation of the phrenic nerves, three stimulations at increasing output intensity (60, 70, 80, 90, 95, and 100%) were delivered with each stimulation separated by 30 seconds. The amplitude of the resulting $P_{DI,TW}$ at submaximal output intensities were compared against the twitches at 100% output intensity to determine if stimulation was maximal. Participants who did not display maximal stimulation, (*i.e.*, those who had a $P_{DI,TW}$ amplitude at 100% stimulator output that was greater than 1 coefficient of variation compared to $P_{DI,TW}$ at 95% stimulator output) were excluded from the study.

Diaphragm contractile function and D-VA were measured from potentiated twitches before (baseline), immediately after exercise, and after 15 and 30 minutes of passive recovery. Blocks of five to eight potentiated twitches were performed before and after exercise. The first two twitch manoeuvres were discarded, and the first three acceptable twitches were used for analysis. Twitches were not included if: *i*) the stimulation was not performed at functional residual capacity; *ii*) the stimulation was contaminated from the participant swallowing; *iii*) cardiac artefact

contaminated the CMAP; or *iv*) notable $EMG_{DI}$ activity was present, suggesting the diaphragm was not in a relaxed state (Laghi *et al.*, 1996). Each potentiated twitch manoeuvre involved two distinct components, a superimposed twitch, and a control twitch. The superimposed twitch was delivered during a maximal inspiratory effort against a semi-occluded breathing apparatus. The control twitch was delivered off the mouthpiece after the inspiratory effort when the participant returned to functional residual capacity. The control twitch was assessed for measures of contractile function of the diaphragm. From each control twitch, the $P_{DI,TW}$ amplitude, contraction time, and half-relaxation time were calculated according to established guidelines (American Thoracic Society/European Respiratory Society, 2002). Additionally, the compound muscle action potential (CMAP) of the control twitch was assessed for peak-to-peak amplitude, onset, and offset as previously described (Ramsook *et al.*, 2021). D-VA was calculated as follows:

$$Voluntary\ Activation\ (\%) = \left[1 - \left(\frac{superimposed\ twitch}{control\ twitch}\right)\right] \times 100\%$$ (Merton, 1954).

To assess the change in $P_{DI,TW}$ and D-VA after exercise, data were expressed relative to baseline. We have previously shown that CMS-evoked twitches can reliably measure both $P_{DI,TW}$ and D-VA in young, healthy adults (Ramsook *et al.*, 2021).

### 3.2.9 Data analysis

Data were converted from analogue to digital with a 16-channel data acquisition system (PowerLab 16/35; ADInstruments, Colorado Springs, CO, USA) and collected using LabChart software (v7.3.7 Pro, ADInstruments). Data were sampled at 10 kHz. The TTE test was broken down into two-minute stages with cardiorespiratory and EMG data sampled from a 30 second window between 60-90 seconds of each two-minute stage. Inspiratory capacity manoeuvres were completed at the end of each two-minute stage to avoid contaminating the breaths during the 60-90 second analysis window. Peak exercise data was determined as the average of the last 30

seconds of exercise, with the exception of peak heart rate which was taken as the highest recorded value during this time and before the final inspiratory capacity manoeuvre.

### 3.2.10 Statistical analyses

Descriptive statistics between groups were compared with an independent samples Student's *t*-test. A repeated-measures ANOVA with Tukey's post-hoc test determined if CMS maximally stimulated the phrenic nerves by comparing $P_{DI,TW}$ at maximal stimulator output intensity against submaximal intensities. A two-way (time and sex) repeated measures ANOVA assessed differences in diaphragm fatigability measures between males and females, and before and after exercise. Sphericity was assessed using Mauchly's test and a Greenhouse-Geisser correction on the *p*-values was applied if the sphericity assumption was violated. Main effects were only interpreted with Tukey's post-hoc test when interaction terms were not significant. If significant interaction effects were found, independent *t*-tests were performed at each time point to identify significant differences by sex. Specifically, we examined if there were significant sex differences in the magnitude of the decrease in either $P_{DI,TW}$ or D-VA at three time points post-exercise. The relationships between voluntary MIP and both $P_{DI,TW}$ and D-VA were tested with linear regression using the 'lm' function in R. Cardiorespiratory variables, EMG, and perceptions of breathing and leg discomfort were also assessed with a two-way repeated measures ANOVA during exercise with Tukey's post-hoc test when appropriate. Participants exercised for different lengths of time during their TTE visit and thus, comparisons were made at baseline, 2 minutes into exercise, 4 minutes into exercise, and peak exercise as all participants have data for these time points. Statistical analyses were performed in R (v.4.1.0).

## 3.3 Results

### 3.3.1 Participants

A total of 32 participants were recruited for this study (18F:14M). Seven (3F: 4M) participants did not show evidence of maximal stimulation with CMS and were excluded from the analysis. Therefore, all data presented are from a sample of 15 female and 10 male participants. Participant characteristics, including pulmonary function and peak incremental exercise responses are summarized in Table 3-1. All participants displayed pulmonary function within normal limits (Gutierrez *et al.*, 2004; Tan *et al.*, 2011) and normal inspiratory muscle strength (Black & Hyatt, 1969). At the time of testing, one female participant was using a monophasic oral contraceptive and four female participants were using an intrauterine device (3 hormonal; 1 non-hormonal).

**Table 3-1. Participant characteristics**

| | Female | Male |
|---|---|---|
| N | 15 | 10 |
| Age, years | 22 ± 3 | 23 ± 3 |
| Height, cm | 163 ± 8 | 177 ± 6 |
| Mass, kg | 60 ± 8 | 73 ± 10 |
| BMI, $kg \cdot m^{-2}$ | 23 ± 2 | 23 ± 3 |
| *Pulmonary Function* | | |
| FVC, L (%predicted) | 4.11 ± 0.85 (100 ± 14) | 5.50 ± 0.68* (98 ± 8) |
| $FEV_1$, L (%predicted) | 3.39 ± 0.55 (95 ± 11) | 4.38 ± 0.61* (94 ± 9) |
| $FEV_1$/FVC (%predicted) | 0.83 ± 0.08 (96 ± 8) | 0.80 ± 0.05 (95 ± 6) |
| TLC, L (%predicted) | 5.29 ± 1.04 (96 ± 12) | 7.15 ± 1.01* (98 ± 10) |
| $PI_{MAX}$, $cmH_2O$ (%predicted) | 93 ± 20 (100 ± 21) | 120 ± 14* (92 ± 11) |
| *Peak incremental exercise* | | |
| Work rate, W | 195 ± 46 | 285 ± 46* |
| $\dot{V}O_2$, $L \cdot min^{-1}$ | 2.40 ± 0.55 | 3.72 ± 0.53* |
| $\dot{V}O_2$, mL $kg^{-1}$ $min^{-1}$ (%predicted) | 40.1 ± 8.9 (114 ± 25) | 51.3 ± 5.8* (111 ± 11) |
| $\dot{V}CO_2$, $L \cdot min^{-1}$ | 2.73 ± 0.49 | 4.13 ± 0.56* |
| RER | 1.15 ± 0.09 | 1.11 ± 0.04 |
| Heart rate, $beat \cdot min^{-1}$ (%predicted) | 185 ± 8 (93 ± 4) | 188 ± 9 (95 ± 5) |
| Breathing frequency, $breath \cdot min^{-1}$ | 50 ± 10 | 51 ± 8 |
| Tidal volume, L | 1.89 ± 0.37 | 2.86 ± 0.41* |
| Minute ventilation, $L \cdot min^{-1}$ | 93 ± 18 | 147 ± 30* |
| $P_{ET}CO_2$, mmHg | 34 ± 4 | 31 ± 7 |

Data are presented as mean ± SD. Abbreviations: BMI, body mass index; FVC, forced vital capacity; $FEV_1$, forced expired volume in 1 second; TLC, total lung capacity; $PI_{MAX}$, maximal inspiratory pressure; $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide production; RER, respiratory exchange ratio; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide; *, $p < 0.05$.

### 3.3.2 Constant load exercise

The average exercise intensity during the constant load exercise test was 83±4%peak work rate in females and 85±3%peak work rate in males ($p$ = 0.165). There was no statistically significant difference in the TTE between females and males (7.6 ± 4.2 *vs.* 9.3 ± 3.5 minutes, respectively, $p$ = 0.295). Cardiorespiratory variables obtained at peak exercise during the constant load exercise test are presented in Table 3-2**.**

**Table 3-2. Cardiorespiratory responses to constant work exercise at peak exercise.**

| | Female | Male |
|---|---|---|
| $\dot{V}O_2$, $L \cdot min^{-1}$ | 2.27 ± 0.53 | 3.54 ± 0.39* |
| $\dot{V}O_2$, $mL \cdot kg^{-1} \cdot min^{-1}$ | 37.6 ± 8.3 | 48.7 ± 5.1* |
| $\dot{V}CO_2$, $L \cdot min^{-1}$ | 2.48 ± 0.5 | 3.67 ± 0.51* |
| RER | 1.11 ± 0.10 | 1.03 ± 0.06 |
| Heart rate, $beat \cdot min^{-1}$ (%predicted) | 177 ± 8 (89 ± 4) | 178 ± 9 (90 ± 4) |
| Breathing frequency, $breath \cdot min^{-1}$ | 49 ± 10 | 48 ± 8 |
| Tidal volume, L | 1.85 ± 0.35 | 2.81 ± 0.38* |
| Minute ventilation, $L \cdot min^{-1}$ | 88.4 ± 19.1 | 135.0 ± 28.9* |
| $W_b$, $J \cdot min^{-1}$ | 230 ± 103 | 425 ± 173* |
| $P_{ET}CO_2$, mmHg | 34.2 ± 4.3 | 30.9 ± 6* |
| Dyspnoea, 0-10 Scale | 7.2 ± 2.5 | 7.2 ± 3.0 |
| Leg Discomfort, 0-10 Borg Scale | 8.3 ± 2.2 | 8.6 ± 1.5 |

Data are presented as mean ± SD. Abbreviations: $\dot{V}O_2$, oxygen uptake; $\dot{V}CO_2$, carbon dioxide production; RER, respiratory exchange ratio; $W_b$, work of breathing; $P_{ET}CO_2$, partial pressure of end-tidal carbon dioxide,*, significantly different from females, $p < 0.05$.

### 3.3.3 Inspiratory muscle electromyography

Individual EMG data can be found in Figure 3-1 and mean EMG data can be found in Table 3-3. $EMG_{DI}$ increased from baseline as exercise progressed (main effect of time; $p < 0.001$) but did not

differ between sexes (main effect of sex, $p = 0.611$). In the extra-diaphragmatic inspiratory muscles (*i.e.,* sternocleidomastoid and scalene), a main effect of time (both $p < 0.001$) and sex ($EMG_{SCM}$ $p = 0.004$ and $EMG_{SCA}$ $p = 0.016$) were observed; however, in neither the $EMG_{SCA}$ nor $EMG_{SCM}$ was an interaction observed (both $p > 0.05$). Overall, female participants had greater activation of extra-diaphragmatic inspiratory muscles during exercise compared to males while diaphragm activation was similar between the sexes.

**Table 3-3. Inspiratory muscle electromyography during exercise.**

| | | Baseline | 2 min | 4 min | Peak |
|---|---|---|---|---|---|
| $EMG_{DI}$, %max$^{\dagger}$ | Female | 9.2 ± 6.4 | 35.9 ± 14.1 | 50.5 ± 12.5 | 53.6 ± 11.9 |
| | Male | 7.3 ± 5.5 | 35.4 ± 11.7 | 44.7 ± 11.9 | 51.7 ± 6.2 |
| $EMG_{SCA}$, %max$^{\dagger, *}$ | Female | 4.1 ± 2.4 | 19.5 ± 8.5 | 28.0 ± 12.6 | 32.2 ± 13.4 |
| | Male | 2.5 ± 1.6 | 10.1 ± 4.2 | 16.3 ± 9.1 | 20.2 ± 10.8 |
| $EMG_{SCM}$, %max$^{\dagger, *}$ | Female | 2.6 ± 1.3 | 7.0 ± 3.0 | 10.0 ± 4.7 | 13.7 ± 4.9 |
| | Male | 1.3 ± 0.8 | 3.0 ± 1.2 | 4.5 ± 1.7 | 10.1 ± 6.5 |

Data are presented as mean ± SD. Abbreviations: $EMG_{DI}$, crural diaphragm electromyography; $EMG_{SCA}$, scalenes electromyography; $EMG_{SCM}$, sternocleidomastoid EMG; $^{\dagger}$, main effect of time ($p<0.05$); $^{*}$, main effect of sex ($p<0.05$).

