Sustained Hypoxia: Respiratory Muscles and Ventilation

Ji, Suk Joon

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Sustained Hypoxia: Respiratory Muscles and Ventilation

by

Suk Joon Ji

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE
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Abstract

The focus of this thesis was to systematically examine the effects of sustained hypoxia on ventilation and the function of the primary breathing muscles in an intact awake mammal. Successful execution of this thesis work relied upon the chronically instrumented awake canine model to test the physiologic questions and hypotheses, collaborative team work to execute the experiments, as well as development of appropriate software tools to analyze the immense physiologic dataset amassed over the years. This thesis is organized as a series of related investigative projects overarching the central theme of sustained hypoxia.

Ventilation in awake canines exhibited the characteristic biphasic pattern during sustained hypoxia, like that of humans and other mammals, with an initial peak followed by a subsequent decline - “roll-off” or hypoxic ventilatory decline (HVD) - to a lesser intermediate plateau. Our findings directly contest the longstanding canine controversy that ventilation does not roll-off in this species. Examination of the principle inspiratory muscle, the diaphragm, during sustained hypoxia revealed a biphasic contraction and neural activation of the costal and crural, with differential segmental function during initial and sustained hypoxia. Persistent effects of hypoxia caused a dramatic loss of the contractile output of the primary inspiratory chest wall muscle, the parasternal intercostal, accompanied by a decline in EMG activity. Immediate response to hypoxia elicited a marked recruitment of the primary expiratory abdominal muscle, the transversus abdominis, however, sustained hypoxia caused the initial acute expiratory abdominal contribution to be nearly abolished. Excitatory and inhibitory influence of hypoxia may largely account for the past discordance of expiratory activity with hypoxia in mammals. Attenuation of central drive appeared to persist following sustained hypoxia affecting the primary respiratory muscles.

We conclude that sustained hypoxic ventilatory roll-off is a universal mammalian characteristic without exception for canines. Attenuation of neural drive with sustained hypoxia is a widespread central phenomenon significantly impacting the primary
respiratory muscles of the diaphragm, the chest wall and the abdominal wall, in a distinct and differential manner.
Acknowledgements

This work relied on the collective efforts of many individuals, both current and past, who have contributed to the success of the projects embodied within this thesis. I would like to take a moment to acknowledge those individuals who have lent their hand towards this scientific endeavor.

First and foremost, I would like to extend my sincere gratitude to my research supervisor, Dr. Paul A. Easton, without whom none of this work would have been possible. For the sake of briefness I won't go into any details, but thank-you for allowing me the opportunity to pursue graduate training in physiology and medicine as a computer scientist, which at that time, the two disciplines seemed worlds apart. Despite the challenges of the transition and the academic journey, it was truly memorable and rewarding. Your unwavering support, mentorship and guidance over the years has been inspiring and influential, allowing me to learn a great deal about science and research, as well as to grow both personally and professionally. I look forward to sharing more exciting endeavors ahead.

I would like to also extend my appreciation to my supervisory committee. Dr. Jim E. Fewell, a distinguished physiologist, who's course, MDSC 604, served as an important stepping stone in making my transition into graduate studies in medicine possible. Thank-you, Dr. Fewell, for sharing your physiology/research expertise and insights, and providing on-going support and encouragement. Dr. Paul J.E. Boiteau (former) and Dr. Christopher J. Doig (current), as Heads of the Department of Critical Care Medicine, who embody great leadership and clinical/scientific acuity, have been instrumental in sharing clinical and physiological insights, and have played a supportive role in my training from its inception. Thank-you Dr. Doig for your constructive feedback and guidances that were right on the mark, and for challenging me with tantalizing statistical questions to gain a deeper appreciation and understanding for statistics. Thank-you Dr. Boiteau for your engaging physiological/clinical questions, appraisals and advising me of the importance
of starting and finishing one's work to completion. I am grateful for the years of commitment shared by my committee members.

An expression of thanks also goes out to Dr. Renaud Leguillette for supporting the committee with his clinical/basic science knowledge in respiratory physiology. A special appreciation to Dr. Sabah N.A. Hussain (McGill University) for acting as the external examiner and sharing his wealth of expert clinical/basic science knowledge pertaining to the muscles of respiration. A very special thanks to my research colleague and very good friend, Ms. Jenny V. Jagers - who was my first teacher in the lab, and also generously acts as a professional reviewer and editor to meticulously go over my data, work and writing. Jenny, this graduate work would not have been possible without having your enthusiasm for science/research and steadfast help and support. I'm very blessed and grateful to have taken this academic path with you, and I look forward to many more exciting journeys ahead.

Also a special thanks to Dr. Naoyuki Fujimura and Dr. Tetsunori Ikekami, clinical research fellows from Japan, for their research support and help in the initial launching of this thesis project. The overlapping years with you two were truly enjoyable as a new graduate student. An extended thanks to Dr. Ronald S. Platt for sharing his time, knowledge and expertise; and Dr. Harvey G. Hawes for initial guidance to the data acquisition system. Acknowledgement also goes out to the surgeons, Dr. Teresa Keiser, Dr. John Kortbeek and Dr. Bruce Rothwell, for the implantation of the sonomicrometry transducers and EMG electrodes. And, a particular appreciation and thank-you goes out to Mrs. Leslie Jacques, Dr. Masato Katagiri and Dr. Maros Pazej for their excellent experimental support and animal care. The prior work done by previous lab students and techs, including the vivarium staff, has been also helpful, so I extend my thanks. Of course, a huge appreciation is reserved for our friendly and respected four legged companions, who's contribution to science is immeasurable.
Last but not least, friends, mentors and family were also significant and instrumental throughout the years of graduate studies and training. Thank you everyone for extending your support!
Dedication

This thesis is dedicated to my family, including my best friend and partner Ji-hye, and to the research community in pursuit of advancing science and medicine.
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<td>Patm</td>
<td>atmospheric pressure</td>
</tr>
<tr>
<td>$T_b$</td>
<td>body temperature</td>
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<tr>
<td>°C</td>
<td>degrees Celsius</td>
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<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>central nervous system</td>
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<td>DRG</td>
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<td>ventral respiratory group</td>
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<td>pontine respiratory group</td>
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<td>CO$_2$</td>
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<td>$F_{\text{ICO}_2}$</td>
<td>fraction of carbon dioxide concentration</td>
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<tr>
<td>$P_{\text{A}CO_2}$</td>
<td>partial pressure of alveolar carbon dioxide</td>
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<td>PCO$_2$</td>
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<td>PaCO$_2$</td>
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<td>ETCO$_2$</td>
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<td>partial pressure of end tidal carbon dioxide</td>
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<td>partial pressure of alveolar oxygen</td>
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<td>PO$_2$</td>
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<td>SpO$_2$</td>
<td>pulse oximeter oxygen saturation</td>
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<tr>
<td>$P_{\text{ET}O_2}$</td>
<td>partial pressure of end tidal oxygen</td>
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<td>VO$_2$</td>
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<td>mmHg or Torr</td>
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<tr>
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<td>N$_2$</td>
<td>nitrogen</td>
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<tr>
<td>--------</td>
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</tr>
<tr>
<td>kg</td>
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<tr>
<td>mm</td>
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</tr>
<tr>
<td>cm</td>
<td>centimeter(s)</td>
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<tr>
<td>mm/microsecond</td>
<td>millimeter(s) per microsecond</td>
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<tr>
<td>m/sec</td>
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</tr>
<tr>
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<td>liter(s) per minute</td>
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<tr>
<td>l/sec or l/s</td>
<td>liter(s) per second</td>
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<tr>
<td>mmHg/min</td>
<td>millimeter of mercury per minute</td>
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<tr>
<td>cmH$_2$O/L/s</td>
<td>centimeter of water per liter per second</td>
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<td>sec or s</td>
<td>second(s)</td>
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<tr>
<td>MHz</td>
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</tr>
<tr>
<td>volt(s)</td>
<td>electrical potential difference or voltage</td>
</tr>
<tr>
<td>PC</td>
<td>personal computer</td>
</tr>
<tr>
<td>AC</td>
<td>alternating current</td>
</tr>
<tr>
<td>A/D</td>
<td>analog to digital</td>
</tr>
<tr>
<td>V$_1$</td>
<td>minute ventilation, inspired</td>
</tr>
<tr>
<td>V$_T$</td>
<td>tidal volume</td>
</tr>
<tr>
<td>f$_R$</td>
<td>frequency</td>
</tr>
<tr>
<td>T$_{TOT}$</td>
<td>total time of the respiratory cycle</td>
</tr>
<tr>
<td>T$_i$</td>
<td>inspiratory time</td>
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<tr>
<td>T$_E$</td>
<td>expiratory time</td>
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<tr>
<td>V$_T$/T$_i$</td>
<td>mean inspiratory flow</td>
</tr>
<tr>
<td>T$<em>i$/T$</em>{TOT}$</td>
<td>inspiratory fraction of respiration</td>
</tr>
<tr>
<td>COS</td>
<td>costal diaphragm segment</td>
</tr>
<tr>
<td>CRU</td>
<td>crural diaphragm segment</td>
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PARA  parasternal intercostal
TA    transversus abdominis
L_o  optimal muscle length
L_BL resting length of the muscle
%L_BL percent change from baseline resting length
SHORT shortening
ECG  echocardiogram
SONO sonomicrometry
EMG  electromyography
Mavg  EMG integrated moving average electromyography
EMG_DIFF baseline to peak difference in electromyography
MUAP(s) motor unit action potential
PIIA  postinspiratory inspiratory activity
PEEA  postexpiratory expiratory activity
P  p-value of a statistical test
NS  non significance
±SD plus and minus the standard deviation
PAH  pulmonary arterial hypertension
HPV  hypoxic pulmonary vasoconstriction
VA  ventilatory acclimatization
VD  ventilatory deacclimatization
HD  hypoxic desensitization
HVD  hypoxic ventilatory decline
HVR  hypoxic ventilatory response
GABA  gamma-Aminobutyric acid
CMRO_2 cerebral metabolic rate of oxygen
NAD+ oxidized nicotinamide adenine dinucleotide
NADH reduced nicotinamide adenine dinucleotide
ETC  electron transport chain
CBF  cerebral blood flow
ECG  electrocardiogram
QRS deflections of ECG: Q-wave, R-wave, S-wave
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<td>functional residual capacity</td>
</tr>
<tr>
<td>PEEP</td>
<td>positive end-expiratory pressure</td>
</tr>
<tr>
<td>UAW</td>
<td>upper airway</td>
</tr>
<tr>
<td>LAW</td>
<td>lower airway</td>
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<tr>
<td>&gt;</td>
<td>greater than</td>
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<td>&lt;</td>
<td>less than</td>
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<td>=</td>
<td>equal to</td>
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<td>approximately</td>
</tr>
<tr>
<td>BASE</td>
<td>baseline room air</td>
</tr>
<tr>
<td>PEAK</td>
<td>initial hypoxia</td>
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<td>PLATEAU</td>
<td>final hypoxia</td>
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Background

Introduction

This thesis was born out of the inquiry and speculation of how we breathe when hypoxia is sustained. Specifically, it is the temporal dynamic changes in ventilation and function of the primary breathing muscles in the context of hypoxia that is unresolved and prolonged that is the focus of this work.

The physiologic effects of hypoxia on respiration have been of longstanding interest among scientists and clinicians given that insufficiency of oxygen is widely implicated in health and disease. For instance, development of hypoxia is a common feature in the physiologic setting of high altitude (Powell et al., 1998) and is the hallmark of clinical respiratory disease and disorders (Sykes et al., 1976), such as severe asthma, chronic obstructive pulmonary disease, pneumonia, obesity hypoventilation syndrome, sleep disordered breathing, etc. The response of the respiratory system to impart changes in ventilatory output for a given change in arterial blood oxygen tension (PaO₂) is characterized by the ventilatory response to hypoxia or hypoxic ventilatory response, which is essential in the homeostatic maintenance of PaO₂ in the blood.

This regulatory response to hypoxia is unimodal where reductions in PaO₂ reflexively cause an increase in ventilation to restore normoxic blood gas levels (Dejours et al., 1963; Weil et al., 1970; Lahiri and Delaney, 1975; Berger et al., 1977). In the early 1970's, however, observations started to surface which demonstrated the potential effects of persistent hypoxia to attenuate the ventilatory response to acute hypoxia (Edelman et al., 1973; Weiskopf and Gabel, 1975). Based on these earlier findings, investigators raised questions as to the significance of the acute hypoxic ventilatory response in long-term control of breathing; thus commencing research into sustained hypoxia. Over the past 38 years, collective evidence from humans and various mammals has established that ventilation, when met with sustained hypoxia, lasting more than few minutes up to several hours, follows a distinct biphasic pattern (Woodrum et al., 1981; Lawson and
Long, 1983; Sankaran et al., 1979; Blanco et al., 1984; Weil and Zwillich, 1976; Easton et al., 1986; Vizek et al., 1987; Brown et al. 1992; for others see review by Mortola, 1996), with an initial increased peak followed by a decline - defined as the "roll-off" or hypoxic ventilatory decline (HVD) - to a lesser intermediate plateau. Although mounting evidence suggests that the biphasic sustained hypoxic ventilatory response is a characteristic feature among mammals, a notable exception has been accorded in the literature where ventilation did not roll-off in canines with constant hypoxia (Cao et al., 1992, 1993). Currently, there is no other experimental evidence to support or refute the apparently controversial canine studies, hence requiring additional investigation.

Although much of the research has focused on ventilation, and the potential mechanism(s) responsible for HVD or roll-off during sustained hypoxia (see review by Mortola, 1996 and Honda and Tani, 1999), relatively little is known about the persistent effects of hypoxia on the muscles of respiration subserving ventilation. Previous studies investigating the effector muscles during prolonged hypoxia have been strictly limited to the assessment of electromyogram (EMG) activity (LaFramboise and Woodrum, 1985; Van Lunteren et al., 1989; Guthrie et al., 1990; Martin et al., 1990; Watchko et al., 1990; Brown et al., 1992; Praud et al., 1993; Vizek and Bonora, 1998), without any additional measurements such as muscle length or shortening, which are required to deduce the actual mechanical consequence or action of a neurally activated muscle. Moreover, these earlier studies generally involved anesthesia and were studied following acute surgical interventions, and thus preventing a normal physiologic assessment of the respiratory muscles. To date, the function of the respiratory muscles with respect to its mechanical and electrical activity during a sustained period of hypoxia has not been examined in any intact mammals.

Employing the chronically instrumented awake canine preparation, this thesis systematically explores the physiologic effects of sustained hypoxia on ventilation, breathing pattern, and the mechanical action and neural activation of the primary inspiratory and expiratory muscles, without the confounding influence anesthetics and/or post operative complications. This work provides the first direct, simultaneous
measurement of muscle length and EMG activity of the costal and crural diaphragm, the parasternal intercostal and the transversus abdominis during a period of prolonged hypoxia lasting 20-25 min in a large, intact, awake animal. Such a period of sustained exposure to hypoxia represented in our canine model is of direct interest and relevance to clinical medicine, as the majority of the patient population experiencing respiratory failure typically present with hypoxia that is sustained for more than several minutes. Our research undertakes the first step towards enhancing our fundamental understanding and knowledge of the physiological and clinical presentation of sustained hypoxia and its impact on ventilation and respiratory muscle function.
Control of Ventilation

The Processor: Central Controller

The respiratory controller, located in the brainstem, is responsible for integrating, interpreting and executing respiration. Two medullary regions have been identified to have distinctive respiratory function, the dorsal respiratory group (DRG) and the ventral respiratory group (VRG), with the pontine respiratory group (PRG) acting to modulate the respiratory region (Figure 1, adapted from Hlastala and Berger, 2001).

![Diagram of brainstem respiratory network](image)

**Figure 1: Pontine-medullary respiratory network in the brainstem**
A: Dorsal view of pons and medulla. B: Brainstem transection at Botzinger Complex and between rostral and caudal VRG. VRG, ventral respiratory group; DRG, dorsal respiratory group; PRG, pontine respiratory group.

The VRG is located in the ventrolateral region of the medulla and is comprised of both inspiratory and expiratory neurons forming a long column which is segregated into the retroambigualis, nucleus ambiguous, and the pre-Botzinger and Botzinger complexes (Bianchi et al., 1995; Feldman and Smith, 1995; Richter, 1996). It is the hyperpolarization and depolarization processes of these inspiratory and expiratory
neurons that determines the neuronal generation of a breath. The rhythmic pattern of breathing has been hypothesized to be caused by the reciprocal nature of a special grouping of inspiratory and expiratory neurons in the pre-Botzinger complex (Smith et al., 1991; Rekling and Feldman, 1998). The DRG, located in the dorsomedial region of the medulla, is primarily responsible for the generation of inspiration, and is comprised mostly of inspiratory neurons some of which have axonal projections descending the spinal cord to innervate the phrenic nerve (Bianchi et al., 1995; Richter, 1996). The DRG is stimulated via the apneustic center in the lower pons and is part of the ventrolateral solitary tract nucleus, which is responsible for integrating the sensory afferent information arising from the chemoreceptors and mechanoreceptors of the respiratory system. A third neuronal region contributing to respiration is the PRG located in the pons which is capable of inspiratory inhibition and respiratory phase related activity (Hlastala and Berger, 2001). Experimental results have shown that these neurons act to inhibit inspiration by decreasing tidal volume, and have a modulating effect on the fine tuning of respiratory rhythm to influence respiratory rate (Michell and Berger, 1975; Oku and Dick, 1992; Ling et al., 1993).

**The Sensors: Chemoreceptors**

Chemoreceptors are sensors which respond to a change in the chemical composition of the blood or other surrounding fluids, namely those of oxygen, carbon dioxide and pH. Once stimulated, chemoreceptors send afferent impulses to the central nervous system (CNS) to bring about a physiological change in the system. The respiratory control system is composed of two types of sensors, central and peripheral (Figure 2, adapted from Tammeling and Quanjer, 1983).
Figure 2: Central and peripheral respiratory chemoreceptors

A: Anatomical location of central chemo-sensitive areas in the medulla (M: Mitchell, S: Schlafke, and L: Loeschcke). B: Anatomical location of peripheral chemosensors (carotid bodies, bifurcation of the common carotid arteries; aortic bodies, arch of the aorta).

Minute-by-minute control of respiration is managed by the central chemoreceptor, thought to be dispersed within the ventral surface of the medulla and is stimulated by changes in PaCO₂ levels (see review by Cherniack and Altose, 1997 and Nattie, 1999). Traditionally, chemo-sensitive regions on the ventral medullary surface have been grossly mapped out as the Mitchell (rostral), Schlafke (intermediate), and Loeschcke (caudal) areas, however, the latest research/evidence points towards the midline raphe nuclei and retrotrapezoid nucleus as the specific locations where the central chemoreceptor cells may reside in the medulla. The central chemoreceptor is surrounded by extracellular fluid and responds to changes CO₂/H⁺ caused by the transport of CO₂ across the blood brain barrier. Specific cell types have yet to be identified to confirm the individual or combination effects of H⁺ and CO₂.

Peripheral chemoreceptors are located in the carotid bodies, at the bifurcation of the common carotid arteries, and in the aortic bodies, near the arch of the aorta (as reviewed in Gonzalez et al., 1994 and Lahiri, 1997). Peripheral chemoreceptor mediated
changes may result from decreases in arterial PO$_2$ and pH, and increases in arterial PCO$_2$; each chemical stimulus can act independently on the peripheral chemoreceptor to elicit distinct chemosensory response, or interact in a manner to potentiate or attenuate another's effect. Carotid bodies in humans, and in some animals, are exclusively responsible for the ventilatory response to hypoxia where ventilation increases with reduction of arterial PO$_2$. It is important to note that carotid bodies sense arterial PO$_2$ and not O$_2$ content. Several animal studies have shown that severe hypoxemia in subjects without carotid bodies depress respiration; this is likely brought about by the inhibitory effect of hypoxia on the respiratory centers in the CNS (Watt et al., 1943; Davenport et al., 1947; Cherniack et al., 1971; Melton et al., 1988). While other studies have revealed the complete loss of hypoxic ventilatory drive in the absence of these chemoreceptors (Holton and Wood, 1965; Morrill et al., 1975; Honda, 1992).

Respiration for the most part is under subconscious control as far as response to changes in the partial pressure of arterial oxygen (PaO$_2$) and carbon dioxide (PaCO$_2$), however, respiratory movements can to a considerable extent be controlled volitionally (during speech/singing, breathing slower/faster, or holding one's breath) until conscious control is either released or can no longer override the urge/chemical drive to breath, i.e. with the development of hypoxia or hypercapnia, as sensed and mediated by the peripheral and central chemoreceptors.

**The Effectors: Respiratory Muscles**

The effectors of the respiratory system, i.e. respiratory pump muscles, are typically recognized as being either inspiratory or expiratory in action (Figure 3, adapted from Schuenke et al., 2010; with respective innervations Figure 4, modified from Hlastala and Berger, 2001), and include the diaphragm, the chest wall muscles, consisting of the internal, external and parasternal intercostals and the triangularis sterni (transversus thoracis), and the four sets of abdominal muscles, internal and external obliques, rectus abdominis, and transversus abdominis. Historically, the accessory neck muscles of inspiration, the sternocleidomastoid and the scalenes, are sometimes also lumped in as
chest wall muscles. Additionally the upper airway valve muscles also play an important role in the regulation of respiration.

**Figure 3: Respiration muscles of inspiration and expiration**

A: Accessory neck muscles: sternocleidomastoid and scalenes; Chest wall muscles: parasternal, internal and external intercostals (not shown, triangularis sterni); Diaphragm; Abdominal muscles: internal oblique, external oblique, rectus abdominis, and transversus abdominis. B: Basic mechanics of quiet respiration. Inspiration being an active process, whereas expiration being predominately a passive process with some expiratory activity.

During the inspiratory phase, the synchronized activation and contraction of the inspiratory muscles serves to bring about a decrease in pleural pressure via the expansion of the ribcage and downward pull of the diaphragm leading to a negative pressure differential between the alveoli and outside of the body which facilitates airflow into the lungs (De Troyer and Loring, 1986, Decramer, 1998; refer to Figure 3B). Expiration is generally characterized as being a passive (relaxation/recoil) process largely brought about by the elastic nature of the lung and the chest wall following inspiration. However, experimental studies in humans and animals have shown expiratory activity coinciding with expiration from both the muscles of the chest wall and the abdominal wall.
(Gilmartin et al., 1987; Arnold et al., 1988; De Troyer et al., 1989; Abe et al., 1996, 1999). Clearly expiration efforts are active during exercise, chemical stimulation and/or voluntary hyperventilation (De Troyer and Loring, 1986; Decramer, 1998), where the expiratory muscles are recruited to contract the ribcage and the abdomen, forcing the diaphragm upwards and increasing intrathoracic pressure to facilitate greater expiratory airflow out of the lungs. The process of inspiratory and expiratory airflow are remarkably regulated by the upper airway patency/resistance which are governed by the multitude of abductor and adductor muscles above the extrathoracic portion of the trachea (Campbell and Davis, 1970; Bartlett, 1989).

Figure 4: Innervation of the respiratory muscles in humans

A: Spinal root level efferent projections. B: Respiratory muscle innervation. ICs, intercostals; C, cervical spinal cord; T, thoracic spinal cord; L, lumbar spinal cord; VRG, ventral respiratory group; DRG, dorsal respiratory group.

Overall, the coordinated action and interaction of the inspiratory and expiratory muscles, along with the upper airway muscles, serves to bring about respiration that
adequately maintains blood gases to facilitate the body’s demands and needs for energy requirements. The following section describes the effector muscles of respiration in greater detail.

**Muscles of Respiration**

*The Upper Airway, as a Respiratory Muscle*

The upper airway (UAW) is comprised of the nasal passage, the nasopharynx, the oropharynx, the laryngopharynx, the larynx, and the extrathoracic portion of the trachea (Sant’ Ambrogio et al., 1995; see Figure 5, adapted from Blausen.com staff). The UAW participates in several physiologic roles, ranging from coughing, swallowing, vomiting, laughing, airway protection, vocalization, thermoregulation, and breathing (Bartlett, 1989). Although well recognized for their roles in vocalization and in protection, their most important function as a respiratory organ is often overlooked. The major respiratory role of the upper airway in mammals is the regulation of resistance to airflow (Bartlett, 1989). This regulation of upper airway patency is very important as subatmospheric pressure in the extrathoracic airway during inspiration tends to collapse the upper airway (Green and Neil, 1955; Sant’ Ambrogio et al., 1995), which could be detrimental as it impedes tidal breathing. Other roles of the upper airway include modification of the pattern of breathing and maintenance of lung volume (Stradling et al., 1987).
Figure 5: Upper airway tract in humans

The nasal passage, nasopharynx, oropharynx, laryngopharynx, larynx, and extrathoracic portion of the trachea.

Laryngeal muscles have an important respiratory function to regulate the vocal cords to modulate airway patency and resistance (Campbell and Davis, 1970), and thus are the organs of focus in this section (see Figure 6, adapted from Hunter and Titze, 2007). These muscles can be divided into two functional groups: the abductors and the adductors. The abductors, chiefly the posterior cricoarytenoids, separate the vocal cords and widen the lumen of the glottis. The adductors, mainly the thyroarytenoids, the interarytenoids and the lateral cricoarytenoids, work to bring the vocal cords close and to narrow the lumen of the glottis. The laryngeal muscles are innervated by the recurrent laryngeal nerve (RLN) except for the cricothyroid tensor muscle which receives its innervation by the external branch of the superior laryngeal nerve (E. SLN) (Campbell and Davis, 1970; Nishino, 2000).
Figure 6: Laryngeal muscles and vocal cords

Schematic of the laryngeal muscles and vocal folds/cords. Abductors: posterior cricoarytenoids (PCA). Adductors: thyroarytenoids (TA), interarytenoids (IA), and lateral cricoarytenoids (LCA). RLN, recurrent laryngeal nerve; E. SLN, external branch of the superior laryngeal nerve.

In human, afferent neural endings, resembling muscle spindles, have been shown in the laryngeal muscles; in contrast with animal studies where attempts to identify proprioceptive receptors were unsuccessful (Campbell and Davis, 1970). Recordings from the RLN in cats during quiet room air breathing report phasic activity only during inspiration following the discharge pattern of the phrenic motorneurons (Green and Neil, 1955). Hypercapnic or hypoxic breathing increases the inspiratory phasic activity along with phrenic discharge (Campbell and Davis, 1970). In general, the abductors are active during inspiration and adductors are active in expiration (Green and Neil, 1955). Adductors play an important protective role to completely shut the vocal cords to prevent aspiration of foreign substances into the lung; as well, these muscles contract during coughing to facilitate forced expiration (Bartlett, 1989).

Considering the many non-respiratory, as well as the respiratory, roles performed by the upper airway, it is not surprising to find that this airway is endowed with many
afferents which feed sensory information to the central nervous system (CNS) (Sant’ Ambrogio et al., 1995). This sensory information is processed and integrated before neuromotor outputs are sent to the upper airway effectors for coordinated activities and actions. Upper airway receptors consists of several types which can be classified as to the specific sensory stimulus to which they respond; the primary stimuli activating these receptors include pressure (positive and negative), chemical, airflow, irritants and temperature (Sant’ Ambrogio et al., 1995). Upper airway afferents play an important role in respiratory homeostasis, particularly concerning the preservation of the upper airway patency during higher levels of ventilation (Bartlett, 1989).

Other important UAW muscles include the nasal dilator muscles, including the alae nasi, which is innervated by the facial nerve, and the genioglossus, an important muscle which runs from the chin to the tongue, which is innervated by the hypoglossal nerve (Nishino, 2000). Roughly there are more than 20 pairs of muscles located around the upper airway, however, only a few muscles have been studied in detail.

**The Diaphragm, Principle Inspiratory Muscle**

The diaphragm, the principle muscle of inspiration, is a large dome shaped, musculotendinous partition that divides the thoracic and abdominal cavities (Kacmarek, 2002; see Figure 7, adapted from Schuenke et al., 2010). Arising from the sternum and anchored to the lower thoracic rib cage, forming the zone of apposition, the diaphragm consists of two segments, the costal and the crural, which are innervated by the phrenic nerve arising from the spinal nerve roots C3, C4 and C5 in humans (Hlastala and Berger, 2001; refer to Figure 4) and C5, C6 and C7 in canines (De Troyer et al., 1982). Upon activation, the diaphragmatic segments contract and pull the central portion downwards into the abdominal cavity resulting in a reduction in intrathoracic pressure and an increase in intra-abdominal pressure; this in turn, expands the lower rib cage to increase the volume of the thoracic cavity (De Troyer and Loring, 1986).
Figure 7: Diaphragm with costal and crural segments

Inferior view of the diaphragm illustrating the central tendon, aortic and vena caval aperture, esophageal aperture, and costal and crural segments.