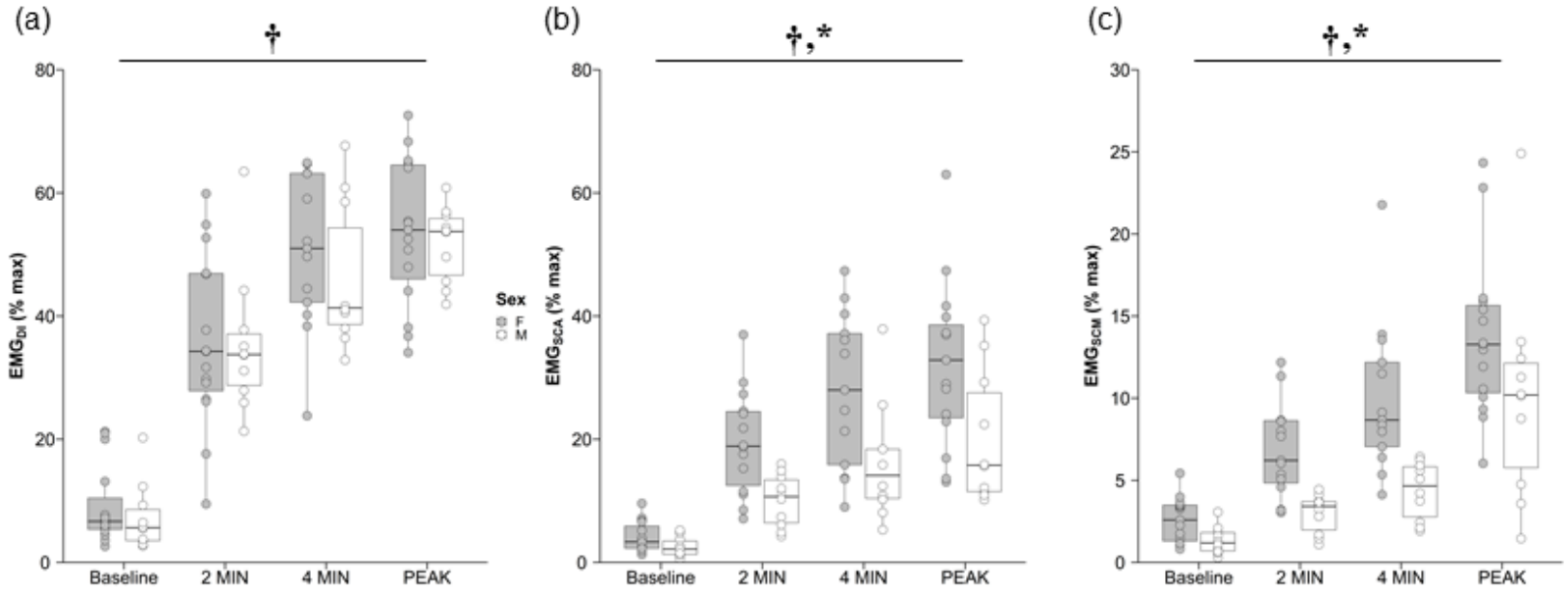


**Figure 3-1. Inspiratory muscle electromyography.**
Abbreviations: F, female; M, males; $EMG_{DI}$, crural diaphragm electromyography; $EMG_{SCA,}$ scalene electromyography; $EMG_{SCM}$, sternocleidomastoid electromyography, †, main effect of time ($p<0.05$); *, main effect of sex ($p<0.05$).

### 3.3.4 Diaphragm neuromuscular function

The $P_{DI,TW}$ response to increasing stimulator output intensity is shown in Figure 3-2. Transdiaphragmatic twitch response to cervical magnetic stimulation. There was no significant difference in $P_{DI,TW}$ between 95 and 100% stimulator output in both males and females (both $p > 0.05$). Three participants (2F:1M) do not have data for the 30-minute post-exercise time point due to catheter discomfort. Their data have been removed from the statistical analyses of diaphragm neuromuscular function. Individual data for amplitude of the $P_{DI,TW}$ is shown in Figure 3-3. Diaphragm contractile function. A main effect of time ($p < 0.001$) and sex ($p < 0.027$) were observed, but no significant interaction ($p = 0.183$). In female participants, baseline $P_{DI,TW}$ was 37.1±10.7 $cmH_2O$ and was reduced to 31.2±8.9 $cmH_2O$ immediately following exercise. $P_{DI,TW}$ increased to 37.7±11.9 $cmH_2O$ after 30 minutes of recovery. Baseline $P_{DI,TW}$ started at 39.8±5.6 $cmH_2O$ in males and decreased to 27.6±5.2 $cmH_2O$ after exercise. After 30 minutes of recovery $P_{DI,TW}$ was 38.1±6.4 $cmH_2O$ in males. D-VA data are shown in Figure 3-4. Diaphragm voluntary activation. Baseline D-VA was not different between females and males (88.2±13.7 *vs.* 88.5±8.2%, $p = 0.95$). We observed main effects of time ($p < 0.001$) and sex ($p = 0.047$) for the change in D-VA, from baseline but no interaction effect ($p = 0.057$). Mechanical and electrical twitch properties, along with voluntary activation data are presented in Table 3-4. There was no significant relationship between the change in MIP and $P_{DI,TW}$ ($R^2 = 0.038$, $p = 0.741$) or D-VA ($R^2 = 0.060$, $p = 0.125$).

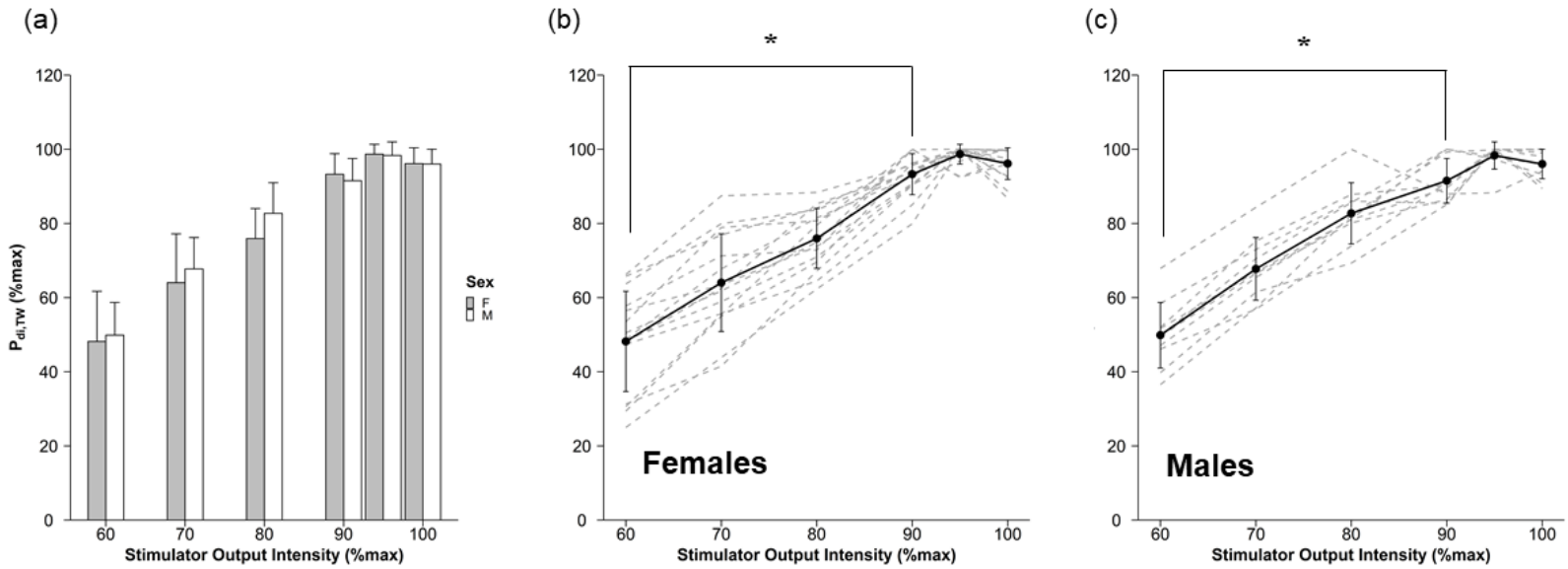


**Figure 3-2. Transdiaphragmatic twitch response to cervical magnetic stimulation.**
Bar graph depicting mean ± SD $P_{DI,TW}$ response as a percent of max $P_{DI,TW}$ in response to increasing intensities of magnetic stimulator output intensity (**a**). Mean (solid line) and individual responses (dashed lines) to increasing stimulator output intensity in females (**b**) and males (**c**). $P_{DI,TW}$ response did not significantly increase from 95 to 100%. Abbreviations: $P_{DI,TW}$. Transdiaphragmatic twitch amplitude, *, significantly different from 100% ($p < 0.05$).

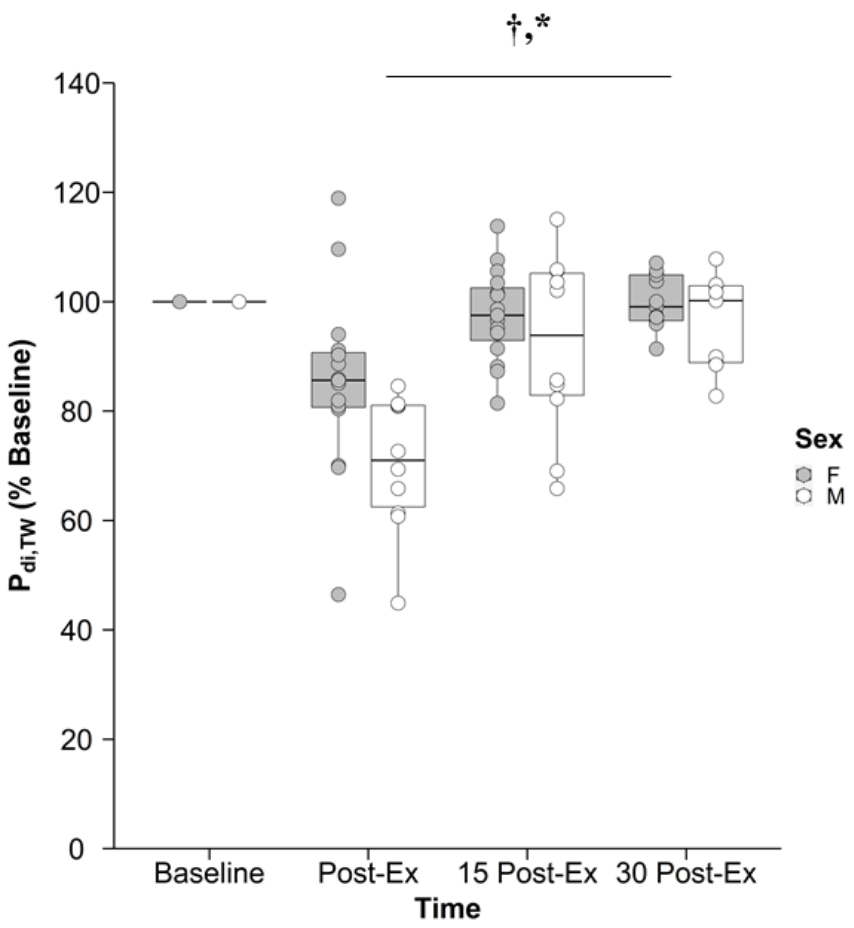


**Figure 3-3. Diaphragm contractile function.**
Change in $P_{DI,TW}$ after exercise expressed as a percent of baseline. $P_{DI,TW}$, transdiaphragmatic twitch pressure; Post-Ex, post-exercise; 15 Post-Ex, 15 minutes post-exercise; 30 Post-Ex, 30 minutes post-exercise; †, main effect of time; * main effect of sex.

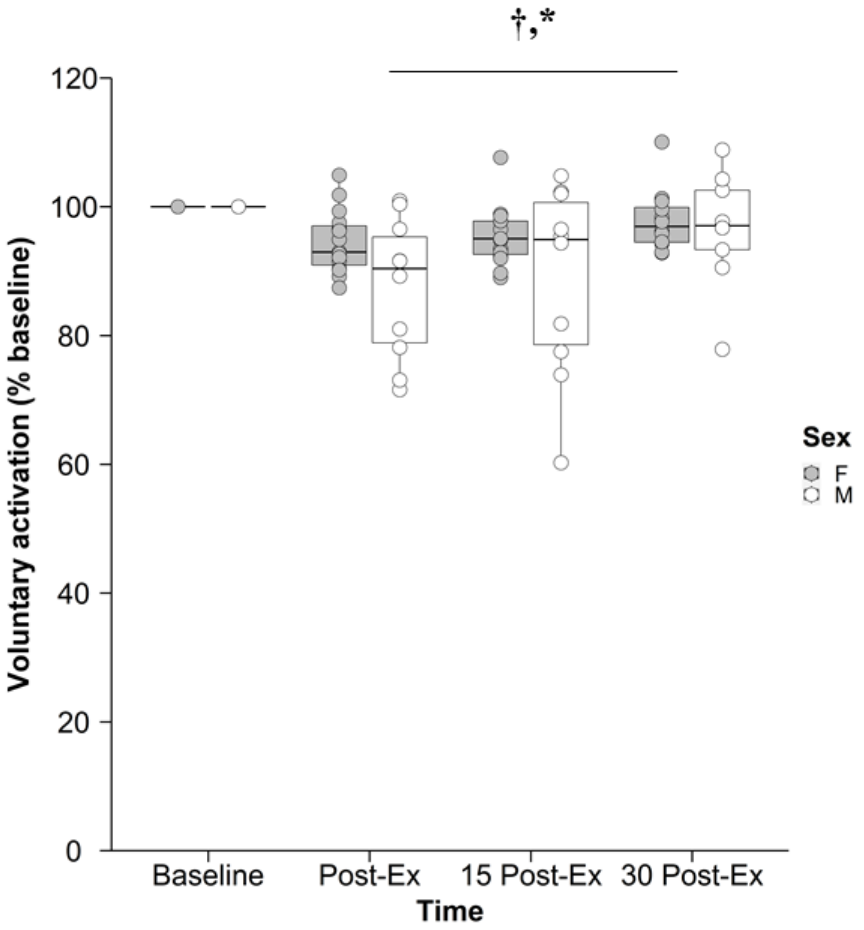


**Figure 3-4. Diaphragm voluntary activation.**
Change in diaphragm voluntary activation after exercise expressed as a percent of baseline. Abbreviations: Post-Ex, post-exercise; 15 Post-Ex, 15 minutes post-exercise; 30 Post-Ex, 30 minutes post-exercise; †, main effect of time; * main effect of sex.

**Table 3-4. Diaphragm neuromuscular function.**

| | *Female* | | | | *Male* | | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | Post-Ex | 15 Post-Ex | 30 Post-Ex | Baseline | Post-Ex | 15 Post-Ex | 30 Post-Ex |
| $P_{DI,TW}$, %baseline †,* | 100.0 ± 0.0 | 85.2 ± 16.7 | 97.6 ± 8.5 | 100.0 ± 4.8 | 100.0 ± 0.0 | 70.3 ± 12.4 | 92.0 ± 16.8 | 96.2 ± 8.7 |
| D-VA, %baseline †, * | 100.0 ± 0.0 | 95.4 ± 4.9 | 95.6 ± 4.6 | 100.0 ± 9.4 | 100.0 ± 0.0 | 87.4 ± 10.8 | 88.9 ± 14.8 | 96.5 ± 9.0 |
| Contraction time, ms † | 127 ± 5 | 114 ±8 | 120 ± 5 | 121 ± 6 | 121 ± 8 | 108 ± 12 | 119 ± 9 | 116 ± 12 |
| Half-relaxation time, ms † | 72 ± 8 | 58 ± 5 | 66 ± 7 | 70 ± 8 | 70 ± 9 | 60 ± 9 | 66 ± 8 | 70 ± 10 |
| CMAP amplitude, mV † | 1.4 ± 0.6 | 1.5 ± 0.7 | 1.5 ± 0.7 | 1.4 ± 0.8 | 1.6 ± 0.7 | 2.0 ± 0.9 | 1.8 ± 0.8 | 1.6 ± 0.7 |
| CMAP onset, ms | 7.5 ± 2.1 | 7.4 ± 1.1 | 7.4 ± 1.4 | 6.9 ± 1.3 | 8.7 ± 3.2 | 7.9 ± 1.2 | 8 ± 1.3 | 7.3 ± 1.3 |
| CMAP duration, ms | 19.5 ± 4.0 | 21.1 ± 3.5 | 22.1 ± 3.4 | 20.3 ± 3.6 | 22.0 ± 2.5 | 21.6 ± 2.1 | 20.5 ± 2.1 | 21.9 ± 2.5 |

Data are presented as mean ± SD. Abbreviations: $P_{DI,TW}$, transdiaphragmatic twitch amplitude; D-VA, diaphragm voluntary activation; CMAP, compound muscle action potential; †, main effect of time ($p < 0.05$); *, main effect of sex ($p < 0.05$).

## 3.4 Discussion

### 3.4.1 Main findings

The purpose of this study was to determine if D-VA would decrease after whole-body exercise and explore whether the magnitude of the decrease would differ between males and females. The main findings of this study are twofold: after whole-body exercise performed at a similar relative intensity *i*) D-VA decreases and contributes to performance fatigability of the diaphragm; and *ii*) the decrease in D-VA is greater in males than females. Previous studies have indicated that the magnitude of diaphragm contractile function loss is greater in males compared to females when performing tasks of similar relative intensity (Guenette *et al.*, 2010; Welch *et al.*, 2018a). We have shown that the magnitude of loss in D-VA is also greater in males compared to females after high intensity, constant load cycling.

### 3.4.2 Diaphragm neuromuscular function

Our findings are in agreement with previous research that shows a loss of contractile function of the diaphragm after high intensity cycling in young healthy adults (Guenette *et al.*, 2010; Welch *et al.*, 2018a). Additionally, we have shown that D-VA decreases after high intensity cycling. A decrease in D-VA has previously been reported after isolated respiratory work (Bellemare & Bigland-Ritchie, 1987; McKenzie *et al.*, 1992; Luu *et al.*, 2020), but to our knowledge, never after high intensity cycling. Notably, the work of McKenzie *et al*., (1992) found a decrease in D-VA after expulsive contractions, and no significant change in activation after an inspiratory task. More recently however, Luu *et al*., (2020) observed significant supraspinal fatigue of the inspiratory muscles measured by transcranial magnetic stimulation. These incongruent results may be due to differences in methodologies. For example, McKenzie *et al.*, (1992) used electrically-evoked twitches and Luu *et al*., (2020) used transcranial magnetic stimulation to induce cortically-evoked

twitches, whereas we used CMS to stimulate the phrenic nerves. Aspects of fatigability are task specific. While fatiguing inspiratory tasks can offer important insights into the physiological responses to periods of elevated respiratory work, it is also important to consider how the body responds to more general tasks such as whole-body exercise. Thus, it is possible that our exercise task elicits a different response than isolated inspiratory manoeuvres that can result in a decrease in D-VA. Moreover, individual differences in the response to whole-body exercise can exacerbate respiratory muscle fatigue. As an example, one female participant showed a large decline in $P_{DI,TW}$ after exercise (46%baseline). This participant also showed evidence of exercise induced arterial hypoxaemia as their $S_PO_2$ was reduced from 97% at baseline to 88% at peak exercise. A similar reduction in $S_PO_2$ was not observed in a male participant with a similar decrease in $P_{DI,TW}$ (44%baseline). These individual differences speak to the integrative and variable nature of physiology and the multifactorial causes of respiratory muscle fatigue in humans.

### 3.4.3 Sex differences in voluntary activation

As alluded to previously, sex differences in diaphragm fatigue have primarily focused on changes in contractile function whereas this study was focused primarily on D-VA. While both males and females showed a decrease in D-VA after exercise, males demonstrated a greater decrease than females. The previous studies examining the change in D-VA after breathing tasks have primarily included male participants and the results are not applicable to more common tasks such as whole body exercise. There are sex differences in respiratory anatomy and physiology that impact the integrative physiological responses to exercise (Molgat-Seon *et al.*, 2018b). Germane to the current study is the apparent blunted response to the inspiratory muscle metaboreflex as shown in recent studies (Smith *et al.*, 2016; Welch *et al.*, 2018b; Geary *et al.*, 2019; Archiza *et al.*, 2021). In these studies, participants performed inspiratory pressure threshold (Welch *et al.*, 2018b; Geary *et al.*,

2019; Archiza *et al.*, 2021) or resistive breathing (Smith *et al.*, 2016) tasks to increase the amount of inspiratory work performed. In all cases, the sympathetic response was suggested to be greater in males. The blunted sympathetic response observed in females may influence respiratory and limb haemodynamics and could offer some resilience to changes in voluntary activation. Feedback from group III/IV afferents have been shown to affect voluntary activation of the quadriceps after fatiguing leg exercise (Sidhu *et al.*, 2017). It stands to reason that if the sympathetic response to inspiratory work is blunted in females, this may have a positive effect to reduce the loss of D-VA. In the present study, we were unable to measure muscle sympathetic nerve activity or surrogates of sympathetic activity (*e.g.*, mean arterial pressure) and thus we are unable to directly comment on the role of sympathetic activity and D-VA as it relates to whole body exercise.

Our observation of a sex difference in D-VA differs from other investigations that have reported no difference in voluntary activation across several skeletal muscles. For example, previous studies examining changes in voluntary activation of the elbow extensors (Yacyshyn & McNeil, 2020), elbow flexors (Hunter *et al.*, 2006), and knee extensors (Ansdell *et al.*, 2019a) have shown no sex difference. One possible explanation is that the type of tasks differs between our study and previous work. The aforementioned studies that do not report a sex difference in voluntary activation had participants perform isolated muscle work whereas the present study used a whole-body exercise task performed at a similar relative intensity. As expected, men performing the same relative exercise intensity produce higher absolute work rates, ventilations, and therefore have a higher absolute $W_b$ relative to females. The higher absolute $W_b$ in males in the present study could have contributed, at least in part, to their decrease in diaphragmatic contractile function ($P_{DI,TW}$) and D-VA. Indeed, it appears that when males and females are matched for absolute

inspiratory muscle strength and perform the same respiratory work during a resistive breathing task, the loss in contractile function appears to be equal (Archiza *et al.*, 2021).

The present study investigated fatigability of the diaphragm; however, the hyperpnoea of exercise is not exclusively performed by the diaphragm. Inspiratory muscles in the neck, including the scalenes and sternocleidomastoid, also contribute to breathing, among several other respiratory muscles. Moreover, expiration becomes an active process as ventilation increases, thereby recruiting abdominal muscles to assist in the emptying of lungs and the regulation of lung volumes. It is possible that the increased recruitment of these extra-diaphragmatic inspiratory muscles (*e.g.*, scalenes and sternocleidomastoid) could have an effect to spare the 'primary' inspiratory muscle (*e.g.*, the diaphragm) from a loss of contractile function with exercise. Previous work from our group has shown that neck inspiratory muscles are recruited differently in males and females, with females showing a greater relative activation during constant load (Mitchell *et al.*, 2018) and incremental (Molgat-Seon *et al.*, 2018a) cycling. Our data from the current study support these earlier findings as we again show similar relative activation of the diaphragm between sexes and greater $EMG_{SCA}$ and $EMG_{SCM}$ in females during exercise relative to males. It remains to be seen if manipulating the recruitment patterns of respiratory muscles can influence diaphragm fatigability.

### 3.4.4 Methodological considerations

We used CMS to stimulate the phrenic nerves as we have shown CMS-evoked twitches can reliably measure D-VA (Ramsook *et al.*, 2021). One concern with using CMS is the limited output of the stimulator compared to electrical stimulation, resulting in submaximal stimulations in some individuals. To address this issue, we only included participants who displayed a plateau in the

$P_{DI,TW}$ response to increasing stimulator output intensity. Therefore, we believe it is likely that all the participants in the current study were maximally stimulated by the CMS technique.

We tested female participants randomly throughout their menstrual cycle and did not exclude females that were on oral contraceptives or intrauterine devices. It is unknown if menstrual cycle phase, oral contraceptive use, or intrauterine devices affect the contractile function of the diaphragm or D-VA. We acknowledge that these measures are task and muscle specific, and further work specifically examining the effect of menstrual cycle phase and intrauterine device usage on D-VA is warranted. Finally, this study was conducted in a young healthy group and the results cannot be extrapolated to other populations such as older adults or those with chronic conditions.

### 3.5 Conclusions

In summary, we have shown that D-VA decreases after high-intensity, constant load cycling in both males and females. Our findings suggest that diaphragm fatigability is influenced by both contractile and neural mechanisms after exercise. Furthermore, the decrease in D-VA was greater in males compared to females when exercising at the same relative intensity. This provides further evidence that the female diaphragm is more resistant to fatigue when performing high intensity exercise. Future studies are needed to determine if the decline in D-VA is related to the inspiratory muscle metaboreflex and to determine if our observed sex differences persist in older adults and individuals with chronic cardiorespiratory conditions.