The costal and crural diaphragms are recognized as being two discreet muscles and have many points of differentiation. They arise from two separate embryological sites, the costal originating from the body wall of the cervical segments, whereas the crural from the mesentery of the esophagus (Pickering and Jones, 2002). The costal and crural segments receive separate blood supplies and discrete motor innervations (Briscoe, 1920; Ogawa et al., 1958; De Troyer et al., 1982). Specifically, Hammond and colleagues (1989) in canines reported that the phrenic nerve splits into four discrete branches to innervate the anterior, medial and posterior segments of the costal diaphragm (SC1, SC2 and SC3) and the crural diaphragm (Cr) (Figure 8, adapted from Hammond et al., 1989). Therefore the two phrenic nerves innervate eight discrete segments of the diaphragm which theoretically could be individually activated by the central respiratory controller. In general, the diaphragm consists of 3 muscle fiber types (Type I, Type IIA and Type IIB) with differences in the proportion of each fiber type occurring between diaphragmatic segments (ventral/sternal, medial/costal and dorsal/lumbar) (Gordon et al.,
1989; Kilarski and Sjostrom, 1990). Furthermore, Reid et al. (1989) studying rodents reported that there was also a difference in the distribution of fiber types between the thoracic surface and the abdominal surface of the diaphragm.

**Figure 8: Diaphragm segmental innervation in canines**

A: Costal and crural diaphragm segmental innervation. B: Branching of the phrenic nerve. SC1, anterior sternal costal; SC2, medial sternal costal; SC3, posterior sternal costal; Cr, crural; L, left; R, right.

The costal diaphragm with its attachment to the ribs primarily exerts an inspiratory influence on the lower rib cage (to expand the lower rib cage), whereas the crural, having no insertion on the ribs, does not have an inspiratory effect on the lower ribcage when activated independently (De Troyer et al., 1982). The crural segment functions both as a gastrointestinal sphincter and as a respiratory muscle (Pickering and Jones, 2002), while the costal segment functions as a dedicated inspiratory muscle. Differential activity and function of the costal and crural diaphragm have been previously reported during both resting and chemical stimulus driven breathing (hypercapnia or hypoxia) (Newman et al., 1984; Van Lunteren et al., 1985; Fitting et al., 1986; Road et al., 1986b; Easton et al., 1987; Darian et al., 1989; Torres et al., 1989; Easton et al., 1994), as well as during automatic reflexic events such as thermal panting and emesis (Easton et al., 1994; Abe et al., 1994).
Despite being anatomically and functionally different muscles, the coordinated action of the costal and crural diaphragm work together to elicit respiration. The diaphragm, rather than being a single muscle acting as a piston, can be better understood as a complex musculotendinous structure consisting of two muscle groups with segmental innervation and blood supply, heterogeneous fiber-type composition, and differential function.

**The Chest Wall Muscles, Inspiratory and Expiratory Muscles**

The chest wall muscles, the parasternal, internal and external intercostals, are located between adjacent rib interspaces. Their discrete muscle location and fiber orientation distinguishes the intercostal muscle groups from each other (Decramer, 1998; see Figure 9, adapted from Schuenke et al., 2010). Parasternal intercostals function as a major inspiratory muscle (De Troyer, 1991), while the internal and external intercostals function as either inspiratory or expiratory muscle depending on their topographical location within the chest wall (De Troyer et al., 1999). Despite the accumulated evidence of the gradient of internal and external intercostal activity exhibiting both inspiratory and expiratory action (De Troyer et al., 2005), the internal intercostals are often exclusively considered as expiratory muscles, whereas the external intercostals are considered as inspiratory muscles (as depicted in Figure 9). Nonetheless during inspiration, intercostal muscles with inspiratory action contract to bring about an upward and outward movement of the ribs, similar to that of a bucket handle being pulled upwards, resulting in an increase of the volume of the thorax (De Troyer and Loring, 1986). During expiration, intercostal muscles with expiratory action contract to pull the rib cage downward and inward, reducing the thoracic volume (De Troyer and Loring, 1986). A further detailed account of each of the intercostal muscle groups are provided below.
Figure 9: Chest wall inspiratory and expiratory intercostal muscles

External intercostals are traditionally recognized as inspiratory muscles and internal intercostals as expiratory muscles. Parasternal intercostals lie between the costal cartilages along each side of the sternum and have inspiratory action.

The parasternal intercostal is well-recognized as being a primary inspiratory muscle of the chest wall and is active even during quiet breathing, closely mimicking the movement of the diaphragm (Easton et al., 1999a). The parasternals are located between the cartilaginous portions of each of the upper ribs and the anatomy of the parasternal intercostal reveals that the muscle fibers that make up the parasternals are the same as those of the internal intercostals (Decramer, 1998; refer to Figure 9). The parasternals create the predominant lateral chest wall movement in resting, quiet breathing and is active during inspiration along with the diaphragm (De Troyer, 1991). Contraction of the parasternals diminishes the angle between the superior border of the ribs and the sternum and functions to raise the ribs (De Troyer and Loring, 1986). The onset and peak EMG activity of the parasternal is consistent with and closely related to inspiratory air flow (Easton et al., 1999a). Moreover, the parasternal intercostals have the greatest mechanical advantage cranially, which then diminishes progressively in a caudal direction until the 8th interspace - where beyond, the parasternals do not contribute towards inspiratory
pressure generation (De Troyer et al., 2005; see Figure 10, adapted from De Troyer et al., 2005). Upon selective denervation of the parasternal muscles, the activity of the external intercostals are greatly increased to compensate for the loss of the parasternals and the cranial displacement of the ribs is substantially reduced suggesting that the parasternals significantly contribute to the act of breathing (De Troyer and Farkas, 1990).

![Figure 10: Gradient of chest wall intercostal muscle action](image)

**Figure 10: Gradient of chest wall intercostal muscle action**

Selective intercostal muscle action (parasternal, external and internal) from second to tenth interspaces as indexed by changes in airway pressure. ICs, intercostals.

The externals run between the ribs with fibers sloping downwards from upper to lower ribs moving medially inwards (i.e. towards the sternum and away from the spine) around the rib cage (De Troyer and Loring, 1986; see Figure 11A, adapted from Decramer, 1998). The external intercostals are either active in inspiration or expiration depending on their topographical location on the rib cage (De Troyer et al., 1999). For instance, external intercostals in the dorsal portion of the rostral interspaces have a large
inspiratory mechanical advantage, but this advantage decreases both in the ventral and caudal directions such that it is reversed into an expiratory advantage (De Troyer et al., 2005; refer to Figure 10). Experimental evidence suggests that the external intercostals are not obligatory respiratory muscles, but nevertheless play an important role. When the external intercostals are denervated, respiration does not cease, nor does it differ from the control state, suggesting that the parasternal intercostals and the diaphragm are sufficient to carry out inspiration (De Troyer et al., 1991). Moreover, the activity of the external intercostals are highly variable in comparison to the parasternal intercostals and may be involved in non-respiratory functions such as regulation of posture (Easton et al., 1998). Nonetheless, these muscles can be actively recruited during times of increased ventilatory demand and during compensatory requirements with functional loss or dysfunction of other respiratory muscles. Externals also differ from other intercostals muscles in their postinspiratory activity, consistent with their different composition and muscle spindle content (Easton et al., 1999a).

Figure 11: Anatomy of the intercostals and the triangularis sterni muscle
A: Fiber orientation of the intercostal muscles (parasternal, external and internal). B: Schematic of the triangularis sterni (transversus thoracis) muscle.

Although less is known about the internal intercostals, it is generally accepted that they are functionally recruited during active breathing. These muscles run between the
ribs with their fibers sloping downwards from upper to lower ribs moving laterally outwards (i.e. away from the sternum and towards the spine) around the rib cage (De Troyer and Loring, 1986; refer to Figure 11A). The internal intercostals in the ventral portion of the caudal interspaces have an expiratory mechanical advantage, but this advantage decreases in the dorsal and cranial direction such that it is reversed into an inspiratory advantage (De Troyer et al., 2005; refer to Figure 10). The internals are not overly associated with rib cage movement but rather play an important role in stabilizing the chest wall to maintain its tone during breathing (Decramer and De Troyer, 1986).

The triangularis sterni (transversus thoracis) is an expiratory muscle of the chest wall; this muscle is attached to the upper cartilaginous portion of the ribs and the inner side of the sternum (Decramer, 1998; Hlastala and Berger, 2001; see Figure 11B, adapted from Decramer, 1998). When activated the triangularis sterni contracts to pull the ribs downwards, reducing the thoracic volume and causing a rise in intrathoracic pressure, to facilitate expiratory airflow.

All muscles of the chest wall are innervated by the intercostal nerves arising from the thoracic segment of the spinal cord (Schuenke et al., 2010; refer to Figure 4). The blood supply and innervations of each intercostal travels along the corresponding rib, with the artery in the center along the shaft of the rib, and the vein above and nerve below.

**The Abdominals, Expiratory Muscles**

All four abdominal muscle groups, the rectus abdominis, external and internal obliques and transversus abdominis (Figure 12, adapted from Schuenke et al., 2010), are expiratory muscles that, when activated, pull the lower ribs downward while compressing the abdomen, increasing the intra-abdominal pressure in an effort to force the diaphragm upwards into the thoracic cavity, aiding in expiratory airflow (Kacmarek, 2002). With greater expiratory activity (active/forced expiration), the diaphragm may be stretched beyond its end-expiratory length to facilitate greater inspiratory force generation as well.
as passive relaxation of the diaphragm (De Troyer and Loring, 1986). This “accessory inspiratory” action of the abdominals greatly improves the efficiency of the diaphragm’s force generating capacity (Abe et al., 1996; De Troyer, 1983; Kacmarek, 2002).

![Diagram of abdominal wall expiratory muscles](image)

**Figure 12: Abdominal wall expiratory muscles**

A: Anatomy of the expiratory abdominal muscles (transversus abdominis, internal oblique, external oblique, and rectus abdominis). B: Abdominal expiratory muscle action during active/forced expiration (diaphragm end-expiratory lengthening and passive relaxation).

Among the four muscle groups, the transversus abdominis, the most inner abdominal layer, is the most active during expiration, followed by the internal oblique, external oblique, and with minimal contribution, rectus abdominis (Abe et al., 1996). Depending on the species and position, the transversus abdominis has been reported to be phasically active, intermittently active and inactive during quiet resting breathing (Gilmartin et al., 1987; Arnold et al., 1988; Estenne et al., 1988; De Troyer et al., 1989; De Troyer et al., 1990; Abe et al., 1996). With increases in ventilatory demands, however, the transversus abdominis, as well as the internal and external obliques, increase in activity to cause expiration to become active (Estenne et al., 1988; Ninane et al., 1992; Ninane et al., 1993, Abe et al., 1996). These muscles play an important role in
non-respiratory actions as well, as they are involved in speech, coughing, emesis, defecation and posture (De Troyer and Loring, 1986).

All abdominal muscles are innervated by the abdominal respiratory motorneurons from the lower thoracic and upper lumber spinal cord segments (Schuenke et al., 2010; refer to Figure 4).

*The Accessory Neck Muscles, Inspiratory Muscles*

Accessory neck muscles of inspiration include the scalene and sternocleidomastoid (refer to Figure 3). These muscles insert onto the first and second ribs and are activated to help elevate the thorax and also to stabilize the upper ribs during inspiration (Kacmarek, 2002; De Troyer and Loring, 1986). The accessory neck muscles are innervated by the cranial nerve IX and cervical nerves (Schuenke et al., 2010; refer to Figure 4).

**Breathing Circuits**

Breathing-circuits used for the physiological assessment of respiratory and other physiologic variables both in the clinical and laboratory setting, are of two main types: open and closed. The chosen circuit dictates the principle design and implementation of the circuit, as well as the method of breathing. The two circuits are described below as it pertains to humans or animals which permit the use of a breathing circuit.

*Closed Breathing Circuit*

A closed circuit, commonly employed in the rebreathing method, consists of a low dead-space, low-resistance breathing circuit that is closed to the atmosphere, providing a positive feedback loop (Rebuck and Campbell, 1974). The subject breathes within the circuit which is attached to a gas reservoir bag or balloon containing the specific gas mixture to be utilized in the study (carbon dioxide, oxygen, nitrogen and/or
other gases in balance). A degree of sophistication can be added to circuit to precisely control the mixture of gas in the reservoir using a manual- or servo-controlled technique (Akiyama and Kawakami, 1999). These techniques may be incorporated to regulate gas sources from a pressurized tank (CO₂, O₂, N₂). In addition, a CO₂ absorber is required in the case of a hypoxic rebreathe method (Rebuck and Campbell, 1974). The breathing circuit may connect the subject to the reservoir either directly through a shared (single) inspiratory-expiratory limb or using separate (dual) limb for inspiration and expiration (Akiyama and Kawakami, 1999). As the subject breathes, the gas mixture in the reservoir is continually modified as a consequence of the subject’s oxygen consumption or carbon dioxide production (Rebuck and Campbell, 1974). Therefore the stimulus to drive breathing increases in a steady, linear fashion making the closed method a very attractive technique to assess ventilatory drive. Furthermore, the closed circuit’s ability to equilibrate the subject, the circuit, and the gas reservoir establishes the “open loop” condition described by Rebuck and Campbell (1974). This “open loop” condition effectively stabilizes the partial pressure of gases in the alveolar, arterial-venous, and the reservoir bag, a major criterion that makes the closed circuit method so attractive for the assessment of chemical control of breathing (Akiyama and Kawakami, 1999). The system is also considered to be efficient as it redirects the expired air back into the gas reservoir and it is not accompanied by heat loss to the atmosphere as the circuit is completely closed. However, this also means that condensation should be controlled for and the circuit should be kept dry between tests.

*Open Breathing Circuit*

In contrast, an open circuit, as the name implies, is a low dead space, low resistance breathing circuit that is open to the atmosphere, usually on the expiratory limb when employing a dual limb design. Some open circuits that use a single shared limb may use a controlled valve to expel air to the atmosphere during the expiratory phase (Akiyama and Kawakami, 1999). In an open circuit, the gas reservoir is connected to the inspiratory (or the shared) limb and the delivery or regulation of the inspired gas concentrations are effectively achieved through pressurized gas sources (O₂, CO₂, N₂)
that are either fed into the gas reservoir or the inspiratory circuit via manual or servo-
control (Akiyama and Kawakami, 1999). Since expired air is expelled out into the
atmosphere, the design is simple and convenient; in that, expired air does not need to be
accounted for in the circuit. Despite this convenience, it is also associated with some
drawbacks and concerns. The open circuit can be affected by some degree of heat loss
and variance in the rate of breathing or equilibration of inspired gas. Related to the
equilibrium of inspired gas is metabolic rate and thus a single, precise, universally timed
progressive, or step, strategy to tightly control the experimental protocol to stimulate
breathing across all study subjects is not feasible. Nevertheless, the simplicity of the open
circuit arrangement is very effective in allowing the operator to independently control the
concentration and volume of gas supplied to the subject (Edelman et al., 1973).

**Hypoxic Ventilatory Response Techniques**

The well-known physiologic techniques for the assessment of the ventilatory
responses to hypoxia, or hypoxic ventilatory response (HVR), is detailed in this section.
Since various studies involving the assessment of hypoxia on respiration and other
physiologic parameters employ a wide range of techniques that differ between animal
species and experimental protocols, it is imperative to understand and recognize which
method is involved in order to accurately interpret and compare the results across
different studies. Assertions are made as to the type of breathing circuit employed and the
rational and limitations of the individual techniques in assessing the response to hypoxia.
The techniques stated within pertain to humans or animals which permit the use of a
breathing circuit.

**Acute Response: Transient Technique**

The transient test, classically utilized by Edelman et al. (1973), is founded on the
principle that the ventilatory response to rapidly changing transient hypoxic stimuli (~15
seconds) will exclusively represent the activity of the peripheral chemoreceptor and
effectively avoid the ventilatory effects of the central compartment with an associated
time delay of ~120 seconds (Kronenberg et al., 1972). In this technique the subject breathes comfortably on an open breathing circuit fitted with a 3-way valve on the inspiratory limb which allows for the quick switching between either a gas reservoir (room air) or a pure nitrogen source (tank). After several minutes of baseline room air breathing, the subject, during expiration, is abruptly switched into pure nitrogen for several breaths to elicit a transient fall in saturation of oxygen (SaO$_2$) or end tidal oxygen tension (P$_{ET}$O$_2$) which in turn drives ventilation. To obtain a wide-range of levels of hypoxic stimulus, different number of breaths of nitrogen are inhaled until the target SaO$_2$ or P$_{ET}$O$_2$ is reached while ventilation is recorded (Edelman et al., 1973). Although it has been consistently reported that the ventilatory response to transient hypoxia is slightly higher than the reported values for steady-state responses, the transient breathing test has been criticized for having large breath variability and a brief response, raising questions as to whether the hypoxic response has been fully developed (Weil and Zwillich, 1976). On the other hand, transient breathing tests have been reported to correlate well with the steady state responses and thus making it feasible for assessing the ventilatory response to hypoxia (Weil and Zwillich, 1976).

**Acute Response: Single Breath Technique**

Single breath assessment of the ventilatory response to hypoxia shares the same rational as the transient breath test and only differs on the principle method associated with the test. The single breath test is a modified version of the transient test utilized by Edelman et al. (1973). In the single breath test as employed by Kronenberg et al. (1972), the subject initially breathes room air on an open circuit that allows for quick switching (via a 3-way valve) between room air (atmosphere) and reservoir bag (hypoxic gas). After baseline control measurements, the subject, upon instruction, voluntarily gives a full exhalation (when the circuit is turned into the hypoxic bag) allowing a single, vital, capacity breath of the hypoxic gas mixture to be inhaled. After a single maximal inspiration, the hypoxic bag is cut off from the circuit to allow subsequent breaths to be taken of room air, and ventilation is recorded for 20-30 sec. Although the single breath test shares the same restrictions as the transient test, it is further limited, in that multiple,
repeated, vital capacity maneuvers are required to measure the response over a range of arterial oxygen tension (PaO$_2$) and, moreover, it requires subject training and cooperation (Kronenberg et al., 1972).

**Acute Response: Progressive Technique, Two Types**

**Open Circuit - Progressive Isocapnic/Poikilocapnic Hypoxia**

In open circuit progressive isocapnic hypoxia, the subject breathes from an inspiratory limb connected to a gas reservoir of room air. After control measurements, pure nitrogen is continuously added to the inspiratory limb of the breathing circuit to progressively drop the expired oxygen tension (P$_{ET}$O$_2$) from 140 Torr – 40 Torr, while at the same time CO$_2$ is also added to the inspirate to maintain ETCO$_2$ equal to baseline control levels (Weil et al., 1971). Ventilation, P$_{ET}$O$_2$, P$_{ET}$CO$_2$ and SaO$_2$ are continually monitored and recorded throughout the entire run to guide the control of gas sources being added to the inspirate. This open circuit progressive isocapnic hypoxia technique was originally proposed by Weil et al. (1970), as a means to provide a more rapid ventilatory response assessment to the rather slower steady state techniques described in the late 1950s (Weil et al., 1971). The impetus for the change stemmed from the fact that ventilatory response to hypoxia is associated with a rather short time constant (~18 sec) justifying the response to be correctly assessed using a more rapid P$_{ET}$O$_2$ or PaO$_2$ reduction strategy than its former steady-state response techniques (Weil et al., 1971). The open circuit isocapnic progressive hypoxia test is favorable in that it offers the experimenter the complete flexibility to control the gas sources on the inspiratory limb to rapidly reduce the P$_{ET}$O$_2$ or PaO$_2$ to target levels while maintaining tight control of the P$_{ET}$CO$_2$. Yet, because of the persistent hypoxia, ventilation is affected by central inhibition, and the test's major weakness is that the reduction in oxygen (O$_2$) cannot be tightly regulated across subjects, hence the ventilatory response to hypoxia is more variable, less reproducible, and subject to operator error.
A simple variant of the progressive isocapnic hypoxia technique without the addition of CO₂ to the inspirate allows PaCO₂ and P_{ET}CO₂ to freely "float" and settle to natural levels, and thus permits the assessment of the ventilatory response to poikilocapnic hypoxia (without CO₂ control). Many investigators prefer the assessment of the ventilatory response to poikilocapnic hypoxia as this technique better represents the physiologic response to hypoxia - where hypocapnia would naturally result from the hypoxic induced hyperventilation. However, the limitation of this method is that poikilocapnia does not allow the investigator to single out the effects of hypoxia on the ventilatory response by "clamping" down CO₂, as is the case during progressive isocapnic hypoxia.

Closed Circuit - Progressive Isocapnic Hypoxia

Progressive isocapnic hypoxia, using a closed circuit strategy, is based on the modification of the CO₂ rebreathe technique originally proposed by Read (Rebuck and Campbell, 1974). After room air control breathing, the subject breathes through a low resistance circuit connected to the low pressure rebreathing bag (6L) containing a premixed gas mixture of 7% CO₂, 24% O₂ balanced N₂. Arterial oxygen tension (reflected by PaO₂, P_{ET}O₂, and SaO₂) is allowed to fall until the targeted P_{ET}O₂ of ~30-40 Torr (~SaO₂ 50-60%) is achieved as a response to the subject’s oxygen consumption. P_{ET}CO₂ is maintained constant at controlled levels throughout the assessment by eliminating the metabolic production of CO₂ added to the rebreathing bag using a CO₂ absorber. Once “open loop” conditions (Rebuck and Campbell, 1974) are achieved, P_{ET}O₂ and PaO₂ gradually fall linearly with time to drive ventilation. The major benefit of the progressive isocapnic rebreathe technique is in its ability to achieve complete equilibrium between the subject and the circuit to provide a nice steady linear hypoxic drive to stimulate breathing, there are minimal operator errors and the results are replicable, reliable and well controlled by the subject, and the test is easy and convenient, making it feasible in the clinical setting. However, one drawback exists, the slow nature of the test (~10-20 min) causes the magnitude of the ventilatory response to be underestimated due to the central inhibitory effect on ventilation (Weil et al., 1971).
Acute or Sustained Response: Steady State Technique, Two Types

Multiple Step Reduction - Steady State Acute Hypoxia

To assess the hypoxic ventilatory drive using a multiple step reduction approach, the subject breathes on a open circuit connected to a gas reservoir where PaO₂ or SaO₂ is lowered in a stepwise fashion consisting of multiple steps (each step: sec to min) to reach a target PaO₂ or SaO₂ level over a predetermined time duration (Steinback and Poulin, 2007). P_{ET}CO₂ can either be maintained by adding CO₂ to the inspirate or left to fall with hypoxic hyperventilation. This multiple step reduction strategy allows the experimenter to pre-standardize and systematically drop the PaO₂ or SaO₂ to the target hypoxic level. Each steady step reduction allows time for ventilation to stabilize and thus provides an added degree of stability to the measurement of the HVR. This technique by and large yields data comparable to the other acute measurement techniques (Weil and Zwillich, 1976) with the added benefit of standardizing the drop in arterial oxygen levels to a certain extent.

Single Step Reduction - Steady State Acute/Sustained Hypoxia

Single step reduction strategy involves the measurement of ventilation during a square change in F_{I}O₂ to achieve a step drop in PaO₂ or SaO₂ over a predetermined hypoxic duration (~5-60 min or more) (Weil et al., 1971). This is a common technique employed in the literature to assess the ventilatory response to sustained hypoxia both in humans and in mammals (Weil and Zwillich, 1976; Woodrum et al., 1981; Easton et al., 1986; Vizek et al., 1987; Long et al., 1993). Single step reduction of PaO₂ or SaO₂ can be made after baseline breathing by abruptly turning the subject into an open breathing circuit connected to a premixed hypoxic gas reservoir containing F_{I}O₂ ~6-14% balanced N₂. After introducing the hypoxic gas mixture, there is a ~1-2 min time delay before arterial oxygen tension reaches the targeted hypoxic level (i.e. moderate hypoxia: ~80% SaO₂) (Weil et al., 1971). Target O₂ level is maintained by titrating an O₂ source attached
to the inspiratory limb, $P_{ETCO_2}$ may also be controlled to maintain isocapnic conditions or left unmanaged to achieve poikilocapnic conditions. The initial ventilatory response during the first couple minutes (~1-3 min) provides a measure of the acute hypoxic response, and the subsequent decline in ventilation reflects the attenuating effects of sustained hypoxia with respect to time and magnitude (Easton et al., 1986). The single step reduction technique is simple to implement, allows for the control of gas sources to the inspirate, is reproducible and accurate, and precisely captures the dynamic changes in ventilation which other assessment methods fail to account for and thus is the most stable. The major limitation of the steady state method is that it does not permit the measurement of ventilation over a range of different arterial blood oxygen levels as well as repeated steady state measurements are also affected by central inhibition.

**Chronic Response: Employing the Acute Techniques**

Since the steady-state techniques employ the breathing circuit, they are only feasible for a couple of hours. Continuous measurement of breathing pattern over the chronic hypoxic exposure is thus not practical on a breathing circuit. Given such restrictions, physiologists have resorted to employing the available acute strategies individually or across multiple time spans (hours to days) to cover the chronic hypoxic duration of interest. Accordingly, researchers have ascended to various altitudes to study the changes in acute ventilatory response to hypoxia over multiple days (Rahn and Otis, 1949). Physiologists have also conducted studies by exposing animals to chronic hypoxia within a closed chamber and then assessing their acute hypoxic ventilatory drive (Olson and Dempsey, 1979). Other investigators have studied the effects of chronic hypoxia by recruiting different study subjects that range from lowlander, highlanders and natives of high altitude to compare the acute HVR between the individual groups all with the available acute strategies (Dempsey and Forster, 1982; Weil et al., 1971).
Partial pressure of carbon dioxide in the arterial blood (PaCO₂) is a profound modulator of ventilation. PaCO₂ imparts its influence on ventilation through its direct action on peripheral and central chemoreceptors of the respiratory system (Honda and Tani, 1999). When considering the systemic ventilatory response imparted by changes in arterial CO₂ tension, the majority of the response is directly mediated by the central chemoreceptor compartments within the brainstem, with the rest being peripheral chemoreceptor influence, contributing around 20%-30% of the total response (Bisgard and Neubauer, 1995). More notably, PaCO₂ is known to interact with PaO₂ in a hyper-additive or multiplicative manner that drastically alters the ventilatory response to hypoxia. Previous studies have demonstrated this multiplicative interaction of PCO₂ and PO₂ on the HVR (Edelman et al., 1973; Weil et al., 1971); in these studies, the ventilatory response to change in PaO₂ was noticeably accentuated with increases in PaCO₂ (hypercapnia), while the decrease in PaCO₂ (hypocapnia) had the opposite effect; markedly attenuating the response to hypoxia.

There are numerous ways in which uncontrolled arterial PCO₂ can affect ventilation and confound the results obtained from the assessment of the HVR. Ventilatory response to hypoxia elicits an increase in ventilation accompanied by a fall in PaO₂ (Gonzalez et al., 1994; Lahiri, 1997). The resulting hyperventilation causes a concomitant decrease in PaCO₂, causing a time-dependent arterial and cerebrospinal alkalosis that reduces the activity of both the peripheral chemoreceptor and the central chemoreceptor (Bisgard and Neubauer, 1995). With reduced activity of the chemoreceptors, the resulting HVR becomes “blunted” and thus the acute response is not fully expressed. Hypoxia may also promote cerebrospinal alkalosis through its action on the cerebral artery to increase cerebral blood flow, promoting the “washout” of CO₂ (alkalosis) (Honda and Tani, 1999). The resulting alkalosis acting on the central chemoreceptor results in an inhibitory action on ventilation that may further decrease ventilatory output, but vasoconstriction effects of alkalosis on cerebral blood flow (Easton et al., 1986) must also be considered. Hypoxia is also known to cause a decrease
in the metabolic rate (hypometabolism) (Gutier, 1996), especially in small animals and newborns; this potential drop in metabolic production of CO₂, secondary to hypometabolism, might further contribute to the decrease in the arterial CO₂ tension to further attenuate ventilation (Mortola, 1996). Taken together, these attenuating effects of uncontrolled PaCO₂ on ventilation affect both the acute hypoxic response and the prolonged hypoxic response, causing ventilation to be attenuated and underestimated throughout the entire assessment period.

Because of the potent effects of CO₂, ventilatory response to hypoxia is typically assessed under conditions of isocapnia, in which CO₂ tension is held constant (Weil and Zwillich, 1976). With CO₂ tension remaining unchanged, the ventilatory response to hypoxia can be examined, independently without the confounding influence of CO₂ tension on ventilation. Controlling the arterial CO₂ tension during the assessment technique can be achieved with relative ease, in theory and in principle, however it is much more challenging in practice, especially, if manually controlled by the operator. Isocapnia during the assessment technique can be maintained at eucapnic (control) or hypercapnic levels. As CO₂ is a potent modulator of ventilation, even a little too much allowance of CO₂ into the inspirate to cause a slight increase in P_{ET}CO₂ (or PaCO₂) can markedly accentuate the ventilatory response; likewise even a slight fall in P_{ET}CO₂ (or PaCO₂) would cause ventilation to be attenuated. Therefore, the operator must be vigilant and diligent throughout the duration of the assessment to avoid inadvertently confounding the outcome of the test. When the P_{ET}CO₂ is left unmanaged a poikilocapnic condition is achieved, and the decrease in PaCO₂ secondary to an increase in ventilation is unfavorable if the aim of the assessment is purely examining the effects of hypoxia on ventilation. On the other hand, some investigators argue in favor of the poikilocapnic conditions as it is thought to more closely reflect the normal physiological response (Steinback and Poulin, 2007).
Physiologic Response to Hypoxia

Oxygen Requirement

All mammals depend upon oxygen for survival. Cellular respiration is an aerobic process requiring oxygen as fuel to produce the energy substrate, adenosine triphosphate (ATP), which is necessary to support cellular, tissue and organ development and function, and thus supporting the life of the organism. The response of the respiratory system to impart changes in ventilatory output for a given change in arterial blood oxygen tension (PaO$_2$) is characterized by the ventilatory response to hypoxia or hypoxic ventilatory response (HVR). Mammals, including humans, exclusively rely on the HVR to meet the energy requirements of the body and to maintain adequate PaO$_2$ in the arterial blood. Generally speaking, hypoxia accompanied by a reduction in PaO$_2$ drives the respiratory system to cause an increase in ventilation, and thus works to return PaO$_2$ back to normoxic levels (Gonzalez et al., 1994; Lahiri, 1997). HVR involves the effectors of respiration compensating for the reductions PaO$_2$ through their actions on ventilation. Although hypoxic stress-induced changes in ventilation are associated with some cost of breathing (oxygen consumption), the incurred cost in most circumstances is well capitalized in the process of restoring PaO$_2$ to or near normoxic levels. The HVR works to supply the oxygen needed to meet the energy requirements of the body.

Ventilatory Response

Ventilatory response to hypoxia depends on the nature and pattern of the hypoxic stimulus. Experimental studies have revealed and identified many distinct time-dependent ventilatory responses to hypoxia (as reviewed in Mortola, 1996 and Powell et al., 1998). Typically, these responses are characterized as either being acute, sustained or chronic.

Acute response is the immediate increase in ventilation activity at the onset of hypoxia; this response can be attained by examining the change in ventilation that ensues within one or more breaths of PaO$_2$ changing at the carotid bodies (Dejours et al., 1963;
Weil et al., 1970; Lahiri and Delaney, 1975). When the relationship of increasing ventilation is plotted as a function of decreasing PaO$_2$, a resultant hyperbolic acute ventilatory response curve is revealed (Figure 13A, adapted from Berger et al., 1977); the shape of the curve reflects the hypoxic sensitivity of the respiratory system to acute hypoxia, which in turn, determines the acute ventilatory response for a given hypoxic stimulus (PaO$_2$) (Weil et al., 1970). This curve depicts that as PaO$_2$ falls slightly (20 mmHg) below normoxia (100 mmHg), there is only a small increase in breathing. It isn’t until PaO$_2$ falls below 60 mmHg where appreciable increases in breathing occur. Further decreases in PaO$_2$ cause marked increases in breathing because the hyperbolic curve slopes prominently in this PaO$_2$ range (< 50 mmHg). At very severe hypoxic levels (< 40 mmHg), no further increase in breathing occurs, and eventually hypoxia acts on the CNS to depress ventilation (Morrill et al., 1975; Van Beek et al., 1984). Depressive effects of severe hypoxia on CNS can be so harsh as to cause cessation of breathing.

![Figure 13: Acute hypoxic ventilatory response](image)

A: Ventilatory response to hypoxia expressed as arterial oxygen tension (PaO$_2$, hyperbolic curve). B: Ventilatory response to hypoxia expressed as arterial oxygen saturation (SaO$_2$, linear curve).
An alternative method to quantify ventilatory sensitivity to hypoxia is to generate an acute HVR curve by means of oxygen saturation (SaO₂) in placement of PaO₂ (Berger et al., 1977). This is a linear relationship between increase in ventilation over a reduction in SaO₂ within the given range of 100% - 60% (Figure 13B, adapted from Berger et al., 1977). Since the curve is linear, simple computation of the slope can provide a direct and effective measure of the magnitude of hypoxic ventilatory sensitivity; hence, the greater the slope, the greater the acute hypoxic ventilatory drive. Moreover, measurement of SaO₂ can easily and non-invasively be attained using a pulse oximeter.

Sustained response is the change in ventilation that occurs over continuous exposure to hypoxia for several minutes to hours (Figure 14, adapted from Mortola, 1996). Ventilatory response to sustained hypoxia is biphasic in pattern, where an initial increase in ventilation is followed by a ventilatory decline that is age and species dependent. Although studies show varied results, the bi-phasic response to sustained hypoxia has been successfully demonstrated in both humans (Rigatto and Brady, 1972; Sankaran et al., 1979; De Boeck et al., 1984; Weil and Zwillich, 1976; Easton et al., 1986; Georgopoulos et al., 1989; Masuda et al., 1989; Yamamoto et al., 1994) and animals (LaFramboise et al., 1981; Blanco et al., 1984; Bureau et al., 1984; Saetta and Mortola, 1987; Vizek et al., 1987; Vizek and Bonora, 1998; Freedman et al., 1988). The decline in ventilation following the initial hyperventilation has been termed the hypoxic ventilatory decline (HVD) or simply referred to as the ventilatory “roll off” phenomenon (Weil and Zwillich, 1976; Easton et al., 1986; Vizek et al., 1987; Tatsumi et al., 1992).
Figure 14: Sustained and chronic hypoxic ventilatory response

Time course of the ventilatory response to hypoxia expressed over minutes-hours, days-weeks and years-many years.

Chronic response to hypoxia is the change in ventilation that occurs over long-lasting periods of hypoxic exposure that extends days, weeks, or years (refer to Figure X). Ventilatory response to chronic hypoxia can be classified into three distinct responses: ventilatory acclimatization (VA); ventilatory deacclimatization (VD); hypoxic desensitization (HD). Classic example of VA is observed at high altitude; it is the occurrence of a gradual time-dependent increase in ventilation and ventilatory O\textsubscript{2} sensitivity serving as an adaptive mechanism to low oxygenated environments (Dempsey and Forster, 1982; West, 1988; Sato et al., 1992). VD occurs when normoxia is acutely re-established after exposure to chronic hypoxia, ventilation and O\textsubscript{2} sensitivity to hypoxia does not immediately return to control levels; hence, hyperventilation persist in normoxia (Dempsey et al., 1979; Dempsey and Forster, 1982; Engwall and Bisgard, 1990). HD is observed in subjects with chronic exposure to hypoxia for years or a lifetime, humans show a “blunted” HVR, where ventilation is suppressed in comparison to normal subjects acclimatized to altitude for shorter periods of time (Sorensen and Severinghaus, 1968; Forster et al., 1969; Weil et al., 1971).
Airway and Lung Response

The upper (extrathoracic) airway of the respiratory system are involved in the maintenance of airway patency, airflow and thermoregulatory functions (Nishino, 2000). Similarly, lower (intrathoracic) airways in the lung serve similar roles to determine airflow and tone, with minimal contribution to heat exchange (McFadden and Ingram, 1986). Since airflow can be hampered by changes in airway resistance, both the upper and lower airways play a crucial role in determining ventilation.

Hypoxia increases upper airway and decreases lower airway caliber (Fontan, 1995). The increased patency of the upper airway is not only important to facilitate airflow, allowing greater tidal breathing, but also serves an important function in preventing the collapse of the upper airway with the larger negative pressures generated by the inspiratory muscles during hypoxia stimulated breathing (McFadden and Ingram, 1986). Increases in contraction of the intrathoracic smooth muscles are thought to be important in maintaining the caliber of the lower airways with the accompanying large increases in lung volume with hypoxic breathing (Fontan, 1995).

With respect to the lungs, classic studies in humans and animals suggests an increase in end-expiratory lung volume, i.e. functional residual capacity (FRC), with hypoxia (Bouverot and Fitzgerald, 1969; Garfinkel and Fitzgerald, 1978). The functional consequence of a substantial increase in FRC in diseased states such as COPD is well-recognized to cause a decrease in lung compliance, reduction in end-expiratory length and force-generating capacity of the diaphragm, as well as diminished inspiratory capacity and tidal volume (De Troyer and Loring, 1986). The functional significance of an increase in FRC with hypoxia is unclear, however, oxygenation may improve due to an increase in the surface area of the lungs available for gas-exchange. On the other hand, the exact mechanical consequence of a hypoxic induced increase in FRC on the diaphragm and its effects on force/pressure generation, at present, is unknown.
Metabolic Response

Hypoxic hypometabolism is defined as the fall in metabolic rate accompanying the exposure to hypoxia which has been predominately and consistently reported to occur in newborns and in small bodied animals (Gautier, 1996). This fall in metabolic rate also causes a concomitant reduction in body temperature ($T_b$) and oxygen consumption ($VO_2$) (Gautier, 1996; Mortola, 2005). The reduction in metabolism is noticeably greater in newborns and small animals exposed to cold than in any other group (Mortola, 2004). Hypometabolism is less commonly observed in large adult mammals in thermoneutral environments; yet a drop in metabolic rate with hypoxia is readily induced and observed with cold exposure (Mortola and Gautier, 1995). This fall in metabolic rate is well-recognized to be facilitated by thermoregulatory mechanisms which cause a shifting of the set point to a lower level below $T_b$ (“regulated hyperthermia”) (Gautier, 1996; Rollins and Fewell, 1998). Thus newborns and small animals, and even large adult mammals exposed to cold environments (where higher thermogenic requirements are needed to maintain $T_b$), exhibit a fall in metabolism with hypoxia induced shifting of the thermoregulatory set point.

Hypoxic hypometabolism (via shifting the thermoregulatory set point below $T_b$) might explain some of the observed reductions in the hypoxic ventilatory response (HVR) with time (“blunted” HVR) (Mortola and Gautier, 1995; Gautier, 1996). Experimental studies have demonstrated a direct relationship between changes in metabolic rate and ventilation during the acute hypoxic response, whereby an increase in one will cause an increase in the other (Mortola, 2005). The decrease in metabolic rate accompanying hypoxia has effects on ventilation, which reduces the full magnitude of the acute hypoxic response (Mortola, 1995).

Interestingly, the time course response of the hypoxic hypometabolism and the paralleled reduction in $T_b$ has a relatively fast onset and rate of decrease. In adult rats, significant reductions in $T_b$ with hypoxia were reported after the first 6 minutes and continued to decrease for up to 60 minutes (Rollins and Fewell, 1998). Since the decrease
in $T_b$ during hypoxia directly tracks changes in metabolic rate, this provides indirect evidence to support the relatively fast onset and rate change of hypometabolism with hypoxia. Therefore, the reduction in metabolic rate that occurs on the back drop of hypoxia (hypoxic hypometabolism) is well within the timeframe of sustained hypoxia (~20-60 minutes) to modulate the biphasic ventilatory response to persistent hypoxia. However, it is important to emphasize that hypoxic hypometabolism is less apparent or does not occur in large adult mammals (Mortola, 1995), and thus, likely, would not impart any significant modulatory effects on the HVR, acutely or sustained. Nevertheless, hypoxic hypometabolism can be induced with cold exposure even in large animals (Mortola and Gautier, 1995), and in such circumstance the HVR may be affected by hypometabolism. As to exactly how much the HVR is modulated or attenuated by the pure effects of hypoxic hypometabolism in large mammals with cold exposure has not been clearly established.

Taken together, the hypoxic ventilatory roll-off or HVD with constant exposure to hypoxia may be further attenuated by hypoxic hypometabolism in newborns or small bodied mammals. However, such modulatory influence of hypoxic hypometabolism does not likely affect the ventilatory response to sustained hypoxia following a biphasic pattern in large adult mammals in the thermoneutral environment.

**Cardiovascular Response**

Cardiovascular responses to acute hypoxia include alterations in heart rate, arterial blood pressure, cardiac output, and systemic vascular resistance. In humans, circulatory responses to hypoxic gas mixtures were associated with tachycardia, increase in stroke volume and cardiac output, and a decrease in systemic vascular resistance, but without a change in mean arterial blood pressure (Kontos et al., 1966). Likewise, in anesthetized canines, cerebral hypoxia caused a marked increase in heart rate, atrial and ventricular contractility, systemic vascular resistance, and arterial blood pressure (Downing et al., 1963). Recent animal studies, both in vivo and in vitro, also allude to the existence of oxygen sensing neurons in the posterior hypothalamus, which when
activated by hypoxia increase sympathetic activity to the heart and blood vessels to cause alterations in cardiovascular function (Neubauer and Sunderram, 2004).

In response to alveolar hypoxia, hypoxic pulmonary vasoconstriction (HPV) facilitates ventilation-perfusion matching and thus optimizes pulmonary gas exchange (Weissmann et al., 2001). HPV response can occur acutely or over a prolonged period of time. For the prolonged response, hypoxia causes an initial rapid vasoconstriction response (peak at ~4-6 min) followed by a subsequent vasodilatation to baseline control levels (~15-20 min), and then a secondary progressive vasoconstriction that becomes persistent overtime. It is unsure whether the primary and secondary vasomotor responses to sustained alveolar hypoxia are regulated by identical or independent mechanisms; however, the secondary sustained response is thought to lead to the vascular remodeling process that occurs with chronic hypoxia. Other circulatory alterations during sustained exposure to hypoxia have been shown where the time course change in heart rate followed a biphasic response that paralleled changes in minute ventilation (Tanaka et al., 1992).

Chronic hypoxia has been reported to cause a progressive increase in heart rate, elevated blood pressure, increase in cardiac output, and a decrease in systemic vascular resistance (Thomson et al., 2006; Tamisier et al., 2007). Despite the cardiovascular adjustments, chronic hypoxia leads to pulmonary arterial hypertension (PAH) as a result of vasoconstriction, polycythemia, and vascular remodeling with medial thickening - consequent to smooth muscle hypertrophy (Watanabe, 1987; Janssens et al., 1991). Medial thickening of vessels decreases vascular compliance which has been shown to impair vasodilator function. But most notably, PAH is the principle cause of right ventricular hypertrophy and this can eventually lead to heart failure and death.

Cardiovascular response to acute, sustained and chronic hypoxia by and large serves a protective function to facilitate better gas exchange, to enhance oxygen delivery to tissues, and maintain blood pressure within a physiological range. However, such defense mechanisms which are prolonged can in turn produce results that paradoxically
worsen systemic oxygenation and oxygen delivery to tissues. Thus, chronic hypoxia could cause a pathophysiological conditioning of the cardiovascular system to hamper oxygen delivery to the body.

**Redox State and Response**

Redox state of a cell is defined as the balance between the oxidized and reduced forms of nicotinamide adenine dinucleotide (NAD+ and NADH, respectively) (Leach et al., 2002). This ratio of NAD+/NADH provides a measurement that reflects both the metabolic activity and the health of the cell. Normally, NAD+ is reduced to NADH within the metabolic pathway of glycolysis and the Krebs cycle during cellular respiration. Once in its reduced form, NAD+ is regenerated by oxidation of NADH at complex I of the electron transport chain (ETC). During the oxidative process, electrons (from NADH) are transferred down the ETC to oxygen which serves as the terminal acceptor driving adenosine triphosphate (ATP) generation via oxidative phosphorylation. In theory, hypoxia may cause an inadequate supply of oxygen available to act as the final electron acceptor and could hamper the rate of NADH to NAD+ turnover. Such impairment would result in a concomitant increase in NADH to bring about a decrease in NAD+/NADH ratio, and hence alter the redox state of the cell to reflect a reduced metabolic activity.

In vitro examination of the effects of hypoxia on the redox state of a superfused cerebral cortex slice has been studied (Garofalo et al., 1988). The investigators reported a nonsignificant increase in NADH and a decrease in NAD+/NADH ratio in mild hypoxia; however, with severe hypoxia, NADH increased over 200% resulting in a significant decrease in NAD+/NADH ratio. Likewise, although with complete anoxia lasting for 5 min, investigators studying the redox state in the brain in vitro using rat models reported a seven fold decrease in the NAD+/NADH ratio from normoxia to anoxia, again implying a marked increase in NADH (Merrill and Guynn, 1982).
In vivo studies, unlike in vitro preparations, employ homeostatic mechanisms in the event of hypoxia to ensure continuous delivery and supply of oxygen to the cells. Specifically, tight regulation of oxygen homeostasis within the brain is highly dependent on the regulation of cerebral blood flow (CBF) (Acker and Acker, 2004). Other cardiovascular responses such as increased heart rate, increased cardiac output, and systemic vasodilatation further support the maintenance of systemic oxygen tension (Downing et al., 1963). Johannsson and Siesjo (1975) in anesthetized artificially ventilated rats measured CBF and cerebral metabolic rate of oxygen (CMRO₂) (i.e. rate of oxygen consumption in the brain) during severe hypoxia (22 mmHg) for 15-25 min. When PaO₂ was reduced to 22 mmHg, they reported a four to six fold increase in CBF without any changes in CMRO₂ over the duration of hypoxia. Their study concluded that the tight maintenance of cerebral energy state, even at extreme degrees of hypoxia, was coupled by the increase in CBF. In awake sheep, Iwamoto et al. (1991) reported a 200-250% increase in CBF with a consequent increase in CMRO₂ of 25-60% with 3.5 hours of prolonged moderate hypoxia (PaO₂ 40 mmHg). Again CBF corresponded well with the fall in PaO₂ as a compensatory mechanism to prevent cerebral oxygen deficiency. Moreover, the paradoxical increase in cellular oxygen consumption that appeared in response to hypoxia was thought to be caused by a temporary rise in blood temperature due to stress induction.

Since both in vivo studies did not show a reduced CMRO₂ during moderate to severe hypoxia, this likely implies that, unlike in vitro studies, reduction in mitochondrial redox state (NAD+/NADH ratio), i.e. insufficiency of oxygen, at least in the brain, does not occur in vivo during hypoxia.
Sustained Hypoxic Response

Sustained Hypoxic Ventilation

Commencement

It is well known that ventilatory response to acute hypoxia causes an increase in ventilation. However, studies leading investigators to consider the potential effects of persistent hypoxia to attenuate the ventilatory response to hypoxia did not surface until the early 1970’s. In 1973, Edelman and colleagues comparing the ventilatory response to transient and steady state hypoxia in humans, showed that, although qualitatively similar in their response, the transient response to hypoxia was significantly greater quantitatively by 18%. In a 1975 study by Weiskopf and Gabel, the ventilatory response in humans at a given PaO$_2$ was found to be greater during the production of progressive isocapnic hypoxia (~5 min) than during the succeeding progressive reversal from this hypoxic state (Figure 15, adapted from Weiskopf and Gabel, 1975). Taken together, these studies clearly reveal the attenuating effects of persistent hypoxia on ventilation and raised questions concerning the importance of the study of the acute hypoxic response to the long-term control of breathing; and from these two studies commenced the start of sustained hypoxia research.
Figure 15: Attenuation of ventilation during acute progressive hypoxia

Ventilatory response in a single subject during development (progression, solid line) and reversal (regression, broken line) of acute progressive isocapnic hypoxia. Note: attenuated ventilation during the reversal of acute progressive hypoxia.

Newborn Studies

Sustained hypoxia effects on ventilation have been studied in pre-and full-term infants (Rigatto and Brady, 1972; Rigatto et al., 1975; Rigatto, 1979; Sankaran et al., 1979; De Boeck et al., 1984; Martin et al., 1998) and in newborns of various animal species including monkeys (LaFramboise et al., 1981; LaFramboise et al., 1983; LaFramboise and Woodrum, 1985), kittens (Blanco et al., 1984; Bonora et al., 1984; Rigatto et al., 1988), lambs (Bureau et al., 1984; Bureau et al., 1986), piglets (Martin et al., 1990), rats (Saetta and Mortola, 1987; Eden and Hanson, 1987), and rabbits (Martin-Body and Johnston, 1988; Wangsnes and Koos, 1991). In response to inhalation of low F\textsubscript{2}O\textsubscript{2} (~7-15%), there is an initial increase in ventilation to peak (~1-3 min) followed by a subsequent decline approaching or dropping below baseline control levels (~5-30 min).
(Figure 16, preterm infant adapted from Sankaran et al., 1979). This biphasic pattern of change in minute ventilation ($V_1$) has been documented successfully in both anesthetized and unanesthetized spontaneously breathing mammals, ruling out the depressive effects of anesthesia as a sole causative factor in producing the biphasic response. Moreover, because the observations of the prompt fall in ventilation below baseline values resolve in newborns by 7-28 days of age (Rigatto et al., 1975; Woodrum et al., 1981; Walker, 1984; Bureau et al., 1984; Mc Cooke and Hanson, 1985; Eden and Hanson, 1987); it was speculated that the biphasic response would mature with age, such that, in adults, it would become unimodal and sustained. Subsequent studies demonstrated that the biphasic ventilatory response to sustained hypoxia is a characteristic feature in adults as well, however, with a notable difference in the timing and magnitude of the ventilatory decline.

Figure 16: Ventilatory response to sustained hypoxia in preterm infants
Ventilatory response to sustained hypoxia ($F_{O_2}$ 15%) lasting 5 min in a single preterm infant. Note: maintenance of steady-state hypoxia and marked decline in ventilation below baseline levels.
Adult Studies

Ventilatory response to sustained hypoxia in adults was first reported in 1976 by Weil and Zwillich. In their study, 4 adult human subjects were subjected to 40 min of sustained isocapnic moderate hypoxia (45 mmHg PaO₂) which caused an initial increase in ventilation to peak within 3-5 min, but thereafter ventilation progressively fell reaching a plateau level - termed the “roll-off” or hypoxic ventilatory decline (HVD) - by 15-30 min (75% relative to peak). Likewise, Easton et al. (1986) characterized the ventilatory response to sustained hypoxia (F_iO_2 ~8-10%, SaO_2 ~80%) in adult humans as being biphasic in pattern, reaching an intermediate level above baseline during 20-60 min period of persistent hypoxia (Figure 17, adapted from Eaton et al., 1986).

![Ventilatory response to sustained hypoxia in adult humans](image)

**Figure 17: Ventilatory response to sustained hypoxia in adult humans**
Ventilatory response to sustained hypoxia (F_iO_2 8-10%, SaO_2 ~80%, 26 min) in a single adult subject. Note: maintenance of steady-state hypoxia, isocapnic condition and biphasic ventilatory pattern.
In the aforementioned study, Easton and colleagues reported breathing pattern parameters accompanying $V_I$ revealing that the initial increase in $V_I$ is brought about by an increase in $V_T$ and $f_R$, while the secondary fall in ventilation is accounted for primarily by a decrease in $V_T$. Moreover, they also noted that the initial ventilatory response related closely with the ensuing HVD. Thus qualitatively similar to newborns, ventilatory response to sustained hypoxia in adults is clearly biphasic in pattern, however, with a prominent quantitative difference in the timing and magnitude of the ventilatory decline (Easton et al., 1986); nevertheless, both responses might share some common mechanistic features (Powell et al., 1998).

Successive investigations have consistently reported a biphasic ventilatory response to sustained hypoxia in adult humans lasting up to 15-60 min (Easton et al., 1988; Easton and Anthonisen, 1988a; Easton and Anthonisen, 1988b; Long et al., 1989; Georgopoulos et al., 1989; Masuda et al., 1989; Yamamoto et al., 1994). In addition, HVD has been shown to occur with repetitive isocapnic hypoxia (ten 2-min episodes of hypoxia separated by 2-min periods of normoxic breathing) and at high altitude even during chronic exposures to hypoxia; hence, continued sustained hypoxic exposure need not account for the HVD, and HVD is independent of the process of ventilatory acclimatization (McEvoy et al., 1996; Sato et al., 1992). Further evidence for the biphasic ventilatory response to sustained hypoxia in adults have been shown in unanesthetized and anesthetized adult animals, including cats (Vizek et al., 1987; Tatsumi et al., 1992; Long and Anthonisen, 1995), rats (Vizek and Bonora, 1998; Maxova and Vizek, 2001; Tabata et al., 2001; Marczak et al., 2004), mice (Huey et al., 2000; Palmer et al., 2013), goats (Freedman et al., 1988; Gershan et al., 1994), and ponies (Brown et al., 1992), all displaying an initial increased ventilation followed by a decline or roll-off down to or above baseline levels.

**Mammalian Controversy**

With the collective evidence in support of the existence of a biphasic sustained hypoxic ventilatory response in humans and across numerous animal species, it may be
hypothesized that such a response might be a mammalian characteristic without species exception. However, a notable exception has been previously reported in canines, where ventilation did not exhibit the characteristic biphasic response pattern to sustained hypoxia common to that of humans and other animals (Cao et al., 1992, 1993); hence, no roll-off or HVD. To the best of our knowledge, the two studies by Cao and colleagues (1992, 1993) are the only published literature that specifically examined the ventilatory response to sustained hypoxia in canines. In their first study, unanesthetized awake canines were subjected to 20 min of sustained isocapnic hypoxia (80% $\text{SaO}_2$) breathing through an endotracheal tube. Results of their study showed that, in both conditions of un-resistive and resistive tracheal loading tests during sustained hypoxic exposure, canines did not show a roll-off or HVD. A subsequent study, using a similar sustained hypoxia protocol, demonstrated that neither mask breathing or tracheal unloaded breathing resulted in a decline in ventilation, however, tracheal loaded breathing in this study (but not in their former study) caused ventilation to roll-off during sustained hypoxia. No other studies have followed up on this apparent controversial discrepancy between canines and other mammalian species; hence, the question of whether HVD is a universal mammalian characteristic, or canines are a notable exception to this typical biphasic ventilatory response to sustained hypoxia, remains to be revisited and settled.

**Mechanism of Hypoxic Ventilatory Decline**

The nature of the biphasic ventilatory response to sustained hypoxia is not thoroughly understood, and thus has been a topic of considerable interest. The initial rapid increase in ventilation upon exposure to hypoxia is well recognized as being due to peripheral chemoreceptor stimulation; however, the mechanism explaining the secondary decline in ventilation, i.e. roll-off or HVD, remains mostly unresolved. Several hypotheses have been proposed and tested, each receiving both positive and negative support.
Peripheral Chemoreceptor Activity

Although controversial, immaturity of the peripheral chemoreceptors and a time dependant reduction in carotid body sensitivity has been postulated to account for the biphasic response during sustained hypoxia.

In newborn kittens, less than 10 days old, activity of the carotid sinus nerve has been found to decline during sustained hypoxia (Marchal et al., 1992); however, this decline in peripheral chemoreceptor activity diminished and was sustained in kittens of 8 weeks of age. Significance of age dependant maturity of the peripheral chemoreceptors is thought to be species dependent since similar findings could not been seen in newborn piglets (Davis et al., 1988; Rosen et al., 1993). These findings led to the belief that, at least in newborns, with species exceptions, immaturity of the peripheral chemoreceptors might partly account for the HVD, amongst other factors such as reduction in metabolic rate (Schwieler, 1968; Blanco et al., 1984; Saetta and Mortola, 1987; Neubauer et al., 1990). In contrast to newborn subjects, in adults where the carotid bodies have matured, it has been suggested that a reduction in peripheral chemoreceptor sensitivity to hypoxia could bring about a time dependent decline in carotid sinus nerve activity to explain for the genesis of the HVD. Although plausible, there is little evidence in support of the time dependent sensitivity changes in the peripheral chemoreceptors with the exception of rabbits (Li et al., 1990) and indirect human observations (Bascom et al., 1990).

On the contrary, experimental studies against the peripheral origin of HVD - and thus, in support of a central origin - have been reported: hypoxic peripheral chemoreceptor sensitivity is unaltered despite the occurrence of ventilatory depression during sustained hypoxia in awake humans (Sato et al., 1992); absence of ventilatory depression in anesthetized cats during sustained isolated peripheral chemoreceptor hypoxic stimulation (Neubauer et al., 1990); ventilatory depression in anesthetized carotid body denervated animals (Neubauer et al., 1985); and, decline in phrenic nerve discharge despite sustained carotid sinus nerve activity in anesthetized cats during sustained hypoxia (Vizek et al., 1987). Although the existing data suggests that the origin
of HVD is likely not directly peripheral, there is good evidence that the peripheral chemoreceptor input may be required to produce the ventilatory depression. Several studies are in support of this viewpoint, especially, in awake intact preparations and patients, since HVD does not occur when the carotid bodies are absent (Long et al., 1993; Honda, 1992).