# Chapter 4: Voluntary activation of the diaphragm after inspiratory pressure threshold loading

## 4.1 Introduction

During dynamic fatiguing tasks, such as whole-body exercise (Johnson *et al.*, 1993; Babcock *et al.*, 1995), resistive breathing (McKenzie *et al.*, 1997), or pressure threshold loading (Welch *et al.*, 2018b; Geary *et al.*, 2019), the human diaphragm can experience a loss in contractile function, resulting in a temporary loss in the diaphragm's ability to generate pressure. A temporary loss in a muscle's ability to generate force or pressure after a task can be described as performance fatigability. In addition to a decline in pressure generation, voluntary activation can also be used to measure an aspect of performance fatigability associated with neural mechanisms (Enoka & Duchateau, 2016). Previous research examining diaphragm fatigability have focused on the decrease in contractile function, while changes in D-VA have received less attention. Recently, it has been shown that after sustained inspiratory efforts, male participants show a decrease in overall inspiratory muscle voluntary activation (Luu *et al.*, 2020). However, the study by Luu *et al* (2020) only included male participants, and therefore it is unknown whether a decrease in voluntary activation can contribute to performance fatigability in female participants.

It is well established that there are sex differences in fatigability of skeletal muscles (Hunter, 2016). In general, females are less fatigable than their male counterparts; however, this varies based on the type of task performed (Hunter, 2016). In the diaphragm, after high intensity cycling (Guenette *et al.*, 2010; Welch *et al.*, 2018a) and isolated inspiratory work (Welch *et al.*, 2018a; Geary *et al.*, 2019), females are less fatigable than males. These aforementioned studies focused on the contractile function of the diaphragm, measured through changes in the

transdiaphragmatic twitch pressure ($P_{DI,TW}$) and did not measure the D-VA. Therefore, it is unknown whether there is a sex difference in the neural mechanisms that contribute to diaphragm fatigability.

The purpose of this study was to investigate whether D-VA changes after a single bout of IPTL and explore whether changes in D-VA differ between males and females. IPTL was chosen as it is a fatiguing task that specifically targets the respiratory muscles as opposed to whole body exercise, where a competition for finite resources between respiratory and locomotor muscles can influence fatigability. We hypothesized that IPTL would result in a decrease in D-VA from baseline and the decrease in D-VA would be greater in males compared to females.

## 4.2 Methods

### 4.2.1 Ethical approval

Written informed consent was obtained from all participants prior to testing. All experimental procedures were approved by The University of British Columbia and Providence Health Care Research Institute Ethics Board (UBC-PHC REB number: H19-00725) and conformed to the *Declaration of Helsinki* apart from registration in a database.

### 4.2.2 Participants

Using G*Power (3.1.9.6) we determined a total sample size of 16 participants, evenly distributed between sexes, would be required, assuming an effect size = 0.4, $\alpha = 0.05$, power = 0.80, a correlation among repeated measures ($\rho) = 0.5$, and a non-sphericity correction ($\varepsilon$) = 0.70. Healthy never smokers with no symptoms or history of cardiovascular or respiratory disease and normal pulmonary function (*i.e.,* $FEV_1$ to FVC ratio > 0.70 and $FEV_1$ >80% predicted) were included in this study. Females were tested randomly throughout their menstrual cycle and were not excluded if they were using oral contraceptives or an intrauterine device. Many participants (M:9, F:10) also

participated in a previous study in our laboratory, which examined changes in D-VA after whole body exercise (see *Chapter 3*).

### 4.2.3 Experimental overview

Participants reported to the laboratory on two separate occasions separated by at least 48 hours. Participants were instructed to avoid caffeine for 8 hours and exercise for 24 hours prior to each testing session. Session 1 involved anthropometric measurements and pulmonary function tests (Vmax Encore 229 with V62J Autobox; CareFusion, CA, USA), which were performed in accordance with standard recommendations (Miller *et al.*, 2005; Wanger *et al.*, 2005). Inspiratory muscle strength was measured using an occluded mouthpiece connected to a calibrated differential pressure transducer (DP15-34; Validyne Engineering, CA, USA) as recommended (Laveneziana *et al.*, 2019b). Pulmonary function and inspiratory muscle strength results were presented in absolute units and as a percentage of predicted values (Black & Hyatt, 1969; Gutierrez *et al.*, 2004; Tan *et al.*, 2011). Following this, participants were familiarized with the scales used to record dyspnoea during IPTL as well as the IPTL protocol itself. Session 2 was the primary testing session where participants engaged in IPTL until task failure.

### 4.2.4 Inspiratory muscle electromyography

$EMG_{DI}$ was measured using a multipair electrode catheter (Guangzhou Yinghui Medical Equipment Co. Ltd, Guangzhou, China) and placed based on the strength of the $EMG_{DI}$ signal as previously described (Luo *et al.*, 2008). $EMG_{SCA}$ and $EMG_{SCM}$ were collected by placing bipolar surface electrodes (Dual EMG Wet Gel Ag/AgCl electrodes; Noraxon, AZ, USA) at the midpoint between the mastoid process and medial clavicle along the long axis of the sternocleidomastoid for $EMG_{SCM}$, and at the level of the cricoid process within the posterior triangle of the next for $EMG_{SCA}$ with a wireless surface electromyography system (MyoSystem 1400A, Noraxon, AZ,

USA). Raw $EMG_{DI}$ signals were collected using an amplifier (bio-amplifier model RA-8, Guangzhou Yinghui Medical Equipment Co. Ltd.), converted from analog to digital (Power Lab 16/35, ADInstruments, CO, USA) and collected using LabChart Pro software (v8.1.19, ADInstruments). All EMG signals were sampled at 10 kHz and band-pass filtered (20-500 Hz) before processing. Filtered EMG were converted to root-mean square calculated using a 100 ms moving average window. EMG data during IPTL are presented relative to the maximal EMG of each inspiratory muscle achieved during a maximal inspiratory manouvre (*e.g.,* inspiratory capacity, maximal inspiratory pressure, or maximal voluntary ventilation manouvre). EMGdi was also used to assess the parameters of the CMAP. A custom MATLAB (R2021a; MathWorks, MA, USA) script was used to process raw $EMG_{DI}$, filtered through a fourth order Butterworth filter (cut-off frequencies: 20 – 500 Hz) and rectified to calculate CMAP peak-to-peak amplitude. Peak-to-peak amplitude was calculated as the sum of the two rectified peaks. CMAP onset was defined as the first point before the first peak that exceeded a threshold calculated as the mean of the rectified EMG signal 40 milliseconds before the simulation +2 standard deviations. CMAP offset was defined as the first point after the second rectified peak that fell below the same threshold and CMAP duration was the difference between onset and offset. All EMG data were visually inspected and verified. Twitches were excluded if the CMAP signal was contaminated with cardiac artefact.

### 4.2.5 Respiratory pressures

The catheter used to record $EMG_{DI}$ was also used to measure $P_{GA}$. In addition, a second balloon catheter measured $P_{OES}$ (no. 47-9005; Cooper Surgical, CT, USA). The position of the oesophageal catheter was verified via the occlusion test (Baydur *et al.,* 1982). Mouth pressure ($P_{MO}$) was measured through a port in the mouthpiece. Catheters derived pressures and $P_{MO}$ measurements

were connected to calibrated differential transducers (DP15-34; Validyne Engineering, CA, USA). $P_{DI}$ was calculated as the difference between $P_{GA}$ and $P_{OES}$. Pressure-time products of the diaphragm ($PTP_{DI}$) were calculated by the numerical integration of an ensemble-averaged $P_{DI}$ trace during inspiration over a 30-second period during each minute of IPTL multiplied by the $f_b$ during that minute.

### 4.2.6 Inspiratory pressure threshold loading

Participants breathed on a custom-built IPTL device described previously (Boyle *et al.*, 2020). Participants would inspire with sufficient negative pressure to lift a weighted plunger to allow for airflow while expiration was unimpeded. The breathing pattern was set to a prolonged duty cycle (0.7), at a $f_b$ of 15 breaths·min$^{-1}$ while targeting 60% of their maximum $P_{DI}$ in order to promote diaphragm fatigue. The goal was to create a tension-time index of the diaphragm ($TTI_{DI}$), calculated as the product of the inspiratory duty cycle and the ratio of the mean $P_{DI}$ generated per breath to the maximal $P_{DI}$ generated, exceeding 0.2 to impede blood flow (Bellemare *et al.*, 1983) and promote fatigue (Bellemare & Grassino, 1982). Participants were given auditory feedback in the form of a metronome with distinct inspiratory and expiratory tones to assist matching the required duty cycle and visual feedback in real time to target $P_{DI}$. End-tidal partial pressure of $CO_2$ ($P_{ET}CO_2$) was monitored throughout the task (VacuMed model 17630; CA, USA) and in the event of hypocapnia, defined as a decrease in $P_{ET}CO_2$ of >2 mmHg from baseline, a mixture of 5% $CO_2$ (balance $N_2$) was introduced into the inspiratory circuit via a mixing chamber. Heart rate was continuously recorded using a commercially available heart rate monitor (Polar H10, Polar Electro, Kempele, Finland). Dyspnoea intensity was evaluated at rest, during every minute of IPTL, and at task failure. Dyspnoea was evaluated using the 0-10 modified category-ratio scale (Borg, 1982), anchored such that '0' represented "no breathing discomfort at all" and '10' represented "the most

intense breathing discomfort you have ever experienced or could imagine experiencing". Participants were coached to adopt a diaphragmatic breathing pattern to emphasize diaphragm use before beginning the IPTL task (Ramsook *et al.*, 2016). No coaching or encouragement on the respiratory muscle activation pattern was given during the task as it would interfere with our ability to explore the role of respiratory muscle recruitment on diaphragm fatigability. Task failure was defined as either *i*) an inability to reach or maintain the target $P_{DI}$ for the duration of the inspiration or *ii*) the participant felt they were no longer able to continue, and voluntarily terminated the task.

### 4.2.7 Diaphragm neuromuscular function

Cervical magnetic stimulation (CMS) was delivered by a magnetic stimulator (MagStim $200^2$, The MagStim Company Ltd., Whitland, Wales), which stimulated the phrenic nerves as previously described (Ramsook *et al.*, 2021). All stimuli were delivered at functional residual capacity, indicated by the participant giving a hand signal "at the end of a breath out" and later confirmed by checking the $P_{OES}$. To determine if CMS elicited a maximal stimulation of the phrenic nerves, a recruitment curve was developed for each participant by eliciting three unpotentiated twitches at increasing stimulator output intensity (60%, 70%, 80%, 90%, 95%, and 100%). Stimulation was considered maximal if the difference in $P_{DI,TW}$ between maximal and submaximal output intensities was less than the individual coefficient of variation for the measurement. Interpolated twitches were performed in blocks before IPTL, immediately after completing IPTL, as well as 15 min and 30 min post task failure to assess diaphragm fatigue and voluntary activation. Each interpolated twitch manouvre began with a maximal inspiratory effort from functional residual capacity against a semi-occluded mouthpiece apparatus, during which a single twitch was delivered via CMS (superimposed twitch). Following this effort, a control twitch was delivered when the participant

returned to functional residual capacity. This technique has previously been shown to be a reliable means of measuring D-VA (Ramsook *et al.*, 2021). Voluntary activation was calculated as:

$Voluntary\ Activation\ (\%) = \left[1 - \left(\frac{superimposed\ twitch}{control\ twitch}\right)\right] \times 100\%$ (Merton, 1954).

Diaphragm contractile function was assessed by comparing the $P_{DI,TW}$ amplitude of the control twitch before and after IPTL.

### 4.2.8 Statistical Analyses

Participant descriptive characteristics were compared using an independent samples Student's *t*-test. Time to task failure (TTF) and cumulative diaphragm work were first tested for normality using the Shapiro-Wilk test. Both outcomes violated normality and sex differences between TTF and cumulative diaphragm work were tested with the Mann-Whitney U test. A one-way repeated measures ANOVA determined if CMS maximally stimulated the phrenic nerves in each group. $P_{DI,TW}$ during the recruitment curve protocol at submaximal stimulator output intensities were compared against the $P_{DI,TW}$ at 100% stimulator output intensity with Dunnett's *post-hoc* test. A two-way (time and sex) repeated measures ANOVA assessed differences in diaphragm fatigability measures between males and females, and before and after exercise. Main effects were only interpreted with Tukey's post-hoc test when interaction terms were not significant. When significant interaction effects were found, Tukey's *post-hoc* tests were performed at each time point to identify significant differences by sex in accordance with our *a priori* hypotheses. To minimize multiple comparisons and ensure an equal number of observations at each time point, variables during IPTL, including EMG, heart rate and dyspnoea, were compared at baseline, the first minute of IPTL and the last minute of IPTL using a repeated measures ANOVA. Simple linear regression tested the relationship between fatigability measures (D-VA and $P_{DI,TW}$ after IPTL) and select measures including TTF, cumulative diaphragm work, and the average EMG from the final

stage of IPTL. The relationship between voluntary MIP and $P_{DI,TW}$ and D-VA were also assessed with linear regression. All linear regressions were tested using the 'lm' function in R. Statistical analyses were performed in R (v.4.1.0). A $P$ value < 0.05 was considered significant and data are presented as mean ± SD.