**Central Chemoreceptor Activity**

One of the earliest postulates to explain the central origin of HVD was a reduction in the level of central chemoreceptor stimulation. This was thought to be mediated by a decline in brain PCO$_2$ levels due to initial hypoxic hyperventilation or a hypoxia induced increase in cerebral blood flow. In both cases, reduced stimulus (i.e. lowered PCO$_2$ levels) at the site of central chemoreceptors would bring about a depression in ventilation. Although straightforward, hyperventilation induced hypocapnia by “blowing off” CO$_2$, as a mechanism for HVD, is unlikely since the biphasic ventilatory response during sustained hypoxia still occurs during isocapnic conditions (Easton et al., 1986; Masuda et al., 1989; Long et al., 1993; Yamamoto et al., 1994). Alternatively, there are two ways that hypoxia can augment cerebral blood flow to cause cerebral CO$_2$ washout; hypoxia can act on the peripheral chemoreceptor to increase cardiac function and/or alternatively hypoxia can act directly to induce cerebral vasodilation. In both cases, CO$_2$ washout leads to central alkalization and reduced ventilatory drive.

**Cerebral Lactic Acidosis**

Another proposed central mechanism is brain lactic acidosis and its associated involvement in generating ventilatory depression. While cerebral acidosis is thought to stimulate ventilation by acting on the central chemoreceptors, studies have revealed an increase in lactic acid production in the medulla despite the development of HVD during sustained hypoxia (Neubauer et al., 1988; Xu et al., 1991). To test the significance of cerebral lactic acidosis in mediating ventilatory depression, ventilatory response to progressive brain hypoxia was studied after administration of dichloroacetate to prevent
lactic acid formation in cats (Neubauer et al., 1988). This treatment effectively abolished the HVD; however, similar treatment was ineffective in attenuating the ventilatory depression in humans (Georgopoulos et al., 1990). Although the evidence is very scarce, such findings thus far suggest that, at least in humans, HVD is probably not mediated by cerebral lactic acidosis. Further studies are warranted to determine the effects of brain lactic acidosis on the sustained hypoxic ventilatory roll-off.

Direct CNS Hypoxic Depression

Investigators have also considered the direct effects of hypoxia on CNS function and neuronal activities as a possible explanation of the central origin of HVD. Although severe hypoxia (~25 mmHg) has been reported to be associated with neuronal dysfunction of metabolic activities secondary to insufficient energy substrates at the level of the medulla (Neubauer et al., 1990), HVD is a consistent finding with mild to moderate hypoxia insufficient to produce detectable changes in energy substrates (Weil, 1994). Also, depressed CNS function fails to account for the well preserved ventilatory response to hypercapnia during sustained hypoxia (Long et al., 1994). Thus, with the exception of severe hypoxia, it is unlikely that depressed CNS function, secondary to metabolic dysfunction, is involved in the generation of the HVD. Alternatively, hypoxia acting on certain neurons within the respiratory control center have been reported to cause membrane hyperpolarization and reduce neuronal activity (Neubauer et al., 1990). Although these events do occur and may serve a protective function, the neuronal responses alone are considered to be too transient to account for the slow recovery (30-60 min) of the hypoxic ventilatory response after sustained hypoxia, and hence fail to fully explain the HVD (Easton et al., 1988). Nevertheless, persistent membrane hyperpolarization - diminished activity of the respiratory neurons - may play an important role in the genesis of HVD, if mediated by a time dependent presence of inhibitory neurochemicals.
Central Respiratory Drive Inhibition

Of all central mechanisms hypothesized thus far, neurochemical inhibition of central respiratory drive has received the greatest amount of support for the genesis of the HVD. In this framework, hypoxia induces changes in the concentration, synthesis and release of inhibitory neuromodulators/neurotransmitters as an active event involving sensory elements (Weil, 1994; Neubauer, 1990; Honda and Tani, 1999). Specific location and the type of sensors involved are presently unknown, as the process could involve either direct stimulation of hypoxia on CNS neurons and/or indirect stimulation through the peripheral chemoreceptor (Bisgard and Neubauer, 1995). Regardless of the actual sensors involved, sensory components appear to play an integral role in activating a central mechanism which modulates neuromodulator/transmitter content. In any event, the involvement of inhibitory neuromodulator/transmitter in the genesis of HVD is strongly favored considering its intrinsic ability to account for both the selective and persistent effects of HVD (Long et al., 1994; Easton et al., 1988). The inhibitory neuromodulator/transmitter hypothesis has received the greatest attention and support with a considerable amount of research effort carried out to find the putative neuromodulator. To date, several prospective neuromodulators have been proposed and challenged as to their role in the HVD, including opioids (Kagawa et al., 1982; Steinbrook et al., 1985; Chernick and Craig, 1982), adenosine (Yamamoto et al., 1994; Easton et al., 1988; Gershan et al., 1996), gamma-Aminobutyric acid (GABA) (Kazemi and Hoop, 1994; Yamada et al., 1981; Dahan et al., 1991; Sica et al., 1993), and dopamine (Goiny et al., 1991; Long and Anthonisen, 1995; Pedersen et al., 1997; Van Beek et al., 1984). Although these neuromodulators have been implicated to have an inhibitory influence to depress ventilation, none of the agents on their own can completely explain the HVD.
**Respiratory Muscle Activity**

Although many experimental studies have investigated the biphasic ventilatory response and its possible mechanisms, there is relatively little information available on the impact of sustained hypoxia on the effector muscles themselves, i.e. the respiratory muscles. In the past, respiratory muscle activity during sustained hypoxia have been examined in a limited number of studies in newborn and adult animals, as well as in adult humans.

**Newborn Studies**

In newborn animals, assessment of respiratory muscle activity, in general, reported a biphasic pattern, however, the time and magnitude of the profile varied considerably depending on the state of the animal (awake or anesthetized), arterial CO\textsubscript{2} levels (isocapnia or poikilocapnia), as well as the specific muscle and species in consideration. LaFramboise and Woodrum (1985) assessing the crural diaphragm activity in awake newborn monkeys showed that the EMG activity, despite falling from a peak, remained above baseline during 5 min of poikilocapnic hypoxia. Similarly, in anesthetized kittens, costal and crural diaphragm showed a peak response declining to an intermediate plateau and did not return to baseline activity levels with 5 min of poikilocapnic hypoxia (Guthrie et al., 1990). These observations are in contrast to a the fall in the crural diaphragm EMG activity from peak below baseline in anesthetized newborn piglets exposed to 5 min of poikilocapnic hypoxia (Watchko et al., 1990). On the other hand, Martin et al. (1990) reported a bimodal costal diaphragm EMG activity in anesthetized newborn piglets that fell to pre-hypoxic levels by 10 min of sustained hypoxia. Furthermore, accounts of expiratory muscle activity rolling-off in newborn subjects has also been found in the transversus abdominis and external oblique expiratory muscles (Watchko et al., 1990; Praud et al., 1993). However, for the external oblique, the roll-off only occurred during sustained hypoxic breathing when accompanied with hypocapnia, but not with isocapnic conditions.
Adult Studies

In adult humans, only two studies have examined the muscle activity of the primary inspiratory muscles during sustained hypoxia, however, employing surface electrodes. Okabe et al. (1993) examined the parasternal intercostal chest wall muscle EMG activity during 20 min of isocapnic sustained hypoxia, and noted that the muscle rolled-off with sustained hypoxia to an intermediate plateau following an immediate peak response. Similarly, McEvoy et al. (1996) using surface electrodes reported a biphasic EMG activity pattern in the costal diaphragm during a 20 min period of sustained hypoxia. Although surface electrodes used in these studies provide a relatively easy, non-invasive and safe measurement of respiratory muscle EMG activity in humans, its inherent limitation is that the resultant EMG signal is often noisy and contaminated with other muscle groups in the vicinity preventing the accurate quantification and assessment of muscle activity.

Studies examining respiratory muscle activity in adult animals during sustained hypoxia are scarce, with reports only existing in cats, rats and ponies (Van Lunteren et al., 1989; Brown et al., 1992; Vizek and Bonora, 1998). Van Lunteren et al. (1989) in anesthetized cats demonstrated that 10 min of poikilocapnic hypoxia elicited a biphasic EMG activity response in the costal diaphragm reaching an intermediate plateau above baseline; whereas the triangularis sterni, an expiratory chest wall muscle, was inhibited below baseline levels with exposure to constant hypoxia without an initial peak hypoxic response. In awake ponies, chronic hypoxia (48 hour duration) elicited a biphasic EMG activity response of the costal diaphragm and transversus abdominis; however, within 30 min of constant hypoxia, costal diaphragm EMG remained above while transversus abdominis EMG fell below baseline activity levels (Brown et al., 1992). In rats, the effects of sustained hypoxia on the costal diaphragm activity has been inconsistent and state dependent. Maxova and Vizek (2001) did not observe a biphasic EMG activity of the costal diaphragm in awake rats during 20 min of sustained poikilocapnic hypoxia. Yet, in another study in anesthetized rats, costal diaphragm EMG activity after reaching peak rolled-off with poikilocapnic hypoxia held for 10 minutes (Vizek and Bonora, 1998).
Summary

Based on the limited data available in animals and humans, respiratory muscle activity response to sustained hypoxia appears to vary both qualitatively and quantitatively depending on the species and maturity, the specific inspiratory and expiratory muscles under consideration, as well as the influence of anesthesia and alteration in arterial CO\textsubscript{2} levels. Previous studies have been limited strictly to the assessment of muscle EMG activity without any additional measurement, such as changes in muscle length and shortening, in order to deduce the actual mechanical consequence or action of a neurally activated muscle. No studies to date have systematically examined the major inspiratory and expiratory breathing muscle groups from a single animal species; hence raising difficulties in the interpretation and understanding the effector system as a whole and their contribution to ventilation.

Research Rational

Canine Controversy

Ventilatory response to sustained hypoxia continues to be a subject of great interest for both basic and clinical researchers. Despite the collective efforts of past investigators examining the origin of the sustained hypoxic ventilatory decline or roll-off in mammals, the exact mechanism remains incomplete and unresolved. Moreover, controversy still surrounds the issue of whether canines do or do not exhibit the characteristic, possibly mammalian, biphasic ventilatory response to sustained hypoxia. Among the selective large animal research labs in the world, the expertise in working with intact awake canines sets our lab in a unique position to address this apparent controversy. In contrast to previous canine studies by Cao et al. (1992, 1993), our study avoids any confounding effects of tracheal instrumentation by studying the ventilatory response to sustained hypoxia in canines with an intact upper airway, breathing through a snout mask. The results of our study will provide direct evidence as to whether
ventilation will roll-off during sustained hypoxia in intact awake canines, and will validate whether this species is a viable model for studying the biphasic ventilatory response and its characteristic changes in breathing pattern.

**Respiratory Muscle Function**

**Limitations**

Despite the important contribution of the respiratory muscles subserving ventilation, relatively little is known about the consequences of sustained hypoxia on the effectors of respiration. Previous studies evaluating EMG activity of individual respiratory muscles in humans and animals have shed some light on this, however, these earlier studies generally involved immature or small animals, expressing a quantitatively different ventilatory response to sustained hypoxia as that of large adult mammals; had removed the important respiratory function of the upper airway via tracheostomy; and/or involved indirect measurement techniques using surface EMG electrodes. In addition, most animal studies in the past that did succeed in measuring respiratory muscle EMG activity during sustained hypoxia did so in an acutely anesthetized preparation.

**Anesthesia**

The effects of anesthesia to depress ventilation as well as to significantly alter the ventilatory response to hypoxia is well-recognized (Hickey and Severinghaus, 1981; Pavlin and Hornbein, 1986). Anesthesia has been also reported to cause a significant reduction in FRC (Hickey and Severinghaus, 1981; Pavlin and Hornbein, 1986), an increase in the resting pre-contraction length of the diaphragm (Fitting et al., 1987), and a loss of diaphragm tonic and postinspiratory inspiratory activity (PIIA) (Fitting et al., 1987; Muller et al., 1979). With respect to the neural control of the respiratory muscles, the state altering influence of anesthetics have been documented to cause a significant diaphragmatic contribution to ventilation with a selective suppression of the chest wall inspiratory muscle during eupnoea, as well as a preferential recruitment of the abdominal
expiratory muscles (Warner et al., 1995; Warner et al., 1992). Such alterations in ventilation, thoracopulmonary mechanics and respiratory muscle function with anesthesia would certainly disrupt the normal physiologic response and activity of the respiratory muscles during sustained hypoxia, and thus amplifying the need for a study in awake mammals without the confounding influence of anesthetics.

Muscle Length and Shortening

Although respiratory muscle EMG activity reflects the neural drive arising from the central respiratory controller, EMG alone is not a reliable or accurate indicator of contraction or force output of muscle. In the diaphragm, reflex inhibition is known to occur following surgical implantation of sonomicrometry transducers and fine wire EMG electrodes, where effective muscle shortening is not guaranteed even in the presence of phasic EMG activity 7- to 10-days post-operatively (Easton et al., 1989). Moreover, contractile shortening of a respiratory muscle depends on numerous mechanical factors beyond electrical activation, including pre-contractile length, mechanical load and impedance, coordination and interaction with other muscles, muscle health and integrity, etc. Since EMG activity is not a direct correlate of the mechanical change, having measurements of length change reinforces the presumption of normal EMG activity by concurrent evidence that each muscle is actually shortening and/or functioning as expected (Easton et al., 1993). In order to precisely and accurately quantitate and evaluate the function of the respiratory muscles, with respect to its mechanical and electrical activity, experimental studies need to include the examination of the electrical activity, i.e. EMG, along with its corresponding mechanical consequence, i.e. muscle length and shortening. To date, investigative studies examining the effects of sustained hypoxia on the mechanical action and EMG activity of the respiratory muscles has not been undertaken in any awake or anesthetized mammals.
**Instrumented Canines**

Much is still lacking in our basic understanding and knowledge of the effects of sustained hypoxia and its impact on the various respiratory muscle groups subserving ventilation. Thus accordingly, the current literature body requires a thorough examination and characterization of respiratory muscle function, i.e. muscle length, shortening and EMG activity, in a large, intact, spontaneously breathing, awake mammal during sustained hypoxia. The chronically instrumented canines, permitting a direct, *in vivo* measurement and assessment of the normal physiologic function of the respiratory muscles, would serve as a viable model for systematically studying the essential inspiratory and expiratory muscles groups within a single animal species.
Objectives and Hypothesis

Aims of Research

The focus of this thesis was to systematically investigate the effects of sustained hypoxia on ventilation and respiratory muscles in fully conscious, spontaneously breathing, large intact canines, chronically instrumented with sonomicrometry transducer and bipolar fine wire EMG electrodes.

Employing the very unique and novel, chronically instrumented, awake canine model, which permits the adequate study of the normal physiologic ventilatory response and \textit{in vivo} intramuscular function of the respiratory muscles, our principle research aims were as follows:

\textbf{Aim #1:} Determine if canines exhibit the characteristic mammalian biphasic ventilatory response to sustained hypoxia, like humans and other animals

\textbf{Aim #2:} Elucidate the mechanical action and neural activation of the primary inspiratory and expiratory muscles during the constant state of hypoxia

General Hypothesis

Biphasic ventilatory response to sustained hypoxia is a universal mammalian characteristic without species exception, and thus ventilation in canines following an initial hyperventilation will roll-off with constant exposure to hypoxia. During sustained hypoxia, attenuation of central drive is a global phenomenon and we expect the major respiratory muscle groups, both inspiratory and expiratory in action, to roll-off in a temporal manner directly contributing to the biphasic changes in ventilation. However, given the discrete structural and mechanical characteristics of individual respiratory muscle groups, and their capacity for differential activation and function, we expect
different muscles to reveal a distinct mechanical action and EMG activity pattern during the sustained hypoxic roll-off.

**Experimental Pre-conditions**

To elicit and evaluate the natural physiologic ventilatory and respiratory muscle response at rest and during chemical stimulant by sustained hypoxia, the animals undergoing the study must be intact and normal, i.e. free of any confounding effects of surgical interventions, post-operative complications and/or anesthetics, and in a fully conscious, awake state, breathing spontaneously without any distress or discomfort. Furthermore, precise and accurate examination of individual respiratory muscle function in canines and other mammals necessitates an *in vivo* simultaneous intramuscular assessment of the electrical activity along with its mechanical correlate, such as muscle length and shortening.

During the series of original investigations undertaken in this presented thesis project, these pre-conditions were realized by utilizing the chronically instrumented awake canines, which provided direct, simultaneous measurement of respiratory changes in length, shortening and EMG activity of the primary inspiratory and expiratory muscles, namely that of the costal and crural diaphragm, the parasternal intercostal, and the transversus abdominis.

**Specific Questions and Individual Projects**

Employing our chronically instrumented, awake canine model, we elected to pose and address a series of fundamental research questions overarching the theme of sustained hypoxia and its effects on ventilation and respiratory muscle function. The proposed research questions and the title of each investigation are summarized below.

Do canines express the characteristic biphasic ventilatory response to sustained hypoxia? How does breathing pattern reflect changes in ventilation? Will ventilation roll-
off due to a volume effect with minimal alterations in respiratory timing, like humans? Will attenuation of central drive occur in the costal diaphragm as indexed by EMG activity? These questions are investigated in the first project, **Ventilation and diaphragm activity during sustained hypoxia in awake canines.**

By direct measurement of the intact diaphragm, how does the principle inspiratory muscle respond to sustained hypoxia? Will the costal and crural diaphragm both roll-off during the constant exposure to hypoxia? Does the costal and crural diaphragm exhibit the capacity for differential segmental activation and function during initial acute and sustained hypoxia? We examine these questions in the second project, **Costal and crural diaphragm function during sustained hypoxia in awake canines.**

The diaphragm does not function alone during inspiration, the primary chest wall inspiratory muscle, the parasternal intercostal, is an obligatory inspiratory muscle that acts in concert with the diaphragm. Will the attenuation of central drive extend to the parasternal chest wall muscle with persistent hypoxia? What is the relative neuromechanical relationship of the parasternal intercostal during sustained hypoxia? These queries are addressed in **Parasternal intercostal function during hypoxia in awake canines,** the third project.

Classic studies suggests that expiratory neuronal and nerve activity is inhibited by hypoxia. Does hypoxia cause inhibition of expiratory abdominal activity in a fully conscious, spontaneously breathing, intact canine? What is the effect of sustained hypoxia on the activity and action of the primary abdominal expiratory muscle, the transversus abdominis? These inquiries are explored in the fourth project entitled **Abdominal muscle action during sustained hypoxia in awake canines.**
Experimental Methods

The investigations of this thesis critically relies upon the chronically instrumented canine preparation for simultaneous direct in vivo measurements of muscle length and EMG activity of the primary respiratory muscles along with breathing pattern variables (Figure 18). The sonomicrometry transducers and fine wire bipolar EMG electrodes were implanted in three inspiratory muscles, the costal and crural diaphragm and parasternal intercostal, and one expiratory muscle, the transversus abdominis. The costal and crural diaphragm and the transversus abdominis were implanted on their abdominal side via midline laparotomy, where as the parasternal intercostal was implanted by exposing the muscle on the outside of the thorax. The animals were fully recovered prior to experimentation, and all signals were continuously recorded and digitally stored onto a computer for review and analysis using an customized, in-house, data acquisition software and suite of analysis programs.

Figure 18: Awake canine model
Schematic of the canine model with proximal locations of the primary inspiratory and expiratory muscles under investigation indicated by arrows.
Surgical Implantation

All aspects of this project were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines.

Each mongrel canine had pairs of sonomicrometry transducers and bipolar fine wire electromyogram (EMG) electrodes (Figure 19, left panel) implanted into the left costal and crural diaphragm segments, parasternal intercostal muscle, and transversus abdominis muscle. Animals were studied after full recovery of postoperative diaphragm segmental shortening. This technique of chronic sonomicrometry and EMG implantation, and the 7-10 day progressive recovery of diaphragm segmental shortening, has been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). Implantation of transducers and electrodes were performed under general anesthesia with thiopental sodium induction (15 mg/kg) and maintained with halothane.

The left hemidiaphragm was exposed through a midline abdominal incision, and ultrasonic transducers were implanted between muscle fibers on a flat portion of each of the costal and crural segments of the left hemidiaphragm (Figure 19, middle panel). Costal transducers were placed in the lateral portion of the segment, approximately midway between central tendon and chest wall in the region corresponding roughly to the second sternocostal branch of the phrenic nerve. Crural transducers were placed in the posterior, perivertebral region of the segment. Opposing transducers in each pair were inserted ~10-15 mm apart. On each segment, immediately adjacent to each transducer, a fine-wire stainless steel bipolar EMG electrode was attached. In the same procedure, sonomicrometry transducers and EMG electrodes were implanted in the left transversus abdominis muscle aligned in the same cross-sectional plane, midway between inferior costal margin and iliac crest, in the plane of the anterior axillary line. In a subsequent surgery, about one week apart, ultrasonic transducers and EMG electrodes were implanted between muscle fibers of the parasternal intercostal muscles of the 2nd to 4th intercostal space, ~1-3 cm lateral to the edge of the sternum, by incision of the skin and deflection over the sternum (Figure 19, right panel).
Figure 19: Surgical implantation of transducers and EMG electrodes
Left: Schematic of the ultrasound transducer and EMG electrode. Middle: Costal and crural diaphragm implantation. Right: Parasternal intercostal implantation.

All implants were secured by fine, synthetic, nonfibrogenic sutures (Prolene, Ethicon, Somerville, NJ), the implanted wires were externalized by a subcutaneous skin tunnel, and the animals were allowed to recover. In general, the animals were awake and ambulatory within 3-6 hours of each operation, and freely active and feeding normally within 1-2 days. Experiments were conducted a mean of 28 days post-implantation (range 8-70 days).

Measurement Techniques

All measurements of ventilation and respiratory muscle function were made with the animals awake and breathing quietly at a laboratory temperature of ~18-20 °C, while lying in right lateral decubitus position which placed the implanted muscles in a non-dependent position. The animals were relaxed and familiar with the location, routine and personnel of the recordings.
Breathing Pattern Variables

The animals were breathing spontaneously through a tightly fitted snout mask. The mask was attached through a 2-way non-rebreathable valve to a low resistance (1 cmH₂O/L/s) open breathing circuit (described in Background: Open Breathing Circuit), which incorporated a pneumotachograph (Fleisch #2) and a piezoelectric differential pressure transducer (Model 163PC01D36, Honeywell Microswitch) to provide measurements of airflow. On the expiratory limb, end tidal CO₂ (ETCO₂) was sampled and analyzed continuously by an infrared CO₂ analyzer (Model CD-3A, AMETEK/Thermox Instrument Division, Pittsburgh, PA). On the inspiratory side, fractional concentration of inspired O₂ (FᵢO₂) was continuously sampled and analyzed by an O₂ analyzer (Model S-3A/1, AMETEK/Thermox Instrument Division, Pittsburgh, PA). The inspiratory limb could be switched, without alerting the animal, from room air to a large reservoir of pre-mixed gas of low FᵢO₂ (8-10%). In addition, supplemental pressurized sources of O₂ and CO₂ were attached to the inspiratory limb to allow the experimenter to precisely titrate FᵢO₂ and ETCO₂ during the study. To relate the level of hypoxia, oxygen saturation (SpO₂) was continually measured by a pulse oximeter (Model Ohmeda Biox 8700, Rexdale, ON, Canada) by attaching a light sensitive analysis probe onto a shaved tendo calcaneous on the animal’s hind limb.

Sonomicrometry

Dynamic measurements within the respiratory muscles of the changing distance between the sonomicrometry transducers of each pair was provided by measuring the speed of transmission of ultrasonic waves using a sonomicrometer (Model 120, Triton Technology, Sand Diego, CA). Technique of muscle length measurements via sonomicrometry has been described in detail elsewhere (Easton et al., 1989; Newman et al., 1984). Each transducer in a pair consisted of a central piezoelectric ceramic plate surrounded on both surfaces by a biconvex epoxy lens. When electrically excited at a rate of 1537 Hz, the emitter piezoelectric transducer resonates, radiating ultrasound waves into the surrounding muscle where some waves strike and deform the receiving
transducer to produce a measurable voltage. A quartz crystal clock oscillator, with a resonance frequency of 1.58 MHz, measures the transit time of the ultrasound waves, and because the conduction velocity in muscle is known (approximately ~1.58 mm/microsecond or 1580 m/sec), the sonomicrometer provides the intertransducer distance. The output signal of the sonomicrometer was offset, amplified and then sampled to computer. The sonomicrometer subsystem is shown in Figure 20.

![Sonomicrometer Subsystem Schematic](image)

**Figure 20: Sonomicrometer subsystem schematic**

**Electromyography**

For measurements of respiratory muscle EMG activity, the fine wire bipolar electrode pairs, consisting of a 36 gauge, Teflon-coated, monel wire from Kooner, from each muscle were connected to an AC differential pre-amplifier (Model 1700, AM Systems, Everett, WA / Mark III, TECA, White Plains, NY). Power line interference was abolished by careful shielding techniques and the use of differential preamplifiers with a high common mode signal rejection of 110 dB. Thereafter the signal was amplified and filtered to attenuate both movement artifact and sonomicrometry noise, and to perform
anti-alias filtering, using a 6-pole, low-pass Bessel band-pass filter at >600 Hz and a matching, 6-pole, high-pass filter at <40 Hz (Model 746, LT-4, Frequency Devices Incorporated, Haverhill, MA). The EMG signals were then rectified and processed by passage through resistance-capacitance, leaky "integrators" with a time constant of 100 ms (CWE Systems, Massachusetts), to provide moving averages of the EMG (Mavg EMG). The electromyographic subsystem is illustrated in Figure 21. Electrocardiogram was obtained using surface electrodes attached to the animal's left front and back legs.

![Laboratory Electronical Systems: Electromyographic Subsystem](image)

**Figure 21: Electromyographic subsystem schematic**

**Data Acquisition**

Using computer software for data acquisition (DataSponge, Bioscience Analysis Software, Calgary, AB, Canada), all signals were monitored in real time on the computer
display and simultaneously collected at 100 Hz to hard disk on a microcomputer equipped with a single board A/D system (Model MIO-16-H-9, National Instruments, Galveston, TX) (Figure 22).

Figure 22: Data acquisition system and physiologic variables
Canine subject with costal diaphragm implants. Physiologic variables are collected using a data acquisition system. Length, muscle length; EMG, electromyogram; ETCO₂, end-tidal carbon dioxide; SpO₂, pulse oximeter oxygen saturation; ECG, electrocardiogram.

Experimental Protocol

The intact awake canine sustained isocapnic hypoxia protocol has been derived from previously published sustained hypoxia studies in humans, and is described fully in a previous publication (Easton et al., 1986). To evaluate ventilation and respiratory muscle function during sustained isocapnic hypoxia, the study sequence was as follows: 1) a control period of room air, resting, baseline breathing of 6-8 min; 2) an abrupt step
A decrease in $F_{I}{O}_2$, which lowered $SpO_2$ to $\leq 80$ in 1-2 min; 3) maintenance of $SpO_2$ at $80 \pm 2\%$ for 20-25 min with constant $ETCO_2$ equal to control levels; and 4) a recovery period of room air breathing of 5-7 min (Figure 23).

**Figure 23: Sustained isocapnic hypoxia experimental protocol**

During sustained hypoxia, the target $SpO_2$ was 80%, and $SpO_2$ variability was restricted by the operator to the range 78-82%, a sustained desaturation that we abbreviate as $80 \pm 2\%$. During the abrupt introduction of hypoxia, supplemental $CO_2$ was added via the pressurized source to the inspired limb of the breathing circuit to bring $ETCO_2$ back to a level approximating $ETCO_2$ during the preceding baseline room air period. We did not attempt to anticipate and exclude any minor dips in $ETCO_2$ at the beginning of hypoxic hyperventilation because complete prevention of any transient fall in $ETCO_2$, at the onset of hypoxia, would have entailed a slight risk of transient overcorrection and spurious increase in inspired minute ventilation during initial hypoxic exposure. After $SpO_2$ had been stabilized at $\leq 82\%$, it was maintained at that level through adjustments in $F_{I}{O}_2$ as necessary, utilizing the pressurized $O_2$ source on the inspiratory limb. This experimental protocol is based upon the single step reduction, steady-state technique to assess the ventilatory response to sustained hypoxia (described in Background: *Single Step Reduction - Steady State Acute/Sustained Hypoxia*).
Data Analysis

After acquisition and storage on disk, data was reviewed and analyzed using programs previously written by supervisor (Dr. Paul A. Easton), as well as software developed by the candidate. These programs provide interactive visual display of the signals during analysis to allow for careful examination of each breath.