## 4.3 Results

### 4.3.1 Participants

A total of 12 healthy males and 15 females were recruited to participate in this study. During the data analysis phase, a total of 6 participants (2 male and 4 females) failed to display a plateau in their individual CMS recruitment curve and were excluded from the analysis. This left 10 males and 11 females included in the final analysis. All participants had pulmonary function within normal limits (Table 4-1).

**Table 4-1. Participant characteristics.**

| | Female | Male |
|---|---|---|
| N | 11 | 10 |
| Age, years | 23 ± 5 | 23 ± 3 |
| Height, cm | 164 ± 7 | 178 ± 6* |
| Mass, kg | 62 ± 9 | 74 ± 9* |
| BMI, kg $m^{-2}$ | 23 ± 2 | 23 ± 3 |
| *Pulmonary function* | | |
| FVC, L (%predicted) | 4.19 ± 0.97 (100 ± 16) | 5.72 ± 0.74* (101 ± 9) |
| $FEV_1$, L (%predicted) | 3.39 ± 0.65 (95 ± 13) | 4.49 ± 0.62* (95 ± 9) |
| $FEV_1$/FVC (%predicted) | 0.82 ± 0.08 (95 ± 8) | 0.78 ± 0.05 (93 ± 6) |
| TLC, L (%predicted) | 5.36 ± 1.14 (96 ± 13) | 7.50 ± 1.04* (101 ± 10) |
| $PI_{MAX}$, $cmH_2O$ (%predicted) | 98 ± 31 (106 ± 33) | 126 ± 27* (97 ± 21) |

Data are presented as mean ± SD. *Abbreviations*: BMI, body-mass index; FVC, forced vital capacity; $FEV_1$, forced expired volume in 1 second; TLC, total lung capacity; $PI_{MAX}$, maximal inspiratory pressure; *, $p < 0.05$. Values are mean ± SD.

### 4.3.2 Inspiratory pressure threshold loading

Baseline $P_{DI,MAX}$ was not different between sexes (F:127 ± 32, M: 139 ± 30 $cmH_2O$, $p = 0.410$). Female participants on average targeted 76 ± 19 $cmH_2O$ and males targeted 83 ± 18 $cmH_2O$ ($p$ =

0.418). At the start of the task, some participants (F: 2; M: 2) had difficulty maintaining the desired $f_b$ (*i.e.,* a $f_b > 15$); however, they were able to correct this as the task proceeded. During the final minute of IPTL, both pressure generation and breathing pattern began to deviate from the desired protocol. Nevertheless, a $TTI_{DI}$ above 0.2 was present from the beginning of the task (F: 0.47 ± 0.08; M: 0.46 ± 0.05) and at the end of the task (F: 0.37 ± 0.06; M: 0.40 ± 0.07) and was maintained throughout the task for each participant. Time to task failure was 15.6 ± 14.8 min in females and 12.4 ± 11.9 min in males ($p$ = 0.557; **Error! Reference source not found.**). Cardiorespiratory variables measured at baseline, during the first minute of IPTL and at peak are presented in Table 4-2. Dyspnoea increased throughout the IPTL task (main effect of time, $p < 0.001$) but was not different between sexes ($p$ = 0.426). At the end of the IPTL task, the cumulative diaphragm pressure was not different between females (28,597 ± 28,912 $cmH_2O$ s) and males (29,032 ± 28,892 $cmH_2O$ s, $p$ = 0.97; Error! Reference source not found.**)**

**Table 4-2. Cardiorespiratory responses during inspiratory pressure threshold loading.**

| | | Baseline | First minute | Peak |
|---|---|---|---|---|
| Time to task failure, min | Female | -- | -- | 15.61 ± 14.84 |
| | Male | -- | -- | 12.44 ± 6.83 |
| Heart rate, beat·min$^{-1}$ * | Female | 68 ± 14 | 80 ± 15 | 93 ± 25 |
| | Male | 65 ± 12 | 89 ± 10 | 90 ± 7 |
| $SpO_2$, % * | Female | 97.9 ± 1.4 | 97.4 ± 0.7 | 96.5 ± 2.4 |
| | Male | 96.9 ± 1.7 | 97.1 ± 0.5 | 95.3 ± 3.0 |
| $P_{ET}CO_2$, mmHg * | Female | 35.1 ± 5.7 | 33.3 ± 4.7 | 32.9 ± 3.9 |
| | Male | 33.9 ± 3.1 | 30.0 ± 2.9 | 35.7 ± 4.7 |
| Dyspnoea, 0-10 scale* | Female | 0.1 ± 0.3 | 1.4 ± 1.6 | 7.3 ± 2.4 |
| | Male | 0 ± 0 | 1.9 ± 2.5 | 8.2 ± 2.3 |
| $f_b$, breath·min$^{-1}$* | Female | 15.0 ± 5.0 | 16.5 ± 1.5 | 20.3 ± 4 |
| | Male | 17.3 ± 4.8 | 16.0 ± 1.4 | 17.3 ± 2 |
| $T_I$, seconds* | Female | 2.14 ± 0.71 | 2.91 ± 0.26 | 2.3 ± 0.50 |
| | Male | 2.18 ± 0.62 | 2.88 ± 0.26 | 2.77 ± 0.33 |
| $T_I/T_{TOT}$ * | Female | 0.51 ± 0.17 | 0.80 ± 0.06 | 0.76 ± 0.08 |
| | Male | 0.59 ± 0.14 | 0.76 ± 0.04 | 0.79 ± 0.05 |

Data presented are mean ± SD. *Abbreviations*: $S_PO_2$, oxyhaemoglobin saturation; $P_{ET}CO_2$, partial pressure of end tidal carbon dioxide; $f_b$, breathing frequency; $T_I$, inspired time; $T_{TOT}$, total breath time, *, main effect of time $p < 0.05$

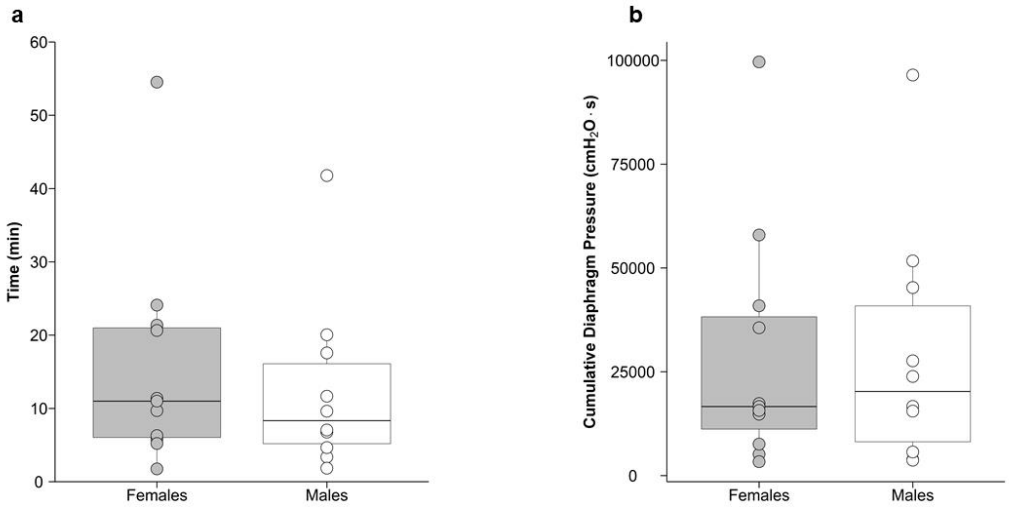


**Figure 4-1. Time to task failure and cumulative diaphragm work after inspiratory pressure threshold loading.**
Box and whisker plot and individual data showing time to task failure (A) and cumulative diaphragm pressure output at task failure. No significant differences between sexes were observed.

### 4.3.3 Inspiratory muscle electromyography

Box and whisker plots along with individual values for inspiratory muscle EMG are presented in Figure 4-2. A main effect of sex was not observed across all inspiratory muscles (all $p > 0.05$); however, a main effect of time was observed in $EMG_{DI}$ ($p < 0.001$), $EMG_{SCA}$ ($p < 0.01$) and $EMG_{SCM}$ ($p < 0.01$). The extra-diaphragmatic inspiratory muscles measured both increased substantially from rest to the first minute of IPTL and continued to increase until task failure. $EMG_{DI}$ increased at the start of the IPTL task and did not change at task failure ($p = 0.325$; Table 4-3).

**Table 4-3. Inspiratory muscle electromyography during inspiratory pressure threshold loading.**

| | | Baseline | First minute | Peak |
|---|---|---|---|---|
| $EMG_{DI}$, %max† | Female | 11.1 ± 6.7 | 51.3 ± 23.1 | 53.8 ± 17.4 |
| | Male | 13.6 ± 8.7 | 64.8 ± 20.0 | 51.7 ± 20.7 |
| $EMG_{SCA}$, %max† | Female | 3.4 ± 2.1 | 42.1 ± 23.1 | 55.7 ± 20.4 |
| | Male | 1.4 ± 1.1 | 43.8 ± 20.7 | 52.8 ± 14.2 |
| $EMG_{SCM}$, %max† | Female | 2.3 ± 1.8 | 34.2 ± 23.8 | 43.3 ± 21.7 |
| | Male | 1.4 ± 1.1 | 32.6 ± 21.0 | 41.2 ± 20.7 |

Data are presented as mean ± SD. *Abbreviations:* $EMG_{DI}$, crural diaphragm electromyography; $EMG_{SCA}$, scalenes electromyography; $EMG_{SCM}$, sternocleidomastoid EMG; †, main effect of time ($p < 0.05$).

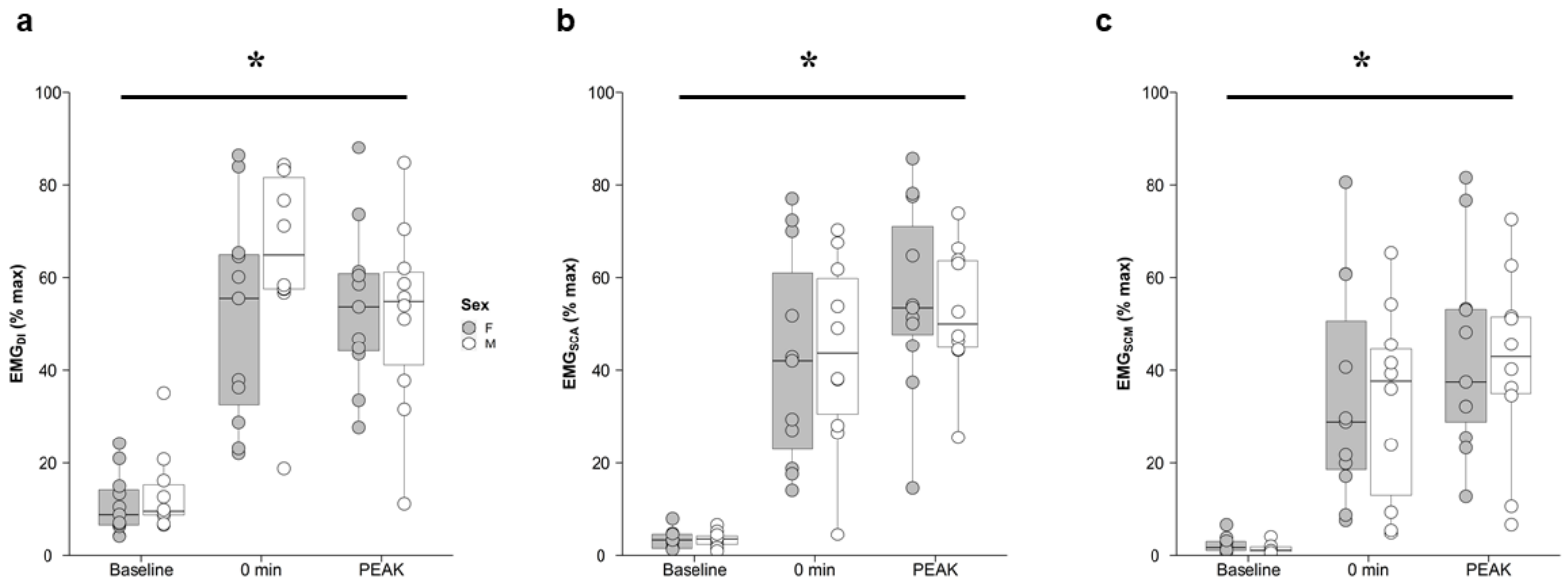


**Figure 4-2. Inspiratory muscle electromyography during inspiratory pressure threshold loading.**
Box-and-whisker plot and individual data of $EMG_{DI}$ (**a**), $EMG_{SCA}$ (**b**) and $EMG_{SCM}$ (**c**) at baseline, and during IPTL. *Abbreviations*: F, females; M, males; $EMG_{DI}$, crural diaphragm electromyography; $EMG_{SCA}$, scalenes muscle electromyography; $EMG_{SCM}$, sternocleidomastoid muscle electromyography; *, main effect of time ($p < 0.05$); IPTL, inspiratory pressure threshold loading.