Program Development

The latest in-house data acquisition and analysis software written by the candidate, DataSponge™7 (Bioscience Analysis Software, Calgary, AB), extends the capability of the lab to collect, visualize and process high-precision, multi-channel, real-time, biological signals (12-bit, 4096 AD value, 16 channels) on a state-of-the-art operating system (Windows 7/8 and beyond) and computing platforms (32/64-bit architecture). DataSponge™7 incorporates several new features and components unmet by predecessor versions, including hours of continuous, high-throughput, biological signal recording and storage (up to +12 KHz), inspection and processing of high-resolution raw EMG signals (at the level of individual MUAPs), brand new graphical user interface and design (Aero Interface: new toolbars, menus, buttons, etc), and support for the latest line of National Instruments data acquisition drivers (NI-DAQmx 9.x) along with analog-to-digital boards. DataSponge™7 has progressed the ability to reach the full potential of our data collection and computing needs opening up new avenues of research (refer to Appendix: Software Programming for a detailed description of the development work).

Signal Pre-processing

Employing the new data acquisition and analysis software digital multi-channel signal recordings were converted into multi-channel daughter data files and manually reviewed for movement artifacts, transient signal disconnections, apneas, and sighs, all of which were deleted from individual breath-by-breath analysis. To allow for an uncomplicated analysis of respiratory EMG signals, QRS complexes were identified and
excised digitally using the aforementioned software, and then reviewed for consistency and accuracy of electrocardiographic (ECG) noise removal by verification against the ECG signal recorded during the experiment (Figure 24).

**Figure 24: Removal of ECG artifact from parasternal intercostal signal**
A: Original parasternal EMG signal with ECG noise/contamination. B: Parasternal EMG signal after removable of ECG artifacts.

**Whole Breath Analysis**

These pre-processed signals were then subsequently analyzed using customized analysis software programs previously written by the supervisor (KRA/KRF, Bioscience Analysis Software, Calgary, AB) to generate tidal breath, or whole breath, respiratory variables. Accordingly, the flow signal was evaluated for respiratory timing and digitally integrated to compute minute ventilation ($V_I$), tidal volume ($V_T$), respiratory frequency ($f_R$), inspiratory time ($T_I$), expiratory time ($T_E$), mean inspiratory flow ($V_T/T_I$), and inspiratory fraction of respiration ($T_I/T_{TOT}$), were calculated breath-by-breath. The software also calculated tidal breath values of respiratory muscle shortening and EMG
activity. These calculations have been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). In context, the timing of expiration was determined from the airflow tracing, and the onset and termination of individual muscle shortening and EMG activity for each breath was measured relative to the beginning and end of expiratory airflow (Figure 25). Using the sonomicrometry length data from each implanted muscle, the computer calculated for each breath the baseline end-inspiratory resting length of the muscle, titled L_{BL}, and the shortening (SHORT) for each breath expressed as a percentage change from resting length, entitled %L_{BL}. Moving average EMG activity from each muscle was quantified arbitrarily per breath as the maximum difference in volts between baseline and the peak height of the moving average signal, expressed as EMG_{DIFF}. 

ETCO₂ and SpO₂ were also analyzed by the software breath-by-breath based on respiratory timing. Individual ETCO₂ values were converted for altitude to sea level (Patm = 760 mmHg).

Figure 25: Tidal whole breath analysis of respiratory parameters
Breathing pattern and muscle parameters derived from airflow and muscle signals (costal diaphragm illustrated). L_{BL}, end-expiratory resting muscle
length; SHORT, muscle shortening; EMG\textsubscript{DIFF}, baseline to peak difference of moving average EMG activity.

**Intrabreath Analysis**

Separate from the tidal breath values described, the aforementioned analysis programs are capable of calculating the within breath development or "shape" of inspiratory airflow, shortening and EMG activity for each breath. Within each individual breath as defined by airflow, the peak inspiratory airflow, shortening and EMG activity were identified. Throughout the duration of each breath ($T\textsubscript{TOT}$), inspiratory flow, shortening and EMG activity were expressed as a percent of the maximum value at successive 5% "slices" of $T\textsubscript{TOT}$ (%$T\textsubscript{TOT}$), starting from the baseline value at the onset of each breath. This percent maximum normalized intrabreath profile for each breath, time standardized as %$T\textsubscript{TOT}$ in 5% intervals or 20-bin values, offered a convenient method for assessing and comparing the postinspiratory inspiratory activity (PIIA) of the costal and crural diaphragm during resting and hypoxic-stimulated ventilation (refer to Concurrent and Future Studies: *Sustained Hypoxia*).

**Analysis Periods**

Measurements of respiratory variables was continuous throughout the protocol, but for clarity of presentation, variables are summarized over corresponding periods of each study (Figure 26). Mean values of ventilation, components of breathing pattern ($V_i$, $V_T$, $f_r$, $T_i$, $T_e$, $V_T/T_i$, $T_i/T\textsubscript{TOT}$, $SpO_2$, $ETCO_2$), and respiratory muscle parameters ($L\textsubscript{BL}$, SHORT, and EMG\textsubscript{DIFF}) were calculated from the continuous measurements during the following periods: BASE, PEAK, PLATEAU, and RECOVERY, as defined earlier by Easton et al., (1986, 1988). Period BASE was the final 5 min of resting ventilation with normal $SpO_2$ before the introduction of hypoxia. Periods PEAK and PLATEAU were from opposite ends of the 20-25 min period of sustained hypoxia at $80 \pm 2\%$ $SpO_2$: PEAK represented the first 3 min of hypoxic ventilation, immediately after $SpO_2$ had descended to a level of $\leq 82\%$, while PLATEAU represented the last 5 min of hypoxic.
ventilation, preceding the relief of hypoxia. Period RECOVERY represented the first 5 minutes of room air breathing after hypoxia was abruptly relieved.

**Figure 26: Sustained isocapnic hypoxia analysis protocol**

**Statistical analysis**

After calculation, mean values were exported for review to spreadsheet software (Microsoft Excel, Microsoft, Redmond, WA), to graphic software to output figures (CorelDRAW X6, Corel Corporation, Ottawa, ON), and to PC version of SAS for statistical analysis (SAS Version 9.1, SAS Institute, NC). Group mean values for parameters of breathing pattern and muscle length, SHORT and EMG activity were tested across the four periods (BASE, PEAK, PLATEAU, RECOVERY) by two-way analysis of variance with repeated measure on a single factor (Keppel, 1982; Steele and Torrie, 1980). Multiple comparison testing of the group mean values of the individual periods was performed using Tukey’s or Student-Newman-Keuls test (Steele and Torrie, 1980). At individual periods, group mean SHORT or EMG activity between two muscle groups were compared using a Student's paired t-test. P-value of less than 0.05 was considered statistically significant; actual P-values are reported for all analysis of variance and pair-wise t-test comparisons.
Results

Individual Projects

#1: Ventilation and Diaphragm Activity during Sustained Hypoxia in Awake Canines

#2: Costal and Crural Diaphragm Function during Sustained Hypoxia in Awake Canines

#3: Parasternal Intercostal Function during Sustained Hypoxia in Awake Canines

#4: Abdominal Muscle Action during Sustained Hypoxia in Awake Canines
Project 1: Ventilation and Diaphragm Activity during Sustained Hypoxia in Awake Canines

Summary

In humans, isocapnic hypoxia sustained for 15-30 minutes elicits a biphasic ventilatory response with an initial increased peak followed by a decline or "roll-off" to a lesser, intermediate plateau. However, it is uncertain if this hypoxic roll-off is common for all mammals, as it has been reported that canines do not exhibit this typical mammalian response to hypoxia. Furthermore, activity of the diaphragm reflecting changes in the central or phrenic neuromotor output has not been directly examined in the canine species during sustained hypoxia. Therefore, we examined the effects of moderate sustained isocapnic hypoxia (SpO$_2$ 80%) lasting 20-25 minutes in thirteen adult, awake, intact canines, after complete recovery following surgical implantation of bipolar fine-wire EMG electrodes in the costal diaphragm.

The ventilatory response to sustained isocapnic hypoxia in these spontaneously breathing, intact, awake canines was not maintained: after an initial brisk response, ventilation declined significantly to an intermediate plateau. The hypoxic ventilatory decline occurred via a decrease in tidal volume, without change in breathing frequency. Independent of airflow, costal diaphragm EMG showed a biphasic activity pattern mirroring that of ventilation which concurrently declined during the constant exposure to sustained isocapnic hypoxia.

Our results demonstrate that the biphasic ventilatory response to sustained hypoxia typical of humans and other mammals was clearly exhibited in this group of intact canines. As well, neuromotor output to the costal diaphragm as indexed by EMG activity declined in parallel with ventilation during sustained hypoxia, consistent with a decrease in phrenic activity. These results suggest that the biphasic ventilatory response during sustained hypoxia is a mammalian characteristic, including that of canines, which
reflects an attenuation of central drive to the principle inspiratory muscle, the costal diaphragm.
Introduction

It is well known in humans that isocapnic hypoxia sustained for 15-30 minutes elicits a biphasic ventilatory response with an initial hyperventilatory response followed by a fall in ventilation - “roll-off” or hypoxic ventilatory decline (HVD) - to a lesser, intermediate plateau (Weil and Zwillich, 1976; Easton et al., 1986; Easton et al., 1988; Long et al., 1989; Georgopoulos et al., 1989; Masuda et al., 1989; Yamamoto et al., 1994). However, the distribution of this fundamental characteristic of hypoxic chemical control among other mammals is puzzling.

Besides humans, the sustained hypoxic ventilatory decline seems to occur in large hoofed mammals such as ponies and goats (Brown et al., 1992; Gershan et al., 1994), felines (Vizek et al., 1987; Tatsumi et al., 1992), and even rodents, although there may be some conflicting evidence concerning rats (Aaron et al., 1993; Gozal et al., 1996; Vizek and Bonora, 1998; Maxova and Vizek, 2001). But the existing literature accords an unexpected exemption to canines (Cao et al., 1992). In very careful experiments by experienced investigators, the typical human sustained roll-off is not apparent in these medium sized, highly aerobic, hunting mammals. Presumably this dynamic adaption to profound hypoxic insult imparts some survival advantage; less extreme ventilatory output surely lengthens the duration of hypoxia that can be endured and moderates subsequent injury or fatigue of the respiratory musculature. Why then, would a species or group be omitted from this advantageous ventilatory adjustment? (Maxwell et al., 1986; Tatsumi et al., 1992; Gershan et al., 1994; Ogawa et al., 1995).

An alternative explanation might be that some characteristic of a specific experimental preparation obviates or interrupts the innate physiologic process that underlies the sustained roll-off. In previous canine sustained hypoxic experiments, the animals have been tracheostomized (Cao et al., 1992). Assuming that the etiology of the ventilatory roll-off is a primary neurologic process involving central reset or perhaps some equivalent reset in the peripheral carotid body, it is difficult to link the route of airflow to an interruption of a neurologic process of respiratory control. Besides, even
under anesthesia with paralysis some animals retain the HVD (Vizek et al., 1987). There is no obvious mechanism whereby by-passing the upper airway would alter hypoxic sensitivity over time. Coupled with the thorough canine experiments already done to explore any role of tracheostomy (Cao et al., 1993), it seems unlikely that any methodological factor pertaining to route of airflow can explain a species difference in ventilatory decline.

We propose a different hypothesis. It is our contention that HVD is a universal finding common to all mammals. But, the dynamic characteristics of the sustained hypoxic roll-off, including amplitude and timing, may be highly variable and species dependent. In this scenario, if the characteristic HVD of one species, i.e. humans, is presumed to occur in identical fashion in another, e.g. canines, then HVD may not be recognized in an individual experimental protocol. Normal inter-species variation may make some aspects of the hypoxic roll-off very elusive, and difficult to recognize in an experimental protocol optimized for another species. By this logic, the long-standing curiosity that canines are exempt from an otherwise universal mammalian characteristic of the hypoxic response, may be a quirk of experimental protocol. It’s not that the HVD is a characteristic of all mammals except one – rather, canines don’t possess the human HVD.

To aggressively test this species-variant nature of the sustained hypoxia roll-off, experimental techniques for timing and maintenance of the hypoxic stimulus, monitoring and dynamic adjustment of incipient hypocapnia, and resistance and airflow characteristics of the breathing circuit are crucial. To guarantee that our sustained hypoxia recordings in canines could be compared to humans, we re-created exactly the experimental setup of a previous series of human sustained hypoxia experiments (Easton et al., 1986; Easton et al., 1988; Easton and Anthonisen, 1988b). Some mechanical components for hypoxic gas switching and correction of hypocapnia were common to both setups (PAE). We had available the original strip chart records from the earlier human studies (Easton et al., 1986), to ensure exact replication of the timing and presentation of sustained hypoxia. These experiments are a continuation of our original
sustained hypoxia protocol, after a hiatus of more than a decade, except that young seated human volunteers, breathing on a mouthpiece, are replaced by awake, relaxed canine subjects in the lateral decubitus position breathing through a snout mask. Given the pace and complexity of implanted canine studies, the experiments and data reported here were accumulated over a decade.
Methods

*Experimental technique and protocols*

Experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines.

Thirteen mongrel dogs (mean weight 28.9 kg, range 20-41 kg) had EMG wires implanted in costal diaphragm and were studied after full recovery. The technique of chronic EMG implantation and the 7 to 10 day progressive recovery of diaphragm segmental shortening have been described fully elsewhere (Easton et al., 1989). Measurements of resting and hypoxic-stimulated ventilation were made a mean of 28 days post-implantation (range 18-70 days). All implants were secured by fine synthetic non-fibrogenic sutures (Prolene, Ethicon, Somerville, NJ), all implant wires were externalized by a subcutaneous skin tunnel and the animals were allowed to recover.

Measurements were performed with the animals awake and breathing quietly while lying in the right lateral decubitus position which placed the implanted muscles in a non-dependent position. The animals were awake and relaxed, familiar with the location, routine and personnel associated with the experiment. The animals were breathing spontaneously and quietly through a snout mask, which was connected through a one way valve to a low resistance open breathing circuit (<1 cmH$_2$O/L/s) which incorporated a pneumotachograph (Fleisch #2) and a piezoelectric differential pressure transducer (Model 163PC01D36, Honeywell Microswitch) connected across the pneumotachograph to provide measurement of inspiratory airflow. On the expiratory limb, end tidal CO$_2$ (ETCO$_2$) was sampled and analyzed continuously by an infrared CO$_2$ analyzer (Model CD-3A, AMETEK/Thermox Instrument Division, Pittsburgh, PA), and on the inspiratory side, fractional concentration of inspired O$_2$ (F$_{1O_2}$) was continuously sampled and analyzed by an O$_2$ analyzer (Model S-3A/1, AMETEK/Thermox Instrument Division, Pittsburgh, PA). The inspiratory limb could be switched, without alerting the animal, from entrainment of room air to a large reservoir of pre-mixed gas of low F$_{1O_2}$ (8-10%).
In addition, supplemental pressurized sources of O\textsubscript{2} and CO\textsubscript{2} were attached to the inspiratory limb to allow the experimenter to precisely titrate F\textsubscript{I}O\textsubscript{2} and ETCO\textsubscript{2} during the study. To relate the level of hypoxia, oxygen saturation (SpO\textsubscript{2}) was continually measured by a pulse oximeter (Model Ohmeda Biox 8700, Rexdale, ON, Canada) by attaching a light sensitive analysis probe onto a shaved tendo calcaneous on the animal's hind limb.

Continuous measurements were made of inspiratory airflow and costal EMG activity. EMG signals from the bipolar fine wire electrodes were amplified (Mark III, TECA, White Plains, NY) and band-pass filtered (16 Hz-1.6 KHz). The output signals were then rectified and processed by passage through resistance-capacitance, leaky “integrators” with a time constant of 100 ms, to provide moving averages of the EMG of costal diaphragm.

Details of the sustained hypoxia technique have been described fully in a previous publication (Easton et al., 1986). To evaluate ventilation during isocapnic sustained hypoxia the study sequence was as follows: 1) a control period of room air breathing of 6-8 min; 2) an abrupt step decrease in F\textsubscript{I}O\textsubscript{2}, which lowered SpO\textsubscript{2} to ≤82% in 1-2 min; 3) maintenance of SpO\textsubscript{2} at 80 ± 2% for 20-25 min; 4) a recovery period of room air breathing of 5-6 min. During the sustained hypoxia, the target SpO\textsubscript{2} was 80%, and SpO\textsubscript{2} variability was restricted by the operator to the range 78-82%, a sustained desaturation that we abbreviate as 80 ± 2%. During the abrupt introduction of hypoxia, supplemental CO\textsubscript{2} was added via the pressurized source to the inspired limb of the breathing circuit to bring ETCO\textsubscript{2} back to a level approximating ETCO\textsubscript{2} during the preceding room air period. We did not attempt to anticipate and exclude any minor dip in ETCO\textsubscript{2} at the beginning of hypoxic hyperventilation because complete prevention of any transient fall in ETCO\textsubscript{2}, at the onset of hypoxia, would have entailed a slight risk of transient overcorrection and spurious increase in inspired minute ventilation (V\textsubscript{I}) during initial hypoxic exposure. After the SpO\textsubscript{2} had been stabilized at ≤82%, it was maintained at that level through adjustments in F\textsubscript{I}O\textsubscript{2} as necessary, utilizing the pressurized O\textsubscript{2} source on the inspiratory limb.
**Analysis of ventilation and breathing pattern**

Using computer software for data acquisition (DataSponge, Bioscience Analysis Software, Calgary, Canada), all signals were monitored in real time on the computer display and simultaneously collected at 100 Hz to hard disk on a microcomputer equipped with a single board A/D system (Model MIO-16-H-9, National Instruments, Galveston, TX). After acquisition and storage on disk, data were analyzed using software programs written by one author (PAE), adapted to this project by a 2nd author (MJ). These programs provide interactive visual display of the signals during analysis to allow careful examination of each breath. All signals were manually reviewed for movement artifacts, transient signal disconnections, apneas and sighs, all of which were deleted from individual breath-by-breath analysis.

The flow signal was evaluated for respiratory timing and digitally integrated; respiratory frequency ($f_R$), tidal volume ($V_T$), minute ventilation ($V_I$), inspiratory time ($T_I$), mean inspiratory flow ($V_T/T_I$) and inspiratory fraction of respiration ($T_I/T_{TOT}$) were calculated breath-by-breath. The EMG activity was quantified arbitrarily per breath as baseline value in volts, and the maximum difference in volts between baseline EMG and the peak height of the moving average EMG signal expressed as $EMG_{DIFF}$. $ETCO_2$ and $SpO_2$ were also calculated breath-by-breath based on respiratory timing. Individual $ETCO_2$ values were converted for altitude to sea level ($Patm = 760 \text{ mmHg}$). These measurements defined whole breath, or “tidal” breath, activity of inspiratory flow and respiratory timing as well as costal diaphragm EMG activity. These calculations have been described in detail elsewhere (Easton et al., 1988; Easton et al., 1986).

Measurement of all variables was continuous throughout the protocol, but for clarity of presentation, variables are summarized over corresponding periods of each study (Easton et al., 1988; Easton et al., 1986). Mean values of $V_I$, $SpO_2$, $ETCO_2$, $EMG_{DIFF}$ and components of breathing pattern were calculated from the continuous measurements during the following periods: BASE, PEAK, PLATEAU and RECOVERY. Period BASE was the final 5 min of resting ventilation with normal $SpO_2$ before the
introduction of hypoxia. Periods PEAK and PLATEAU were from opposite ends of the 20-25 min period of sustained hypoxia at 80 ± 2% SpO₂: PEAK represented the first 3 min of hypoxic ventilation, immediately after SpO₂ had descended to a level of ≤82%, while PLATEAU represented the last 5 min of hypoxic ventilation, preceding the relief of hypoxia. Period RECOVERY represented the first 5 minutes of room air breathing after hypoxia was abruptly relieved.

**Statistical analysis**

After calculation, mean values were exported for review to spreadsheet software (Microsoft Excel, Microsoft, Redmond, WA), to graphic software to output Figures 27-32 (CorelDRAW X6, Corel Corporation, Ottawa, ON), and to the PC version of SAS for statistical analysis (SAS version 8, SAS Institute, NC).

Statistical analysis of these breath-by-breath values was by two-way analysis of variance with repeated measures on a single factor (Keppel, 1982; Steele and Torrie, 1980) across each of the four periods (BASE, PEAK, PLATEAU, RECOVERY). Multiple comparison testing of the mean values of the individual periods within each protocol was performed using Tukey’s test (Steele and Torrie, 1980). *P*-value of less than 0.05 was considered statistically significant.
Results

We report measurements for breathing pattern in N=13 animals. The typical ventilatory response, with a period of sustained isocapnic hypoxia, is shown for a single canine subject in Figure 27. The record of SpO2 demonstrates that when FIO2 was lowered abruptly, SpO2 dropped to reach the target range of 78-82% in 1-2 min. Thereafter SpO2 was maintained at 80 ± 2% for 20 min, utilizing a dynamic adjustment of FIO2, until the abrupt re-introduction of room air. The minute ventilation increased briskly as SpO2 fell, but hyperventilation was not sustained. Throughout a constant isocapnic low desaturation of SpO2 80 ± 2%, V1 declined moderately to a plateau of about ~80% of PEAK hypoxic V1. PETCO2 dipped unavoidably with the initial burst of hypoxic hyperventilation but was titrated promptly back to, and then maintained at almost, the initial PETCO2 value noted during BASE.

Ventilation and breathing pattern

The mean response of V1 to sustained isocapnic hypoxia of a similar duration for 13 subjects is illustrated in Figure 28. The mean V1 increased significantly from BASE value of 7.58 l/min to PEAK sustained hypoxic value of 17.42 l/min, corresponding to 236% of the initial BASE room air value. Since a small initial dip in ETCO2 was generally present during the first minutes of sustained hypoxia, the PEAK mean value slightly underestimates the magnitude of the canine subjects’ initial hypoxic response. The ventilatory effect when hypoxia was persistent is shown at PLATEAU, where mean V1 was 13.79 l/min or 179% of initial BASE V1. Thus, from PEAK to PLATEAU, through sustained isocapnic hypoxia, there was a very significant decrease in minute ventilation (P<0.01).

Breathing pattern for the N=13 subjects during sustained hypoxia is illustrated in Figures 29, 30, 31. Tidal volume generally accounted for the aforementioned adjustment in V1, as VT increased significantly (P<0.01) from 0.39 liters during BASE to 0.69 liters during PEAK, then decreased significantly (P<0.01) to 0.51 liters during PLATEAU.
Conversely, respiratory frequency increased significantly (P<0.01) from 19.68 breaths/min during BASE to 25.45 breaths/min during PEAK. Thereafter, throughout the hypoxia, \( f_R \) did not change significantly from PEAK to PLATEAU, remaining during the PLATEAU at 139% of the original BASE value. Similarly, from PEAK through PLATEAU, inspiratory time was unchanged (Table 1); since \( V_T \) decreased significantly throughout sustained hypoxia, there was a parallel decrease in mean inspiratory flow from PEAK to PLATEAU (P<0.01), declining from 201% to 163% of the original BASE value.

**Costal diaphragm EMG activity**

Independent of airflow recordings, the EMG activity of the costal diaphragm was measured during sustained isocapnic hypoxia, as illustrated in Figure 32. Although the diaphragm implants are long lasting, durability of implanted electrodes was not universal. Therefore, we report EMG measurements from the costal diaphragm in \( N=10 \) canines. From baseline, costal EMG increased to 152% of BASE during initial PEAK hypoxia. Then costal EMG decreased significantly (P<0.01) to 122% during sustained PLATEAU hypoxia. Accordingly, the moving average \( \text{EMG}_{\text{DIFF}} \) increased significantly (P<0.01) from 3.68 ± 1.07 volts during BASE to 5.30 ± 0.85 volts during PEAK, and then decreased significantly (P<0.01) to 4.43 ± 1.17 volts during PLATEAU (Table 1). These breath-by-breath “tidal” EMG changes generally track, but are not equal to, the significant decline in tidal volume through sustained hypoxia from PEAK to PLATEAU. Although both \( V_T \) and costal EMG visibly dropped in initial RECOVERY to values below the original BASE, mean RECOVERY value over 5 minutes post hypoxia were not different statistically from the starting room air BASE.
Discussion

The two main findings of this study are: 1) canines do exhibit a roll-off of ventilation and diaphragm EMG during sustained isocapnic hypoxia, but 2) canines do not show a classic human decline in hypoxic ventilation. These results support the thesis that some type of decline in ventilatory response to sustained hypoxia is common in all mammals. And, these results also illustrate significant variation and differences in hypoxic ventilatory decline between species.

Based on these results, the timing, character and magnitude of the roll-off, the HVD, is likely species dependent. In humans, the initial brisk response to moderate hypoxia is apparently maintained for several minutes (Easton et al., 1986), then declines by about 15 minutes to an intermediate plateau. By contrast, as seen here in Figure 27, in canines the initial brisk response to hypoxia may be maintained briefly, or not at all, before beginning the decline to an intermediate plateau. This very different character may help explain the apparent absence of the HVD in some old canine experiments (Cao et al., 1992; Cao et al., 1993). If ventilation is averaged over several minutes, with some contribution from variance in ETCO$_2$ replacement, a stepwise decline in ventilation could be difficult to visualize (Cao et al., 1992).

In addition, the roll-off in hypoxic ventilation is made even more elusive in canines because of a much smaller magnitude of decline. In our original, matching, human study (Easton et al., 1986), the initial brisk hypoxic ventilatory response dropped by 1/2, ending at a plateau that was only modestly higher than baseline, specifically 126% of the starting ventilation during room air. By contrast, in these canines, the initial brisk hypoxic ventilatory response dropped by only 1/4, ending at a plateau that was still 79% greater than the original room air breathing. Thus in the canines, there was relatively little roll-off in hypoxic ventilation, compared to humans - even though the magnitude of the original brisk transient hypoxic response was actually much greater in the canines. Specifically, canines showed approximately double the magnitude of the initial peak hypoxic ventilatory response, compared to humans.
Species variation in ventilation during sustained hypoxia

Even if a roll-off or HVD during sustained hypoxia is a fundamental mammalian property, this does not infer that hypoxic roll-off is an identical event in all mammals. Unrecognized heterogeneity in this hypoxic response probably accounts for much of the discordance among various published reports. If we review the reported HVD characteristics across several species, we believe that some pattern can be discerned. We can include at least ponies (Brown et al., 1992), goats (Gershan et al., 1994), cats (Vizek et al., 1987; Tatsumi et al., 1992), rats (Vizek and Bonora, 1998; Maxova and Vizek, 2001), humans (Weil and Zwillich, 1976; Easton et al., 1986; Maxwell et al., 1986; Easton and Anthonisen, 1988b; Masuda et al., 1989; Yamamoto et al., 1994) and canines as reported here. Despite differences in technique, and some protocols including anesthesia, there is a general trend in timing that apparently correlates approximately with mammal body size. Specifically, larger mammals apparently take longer to roll-off during sustained hypoxia, while smaller mammals seem to exhibit a very brief, acute response to hypoxia with a prompt decline. This seems to be illustrated even in the timing differences of the HVD between humans and canines, as seen here in canine experiments that are nearly identical to previous human studies (Easton et al., 1986). However, we cannot offer any worthwhile synthesis regarding the magnitude of either the initial hypoxic response or the proportional ventilatory decline. Comparing previous human and current canine studies, we can only observe that canines exhibited a greater initial hypoxic response followed by a proportionally lesser decline. However, hypoxic protocols in other species are too heterogeneous in $F_{O_2}$ and effects of anesthesia, to offer any coherent synthesis of the relative magnitude of the hypoxic response across species.

Breathing pattern during sustained hypoxia

Changes in breathing pattern observed in this study are similar to those seen in response to sustained hypoxia in adult humans (Easton et al., 1986; Easton and Anthonisen, 1988b; Long et al., 1989; McEvoy et al., 1996) and other mammalian
species (McCooke and Hanson, 1985; LaFramboise and Woodrum, 1985; Vizek et al., 1987; Eden and Hanson, 1987; Rigatto et al., 1988; Gershan et al., 1994; Vizek and Bonora, 1998; Maxova and Vizek, 2001). In these canines, as in humans, changes in ventilation with hypoxia are due essentially to changes in tidal volume. An initial increase in minute ventilation was accompanied by increases in tidal volume ($V_T$) and frequency. The subsequent fall in $V_T$ and parallel decrease in mean inspiratory flow occur with very minimal alteration in any timing parameters. These results strongly suggest that changes in minute ventilation are related to the changes in neural output per breath during hypoxia, without regard for timing.