### 4.3.4 Diaphragm neuromuscular function

In response to increasing stimulator output intensity, there was no significant increase in $P_{DI,TW}$ between 95 and 100% (Figure 4-3). We observed main effects of time ($p < 0.001$) and sex ($p = 0.030$) when examining the change in $P_{DI,TW}$ after IPTL along with a significant interaction effect ($p = 0.007$). Individual $P_{DI,TW}$ responses are presented in Figure 4-4. After the IPTL task, $P_{DI,TW}$ was significantly lower in males compared to females ($p = 0.016$) and this difference persisted after 15 ($p = 0.004$) and 30 minutes of recovery ($p = 0.006$). Both the contraction time (main effect of time, $p < 0.001$) and half-relaxation time (main effect of time, $p = 0.022$) changed after IPTL but did not differ between sexes (both main effect of sex, $p > 0.05$). Contraction time and half-relaxation time shortened in both groups after IPTL but returned to baseline values after 30 minutes (Table 4-4). At baseline, D-VA was not different between sexes (F: 91.0 ± 6.9, M: 90.0 ± 3.8%, $p = 0.683$). When comparing the change in D-VA relative to baseline (Figure 4-4) we observed a main effect of time ($p < 0.001$) but no effect of sex ($p = 0.177$). D-VA declined to 91.4 ± 7.6%baseline in females and 88.2 ± 10.5%baseline within 5 minutes of task failure. In both sexes, D-VA began to approach resting levels after 15 minutes (F: 98.6 ± 6.2%baseline, M: 93.0 ± 5.3%baseline), and 30 minutes of recovery (F: 98.6 ± 3.1%baseline, M: 96.6 ± 7.3%baseline).

D-VA after IPTL normalized to baseline, significantly correlated with TTF ($R^2 = 0.24$; $p = 0.041$;Figure 4-5); however, did not significantly correlate with cumulative diaphragm work ($R^2 = 0.19$; $p = 0.079$). The change in $P_{DI,TW}$ was significantly correlated with cumulative diaphragm work ($R^2 = 0.43$; $p = 0.021$; Figure 4-5), and TTF ($R^2 = 0.40$; $p = 0.030$). Correlations between respiratory muscle activation during the final minute of IPTL and fatigability were also explored. The relationship between $EMG_{SCM}$ and D-VA after IPTL was weak ($R^2 = 0.19$) and not significant ($p = 0.080$). A similar relationship was observed between $EMG_{SCA}$ and D-VA ($R^2 = 0.19$; $p =$

0.077). Neither $P_{DI,TW}$ ($R^2$ = 0.38, $p$ = 0.384) nor D-VA ($R^2$ = 0.046, $p$ = 0.733) correlated with the change in voluntary MIP after IPTL. There was no notable relationship between $EMG_{DI}$ and D-VA ($R^2$ = 0.06; $p$ = 0.409). There were no significant relationships between the change in $P_{DI,TW}$ and $EMG_{DI}$ ($R^2$ = -0.04; $p$ = 0.738), $EMG_{SCA}$ ($R^2$ = 0.02; $p$ = 0.835), or $EMG_{SCM}$ ($R^2$ = 0.00; $p$ = 0.99).

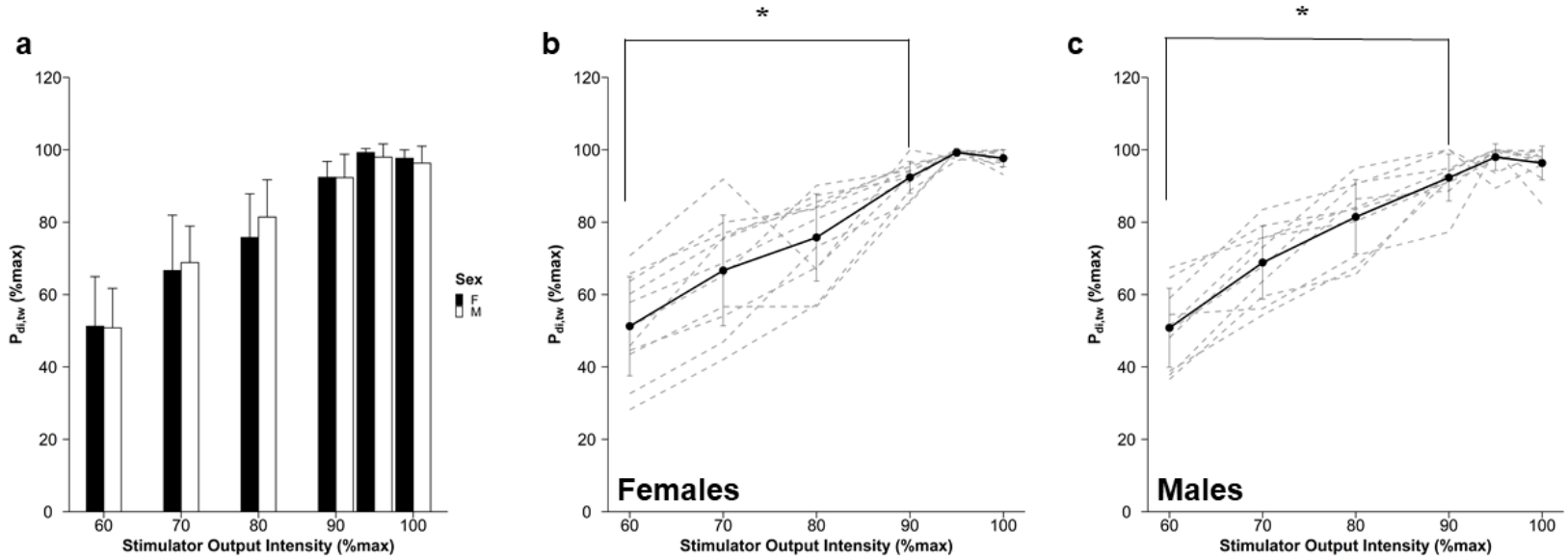


**Figure 4-3. Transdiaphragmatic twitch response to cervical magnetic stimulation.**
Bar graph depicting mean ± SD $P_{di,TW}$ response as a percent of max $P_{di,TW}$ in response to increasing intensities of magnetic stimulator output intensity (**a**). Mean (solid line) and individual responses (dashed lines) to increasing stimulator output intensity in females (**b**) and males (**c**). $P_{DI,TW}$ response did not significantly increase from 95 to 100%. *Abbreviations*: $P_{di,TW}$. transdiaphragmatic twitch amplitude, *, significantly different from 100% ($p < 0.05$).

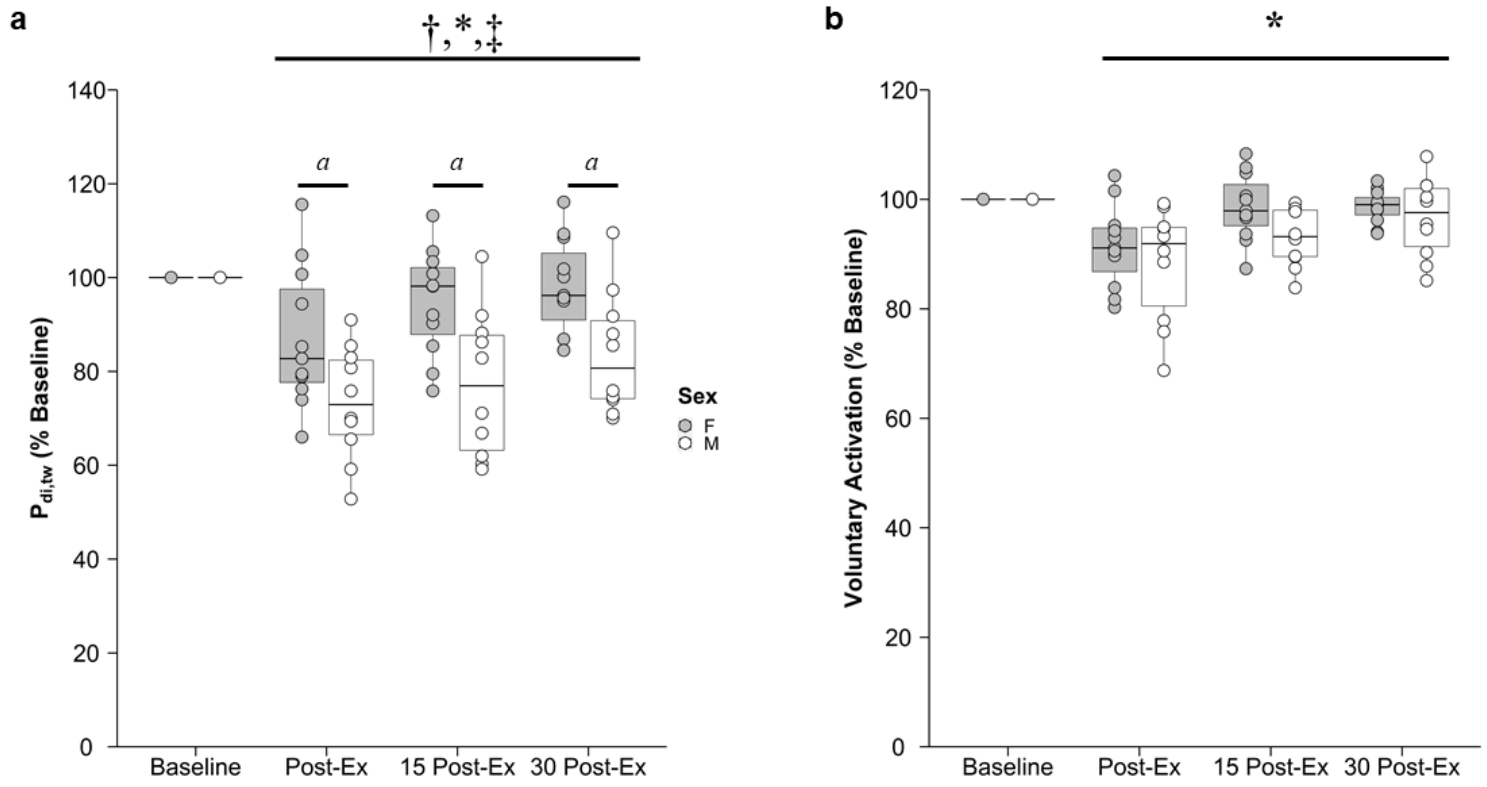


**Figure 4-4. Diaphragm neuromuscular function after inspiratory pressure threshold loading.**
Change in $P_{di,TW}$ (**a**) and change in D-VA (b) after IPTL expressed as a per cent of baseline. *Abbreviations:* $P_{di,TW}$, transdiaphragmatic twitch amplitude; F, females; M, males; †, main effect of sex ($p < 0.05$); *, main effect of time ($p < 0.05$); ‡, interaction effect (sex-time; $p < 0.05$); *a*, different from males at similar time point ($p < 0.05$).

**Table 4-4. Diaphragm fatigability.**

| | *Female* | | | | *Male* | | | |
|---|---|---|---|---|---|---|---|---|
| | Baseline | Post-Ex | 15 Post-Ex | 30 Post-Ex | Baseline | Post-Ex | 15 Post-Ex | 30 Post-Ex |
| $P_{DI,TW}$, %baseline †,*,‡ | 100 ± 0 | 87.1 ± 15.0 | 94.8 ± 11.4 | 98.1 ± 10.4 | 100 ± 0 | 73.3 ± 12.1[a] | 77.3 ± 15.6[a] | 83.8 ±13.0[a] |
| D-VA, %baseline † | 100 ± 0 | 91.4 ± 7.6 | 98.6 ± 6.2 | 98.6 ± 3.1 | 100 ± 0 | 88.2 ± 10.5 | 93.0 ± 5.3 | 96.6 ± 7.3 |
| Contraction time, ms † | 126.7 ± 8.2 | 122.9 ± 12.0 | 124.8 ± 9.2 | 125.4 ± 9.5 | 125.2 ± 9.3 | 118.8 ± 12.7 | 120.0 ± 11.9 | 121.1 ± 10.6 |
| Half-relaxation time, ms † | 72.1 ± 9.2 | 67.3 ± 15.0 | 68.8 ± 12.2 | 71.6 ± 14.1 | 69.0 ± 10.3 | 64.3 ± 12.9 | 66.8 ± 10.2 | 69.2 ± 8.2 |
| CMAP amplitude, mV | 1.8 ± 0.6 | 1.6 ± 0.7 | 1.7 ± 0.5 | 1.8 ± 0.6 | 1.7 ± 0.9 | 1.4 ± 0.7 | 1.5 ± 0.7 | 1.6 ± 0.8 |
| CMAP onset, ms | 7.3 ± 1.3 | 6.7 ± 1.9 | 7.5 ± 1.2 | 7.6 ± 1.5 | 7.9 ± 0.9 | 7.6 ± 1.6 | 8.2 ± 0.8 | 8.0 ± 1.1 |
| CMAP duration, ms | 20.9 ± 3.0 | 19.9 ± 5.2 | 21.0 ± 2.6 | 22.5 ± 3.9 | 20.5 ± 1.9 | 20.0 ± 4.1 | 20.1 ± 3.0 | 19.9 ± 2.9 |

Data are presented as mean ± SD. *Abbreviations:* $P_{DI,TW}$, transdiaphragmatic twitch amplitude; D-VA, diaphragm voluntary activation; CMAP, compound muscle action potential; †, main effect of time ($p < 0.05$); *, main effect of sex ($p < 0.05$); ‡, significant interaction effect ($p < 0.05$), [a], significantly different from females at same time point ($p < 0.05$).

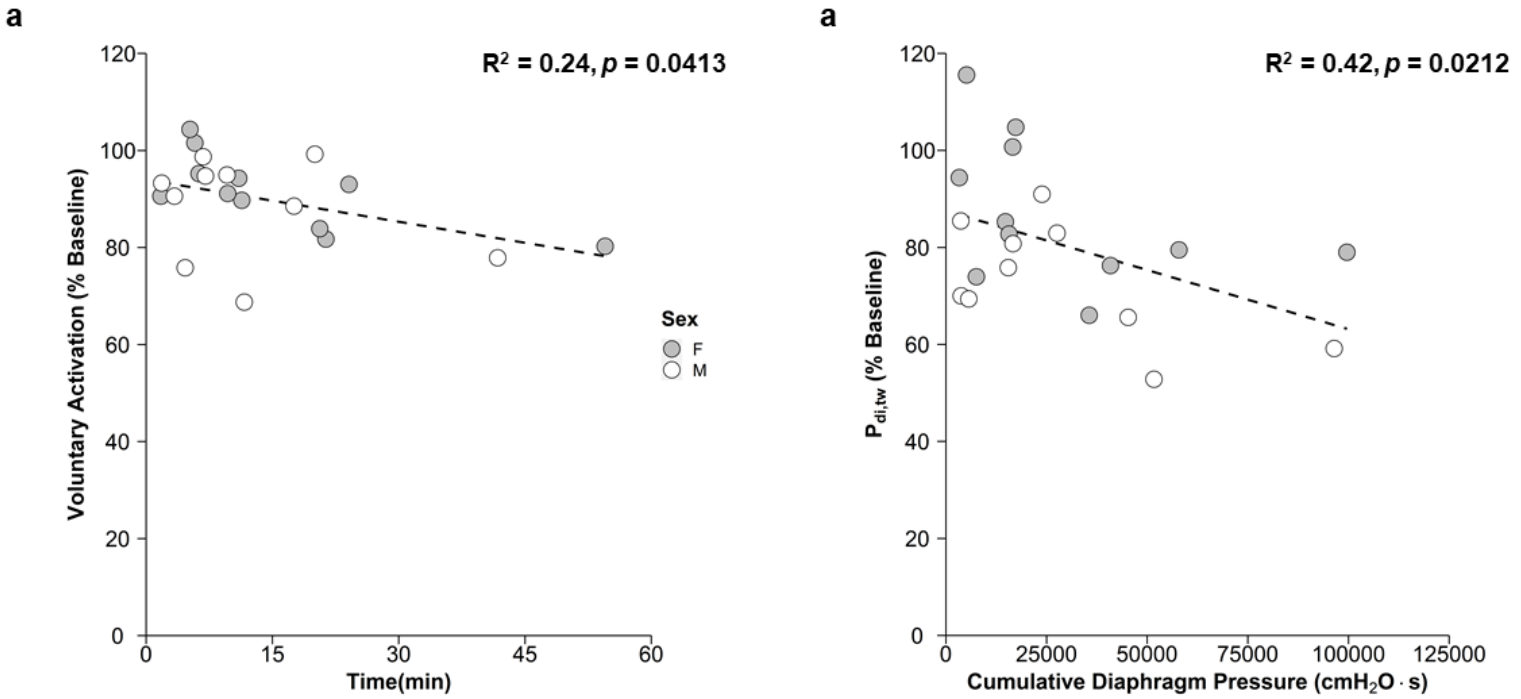


**Figure 4-5. Relationship between diaphragm fatigability and inspiratory pressure threshold loading.**
Scatter plot showing relationship between D-VA after IPTL and TTF (**a**) and $P_{DI,TW}$ after IPTL and cumulative diaphragm pressure output (**b**). Dashed line is a linear regression representing the relationship between the associated variables. *Abbreviations:* $P_{di,TW}$, transdiaphragmatic twitch amplitude; D-VA, diaphragm voluntary activation; IPTL, inspiratory pressure threshold loading; TTF, time to task failure.

## 4.4 Discussion

### 4.4.1 Main findings

The present study endeavoured to determine if D-VA would decline after isolated, fatiguing inspiratory work and to determine if the change D-VA would differ between sexes. The main findings of this study suggest that both contractile and neural mechanisms contribute to diaphragm fatigability in males and females, measured by the change in $P_{DI,TW}$ and D-VA, respectively, during isolated respiratory muscle loading. However, contrary to our hypothesis, we did not observe a difference in the decline in D-VA between sexes. The change in D-VA was significantly correlated with TTF, whereas the change in $P_{DI,TW}$ was correlated with total work performed by the diaphragm. Collectively, these findings suggest that changes in contractile and neural mechanisms contributing to diaphragm fatigability may be affected by different pathways.

### 4.4.2 Inspiratory pressure threshold loading

The IPTL task aimed to induce diaphragm fatigue through limiting blood flow to the diaphragm. In canines, when the $TTI_{DI}$ exceeds 0.2, blood flow to the diaphragm is impeded (Bellemare *et al.*, 1983). The $TTI_{DI}$ of the present study was designed to be approximately 0.42, thereby exceeding the 0.2 $TTI_{DI}$ threshold and creating a task that would be unsustainable for long periods of time. Similar protocols have been used before to induce diaphragmatic fatigue in humans (Welch *et al.*, 2018b; Boyle *et al.*, 2020). Both males and females targeted a similar $P_{DI}$ during IPTL. While males had greater overall inspiratory muscle strength, as measured through maximal inspiratory pressure, the average $P_{DI,MAX}$ was not significantly different between males and females in the present study (F:127 ± 32, M: 139 ± 30 $cmH_2O$, $p$ = 0.410). The duration of the IPTL was, on average, over 3 minutes longer in females compared to males; however, the responses were highly variable and not significantly different between groups. The similar task duration and absolute

load on the diaphragm resulted in similar total cumulative diaphragmatic work in males and females. There is evidence that when matched for strength, females are able to perform an exercise task for a longer duration compared to males. For example, when strength-matched males and females performed intermittent elbow flexor exercise at 50% of maximal voluntary contraction, female participants were able to sustain exercise significantly longer than males (Hunter *et al.*, 2004). In contrast, we observed statistically similar TTF between sexes despite performing similar absolute work. The discrepancy between studies could be related, at least in part, to differences in the muscle of interest and the type of fatiguing task used. In addition to having a fixed criteria for task failure based on the inability to perform inspirations to the required time or pressure, we also allowed participants to voluntarily terminate the task if they chose to. It is possible that motivation could have influenced the TTF in the present study. No verbal encouragement was offered to participants during the IPTL task to maximize task standardization and avoid any bias from the experimenter who was aware of the study hypotheses. In the present study, three female participants and one male participant voluntarily ended the IPTL task rather than approach task failure using our objective criteria. However, excluding these participants did not result in a significantly different TTF or cumulative diaphragmatic work between sexes. The observed variability in IPTL may be related to the task itself. While the goal of the IPTL task was to induce diaphragm fatigue, the task does not exclusively target the diaphragm. The activation of extra-diaphragmatic inspiratory muscles could also influence TTF.

### 4.4.3 Inspiratory muscle electromyography

When performing bouts of inspirations that require the participant to generate substantial negative pressures, such as during inspiratory muscle training, participants will often preferentially recruit extra-diaphragmatic inspiratory muscles (Ramsook *et al.*, 2016). In an effort to encourage

participants to target the diaphragm during IPTL, they were instructed to engage in a diaphragmatic breathing pattern to reach their target $P_{DI}$. They were given no further instruction during the IPTL to explore whether distinct respiratory muscle recruitment patterns emerged between males and females. During resting breathing, the intercostal muscles of the rib cage contribute more to breathing in females compared to males, possibly owing to the shape of the rib cage (Bellemare *et al.*, 2003). Moreover, during whole body exercise, females tend to engage extra-diaphragmatic inspiratory muscles, such as the scalenes and sternocleidomastoids, to a greater degree than their male counterparts (Mitchell *et al.*, 2018; Molgat-Seon *et al.*, 2018a). We did not make a similar observation in the present study as $EMG_{SCM}$ and $EMG_{SCA}$ were similar between males and females. Males appeared to have a greater activation of the diaphragm, evidenced by a numerically greater $EMG_{DI}$ compared to females at the beginning of IPTL; however, by the end of the task, $EMG_{DI}$ values, along with $EMG_{SCA}$ and $EMG_{SCM}$ were nearly the same between sexes. The discrepancy between the present study and our previous work (Mitchell *et al.*, 2018; Molgat-Seon *et al.*, 2018a) is likely attributable to differences in the task (*i.e.*, IPTL vs. the hyperpnoea of whole-body exercise). IPTL requires that participants generate and sustain relatively large negative pressures at a fixed breathing frequency and duty cycle that are not replicated during exercise or other activities of daily living. However, IPTL has the advantage of isolating the respiratory muscles compared to whole body exercise, where locomotor and respiratory muscles must compete for a finite cardiac output. While no significant correlations between respiratory muscle activation and fatigability were observed, there were trends that suggest D-VA may be related to the activity of extra-diaphragmatic inspiratory muscles. It is possible that as the diaphragm loses its ability to contract through neural mechanisms, the extra-diaphragmatic respiratory muscles are activated to a greater degree to achieve the desired negative pressure. Another potential explanation is that

individuals preserve diaphragm function by using their extra-diaphragmatic inspiratory muscles to generate negative pressure to a greater degree. It is possible that the present study was underpowered to explore the relationship between D-VA and inspiratory muscle activity, and future studies are warranted to investigate the role of respiratory muscle activation and fatigability.

### 4.4.4 Diaphragm neuromuscular function

There was a loss in contractile function of the diaphragm following IPTL, as evidenced by a decrease in $P_{DI,TW}$. We found the decrease in $P_{DI,TW}$ to be moderately and significantly correlated to the cumulative work performed by the diaphragm. This is similar to the strong relationship observed between the decrease in $P_{DI,TW}$ and cumulative diaphragm work during exercise reported previously (Archiza *et al.*, 2018). It is worth noting that our observation was independent of sex whereas the previous report only included male participants. In the present study, the decrease in $P_{DI,TW}$ was greater in males compared to females despite performing similar amounts of relative and absolute diaphragmatic work, providing further evidence to support greater diaphragm fatigue resistance, as it relates to contractile function, in females compared with males. This finding is contrary to a previous study conducted where male and female participants, purposefully matched for absolute diaphragm strength based of $P_{DI,MAX}$, performed five minutes of IPTL in normoxia at a fixed intensity and the decrease in $P_{DI,TW}$ was similar between sexes (Archiza *et al.*, 2021). It is possible this difference can be explained by the methods used to measure $P_{DI,TW}$. While Archiza *et al*., used CMS-evoked twitches to measure $P_{DI,TW}$, just as the present study, it is unknown if they included participants who did not exhibit maximal stimulation of the phrenic nerves. If the phrenic nerves were not maximally stimulated, it is possible the $P_{DI,TW}$ response to IPTL could have been underestimated. Nevertheless, these disparate results highlight the fact that task failure, and overall

performance fatigability, are not governed by a single mechanism, but rather a host of factors, which work together to influence performance.