Although the effect of tracheostomy in canines during sustained hypoxia has been carefully studied by Cao et al. (1993), our results here suggest that tracheostomy per se may still have had some effect on this phenomenon. Specifically, the canines in the aforementioned study, even during mask breathing, still had some type of tracheal instrumentation. The breathing pattern of the aforementioned tracheostomized canines during both room air and hypoxia was very different; respiratory frequency was nearly 1/2 and tidal volume during hypoxia nearly double, the values we recorded here from our airway intact canines. It is difficult to completely exonerate the tracheostomy and to conclude that it has no effect on breathing pattern per se. Perhaps through some unrecognized impact on airflow resistance (Cullen et al., 1963; Cavo et al., 1973), respiratory mechanics (Criner et al., 1987), or intrinsic PEEP (Davis and Campbell, 1999; Moscovici da et al., 2002), tracheostomy can influence canine breathing during hypoxia.

**Costal diaphragm EMG changes during sustained hypoxia**

As an additional measure of respiratory output during sustained hypoxia, we implanted EMG electrodes in the costal diaphragm and report the results here. This was intended as a supplementary and independent measure, given the previous uncertainty about ventilation in canines during hypoxia. We can reasonably presume that the changes in costal EMG reflect similar changes in phrenic nerve activity, and respiratory neural output (Lawson and Long, 1983; Laframboise and Woodrum, 1985). These findings of a
biphasic costal EMG during sustained hypoxia are in general agreement with previous observations by Vizek et al. (Vizek et al., 1987) who demonstrated in a very different preparation, namely anesthetized paralyzed ventilated felines, that phrenic nerve activity decreased during the hypoxic roll-off without changing carotid sinus activity. Of particular note in these experiments, is that the costal diaphragm EMG generally tracks the tidal volume decline during hypoxia, but is certainly not identical. We may assume that the costal EMG recorded here faithfully reflects costal diaphragm shortening, but that is not certain. And, the relative contribution of other respiratory muscles including the crural diaphragm during sustained hypoxia is unknown. These uncertainties are the subject of ongoing research with these canines.

Adequacy of experimental technique

We believe that in this type of protocol, it is important to replace ETCO₂ conservatively. We were careful to avoid any artifactual over-stimulation of V₁ through over-enthusiastic replacement of ETCO₂ at the beginning of each hypoxic exposure. Instead, ETCO₂ was allowed to begin to fall before being titrated back to a level equal to or very slightly less than the preceding baseline (BASE), during the first 3 min of isocapnic hypoxia. This permitted a small initial dip in ETCO₂ at the start of each hypoxic challenge. If our conservative approach to ETCO₂ replacement had any disadvantage, it might have imposed a slight underestimation of the magnitude of the initial hypoxic hyperventilation during sustained hypoxia (Easton et al., 1986; Easton and Anthonisen, 1988b). This is not problematic since any small bias is against the demonstration of a roll-off decline in ventilation during sustained hypoxia.

Conclusion

In intact awake canines, ventilatory response to sustained hypoxia is clearly biphasic in response, with an initial peak ventilation followed by a roll-off to an intermediate plateau. Initial increase in ventilation occurred with an increase in both tidal volume and respiratory rate, however, the subsequent ventilatory decline was accounted
for by a reduction in volume with minimal alterations in rate. Concurrently, with persistent hypoxia, time course changes in costal diaphragm EMG activity qualitatively mirrored the biphasic changes in ventilation. We conclude that the biphasic sustained hypoxic roll-off is a mammalian characteristic which also occurs in canines, and reflects an attenuation of central drive to the principle inspiratory muscle, the costal diaphragm.
Acknowledgements

This study was supported by grants from the Canadian Institutes of Health Research. Expert technical assistance was provided by Ms. Leslie Jacques. Excellent research and laboratory support from colleagues Dr. Naoyuki Fujimura and Ms. Jenny V. Jagers. The provision of all suture materials by Ethicon Sutures Ltd., a Johnson & Johnson Company, is gratefully acknowledged.
Figure 27: Ventilation and costal diaphragm EMG activity during room air and sustained isocapnic hypoxia

Time course of ventilation and costal diaphragm EMG activity in response to sustained isocapnic hypoxia in a representative animal. Closed circle, open circle, open diamond, and open triangle represent mean values/20 sec for minute ventilation ($V_1$), $O_2$ saturation ($SpO_2$), costal diaphragm EMG activity ($COS\ EMG_{DIFF}$), and partial pressure of end tidal CO2 ($P_{ET}CO_2$), respectively. BASE, 5 min room air breathing before hypoxia; PEAK, first 3 min after $O_2$ saturation ($SpO_2$) decreased to $80 \pm 2\%$; PLATEAU, final 5 min at $SpO_2\ 80 \pm 2\%$; RECOVERY, 5 min room air breathing after hypoxia.
Figure 28: Minute ventilation during room air and sustained isocapnic hypoxia

Group mean minute ventilation during room air and sustained isocapnic hypoxia (20-25 min) in thirteen animals (N=13). Symbols show group mean values with standard error bars. Minute ventilation ($V_t$) during 5 min room air breathing before hypoxia (BASE); first 3 min after O$_2$ saturation (SpO$_2$) decreased to 80 ± 2% (PEAK); final 5 min at SpO$_2$ 80 ± 2% (PLATEAU); and 5 min room air breathing after hypoxia (RECOVERY). Y-axis shows $V_t$ in l/min. Numerical labels under each symbol are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on $V_t$, $P<0.0001$. †, significant difference between individual periods at $P<0.01$. 
Figure 29: Tidal volume during room air and sustained isocapnic hypoxia

Group mean tidal volume during room air and sustained isocapnic hypoxia (20-25 min) in thirteen animals (N=13). Symbols show group mean values with standard error bars. Tidal volume ($V_T$) during 5 min room air breathing before hypoxia (BASE); first 3 min after $O_2$ saturation ($SpO_2$) decreased to 80 ± 2% (PEAK); final 5 min at $SpO_2$ 80 ± 2% (PLATEAU); and 5 min room air breathing after hypoxia (RECOVERY). Y-axis shows $V_T$ in l. Numerical labels under each symbol are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on $V_T$, $P<0.0001$. ‡, significant difference between individual periods at $P<0.01$. 
Figure 30: Respiratory rate during room air and sustained isocapnic hypoxia

Group mean respiratory rate during room air and sustained isocapnic hypoxia (20-25 min) in thirteen animals (N=13). Symbols show group mean values with standard error bars. Respiratory rate ($f_R$) during 5 min room air breathing before hypoxia (BASE); first 3 min after O$_2$ saturation (SpO$_2$) decreased to 80 ± 2% (PEAK); final 5 min at SpO$_2$ 80 ± 2% (PLATEAU); and 5 min room air breathing after hypoxia (RECOVERY). Y-axis shows $f_R$ in breaths/min. Numerical labels under each symbol are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on $f_R$, $P<0.0001$. †, significant difference between individual periods at $P<0.01$. 
Group mean inspiratory flow during room air and sustained isocapnic hypoxia (20-25 min) in thirteen animals (N=13). Symbols show group mean values with standard error bars. Mean inspiratory flow (VT/TI) during 5 min room air breathing before hypoxia (BASE); first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2% (PEAK); final 5 min at SpO₂ 80 ± 2% (PLATEAU); and 5 min room air breathing after hypoxia (RECOVERY). Y-axis shows VT/TI in l/s. Numerical labels under each symbol are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on VT/TI, P<0.0001. †, significant difference between individual periods at P<0.01.
Figure 32: Costal diaphragm EMG activity during room air and sustained isocapnic hypoxia

Group mean costal diaphragm EMG activity during room air and sustained isocapnic hypoxia (20-25 min) in ten animals (N=10). Symbols show group mean values with standard error bars. Tidal EMG activity of the costal diaphragm (COS) during 5 min room air breathing before hypoxia (BASE); first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2% (PEAK); final 5 min at SpO₂ 80 ± 2% (PLATEAU); and 5 min room air breathing after hypoxia (RECOVERY). Y-axis expresses EMG activity per breath as maximum difference between baseline and the peak height of the integrated moving average EMG signal (EMG\textsubscript{DIFF}) in volts. Numerical labels under each symbol are means expressed as a percent of room air ventilation (%BASE). Significance of the overall effects of hypoxia on COS EMG\textsubscript{DIFF}, P<0.0001. †, significant difference between individual periods at P<0.01.
Table 1: Breathing pattern and diaphragm EMG activity during room air and sustained isocapnic hypoxia
(legend on next page)

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
<th>Hypoxia</th>
<th>Room Air</th>
<th>Effect</th>
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<td>5 min</td>
<td>3 min</td>
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<tr>
<td>SpO₂ (%)</td>
<td>92.84 ± 2.18</td>
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<td>P_{ET}CO₂ (mmHg)</td>
<td>37.18 ± 0.59</td>
<td>36.63 ± 2.60</td>
<td>37.78 ± 2.27</td>
<td>35.64 ± 1.90</td>
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<td>V₁ (l/min)</td>
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<td>Vₜ (l)</td>
<td>0.39 ± 0.06</td>
<td>0.69 ± 0.14</td>
<td>0.51 ± 0.11</td>
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</tr>
<tr>
<td>fₗ (breaths/min)</td>
<td>19.68 ± 6.52</td>
<td>25.45 ± 5.65</td>
<td>27.39 ± 9.79</td>
<td>20.50 ± 7.37</td>
</tr>
<tr>
<td>T₁ (s)</td>
<td>1.36 ± 0.37</td>
<td>1.18 ± 0.27</td>
<td>1.11 ± 0.37</td>
<td>1.32 ± 0.46</td>
</tr>
<tr>
<td>Vₜ/T₁ (l/s)</td>
<td>0.30 ± 0.08</td>
<td>0.60 ± 0.14</td>
<td>0.51 ± 0.17</td>
<td>0.28 ± 0.08</td>
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<td>Tₗ/Tₗ{TOT} (ratio)</td>
<td>0.41 ± 0.05</td>
<td>0.48 ± 0.04</td>
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<td>0.40 ± 0.03</td>
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<tr>
<td>COS EMG_{DIFF} (volts)</td>
<td>3.68 ± 1.07</td>
<td>5.30 ± 0.85</td>
<td>4.43 ± 1.17</td>
<td>3.45 ± 0.84</td>
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</table>
Table 1 (table legend)

SpO₂, pulse oximeter O₂ saturation; PₑTCO₂, partial pressure of end tidal CO₂; Vᵣ, minute ventilation; Vₜ, tidal volume; fᵣ, respiratory rate; Tᵢ, inspiratory time; Vᵣ/Tᵢ, mean inspiratory flow; Tᵢ/TTOT, inspiratory fraction of respiration; COS EMGDIFF, segmental EMG activity per breath quantified as the maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. BASE, room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; RECOVERY, room air breathing after hypoxia. Values are means ± SD for N=13, breathing pattern; N=10, COS EMGDIFF. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Comparisons: SpO₂ (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, PEAK-PLATEAU, NS); PₑTCO₂ (BASE-PEAK, PEAK-PLATEAU, BASE-PLATEAU, BASE-RECOVERY, NS; PLATEAU-RECOVERY, P<0.05); Vᵣ, Vₜ, Vᵣ/Tᵢ, Tᵢ/TTOT, COS EMGDIFF (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, NS); fᵣ (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, BASE-RECOVERY, NS); Tᵢ (BASE-PLATEAU, PLATEAU-RECOVERY, P<0.01; BASE-PEAK, P<0.05; PEAK-PLATEAU, BASE-RECOVERY, NS).
Project 2: Costal and Crural Diaphragm Function during Sustained Hypoxia in Awake Canines

Summary

In humans and other mammals, including canines, isocapnic hypoxia sustained for 20-60 minutes elicits a biphasic ventilatory response with an initial peak followed by a roll-off to a lesser intermediate plateau. During sustained hypoxia, the function of the diaphragm, especially the costal (COS) and crural (CRU) segments, has not been studied. Therefore respiratory changes in length and EMG activity of the COS and CRU segments of the diaphragm with ventilation were recorded in thirteen spontaneously breathing awake canines during moderate levels of sustained isocapnic hypoxia lasting 20-25 min (mean 80±2% SpO\textsubscript{2}).

Phasic inspiratory shortening (SHORT) and EMG activity was observed in the COS and CRU during room air and sustained hypoxia in all animals. Temporal changes in COS SHORT during sustained isocapnic hypoxia tracked the biphasic changes in ventilation. Mean tidal SHORT and EMG activity of the COS and CRU increased significantly with initial hypoxia, however, with constant hypoxia both COS and CRU segmental SHORT and EMG activity markedly declined to an intermediate plateau above baseline. With restoration of room air following sustained hypoxia, mean SHORT and EMG activity of the COS and CRU fell below baseline control levels. Although both segments exhibited a biphasic response, tidal SHORT and EMG activity of the CRU was always greater than the COS. Moreover, initial hypoxia induced a relatively greater recruitment of the COS SHORT and EMG activity compared to the CRU, however, constant hypoxia resulted in an equivalent reduction in SHORT of the COS and CRU despite a visibly lesser decline in CRU EMG activity compared to the COS segment.

We conclude that the biphasic response to sustained hypoxia is expressed in both ventilation and diaphragm function, but there is a clear differential activation of the two diaphragmatic segments. During sustained hypoxia, attenuation of central drive occurs to
a lesser extent for the CRU, which does not translate mechanically; such alteration in CRU mechanics (i.e. neuromechanical disassociation) may reflect the function of the CRU acting as an length adjustor to safeguard the force output of the COS during the significant hypoxic roll-off.
Introduction

In adult humans and other mammals, including canines, isocapnic hypoxia sustained for 20-60 minutes elicits a biphasic ventilatory response, an initial increased peak followed by a “roll-off” to a lesser intermediate plateau (Weil and Zwillich, 1976; Easton et al., 1986; Vizek et al., 1987; Easton et al., 1988; Tatsumi et al., 1992; Long et al., 1993; Praud et al., 1993; Fujimura et al., 2006). Although the initial increase in ventilation is brought about by the actions of the peripheral chemoreceptors, the precise mechanism for the subsequent decline remains largely unresolved; however, the prevalent theory to date suggests an accumulation of net inhibitory modulators as the primary determinant of the central attenuation of respiratory drive in mammals (Mortola, 1996; Honda and Tani, 1999). Moreover, despite the important role of the respiratory muscles in subserving ventilation and thus, maintaining effective gas exchange, little is known about the activity of the these respiratory effectors during the biphasic response to constant hypoxia. In particular, the function of the diaphragm, including the costal and crural segments, during sustained hypoxia has not been studied in any awake intact mammals.

Without any question, the diaphragm, as a principle inspiratory muscle, has intrigued scientists and has been the subject of great physiologic inquiry over the past century. Traditionally, the diaphragm has been considered as a single functional entity, that contracts and causes changes in intrathoracic pressure to subserve ventilation. This classic view of the diaphragm has been challenged as early as the 1920's by Briscoe, who stated that "the diaphragm should not be considered as a single muscle, but should be regarded as consisting of the crural and the costal portions" (Briscoe, 1920). To date, accumulated evidence indeed suggests that the diaphragm consists of two very distinct and separate muscles, consisting of the costal and crural segments, with various characteristic differences. In particular, the costal and crural segments arise from different embryological sites; the costal originating from the third, fourth and fifth cervical segments and the crural developing in the mesentery of the esophagus (Pickering and Jones, 2002). The costal and crural segments have differential fiber type
compositions (Reid et al., 1987; Gordon et al., 1989; Boriek et al., 2001), receive separate blood supplies (Briscoe, 1920), are supplied by discrete phrenic nerve branches (Ogawa et al., 1958; De Troyer et al., 1982; Hammond et al., 1989), and have distinct proprioceptive/sensory innervations (Corda et al., 1965; Duron et al., 1968; Pickering and Jones, 2002). Moreover, experimental evidence in canines demonstrates that the two segments differ with respect to their mechanical characteristics (Road et al., 1986a; Farkas and Rochester, 1988) as well as their individual action on the abdomen and the rib cage (De Troyer et al., 1982).

Beside their distinctive structural and mechanical characteristics, mounting evidence exists in support of the differential activation and function of the costal and crural diaphragm acting as two separate muscles during both non-respiratory and respiratory events. For instance, divergence of costal and crural diaphragm activity has been consistently reported during esophageal distension (Cherniack et al., 1984; Oliven et al., 1989), postural adjustments (Hodges and Gandevia, 2000; Hodges et al., 2001), as well as during swallowing, eructation and emesis (Monges et al., 1978; Titchen, 1979; Abe et al., 1994). Previous animal studies measuring segmental length change and/or EMG activity of the costal and crural diaphragm have also demonstrated the distinct activity of the two segments during spontaneous respiration (Newman et al., 1984; Van Lunteren et al., 1985; Fitting et al., 1986; Road et al., 1986b; Easton et al., 1987; Darian et al., 1989; Torres et al., 1989; Easton et al., 1994). Evidence to date in acutely anesthetized preparations suggests that: peak and velocity of shortening of the crural is greater than the corresponding costal segmental values; onset of crural EMG activity precedes costal EMG activity with associated segmental shortening following a similar pattern; and there is a greater increase in crural segmental shortening or EMG activity during acute chemical stimulation by hypoxia/hypercapnia (Newman et al., 1984; Decramer et al., 1984; Van Lunteren et al., 1984; Van Lunteren et al., 1985; Fitting et al., 1986; Road et al., 1986b; Easton et al., 1987; Darian et al., 1989).

Based on the available evidence, it is conceived that the costal and crural diaphragm may function as two separate muscles during the biphasic ventilatory response
to sustained hypoxia. Several studies have previously examined the influence of sustained hypoxia on the activity of the diaphragm via the phrenic nerve or EMG activity. With the exception of rats (Maxova and Vizek, 2001; Iizuka and Fregosi, 2007) and canines (Smith et al., 1989), previous studies have generally established that sustained hypoxia elicits a biphasic activity response of the diaphragm (LaFramboise and Woodrum, 1985; Vizek et al., 1987; van Lunteren et al., 1989; Guthrie et al., 1990; Watchko et al., 1990; Martin et al., 1990; Brown et al., 1992; Kimura et al., 1994; McEvoy et al., 1996). However, many of these studies involved the use of anesthetics, were conducted in the state of immaturity and/or were studied in the less physiologic supine position for quadrupedal animals. Additionally, the activity of the costal and crural diaphragm during sustained hypoxia has not been simultaneously measured within a single study in adult mammals, and as such has not permitted the direct assessment of the two segments to be made with respect to their neural activation pattern. Most importantly, the actual mechanical consequence of the biphasic neural activity response of the costal and crural diaphragm, and their capacity to function in a differential manner has not been examined in any mammalian species to date.

To the extent that EMG activity is not a direct correlate of the mechanical output of the diaphragm, given its complex action and interaction with the rib cage and the abdomen, as well as different respiratory muscle groups, any assertion of the function of the diaphragm based on EMG activity alone is speculative and prone to misinterpretation. Thus, this investigation was set out to directly measure the respiratory changes in segmental length and EMG activity of the costal and crural diaphragm along with ventilation during moderate levels of sustained isocapnic hypoxia lasting 20-25 min in a fully conscious, adult canine, spontaneously breathing in the right lateral decubitus position. Our awake chronically instrumented animal model avoids any confounding effects of anesthesia on ventilation and respiratory muscle function (Fitting et al., 1987; Warner et al., 1995; Warner and Warner, 1995; Muller et al., 1979) and/or post operative dysfunction of the diaphragm previously reported in humans and animals (Ford et al., 1983; Road et al., 1984; Easton et al., 1989; Torres et al., 1989). Furthermore, simultaneous intramuscular mechanical and electrical recordings of the costal and crural
in the right lateral decubitus position will permit an adequate assessment of the diaphragmatic segments with respect to their capacity to function differentially in a posture that is physiologically natural and conducive for quadrupeds.

Therefore, in our intact awake canines, we hypothesize that the costal and crural diaphragm will invariably and phasically shorten with EMG activation with each inspiratory breath during room air and hypoxic stimulated ventilation. We further hypothesize that the costal and crural diaphragm contractile shortening and EMG activity will exhibit a biphasic response to sustained hypoxia mirroring that of ventilation; however, the peak tidal and relative changes in segmental muscle activity for the two muscles will be unique and distinct during the initial peak and sustained hypoxic roll-off with the capacity to function in a differential segmental manner.
Methods

Implantation of transducers and electrodes

The study and the experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines.

Thirteen mongrel canines (mean weight 28.9 ± 5.57 kg; range 20-41 kg) had pairs of sonomicrometry transducers and bipolar fine wire EMG electrodes implanted in the left costal and crural diaphragm segments. Animals were studied after diaphragm segmental shortening had recovered fully. This technique of chronic sonomicrometry and EMG implantation, and the 7- to 10-day progressive recovery of diaphragm segmental shortening, has been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). Briefly, implantation of transducers and electrodes was performed under general anesthesia with thiopental sodium induction and halothane. The left hemidiaphragm was exposed through a mid-abdominal incision, and ultrasonic transducers were implanted between muscle fibers on a flat portion of each of the costal and crural segments of the left hemidiaphragm. Costal transducers were implanted in the lateral portion of the segment, approximately midway between central tendon and chest wall in the region corresponding roughly to the second sternocostal branch of the phrenic nerve (Hammond et al., 1989). Crural transducers were placed in the posterior, perivertebral region of the segment. On each segment, immediately adjacent to each transducer, a fine wire stainless steel bipolar EMG electrode was attached. All implants were secured by fine synthetic non-fibrotic sutures (Prolene, Ethicon, Somerville, NJ). All implant wires were externalized by a subcutaneous skin tunnel, and the animals were allowed to recover. Measurements of resting and hypoxic-stimulation were made a mean of 28 days post-implantation (range 8-70 days).
**Measurement techniques**

All measurements of ventilation and respiratory muscle function were made with the animals awake and breathing quietly, while lying in right lateral decubitus position which placed the implanted muscles in a non-dependent position. The animals were relaxed and familiar with the location, routine and personnel of the recordings. The animals were breathing spontaneously and quietly through a snout mask, which was connected through a 2-way non-rebreathable valve to a low resistance open breath circuit (<1 cmH\textsubscript{2}O/L/s) which incorporated a pneumotachygraph (Fleisch #2) and a piezoelectric differential pressure transducer (Model 163PC01D36, Honeywell Microswitch) to provide measurement of inspiratory airflow. On the expiratory limb, end tidal CO\textsubscript{2} (ETCO\textsubscript{2}) was sampled and analyzed continuously by an infrared CO\textsubscript{2} analyzer (Model CD-3A, AMETEK/Thermox Instrument Division, Pittsburgh, PA), and on the inspiratory side, fractional concentration of inspired O\textsubscript{2} (F\textsubscript{I}O\textsubscript{2}) was continuously sampled and analyzed by an O\textsubscript{2} analyzer (Model S-3A/1, AMETEK/Thermox Instrument Division, Pittsburgh, PA). The inspiratory limb could be switched, without alerting the animal, from entrainment of room air to a large reservoir of pre-mixed gas of low F\textsubscript{I}O\textsubscript{2} (8-10%). In addition, supplemental pressurized sources of O\textsubscript{2} and CO\textsubscript{2} were attached to the inspiratory limb to allow the experimenter to precisely titrate F\textsubscript{I}O\textsubscript{2} and ETCO\textsubscript{2} during the study. To relate the level of hypoxia, oxygen saturation (SpO\textsubscript{2}) was continually measured by a pulse oximeter (Model Ohmeda Biox 8700, Rexdale, ON, Canada) by attaching a light sensitive analysis probe onto a shaved tendo calcaneous on the animal's hind limb.

Dynamic measurements within the respiratory muscles of the changing distance between the sonomicrometry transducers of each pair was provided by measuring the speed of transmission of ultrasonic waves using a sonomicrometer (Model 120, Triton Technology, Sand Diego, CA) (Easton et al., 1989). The output signal of the sonomicrometer was offset, amplified and then sampled to computer. For measurements of EMG, the fine wire bipolar electrode pairs from each muscle were connected to an AC differential pre-amplifier (Model 1700, AM Systems, Everett, WA). Power line interference was abolished by careful shielding techniques and the use of differential
preamplifiers with a high common mode signal rejection of 110 dB. Thereafter the signal was filtered to attenuate both movement artifact and sonomicrometry noise and to perform anti-alias filtering, using a 6-pole, low pass Bessel filter at 20 Hz-700 Hz (Model 746, LT-4, Frequency Devices Incorporated, Haverhill, MA). The EMG signals were then rectified and processed by passage through resistance-capacitance, leaky "integrators" with a time constant of 100 ms, to provide moving averages of the EMG.

Using computer software for data acquisition (DataSponge, Bioscience Analysis Software, Calgary, AB, Canada), all signals were monitored in real time on the computer display and simultaneously collected at 100 Hz to hard disk on a microcomputer equipped with a single board A/D system (Model MIO-16-H-9, National Instruments, Galveston, TX).

**Experimental protocol**

The details of the sustained hypoxia technique have been described fully in a previous publication (Easton et al., 1986). To evaluate ventilation and respiratory muscle function during sustained isocapnic hypoxia, the study sequence was as follows: 1) a control period of room air, resting, baseline breathing of 6-8 min; 2) an abrupt step decrease in $F_{IO_2}$, which lowered $SpO_2$ to $\leq 80$ in 1-2 min; 3) maintenance of $SpO_2$ at $80 \pm 2\%$ for 20-25 min with constant $ETCO_2$ equal to control levels; and 4) a recovery period of room air breathing of 5-7 min. During sustained hypoxia, the target $SpO_2$ was 80%, and $SpO_2$ variability was restricted by the operator to the range 78-82%, a sustained desaturation that we abbreviate as $80 \pm 2\%$. During the abrupt introduction of hypoxia, supplemental $CO_2$ was added via the pressurized source to the inspired limb of the breathing circuit to bring $ETCO_2$ back to a level approximating $ETCO_2$ during the preceding room air period. We did not attempt to anticipate and exclude any minor dips in $ETCO_2$ at the beginning of hypoxic hyperventilation because complete prevention of any transient fall in $ETCO_2$, at the onset of hypoxia, would have entailed a slight risk of transient overcorrection and spurious increase in inspired minute ventilation during initial hypoxic exposure. After $SpO_2$ had been stabilized at $\leq 82\%$, it was maintained at that
level through adjustments in $F_{t}O_{2}$ as necessary, utilizing the pressurized $O_{2}$ source on the inspiratory limb.

**Analysis of breathing pattern and muscle activity**

After acquisition and storage on disk, data were analyzed using software programs written by one author (PAE), adapted to this project by the 2nd author (MJ). These programs provide interactive visual display of the signals during analysis to allow careful examination of each breath. All signals were manually reviewed for movement artifacts, transient signal disconnections, apneas and sighs, all of which were deleted from individual breath-by-breath analysis.

The flow signal was evaluated for respiratory timing and digitally integrated; minute ventilation ($V_{I}$), tidal volume ($V_{T}$), respiratory frequency ($f_{R}$), inspiratory time ($T_{I}$), mean inspiratory flow ($V_{T}/T_{I}$), and inspiratory fraction of respiration ($T_{I}/T_{TO}$) were calculated breath-by-breath. The software also calculated whole breath, or “tidal” breath, values of respiratory muscle shortening and EMG activity. These calculations have been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). Briefly, the timing of inspiration was determined from the airflow tracing, and the onset and termination of individual muscle shortening and EMG activity for each breath was measured relative to the beginning and end of inspiratory airflow. Using the sonomicrometry length data from each implanted muscle, the computer calculated for each breath the baseline end-expiratory resting length of the muscle, titled $L_{BL}$, and the shortening (SHORT) for each breath expressed as a percentage change from resting length, entitled $\%L_{BL}$. Moving average EMG activity from each muscle was quantified arbitrarily per breath as the maximum difference in volts between baseline and the peak height of the moving average signal, expressed as $EMG_{DIFF}$. $ETCO_{2}$ and $SpO_{2}$ were also calculated breath-by-breath based on respiratory timing. Individual $ETCO_{2}$ values were converted for altitude to sea level ($Patm = 760$ mmHg).
Measurement of all variables was continuous throughout the protocol, but for clarity of presentation, variables are summarized over corresponding periods of each study (Easton et al., 1986; Easton et al., 1988). Mean values of $V_1$ and components of breathing pattern, $\text{SpO}_2$, $\text{ETCO}_2$, SHORT, and $\text{EMG}_{\text{DIFF}}$ were calculated from the continuous measurements during the following periods: BASE, PEAK, PLATEAU, and RECOVERY. Period BASE was the final 5 min of resting ventilation with normal $\text{SpO}_2$ before the introduction of hypoxia. Periods PEAK and PLATEAU were from opposite ends of the 20-25 min period of sustained hypoxia at $80 \pm 2\% \text{SpO}_2$: PEAK represented the first 3 min of hypoxic ventilation, immediately after $\text{SpO}_2$ had descended to a level of $\leq 82\%$, while PLATEAU represented the last 5 min of hypoxic ventilation, preceding the relief of hypoxia. Period RECOVERY represented the first 5 minutes of room air breathing after hypoxia was abruptly relieved.

**Statistical analysis**

After calculation, mean values were exported for review to spreadsheet software (Microsoft Excel, Microsoft, Redmond, WA), to graphic software to output figures (CorelDRAW X6, Corel Corporation, Ottawa, ON), and to PC version of SAS for statistical analysis (SAS Version 9.1, SAS Institute, NC). Mean values of ventilation, breathing pattern, and muscle parameters were tested across the four periods (BASE, PEAK, PLATEAU, RECOVERY) by two-way analysis of variance with repeated measure on a single factor (Keppel, 1982; Steele and Torrie, 1980). Multiple comparison testing of the mean values of the individual periods was performed using Student-Newman-Keuls test (Steele and Torrie, 1980). At individual periods, mean SHORT or EMG activity between the costal and crural segments of the diaphragm was compared using a Student's paired t-test. $P$-value of less than 0.05 was considered statistically significant; actual $P$-values are reported for all pair-wise t-test comparisons. Relative changes in segmental SHORT and EMG were examined for the group as a percentage of baseline (%BASE) and peak (%PEAK) activity during initial and sustained hypoxia, as well as post-hypoxic recovery.
Results

The study was conducted in thirteen healthy awake canines with chronic implantation of sonomicrometry transducers and bipolar fine wire EMG electrodes. Although the implants are long-lasting, durability of implanted transducers and electrodes was not universal. Thus, costal (COS) diaphragm segmental muscle length and EMG recordings are available in N=11 and N=10, respectively; and for the crural (CRU) diaphragm, segmental muscle length and EMG activity are available in N=13 and N=12, respectively.

Ventilation and breathing pattern

Breathing pattern for the N=13 subjects during sustained hypoxia is summarized in Table 2. For the group, mean SpO\textsubscript{2} decreased and was held at target SpO\textsubscript{2} levels of 80 ± 2% from PEAK through PLATEAU, and mean P\textsubscript{ET}CO\textsubscript{2} was maintained isocapnic to BASE control levels throughout sustained hypoxia (BASE-PEAK-PLATEAU, all NS). Accordingly, mean V\textsubscript{T} increased significantly (P<0.01) from BASE value of 7.58 l/min to PEAK hypoxic value of 17.42 l/min, then significantly decreased (P<0.01) to an intermediate PLATEAU value of 13.79 l/min. Tidal volume generally accounted for the aforementioned adjustment in V\textsubscript{T}, as V\textsubscript{T} declined significantly (P<0.01) from PEAK to PLATEAU despite minimal alterations in f\textsubscript{R}. Likewise, from PEAK through PLATEAU, there was a parallel decrease (P<0.01) in mean inspiratory flow, V\textsubscript{T}/T\textsubscript{I}, and effective respiratory timing, T\textsubscript{I}/T\textsubscript{TOT}, as V\textsubscript{T} decreased significantly with sustained hypoxia with a trend for a reduction in T\textsubscript{I}. With RECOVERY room air breathing, SpO\textsubscript{2} returned towards and P\textsubscript{ET}CO\textsubscript{2} remained modestly below initial BASE levels; V\textsubscript{T}, V\textsubscript{I} and V\textsubscript{T}/T\textsubscript{I}, despite reaching statistical significance, visibly fell below starting BASE values; all other ventilatory parameters (f\textsubscript{R}, T\textsubscript{I}, T\textsubscript{I}/T\textsubscript{TOT}) returned promptly back to or near BASE values.
Diaphragm segmental length change and EMG activity

Representative tracing of the segmental length change and EMG activity of the COS and CRU diaphragm during room air and sustained isocapnic hypoxia is illustrated in Figure 33 (A-D). During initial room air, resting BASE ventilation (Figure 33A), COS and CRU invariably shortened with each inspiratory breath along with phasic EMG activity. Within a few minutes of initial hypoxia, corresponding to PEAK (Figure 33B), there was a striking increase in the rate and magnitude of COS and CRU SHORT and EMG activity in concert with inspiratory airflow. However, such extent of COS and CRU SHORT and EMG activity at PEAK did not endure, as isocapnic hypoxia maintained for more than 20 min, as represented by final hypoxia at PLATEAU (Figure 33C), resulted in a reduction in COS and CRU SHORT and EMG activity along with inspiratory airflow. Of note, in this particular animal, although COS EMG activity considerably declined with steady-state sustained hypoxia, the reduction in CRU EMG activity was less dramatic and remained modestly smaller than the activity during initial hypoxia at PEAK (Figure 33B). During final room air, post-hypoxic RECOVERY ventilation (Figure 33D), segmental SHORT and EMG activity of the COS and CRU more or less returned to initial room air levels at BASE.

Time course of costal diaphragm shortening and ventilation

The typical ventilatory and COS diaphragm segmental SHORT response to a period of sustained isocapnic hypoxia is illustrated for a single canine subject in Figure 34. Records of O₂ saturation demonstrates that SpO₂, within minutes, fell to reach target levels of 78-82% upon entertainment of low F₁O₂ gas mixture, and thereafter was maintained for 23 min until the abrupt re-introduction of room air. P_{ET}CO₂ was permitted to drop with initial hypoxic hyperventilation, but was quickly titrated back to baseline room air control levels until recovery room air. As a result, tidal COS SHORT in parallel with V₁ increased briskly as SpO₂ fell, which was subsequently followed by a time dependent roll-off in COS SHORT and V₁ as hypoxia was held constant at levels of 80 ± 2% desaturation. Although COS SHORT and V₁ declined progressively, and in concert,
to reach a new steady-state plateau around ~15-20 min of constant hypoxia, the relative degree of the COS SHORT decline was visibly greater than the fall in V₁. Specifically, COS SHORT fell roughly by 3/4 from its initial peak response, whereas V₁ fell moderately to a plateau of about ~68% of peak hypoxic value. With restoration of room air, SpO₂ returned to initial room air normoxic values, while PₑT₇CO₂ initially dipped prior to settling back to control levels. Consequently, COS SHORT with some variability visibly trended to remain below original baseline, while V₁ initially fell within the first few minutes of recovery room air and then progressively returned to baseline.

**Diaphragm segmental shortening and EMG activity**

*Costal segment.* The group mean segmental SHORT and EMG activity of the COS diaphragm during room air and sustained hypoxia are shown in Figure 35 and summarized in Table 3. From baseline resting levels of contraction, mean tidal SHORT of the COS diaphragm of 3.18 ± 0.59 %LBL at BASE increased significantly (P<0.01) to PEAK hypoxic value of 6.36 ± 1.14 %LBL, corresponding to 221% of initial BASE room air value. The effects of prolonged steady-state hypoxia on the contraction of the COS diaphragm is shown at PLATEAU, where mean tidal SHORT decreased significantly (P<0.01) to an intermediate PLATEAU value of 4.11 ± 0.65 %LBL or 141% of initial BASE value. With RECOVERY room air, mean tidal COS SHORT significantly fell (P<0.05) below original BASE levels of contraction, corresponding to 2.58 ± 0.49 %LBL or 83% of original BASE. Such adjustments in COS SHORT were accompanied by biphasic changes in COS EMG activity, as mean tidal EMG activity increased significantly (P<0.01) from 3.68 ± 0.34 volts during BASE to 5.30 ± 0.27 volts during PEAK (152% BASE), then significantly decreased (P<0.01) to 4.43 ± 0.37 during PLATEAU (122% BASE). During RECOVERY, despite reaching statistical significance, mean COS EMG activity of 3.45 ± 0.26 (95% BASE) remained below original BASE activity levels (BASE-RECOVERY, NS).

*Crural segment.* Independent of the COS diaphragm recordings, segmental SHORT and EMG activity of the CRU diaphragm was simultaneously measured during
room air and sustained hypoxia, as illustrated in Figure 36 and summarized in Table 3. From baseline, mean CRU tidal SHORT increased significantly (P<0.01) from BASE to PEAK with initial hypoxia (4.43 ± 0.42 to 8.47 ± 0.99 %L\text{BL}, P<0.01), corresponding to 195% initial BASE room air value. With constant hypoxia, mean tidal SHORT of the CRU diaphragm significantly decreased (P<0.01) down to a PLATEAU value above baseline (5.42 ± 0.52 %L\text{BL} or 126% initial BASE). Upon restoration of room air, mean CRU SHORT significantly fell (P<0.05) below starting room air BASE during RECOVERY (3.34 ± 0.51 %L\text{BL} or 94% original BASE). Tidal CRU EMG activity generally accounted for the bimodal changes in CRU SHORT, as mean CRU tidal EMG activity increased significantly (P<0.01) from BASE value of 5.03 ± 0.48 volts to PEAK value of 6.47 ± 0.52 volts (131% BASE), then decreased significantly (P<0.01) to PLATEAU value of 5.93 ± 0.55 volts (118% BASE). Post sustained hypoxia, mean CRU EMG activity of 4.79 ± 0.52 volts (94% BASE) at RECOVERY visibly remained below initial BASE activity, despite reaching statistical difference (BASE-RECOVERY, NS).

Differential segmental shortening and EMG activity

Peak response. Direct comparisons of group mean segmental SHORT of the COS and CRU diaphragm during room air and sustained hypoxia is shown in Figure 37. With initial hypoxia, mean tidal COS and CRU SHORT increased in tandem from BASE to PEAK, and then markedly declined from PEAK through PLATEAU with constant hypoxia. However, the extent and magnitude of the muscle SHORT for the two diaphragmatic segments were notably distinct, with CRU exhibiting significantly greater SHORT compared to the COS during resting BASE room air (P=0.0126), initial PEAK hypoxia (P=0.0093), and sustained PLATEAU hypoxia (P=0.0251). During RECOVERY, mean COS and CRU SHORT, although visibly different, did not reach statistical significance (P=0.1531). Likewise, Figure 38 illustrates the group mean comparisons of segmental EMG activity from the COS and CRU diaphragm. Although visibly to a lesser extent than tidal SHORT, mean EMG activity of the COS and CRU segments changed in tandem to exhibit a biphasic pattern with sustained hypoxia. Furthermore, at each individual period of room air and sustained hypoxia, CRU EMG activity was
significantly greater than the EMG activity of the COS diaphragm (BASE, P=0.0176; PEAK, P=0.0492; PLATEAU, P=0.0143; RECOVERY, P=0.0117).

Relative response. Relative degree of COS and CRU segmental SHORT and EMG activity response to initial and sustained hypoxia was also examined, as illustrated in Figure 39 and Figure 40. As a percentage of resting room air BASE, mean COS SHORT relatively increased to a greater extent than CRU SHORT in response to initial PEAK hypoxia (221% vs. 195% BASE, respectively), however, variability existed among the animals in their relative COS and CRU SHORT response as noted by the overlapping error bars (Figure 39A). Such trends for a differential SHORT response of the COS and CRU were more prominently expressed in the relative EMG activity of the two diaphragmatic segments, with mean COS EMG activity proportionally increasing to a greater degree than CRU EMG activity from BASE to PEAK (152% vs. 131% BASE, respectively; Figure 39B). In addition, the relative magnitude of SHORT and EMG activity decline with sustained hypoxia is shown in Figure 40A and 40B for the COS and CRU segments of the diaphragm. As a percentage of initial PEAK activity, constant hypoxia resulted in a proportional and matching decline in the mean SHORT of the COS and CRU diaphragm from PEAK to PLATEAU (68% vs. 68% PEAK). However, the relative decline in the EMG activity of the COS and CRU segments were notably distinct and separate, with mean CRU EMG activity exhibiting a lesser decline compared to the mean COS EMG activity throughout PEAK to PLATEAU hypoxia (91% vs. 83% PEAK, respectively). Moreover, with restoration of room air, mean CRU SHORT trended to visibly decline more than COS SHORT relative to initial room air BASE (73% vs. 83%, respectively), even though mean COS and CRU EMG activity proportionately declined during RECOVERY (95% vs. 94%).
Discussion

Summary

Invariable phasic inspiratory shortening and EMG activity was observed for the costal and crural segments of the diaphragm during room air and sustained hypoxia in all intact awake canines. During ventilatory response to sustained isocapnic hypoxia, we found that: 1) tidal changes in costal diaphragm shortening tracked the time course changes in ventilation; 2) with initial exposure to sustained hypoxia, segmental shortening and EMG activity of the costal and crural diaphragm increased significantly with abrupt desaturation; 3) with steady-state exposure to sustained hypoxia (i.e. constant desaturation) both costal and crural segmental shortening and EMG activity remarkably declined to an intermediate plateau above baseline room air levels; and 4) costal and crural shortening and EMG activity fell below resting baseline levels upon restoration of room air following hypoxia. Despite the biphasic segmental response pattern of the diaphragm, tidal shortening and EMG activity of the crural was greater than the costal at each individual period of room air and sustained hypoxia. Furthermore, the relative recruitment of the costal was visibly greater than the crural during initial hypoxia, as reflected in both shortening and EMG activity, however, sustained hypoxia resulted in a proportional decline in the costal and crural shortening despite a lesser fall in crural EMG activity when compared to the costal.

Ventilatory response

The sustained isocapnic hypoxic condition was successfully attained throughout the study for each animal. In this group of intact awake canines, ventilation clearly exhibited a biphasic pattern that is characteristic of the ventilatory response to sustained hypoxia in adult humans and other mammals (Weil and Zwillich, 1976; Easton et al., 1986; Vizek et al., 1987; Easton et al., 1988; Tatsumi et al., 1992; Long et al., 1993; Praud et al., 1993; Fujimura et al., 2006). The biphasic changes in ventilation were predominately accounted for by the changes in tidal volume, with minimal alteration in
respiratory rate during the sustained hypoxic roll-off. Moreover, changes in ventilation during sustained hypoxia was accompanied by the biphasic changes in the mean inspiratory flow, $V_{I}/T_{I}$, and effective respiratory timing, $T_{I}/T_{TOT}$, and thus reflecting fundamental alterations in the respiratory neuromotor output and relative duration of inspiration governing the activity of the respiratory muscles subserving ventilation.

**Diaphragm activity during resting room air**

In our intact awake canines, costal and crural diaphragm phasically shortened and were electrically active with each inspiratory breath during quiet room air ventilation (Figure 33). As a group, mean tidal shortening of the costal and crural (3.18% and 4.45%) at rest in the present study are compatible with previous studies made in awake canines in the same lateral decubitus positioning (Abe et al., 1994; Easton et al., 1995; Easton et al., 1994; Easton et al., 1993; Fitting et al., 1989), with costal and crural shortening reported in the range of ~3.9 to 6.3% and ~3.4 to 7.5%, respectively. In comparison to the supine anesthetized state, however, our tidal shortening values for the two diaphragmatic segments are somewhat quantitatively smaller than the degree of shortening reported in the same species (Fitting et al., 1986; Newman et al., 1984; Decramer et al., 1984; Road et al., 1986b; Suzuki et al., 1997).

Specifically for the crural diaphragm, a shortening value of 4.45% found in the current study is roughly two fold smaller compared to the acutely anesthetized state previously reported in the range of ~8.1 to 10.6%, while the difference in the costal shortening of our study compared to previous studies under anesthesia was less remarkable. Although we cannot easily reconcile for this difference, Fitting et al. (1987) in awake canines reported the effects of anesthesia to cause a preferential lengthening of the crural segment of the diaphragm at end-expiration, i.e. baseline resting length, without impacting the resting length of the costal. To the extent that lengthening of the crural resting length would act to improve the force-generating capacity of the muscle based on its length-tension characteristics (Road et al., 1986a; McCully and Faulkner, 1983), the prominent crural and lesser costal shortening difference when compared to the
former acutely anesthetized studies may be partly accounted for by these differential length changes occurring in the costal and crural segments of the diaphragm with anesthesia. Accordingly, anesthesia fundamentally alters the segmental contribution of the diaphragm to resting ventilation compared to the state of wakefulness, especially affecting the crural diaphragm.

While direct comparisons of resting EMG activity are difficult to compare across studies due to species differences, experimental and signal processing techniques, and normalization methods involved, our results are emphatic and directly agree with previous investigations reporting on the phasic EMG activity of the costal and crural diaphragm during quiet respiration in anesthetized and awake mammals, including humans (Takasaki et al., 1989; Yasuma et al., 1993; Brice et al., 1990; Darian et al., 1989; van Lunteren et al., 1989). Specifically in the intact awake canines, our peak tidal EMG activity of the costal and crural (3.68 volts and 5.03 volts) at rest expressed arbitrarily in volts following signal amplification are somewhat higher than our earlier studies reported in the range of ~1.48 to 3.19 volts for the costal and ~1.51 to 3.50 volts for the crural segments of the diaphragm (Easton et al., 1995; Abe et al., 1994; Easton et al., 1994; Easton et al., 1993; Easton et al., 1989). Such differences in the absolute peak EMG values are not surprising considering that measurements of EMG activity, although a very precise and reliable technique, are highly sensitive and dependent on various physiological and technical factors. As a result, even with the best experimental controls in place and employing the same implantation and EMG processing techniques, natural variance among animals within a study group will certainly cause the magnitude of peak EMG to vary across individual studies from the same laboratory.

Despite the highly sensitive nature of recording EMG activity from the respiratory muscles, the variance within the group will preside throughout the study permitting the adequate use and assessment of absolute EMG values to compare between individual muscles and/or across experimental conditions. In addition, the validity of the trends expressed through absolute EMG within one study can also be qualitatively compared with absolute EMG trends in other studies, despite differences in the EMG magnitude.
Alternatively, normalization methods can be employed to standardize the absolute EMG values with respect to baseline/peak activity or maximal volitional maneuvers, and thus controlling for the variability among the animals within a study group. In the present study, segmental shortening and EMG activity of the costal and crural diaphragm are reported in both their absolute and relative terms.

**Diaphragm activity with initial hypoxia**

Immediate response to hypoxia resulted in a notable recruitment of both the costal and crural segments of the diaphragm, akin to the acute hypoxic response of the diaphragm reported previously in humans and animals (Darian et al., 1989; Kelsen et al., 1977; Brice et al., 1990; Takasaki et al., 1989; Lopata et al., 1978; Onal et al., 1981; Yasuma et al., 1993; Easton et al., 1995; Road et al., 1986b; Suzuki et al., 1997). In particular, segmental shortening of the costal diaphragm progressively increased in proportion to the degree of hypoxic desaturation reaching peak values at a target SpO$_2$ level of ~80% (Figure 34). Although individual examinations are not reported, the crural segmental shortening exhibited a similar progressive increase with initial abrupt desaturation that reached peak shortening values in concert with the costal diaphragm.

Previously, in anesthetized and awake canines, costal and crural segmental shortening has been reported to increase in a proportional manner during progressive isocapnic hypoxia or stepwise reductions in F$_1$O$_2$ (21%, 15%, 13%, 10%, and 7%) (Easton et al., 1995; Road et al., 1986b; Suzuki et al., 1997). In direct comparison, mean tidal shortening of costal and crural (6.36 and 8.47%) noted in the present study during initial hypoxia corresponding to ~80% SpO$_2$ (Figure 35 and 36) are, by and large, compatible with the costal and crural shortening values (8.38% and 8.49%) reported earlier in awake canines when matched at a similar hypoxic level of ~77% SpO$_2$ (Easton et al., 1995). However, at a comparable F$_1$O$_2$ concentration of ~10% as employed in the present study, costal and crural segmental shortening in our awake animals (6.36 and 8.47%) were an order of magnitude smaller compared against the former anesthetized animals, reporting in the range of ~12 to 22% and ~19 to 20% for the costal and crural
segments, respectively (Road et al., 1986b; Suzuki et al., 1997). Although we cannot provide an adequate explanation, it is worth mentioning that, in direct contrast to quiet breathing where a notable shortening difference only occurred in the crural segment, both the costal and crural diaphragm during the initial hypoxic response shortened less than half of that under the influence of anesthesia. Thus accordingly, our data indicates a greater diaphragmatic contribution to hypoxic ventilation when anesthetized compared to the state of wakefulness; however, it is unlikely that this greater contractile shortening of the diaphragm mechanically translates into greater ventilatory output, as anesthesia is well-recognized to impair the ventilatory response to hypoxia (Hickey and Severinghaus, 1981; Pavlin and Hornbein, 1986).

As well, segmental shortening of the costal and crural diaphragm in response to initial acute hypoxia was accompanied by a paralleled increase in the EMG activation of the two diaphragmatic segments (Figure 35 and 36). Previously, progressive isocapnic/poikilocapnic hypoxia has been reported to cause a linear or hyperbolic increase in the EMG activity of the costal and crural diaphragm in adult humans and animals (Takasaki et al., 1989; Lopata et al., 1978; Onal et al., 1981; Darian et al., 1989; Kelsen et al., 1977; Brice et al., 1990; Yasuma et al., 1993). Our results qualitatively agree with these former EMG studies, and extend the previous work to demonstrate the direct mechanical consequence, i.e. segmental shortening, resulting from the changes in the neural activation of the diaphragm. Therefore our results along with the available evidence suggests that changes in the neuromotor output to the costal and crural segments are directly accountable for the greater contractile output of the diaphragm, and thus facilitating the increase in ventilation seen during the acute hypoxic ventilatory response.

**Sustained hypoxia: diaphragm EMG activity**

Besides the acute hypoxic response, steady-state effects of sustained hypoxia on the phrenic and diaphragm activity has been previously examined in newborns and adults across several mammalian species. Despite the differences in species, maturation and experimental technique, including some protocols involving anesthesia, neuromotor
output to the diaphragm in humans and animals generally follows a biphasic pattern, with initial peak increase followed by a significant decline or roll-off with constant exposure to hypoxia (LaFramboise and Woodrum, 1985; Guthrie et al., 1990; Watchko et al., 1990; Martin et al., 1990; Vizek et al., 1987; Vizek and Bonora, 1998; van Lunteren et al., 1989; McEvoy et al., 1996; Brown et al., 1992; Kimura et al., 1994; Fujimura et al., 2006).

In the present study, the biphasic costal EMG activity that rolls-off from initial peak activity to an intermediate plateau with sustained hypoxia (Figure 35B) are consistent with our previous work in canines (Fujimura et al., 2006) and the work of others in rats, cats, ponies, and humans (van Lunteren et al., 1989; McEvoy et al., 1996; Vizek and Bonora, 1998; Brown et al., 1992). Additional information can be gained from comparing our results for the costal diaphragm with other adult mammals. In our previous study thoroughly examining the ventilatory response to sustained hypoxia in conscious canines, we indicated that the timing of the ventilatory roll-off may be species dependent that may be related to mammalian body size (refer to discussion in Project 1: Species variation in ventilation during sustained hypoxia). Likewise, timing of the costal EMG activity roll-off in our medium bodied canines when compared to other mammals reveals a similar temporal pattern correlating with body size. Specifically, peak costal EMG activity in rats reported by Vizek and Bonora (1998) exhibited a very brief acute response to hypoxia with a prompt decline compared to our canines, whereas Brown et al. (1992) study in ponies expressed a much slower progressive attenuation of diaphragm activity that reached a plateau around the one hour mark. Accordingly, the temporal relationship of ventilation correlating with mammalian body size are closely coupled with the changes in neural activation of the diaphragm during sustained hypoxia.

Here we also extend the work of previous adult studies to demonstrate that the activity of the crural diaphragm, which is anatomically and functionally distinct from the costal, also significantly rolls-off with sustained hypoxia following a biphasic pattern (Figure 36B). For the awake canines in the present study, crural EMG activity following an initial peak augmentation declined to a plateau above baseline control levels with
sustained hypoxia. These findings on one hand agree with the reports made by Smith and colleagues (1989) in awake canines, where crural EMG activity initially increased and then remained above baseline activity levels following constant hypoxia. On the other hand, our results qualitatively differ in that crural EMG activity clearly exhibited a biphasic roll-off, whereas Smith and colleagues did not note a consistent biphasic trend. Several factors may have contributed in making the biphasic crural EMG activity difficult to realize in the former awake canine study. These include, but are not limited to, the small sample size ($N = 4$ for time course response), brief exposure to hypoxia (5 min), and confounding influence of $CO_2$ (arterial/central hypocapnia). To the extent that variability exists among individual animals in their response to initial and sustained hypoxia, the temporal changes in the diaphragmatic and ventilatory roll-off developing over the time course of 20-25 minutes, and the influence of hypocapnia to lessen the magnitude of the initial acute hypoxic response by acting through the central and peripheral chemoreceptors, it is conceivable that the characteristic EMG roll-off in the crural diaphragm may have been effectively masked in the former study. Nonetheless, our results are emphatic in demonstrating a biphasic activity response of the crural diaphragm with sustained isocapnic hypoxia in awake canines.

In addition, our results for the costal and crural diaphragm also qualitatively agree with studies done in newborn animals demonstrating a biphasic EMG activity roll-off (Martin et al., 1990; Guthrie et al., 1990; Watchko et al., 1990; LaFramboise et al., 1985), however, EMG activity of the diaphragm in the present study never fell down to, or below, baseline control levels and expressed a much slower rate of roll-off compared to the non-adult counterparts. Thus, our results suggest that the magnitude and rate of diaphragmatic EMG activity roll-off likely lessens and matures with age, but does not completely resolve in adults. This is consistent with the notion that the ventilatory response to sustained hypoxia fundamentally differs between adults and newborns and that this response likely matures with age (Easton et al., 1986; Bisgard and Neubauer, 1995; Mortola, 1996; Bissonnette, 2000).
Based on the available evidence, significant attenuation of central neural drive to the diaphragm mediates the sustained hypoxic ventilatory roll-off noted in mammals, including that of the canines in the present study. With respect to the mechanism of central attenuation of neuromotor output, a large body of literature suggests the role of net inhibitory neuromodulator production and accumulation which acts centrally to suppress the respiratory drive and ventilatory output during sustained hypoxia (Neubauer et al., 1990; Bisgard and Neubauer, 1995; Mortola, 1996). Our results are in direct agreement with this prevailing thesis and we provide experimental evidence demonstrating the effects of central attenuation affecting both segments of the costal and crural diaphragm in awake canines.

**Sustained hypoxia: diaphragm contraction and shortening**

What remained uncertain until the present study was the significant effects of a central attenuation on the actual mechanical consequence of the diaphragm during the sustained hypoxic ventilatory response. Although the diaphragm may be expected to contract and shorten in response to changes in central neuromotor output as indexed by EMG activity (Easton et al., 1995; Fitting et al., 1986; Easton et al., 1993), any presumptions on the contractile output of a respiratory muscle based solely on EMG activity alone without accompanying length measurements would be merely speculative and prone to inaccurate representation and/or misinterpretation. This is certainly the case for the costal and crural diaphragm, where its length and shortening for a given neural activation is critically dependent on the muscle's length-tension characteristics (Farkas and Dudley, 1988; Road et al., 1986a), the interaction with the rib cage and the abdomen (De Troyer et al., 1982; Decramer et al., 1984), as well as the influence of other respiratory muscles acting mechanically in series or in parallel with the costal and crural segments (Macklem et al., 1983).

To the best of our knowledge, our study is the first to report on the actual mechanical length change and shortening of the diaphragm during sustained hypoxia in any awake or anesthetized mammals. Our results demonstrate that during sustained
isocapnic hypoxia, the time course response of the costal diaphragm shortening tracks the biphasic changes in ventilation, with an initial peak response followed by a roll-off that settles to a plateau above baseline within the final 5 min of constant hypoxia (Figure 34). Such characteristic temporal pattern of costal diaphragm shortening was equally met by a biphasic time course contractile shortening response of the crural diaphragm (individual examination not reported). Thus, sustained hypoxia extended for 20-25 min resulted in a dramatic loss of the segmental shortening of the costal and crural diaphragm, with only 1/2 of initial acute hypoxic response remaining following the constant exposure to hypoxia (Figure 35A and 36A). Since changes in muscle length were recorded simultaneously along with EMG activity from the same region of the costal and crural segments of the diaphragm from each animal (Figure 35B and 36B), we can reasonably conclude that the significant reduction in the costal and crural shortening are largely accounted for by the changes in the neuromotor output to the diaphragm during sustained hypoxia.