D-VA decreased in both males and females after IPTL. The decrease in D-VA was significantly, albeit weakly, correlated with TTF when data were pooled together. A decrease in voluntary activation after isolated respiratory work has been reported before after both inspiratory (Luu *et al.*, 2020) and expiratory efforts (McKenzie *et al.*, 1992) in male participants. To our knowledge, no previous studies have reported changes in D-VA in females after IPTL. Interestingly, we did not observe a sex difference in the change in D-VA after IPTL. This differs from our previous findings using whole body exercise where males experienced a greater loss of D-VA after exercise than females (*Chapter 3*). A lack of sex difference in D-VA after IPTL could be explained by task specificity. For example, while whole body exercise can result in a loss of contractile function, mimicking the hyperpnoea of exercise does not result in a similar decrease in $P_{DI,TW}$ (Babcock *et al.*, 1995). Thus, feedback from locomotor muscles may play a role in controlling D-VA during fatiguing tasks. Blocking afferent feedback via intrathecal fentanyl injection preserved quadriceps voluntary activation compared to exercise where afferent feedback was undisturbed (Sidhu *et al.*, 2017). Perhaps without the added stimulus of additional sympathetic activity from the locomotor muscles, as is present during exercise, the total sympathetic activity was not sufficient to promote a sex difference in D-VA after IPTL. We did not show a difference in heart rate at the beginning or end of IPTL. While heart rate is not a replacement for muscle sympathetic nerve activity, it does provide some indirect evidence that sympathetic responses were potentially similar between males and females. However, we acknowledge that this remains speculative and requires further research with more direct measures muscle sympathetic activity.

### 4.4.5 Methodological considerations

The phrenic nerves can be stimulated using electrical stimulation, transcranial magnetic stimulation, and CMS. One major difference between electrical stimulation and CMS is the output intensity of the stimulator. CMS is more likely to result in submaximal phrenic nerve stimulation compared with electrical stimulation, which makes it difficult to interpret the effects of any fatiguing task on neuromuscular function. We aimed to avoid this limitation by only including participants who showed a plateau in $P_{DI,TW}$ in response to CMS.

A validated measure of sympathetic activity would help explore the relationship between the inspiratory muscle metaboreflex and the change in D-VA. It follows that a blunted inspiratory muscle metaboreflex could attenuate the increase in sympathetic activity during IPTL, and thus mitigate the decline D-VA. We were unable to measure sympathetic activity during IPTL via muscle sympathetic nerve activity or a surrogate such as mean arterial pressure. Future studies are needed to determine if there is a relationship between sympathetic activity and D-VA.

We did not limit testing female participants to a specific phase of the menstrual cycle and did not exclude females currently taking oral contraceptives or using an intrauterine device. While menstrual cycle phase may have an influence on baseline voluntary activation of the knee extensor muscles, there does not appear to be an effect on the change in voluntary activation of the knee extensors after a fatiguing task (Ansdell *et al.*, 2019b). It is unknown if these findings extend to D-VA as well. Moreover, any role of oral contraceptive or intrauterine device use on D-VA is also unknown. Future studies are needed to appreciate how the menstrual cycle may influence D-VA and diaphragm contractile function. Furthermore, the participants in the present study were all young, healthy, never smokers and future work is required to determine if the findings from the present study hold true in other populations.

## 4.5 Conclusions

In summary, we have shown that D-VA decreases after a single bout of fatiguing inspiratory work in both males and females. Both males and females experienced a similar decrease in D-VA, despite males showing a greater loss in $P_{DI,TW}$ compared to females after performing IPTL at similar relative and absolute intensities. These findings support some previous findings that the female diaphragm is less fatigable than males; however, our results also suggest that the loss in contractile function and voluntary activation are governed by unique processes. Finally, our observed sex similarity in the decrease in D-VA is contrary to earlier findings of a sex difference in D-VA after high-intensity whole-body exercise. Together, these findings reiterate fatigability is task-specific with potentially different underlying mechanisms. Future studies are needed to determine the role of sympathetic activity in the loss of D-VA after fatiguing tasks and to further explore if different extra-diaphragmatic inspiratory muscle recruitment patterns can influence diaphragm fatigue. Finally, future studies are needed to understand how whole-body exercise affects diaphragm fatigability differently than isolated inspiratory work.

# Chapter 5: Conclusions

## 5.1 Overall summary

The overall objectives of this doctoral thesis were to provide new insights into the neuromuscular function of the diaphragm by investigating neural contributions to diaphragm fatigue and explore any sex differences therein. The specific objectives of this thesis were threefold. First, we sought to determine if D-VA could reliably be measured from CMS-evoked twitches. Second, we examined whether D-VA contributed to diaphragm fatigability after whole body exercise. Finally, we investigated whether D-VA contributed to diaphragm fatigability after IPTL. Moreover, for the second and third objectives, we explored any potential sex difference in the change in D-VA after each task. Performance fatigability is characterized by an activity-induced loss in the force generating capacity that contributes to task failure. In the laboratory setting, task failure can be quantified as a decrease in maximal strength or the time to failure of a submaximal task (Hunter, 2016). The processes that contribute to a muscle's ability to generate force, and therefore potential sites of fatigue, can be broadly divided into contractile and neural mechanisms. The majority of the existing literature on the topic of diaphragm fatigue has focused on assessing the contractile function of the diaphragm (Mador *et al.*, 1993; Taylor & Romer, 2009; Guenette *et al.*, 2010; Archiza *et al.*, 2018; Welch *et al.*, 2018b; Archiza *et al.*, 2021). Furthermore, sex differences in the structure and function of the human respiratory system are well reported (Sheel *et al.*, 2004; Harms & Rosenkranz, 2008; Sheel *et al.*, 2016; Molgat-Seon *et al.*, 2018b). Additionally, there are well reported sex differences in fatigability (Hunter, 2014). In order to provide proper insight into fatigability, an evaluation of potential sex differences in required. The studies of this thesis were designed to address the knowledge gap regarding neural mechanisms that contribute to diaphragm fatigability.

In *Chapter 2* it was determined that D-VA can be reliably measured in young, healthy humans. After establishing that D-VA measurements are reliable *Chapters 3* and *4* applied this technique to determine if D-VA contributes to fatigability after a functional and fatiguing task, respectively. *Chapter 3* identified that D-VA decreases after high intensity exercise, which was more pronounced in males than females. This has reinforced earlier findings that suggest the female diaphragm is more resistant to fatigue compared to males (Guenette *et al.*, 2010; Welch *et al.*, 2018a), and extended those findings to suggest that females are more resistant to diaphragm fatigue at both neural and contractile sites of fatigue. *Chapter 4* investigated how D-VA changes after IPTL, an isolated fatiguing inspiratory task. After IPTL, neural mechanisms contribute to diaphragm fatigue, as evidenced by a decrease in D-VA. Decreases in D-VA appeared to be related to the total time one spent on the IPTL task whereas decreases in $P_{DI,TW}$ were related to cumulative work performed by the diaphragm. Notably, we did not observe a sex difference in D-VA after IPTL despite a males exhibiting a greater decline in contractile function, measured through changes in $P_{DI,TW}$.

## 5.2 Significance

The findings of this thesis are significant for several reasons. The results of C*hapter 2* addresses a growing concern surrounding the reproducibility of methods in physiology (Wagner, 2017). *Chapter 2* provides evidence that CMS-evoked twitches can reliably measure D-VA both within- and between-sessions. This supports future studies of diaphragm fatigability integrating D-VA measurements into their study design to provide a more comprehensive examination of diaphragm fatigue. The studies presented in *chapters 3* and *4* address a knowledge gap to explore if D-VA contributes to diaphragm fatigue. Moreover, the results of *chapters 3* and *4* provide an examination of D-VA between sexes in humans. Together, the findings in this thesis represent a step forward

in our understanding of human physiology and provide a foundation for future studies to explore the mechanisms through which diaphragm fatigability affects exercise.

### 5.3 Strengths and limitations

While specific strengths and limitations for each individual study have been addressed in their respective chapter, this section will focus on overall strengths and limitations of the thesis as a whole.

A strength of this thesis was our decision to exclude participants who were not maximally stimulated by CMS. Our decision to only include participants who showed a maximal stimulation of the phrenic nerves via CMS was an effort to improve the rigour with which we measured both $P_{DI,TW}$ and D-VA. Participants who did not show maximal stimulation were at risk of underestimating their diaphragm neuromuscular function and could confound the results of the study.

When comparing sex differences and fatigability, one must account for differences in size and absolute muscle force. This is pertinent to this thesis as there is an expected sex difference in respiratory muscle strength, wherein males would be expected to have greater respiratory muscle strength compared to females (Black & Hyatt, 1969). We addressed these individual and sex differences by normalizing fatigability outcomes to the baseline measurement of the individual. This puts the emphasis on the change in D-VA or $P_{DI,TW}$ for each individual.

The female participants included in all three studies presented in this thesis were not limited to testing during a specific phase of the menstrual cycle. We believe this strengthens the generalizability of the study to the female population as females do not limit exercise and training to specific phases of their menstrual cycle. There are no available studies that have investigated whether or not menstrual cycle phase affects the contractile function or voluntary activation of the

diaphragm; however, menstrual cycle phase does not appear to affect changes in voluntary activation of the knee extensor muscles in response to exercise (Ansdell *et al.*, 2019b). Given the negligible changes to voluntary activation between-session, our decision to test females at any given phase of their menstrual cycle, and those using oral contraceptives or intrauterine devices would not influence the results of this thesis.

The primary limitation of the studies performed in this thesis concerns the generalizability of the findings. All participants in this thesis were young and healthy, therefore the findings may not apply to other populations, such as older adults and those living with chronic diseases. A technical limitation pertinent to *chapters 3* and *4* is the absence of muscle sympathetic nerve activity. A measure of muscle sympathetic nerve activity would provide insight into potential mechanisms that influence changes in D-VA, as sympathetic feedback has been shown to affect voluntary activation (Sidhu *et al.*, 2017). Finally, the sample size of the studies in *chapters 3* and *4*, while adequately powered for the primary outcome, may have been underpowered for the exploratory outcomes. With a greater sample size, it would be possible to perform sub-group analyses to further investigate fatigability.

### 5.4 Future directions

While this thesis has addressed several important questions regarding fatigability of the diaphragm, several questions remain. Moreover, the results of this thesis served to generate more questions to comprehensively examine the role of the respiratory muscles during exercise, the inspiratory muscle metaboreflex, and the interactions between the respiratory muscles and the locomotor muscles. The precise mechanisms that govern the decrease in D-VA are still unknown. While it is possible that sympathetic feedback from the muscles, both respiratory and locomotor, may contribute to changes in D-VA, this remains speculative at this moment. As alluded to in the

previous section, the results of this thesis are specific to young, healthy males and females. Future studies are needed to investigate how diaphragm fatigue, and D-VA specifically, is affected by the healthy ageing process as well as those with chronic debilitating conditions. Investigating how different tasks contribute to diaphragm fatigability is another interesting future pursuit for research. In the current thesis, we observed a sex difference in the decrease in D-VA during exercise, where the decrease in D-VA was greater in males compared to females, however; after IPTL, the decrease in D-VA was similar between the sexes. Perhaps there was some interaction between the locomotor and respiratory muscles during exercise that exacerbated the decrease in D-VA in males compared to females. While answering these questions will require a substantial amount of work and specialized experimental design, the findings in this thesis form a solid foundation, upon which future research can build.

## 5.5 Conclusions

The aim of this thesis was to address the gap in knowledge concerning neural mechanisms that contribute to diaphragm fatigability in males and females. We established reliability metrics for measuring D-VA, a technique that can be used to investigate the neural contribution to diaphragm fatigability (*chapter 2)*. We found that D-VA decreases after high intensity, whole-body exercise (*chapter 3*) and isolated inspiratory work (*chapter 4*), thus determining neural mechanisms do contribute to diaphragm fatigability. Furthermore, we noted that after exercise, the decrease in D-VA is greater in males compared to females, however; this sex difference was not present after isolated inspiratory work. Future work is required to identify the mechanisms that influence D-VA and determine how these mechanisms vary between tasks such as exercise and inspiratory pressure threshold loading in males and females.

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