As a principle inspiratory muscle, the diaphragm has been reported to account for up to ~41-75% of tidal volume changes at rest (Sant’Ambrogio et al., 1966; Newman et al., 1984), and possibly to an equal or greater extent during increased ventilatory demand or work. Consequently, the dramatic loss of the costal and crural diaphragm contractile output with sustained hypoxia would impart a significant mechanical effect on intrathoracic pressure generation and thus tidal changes in ventilation. Indeed changes in tidal volume in our awake canines proportionally reflected the changes in the contractile shortening response of the costal and crural diaphragm during the sustained hypoxic roll-off.

*Diaphragm activity during recovery*

With recovery room air breathing following 20-25 min of sustained isocapnic hypoxia, segmental tidal shortening of the costal promptly declined below baseline control levels then gradually returned within 5 min towards initial resting values prior to sustained hypoxia (Figure 34). Likewise, similar temporal response was noted for the
crural diaphragm and its contractile shortening recovery following the constant exposure to sustained hypoxia (individual report not reported). Thus in this canine group, mean tidal shortening of the costal and crural showed a modest and significant decline below original baseline values when averaged over the 5 min period of recovery (Figure 35A and 36A). This segmental shortening occurred with a slight, but non-significant, decline in the tidal EMG activity of the two diaphragmatic segments visibly falling below baseline levels during recovery (Figure 35B and 36B). Moreover, the lesser contractile output of the diaphragm following sustained hypoxia was associated with a reduction in ventilation without changes in respiratory rate.

Overall, our recovery data in awake canines suggests the role of central attenuation of neuromotor activity that persists following sustained hypoxia to impact the contractile output of the diaphragm and thus affecting tidal ventilation. Although contractile shortening of the diaphragm in these awake canines appears to be adequately restored to control levels within a brief period of post-hypoxic room air recovery breathing, there is prior evidence in adult humans (Easton et al., 1988) suggesting that the ventilatory response to subsequent hypoxia may be impaired up to 60 min following the constant exposure to sustained hypoxia of a similar level and duration as applied in the present study. Based on the previous human work and considering that the diaphragm is largely accountable for the tidal volume generation at rest and during stimulated ventilation, our post-hypoxic normoxic data likely does not portray the full recovery of the diaphragm and its subsequent response to hypoxia. Certainly further studies are warranted with respect to the recovery of the hypoxic ventilatory response following sustained hypoxia in awake canines and other mammals, and its impact on the diaphragm.

*Evidence for differential segmental function of the diaphragm*

Considerable evidence has been accumulated in support of the concept that the costal and crural diaphragm are fundamentally distinct muscles with the capacity to function in a differential manner. Indeed, the two segments are supplied by different phrenic nerve branches (Ogawa et al., 1958; De Troyer et al., 1982; Hammond et al.,
and have distinct proprioceptive/sensory afferent innervations (Corda et al., 1965; Duron et al., 1968; Pickering and Jones, 2002), and when stimulated they exert different mechanical effects on the rib cage and the abdomen (De Troyer et al., 1982). Various non-respiratory maneuvers in mammals, such as emesis, swallowing, regurgitation, and eructation (Abe et al., 1994; Titchen, 1979; Monges et al., 1978), and distinct respiratory function in canines, such as panting (Easton et al., 1994), reveal a clear divergence of segmental activity of the costal and crural parts of the diaphragm. Apart from a select number of studies in smaller mammals that have suggested a uniform activation of the diaphragmatic segments (D'Angelo et al., 2010; Boyd and Basmajian, 1963; Sant'Ambrogio et al., 1963; Oyer et al., 1989; Pickering and Jones, 2007), studies in larger mammals support a differential segmental innervation, activation and function of costal and crural diaphragm.

Previous studies measuring the costal and crural segmental length change and/or EMG activity during spontaneous respiration have been mostly conducted in acutely anesthetized preparations. These studies have collectively demonstrated the unique and distinct segmental activity of the costal and crural diaphragm during both resting and stimulated ventilation, and evidence to date in canines and cats suggests that: 1) peak tidal shortening and EMG activity of the crural is greater than the corresponding costal segmental values; 2) onset of crural EMG activity precedes costal EMG activity, with associated onset of segmental shortening following a similar pattern; 3) greater velocity of crural segmental shortening compared to the costal segmental values at rest and hypercapnic stimulated ventilation; and 4) there is a proportionally greater increase in crural segmental shortening/EMG activity during hypoxic/hypercapnic stimulated ventilation (Newman et al., 1984; Fitting et al., 1986; Road et al., 1986b; Easton et al., 1987; Decramer et al., 1984; Darian et al., 1989; Van Lunteren et al., 1985; Van Lunteren et al., 1984). Studies in chronically instrumented awake animals generally agree with the differential segmental activity reported in the anesthetized animals, and further emphasize the distinctiveness of the two diaphragmatic segments with respect to their mechanical and electrical behavior during respiration (Easton et al., 1995; Easton et al., 1993; Torres et al., 1989; Easton et al., 1994; Easton et al., 1999b).
Differential segmental function: peak shortening and EMG activity

In our intact awake canines, direct measurement of shortening and EMG activity with respect to the traditional peak tidal breath values clearly reveal a unique and distinct activity of the costal and crural segments of the diaphragm during both quiet resting and hypoxic stimulated ventilation. Specifically, when the two segments were directly compared, greater contractile shortening in conjunction with EMG activation was noted for the crural as compared to the costal at each individual period of room air and sustained hypoxia (Figure 37 and 38). Such differential segmental activity noted in our awake canines qualitatively agrees and extends to the results of the former anesthetized canine studies, where peak tidal crural shortening has been shown to be significantly greater than the corresponding costal values at rest and during hypoxic simulated ventilation (Newman et al., 1984; Fitting et al., 1986; Road et al., 1986b). However, such predominance of crural compared to costal activity has not always been a general rule in canines, especially in the non-anesthetized state.

In chronically implanted awake canines, peak tidal shortening and EMG activity of the costal and crural has been reported to be generally equivalent whether at rest or with hypercapnic or hypoxic stimulated ventilation (Easton et al., 1993; Easton et al., 1995). Up until the present study, differential segmental peak shortening/EMG activity of the diaphragm during respiration was generally perceived as a trait among canines in the anesthetized state, possibly attributed to a greater EMG activation and shortening of the crural diaphragm and/or increased variability in segmental activity in the conscious animals (Easton et al., 1993). Such presumptions raise a legitimate concern as to whether the differential segmental activity in the prior acute canine studies may have been an artifact due to the effects of anesthesia, and thus fail to adequately represent the normal physiologic function of the diaphragm in a conscious state. In this regard, our results are clear in demonstrating that the crural predominance in peak tidal activity as compared to the costal is not an exemption under the influence of anesthetics, but a characteristic feature of a normal functioning diaphragm in awake canines. In addition, confounding effects of anesthesia which cause an increase in the contractile shortening of the crural
(and thus, contributing to a greater divergence in the segmental activity of the two parts of the diaphragm) was indirectly apparent in our awake canines, as the difference in costal and crural peak shortening were modest and not as striking in our study (~1 to 2%) compared to the anesthetized canines of the past (~4% to 7%) (Newman et al., 1984; Fitting et al., 1986; Road et al., 1986b).

We also observed a certain degree of variability in the predominance of crural peak shortening and EMG activity, where few animals exhibited a greater costal than crural peak tidal shortening and EMG activity at rest and throughout hypoxia. Accordingly, we presume that this natural variability along with a smaller sample size of available length and EMG measurements in the former awake canine studies (Easton et al., 1993; Easton et al., 1995) could have made it difficult to achieve a statistically significant difference in the tidal "whole breath" activity of the two diaphragm segments. In the present study, although the difference in peak shortening and EMG activity were not of a generous amount, the majority of the animals expressed a trend for greater crural than costal peak segmental activity and the study was adequately powered to detect a statistically significant difference between the two segments during room air as well as hypoxic stimulated ventilation. Thus, with a large enough sample size to account for the natural variability in segmental diaphragm activity in the state of wakefulness, we envision that a similar differential peak tidal costal and crural activity would have been realized in the earlier awake canine studies.

Also worth mentioning, a greater predominance of crural than costal segmental peak tidal shortening was observed in our awake canines in the lateral decubitus position which differs from the significantly greater shortening of the costal than of crural segments reported in spontaneously breathing, awake sheep in the standing posture (Torres et al., 1989). Such discrepancy in peak segmental shortening response might be ascribed to the differences in ovine vs. canine diaphragm, posture, and/or variations in the implantation site within the costal and crural diaphragm for the two studies. Nonetheless, our results clearly demonstrate a differential segmental shortening of the costal and crural that is driven by a qualitatively distinct neuromotor output to the two diaphragmatic
segments. Although the exact mechanism responsible for the differential segmental peak activity is unknown, it is conceivable that our findings may be accounted for by a unique central control mechanism invoking discrete neuromotor output to the costal and crural diaphragm and/or an outcome of a segmental reflex that may affect the two parts of the diaphragm in a differential manner.

Other factors may contribute towards the differential segmental contractile activity of the costal and crural diaphragm, including its discrete muscle fiber architecture (Reid et al., 1987; Gordon et al., 1989), resting length at FRC (Farkas and Dudley, 1988; Road et al., 1986a), and/or mechanical action and interaction with the rib cage and abdomen (De Troyer et al., 1982). To the extent that the mechanical effects of the rib cage and the abdomen act differentially on the two segments of the diaphragm, with the costal having to face the additional elastance of the rib cage (De Troyer et al., 1982; Easton et al., 1993) and the crural being a more compliant muscle mostly interacting with the abdomen (Road et al., 1986b), the costal segment may be expected to shorten to a lesser extent even if the neuromotor output were equivalent for the two segments. However, based on the in situ lengths, cross-sectional area, and in vitro length-tension characteristics at FRC, the costal diaphragm has been suggested to exert up to 60% more force than the crural diaphragm (Farkas and Dudley, 1988). Thus, despite the greater peak tidal shortening of the crural compared to the costal diaphragm observed in the present study, it is unlikely that the crural diaphragm is contributing to a greater extent than the costal in regards to intrathoracic pressure generation and movement of tidal air during room air and hypoxic stimulated ventilation.

**Differential segmental function: relative recruitment and roll-off**

Despite the greater peak tidal shortening and EMG activity of the crural diaphragm compared to the costal observed in our intact awake animals, such data expressed in absolute magnitude does not reveal whether one part of the diaphragm was recruited or derecruited more than the other during the biphasic response to sustained hypoxia. Therefore, relative changes in peak tidal shortening and EMG activity of the
costal and crural segments were also assessed as a percentage of baseline and peak activity during the initial and sustained exposures to hypoxia, respectively (Figure 39 and 40). We did not attempt to run pair-wise statistical comparisons on relative values, but rather were interested in noting any trends in the relative response of the costal and crural diaphragm.

**Relative recruitment with initial hypoxia**

With initial hypoxia relative recruitment of the costal and crural diaphragm exhibited a differential segmental pattern with a trend towards greater costal than crural shortening (221% vs. 195% BASE), however, there was some variability within the individual animals’ segmental shortening response as noted by the overlapping error bars (Figure 39A). Such trends for a discrete contractile shortening response of the costal and crural diaphragm occurred in parallel with a remarkably distinct and separate relative EMG activation of the two diaphragmatic segments (152% vs. 131%) (Figure 39B). As a result, in direct comparison to the segmental shortening response that was somewhat variable within the group, segmental EMG activity response in the vast majority of our animals consistently expressed a relatively greater activation of the costal than the crural segment. Our results, demonstrating a modest degree of variability in the segmental shortening response, that did not directly correlate with the notably distinct segmental EMG activation is not at all surprising, and to some extent expected for a respiratory muscle such as the diaphragm.

Although contractile shortening of the costal and crural diaphragm would be generally expected to correlate with the changes in EMG activation, direct mechanical translation of motor output may not be realized given that segmental length change and shortening are ultimately determined by several other factors beside electrical activation. Such factors include but are not limited to, active and passive muscle length-tension characteristics, abdominal load, elastance of the rib cage, alterations in respiratory mechanics, and interactions with other respiratory muscles acting mechanically in series or parallel with the diaphragm. For this reason, a certain degree of variability in the
mechanical shortening would naturally result despite a very distinct and separate motor output to the two segments of the diaphragm. Such results emphasize and reiterates an important concept; accurate assessment of respiratory muscle function requires either a direct or indirect means of measuring the mechanical consequence along with its corresponding electrical activity.

In our intact awake canines, permitting the direct assessment of the respiratory changes in length and EMG activity, initial exposure to sustained hypoxia recruited the costal and crural diaphragm differentially with a trend towards a relatively greater costal than crural segmental shortening, largely accounted for by a notably distinct neural activity response of the two segments. Although the exact mechanism accounting for the differential segmental activity response is uncertain, such findings may be a manifestation of a unique central control mechanism evoking a discrete neuromotor output pattern to the two diaphragmatic segments. To the extent that the costal and crural segmental contraction would act to reduce intrathoracic pressure and thus generate inspiratory airflow, the two segments would act synergistically to facilitate and augment ventilation during the initial acute response to sustained hypoxia.

*Previous studies of relative recruitment*

Similar trends for a relatively greater recruitment of the costal than crural segmental peak shortening with acute steady-state hypoxia has been previously reported by Road et al. (1986b) in anesthetized canines, where direct length measurements of the diaphragm were made by sonomicrometry. However, our results are at variance with the study by Darian et al. (1989) and Gottfried and DiMarco (1986) in anesthetized canines, as the relative increase in peak crural shortening and EMG activity has been shown to be greater than the corresponding costal segment during acute progressive hypoxia.

Although we cannot reconcile the difference in the relative recruitment response of the costal and crural noted in the past anesthetized canine studies, it is worth mentioning that the former study by Road et al. (1986b) employed a similar steady-state
step reduction in $F_O^2$ technique as that of the current study, whereas the later study by Darian et al. (1989) and Gottfried and DiMarco (1986) used a progressive rebreathe technique to elicit hypoxia. It is hard to envision how a step reduction vs. a progressive rebreathe method of entertaining hypoxia might cause a drastic difference in the relative requirement of the two diaphragmatic segments. Also, it is unlikely that differences in position or type of anesthesia was a causative factor since both anesthetized canines were studied in the supine position and employed pentobarbital sodium with a similar dose. Our study also rules out the effects of arterial/cerebral CO$_2$ as a determining factor, since our animals in the isocapnic state also exhibited a relatively greater costal than crural recruitment response to acute hypoxia as noted by Road and colleagues.

Here we extend the earlier findings to demonstrate in intact awake canines a relatively greater costal than crural shortening and EMG activity response to acute steady-state hypoxia. Further studies are warranted to assess the relative chemoresponsiveness of the costal vs. crural diaphragm shortening and EMG activity during acute progressive hypoxia in awake mammals.

*Relative derecruitment with sustained hypoxia*

Relative derecruitment of the costal and crural diaphragm during sustained hypoxia was also examined in our awake canines. As a percentage of initial peak hypoxic activity, segmental shortening of the costal and crural declined in an equal and proportional manner (68% vs. 68% PEAK) (Figure 40A), and thus did not exhibit a distinct derecruitment pattern in response to sustained hypoxia. However, differential segmental activity was clearly noted for the costal and crural diaphragm, with a trend towards a relatively lesser decline in crural than costal EMG activity (91% vs. 83% PEAK) (Figure 40B). If we assume that segmental shortening would generally reflect EMG activity in a relative manner similar to the initial hypoxic response described earlier, then we would expect crural shortening to relatively decline to a lesser extent than the corresponding costal segment during the sustained hypoxic roll-off. Clearly this was not the case in our intact awake animals. Rather what we found was a striking disassociation
in the relative shortening and EMG activity response of the crural diaphragm during sustained hypoxia when compared against the costal diaphragm. The following section expounds upon the neuromechanical disassociation occurring in the crural diaphragm.

**Neuromechanical disassociation with sustained hypoxia**

This is the first report describing the effects of sustained hypoxia causing a neuromechanical disassociation of the crural diaphragm as revealed by the relative changes in the segmental shortening and EMG activity of the muscle. Our findings would be largely accounted for, if the mechanics of the crural diaphragm were to have changed with the constant exposure to hypoxia. This would certainly affect the contractile shortening of the muscle for a given neural activation, and explain the divergence of crural segmental shortening and EMG activity. Conceivably, changes in the mechanics of the crural diaphragm may be brought about by an extrinsic and/or intrinsic mechanism.

**Extrinsic mechanism: mechanical load and impedance**

De Troyer et al. (1982) in anesthetized canines demonstrated that the crural diaphragm mostly interacted with the abdomen. Therefore, it is probable that changes in abdominal load could impede the shortening of the crural to a greater extent than the costal diaphragm. It is also possible that the action of the inspiratory intercostals/accessory muscles may impede the shortening of the crural more than the costal segment. In anesthetized canines, crural consistently lengthened during inspiration following bilateral phrenicotomy, whereas the costal shortened or lengthen during inspiration (Decramer et al., 1984). Similarly, Katagiri and colleagues (1994) in awake canines with complete bilateral hemidiaphragm paralysis showed a greater crural lengthening during inspiration as compared to the costal diaphragm in a representative animal. Based on the available evidence, it appears that the counteracting action of the inspiratory intercostals/accessory muscles against the contraction of the diaphragm likely affects the crural segment more than the costal. Moreover, the costal diaphragm is a much larger muscle relative to the smaller crural diaphragm, it may be plausible that much of
the length change occurring in crural segment may be governed by the costal segment during inspiration. In such circumstances, greater crural activation relative to the costal diaphragm may not directly translate mechanically into greater contractile output of the crural diaphragm. At present, it is uncertain whether, or to what extent, sustained hypoxia would affect the aforementioned extrinsic factors to impede the segmental shortening of the crural compared to costal during spontaneous respiration.

*Intrinsic mechanism: dynamic length adjustment*

Alterations in crural mechanics may equally involve an intrinsic factor relating to the length-tension characteristics of the muscle. It is our contention that the disassociation of the relative shortening and EMG activity of the crural diaphragm predominately resulted from an active mechanism involving a dynamic adjustment of the muscle's resting length to enhance the force output of the costal diaphragm during the sustained hypoxic roll-off.

Such an assertion and hypothesis would require the crural diaphragm to function as both a sensor and an adjustor to reflexively enact a compensatory action on the costal diaphragm. Indeed, the crural diaphragm has been shown to have a richer proprioceptive afferent innervation (i.e. associated with muscle spindles and Golgi tendon organs) than the costal diaphragm in cats (Corda et al., 1965). Similarly, histological identification of diaphragmatic receptors in cats have identified muscle spindles that were exclusively localized in the crural diaphragm (Duran et al., 1978). To the extent that proprioceptive afferent feedback can provide important information about the mechanical state of the muscle, the crural segment should be capable of detecting changes in its length/tension that might transpire as a result of changes occurring in the costal contraction (i.e. force output) and/or configuration (i.e. resting length). Moreover, considering the mechanical arrangement of the costal and crural diaphragm that is partly in series and partly in parallel (Decramer et al., 1984), the crural segment should be capable of enacting a fine dynamic adjustment of muscle length to affect the resting length of the costal through its series component.
In this regard, active reduction in the crural resting length could potentially lengthen the pre-contraction resting length of the costal to enhance its length-tension characteristic and associated force-generating capacity during the sustained hypoxic roll-off. Such compensatory action would indeed place the crural diaphragm at a mechanical disadvantage and consequently account for the neuromechanical disassociation of the crural diaphragm noted in the presented study. As well, it is also worth mentioning that hypoxia has been reported to cause an increase in the end-expiratory lung volume, i.e. FRC, and an inhibition of expiratory abdominal activity (Fregosi et al., 1987; Bouverot and Fitzgerald, 1969; Garfinkel and Fitzgerald, 1978). The net effect of these systemic changes could potentially manifest to cause a reduction in the end-expiratory resting length of the diaphragm. Under these circumstances, the functional capacity of the crural to dynamically sense and act as an adjustor to preserve the resting length of the costal may play a critical role in safeguarding force output of the diaphragm.

Certainly further studies are warranted to assess whether sustained hypoxia indeed causes changes in the resting length of the costal and crural segments of the diaphragm, and whether an active mechanism may be involved in the crural diaphragm reducing its resting length in support of its functional role as a dynamic length adjustor for the costal diaphragm.

**Mechanism of differential segmental EMG attenuation**

How can we reconcile the costal and crural EMG activity that relatively declined or rolled-off in a differential manner with sustained hypoxia? We conceive two mechanisms that could account for the differential neural activity response noted for the costal and crural diaphragm with constant hypoxia. First, our results may be ascribed by a central mechanism involving discrete inspiratory neuronal pools in the medullary respiratory center separately projecting to the costal and crural diaphragm, with differential sensitivity to the central attenuating effects of sustained hypoxia mediated by net inhibitory neuromodulators. Second, it is also possible that the central motor output
may or may not be discrete during the attenuation of central drive, but may be reflexively modulated by proprioceptive feedback mechanism mediated by diaphragm afferents at the spinal or supraspinal level. At present, it is unclear whether discrete populations of medullary respiratory neuronal pools or bulbospinal motor neurons exists that project to the costal and crural segments of the diaphragm via separate branches of the phrenic nerve. With respect to the proprioceptive reflex modulation, the crural would be well suited to respond to the sensory feedback given that mechanoreceptors are predominated located in this segment of the diaphragm. As the costal diaphragm is poorly endowed with sensory organs, it would be expected that any proprioceptive afferent modulation would affect this segment to a lesser extent than the crural segment.

Experimental evidence in support of a proprioceptive afferent modulation of diaphragm peak/tonic EMG activity, especially affecting the crural segment to a greater extent, has been previously reported in humans and animals following bronchoconstriction, obstruction and abdominal loading (van Lunteren et al. 1984; Fitting et al., 1986; Revelette et al., 1992; Muller et al., 1979), as well as during changes in end-expiratory lung volume (van Lunteren 1985; Erickson et al., 1994). Consequently, any extrinsic (i.e. diaphragmatic load or impedance) or intrinsic (i.e. muscle length) factors altering the mechanics or mechanical efficiency of the diaphragm could therefore elicit a proprioceptive reflex mediated modulation of the motor output to the diaphragm. Presumably this would affect the crural relatively more than the costal diaphragm, and thus may account for the differential attenuation of neural drive during sustained hypoxia. In any case, our data clearly demonstrates a distinct neural activity response of the two diaphragmatic segments during the sustained hypoxic roll-off.

**Persistence of neuromechanical disassociation**

With recovery room air breathing, the relative segmental shortening and EMG activity of the two segments declined below initial baseline levels prior to sustained hypoxia, with a trend for a lesser contractile shortening of the crural for a given change in neural activation when compared to the costal diaphragm. Accordingly, for a comparable
decline in the EMG activity of the two diaphragmatic segments (costal and crural: 95% vs. 94%), crural shortening proportionately fell more than the costal segment (73% vs. 83%). Therefore, even at a similar neural activation level, crural when compared to the costal exhibited the characteristic neuromechanical disassociation pattern noted during the sustained hypoxic roll-off (albeit in a qualitatively and quantitative different manner).

On the basis of the crural diaphragm possibly acting as an length adjustor to facilitate costal diaphragm function, our results would indicate that alterations in crural mechanics through a reduction in resting length may still be occurring following the constant exposure to hypoxia. Alternatively, the relatively greater decline in crural shortening compared to costal may be a result of the external forces impeding the contractile action of the crural more so than that of the costal diaphragm. In either case, proprioceptive afferents may have contributed to some extent to increase the segmental EMG activity of the two diaphragm segments with a greater influence on the crural segment. Nonetheless, contractile shortening response of the costal and crural diaphragm in these awake canines were relatively distinct during post-hypoxic recovery room air breathing.

**Conclusion**

During sustained isocapnic hypoxia, costal and crural diaphragm contraction and neural activation exhibited a bimodal pattern, and thus directly contribute to the biphasic changes in ventilation. However, the diaphragm does not function as a single homogenous entity, as segmental shortening and EMG activity at each individual period of room and sustained hypoxic stimulated ventilation was clearly distinct for the costal and crural segments. Moreover, capacity for differential segmental function was further demonstrated by a discrete recruitment pattern of the two diaphragmatic segments, with relatively greater costal than crural shortening and EMG activity during initial hypoxia followed by a proportional decline in segmental shortening output of the two segments despite a lesser decline in crural EMG activity during sustained hypoxia. Such neuromechanical disassociation of the crural diaphragm may reflect the functional
capacity of the crural acting as a length adjustor to safeguard the force output of the costal diaphragm during the significant hypoxic roll-off.
Acknowledgements

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Figure 33: Costal and crural diaphragm segmental length and EMG activity during room air and sustained isocapnic hypoxia (legend on next page)

A

Initial Room Air, BASE 92.2% SpO₂

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<th>COS Mavg EMG</th>
<th>CRU Length</th>
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<tbody>
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0:20 (min:sec) 1 sec

B

Initial Hypoxia, PEAK 81.4% SpO₂

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1:21 (min:sec) 1 sec

C

Final Hypoxia, PLATEAU 81.7% SpO₂

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21:45 (min:sec) 1 sec

D

Final Room Air, RECOVERY 90.3% SpO₂

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1:36 (min:sec) 1 sec
Figure 33 (figure legend)

Representative tracing of inspiratory airflow, costal diaphragm segmental length (COS Length) and integrated moving average EMG activity (COS Mavg EMG), and crural diaphragm segmental length (CRU Length) and integrated moving average EMG activity (CRU Mavg EMG) during room air and sustained isocapnic hypoxia. Traces shown are breaths during initial room air breathing before hypoxia (BASE); initial hypoxia once O$_2$ saturation (SpO$_2$) reached 80 ± 2% (PEAK); final hypoxia preceding return to room air (PLATEAU); and final room air breathing after hypoxia (RECOVERY). Each tracing captures 11 sec worth of data with start time (min:sec) and SpO$_2$ (%) noted at the bottom-left and top-right, respectively. L$_{BL}$ indicates baseline resting muscle length at end-expiration. Note: upward deflection of airflow marks inspiratory airflow, or inspiration; downward deflection of COS and CRU Length reflects segmental shortening and upward deflection of COS and CRU Mavg EMG signal represents segmental muscle activation.
Figure 34: Costal diaphragm segmental shortening and ventilation during room air and sustained isocapnic hypoxia

Typical time course of costal diaphragm segmental shortening and ventilation in response to sustained isocapnic hypoxia in a representative animal. Black circle (top), open circle, black circle (bottom), and open triangle represent mean values/20 sec for costal diaphragm segmental shortening per breath (COS SHORT), O₂ saturation (SpO₂), minute ventilation (V₁), and partial pressure of end tidal CO₂ (P₂CO₂), respectively.
Figure 35: Costal diaphragm segmental shortening and EMG activity during room air and sustained isocapnic hypoxia (legend on next page)

**A**  Costal Diaphragm Shortening

![Bar chart showing costal diaphragm shortening with percentages for BASE, PEAK, PLATEAU, and RECOVERY stages.]

- **BASE**: 100%
- **PEAK**: 221%
- **PLATEAU**: 141%
- **RECOVERY**: 83%

**B**  Costal Diaphragm EMG

![Bar chart showing costal diaphragm EMG activity with percentages for BASE, PEAK, PLATEAU, and RECOVERY stages.]

- **BASE**: 100%
- **PEAK**: 152%
- **PLATEAU**: 122%
- **RECOVERY**: 95%
Group mean costal diaphragm segmental shortening and EMG activity during sustained isocapnic hypoxia in N=11 and N=10 animals, respectively. A: Costal diaphragm shortening (COS SHORT). Tidal segmental shortening of the costal diaphragm during BASE, room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; and RECOVERY, room air breathing after hypoxia. Y-axis expresses shortening per breath as a percentage change from baseline resting muscle length at end-expiration, %Lₐ₉ₐ₇. B: Costal diaphragm EMG (COS EMGDIFF). Tidal EMG activity of costal diaphragm during periods of BASE, PEAK, PLATEAU, and RECOVERY. Y-axis shows muscle EMG activity per breath as maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. Columns show group mean values with standard error bars. Numerical labels are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on COS SHORT and EMGDIFF, both P<0.0001. ¤ and ¶, significant difference between individual periods at P<0.01 and P<0.05, respectively.
Figure 36: Crural diaphragm segmental shortening and EMG activity during room air and sustained isocapnic hypoxia (legend on next page)

A

Crural Diaphragm Shortening

<table>
<thead>
<tr>
<th></th>
<th>BASE</th>
<th>PEAK</th>
<th>PLATEAU</th>
<th>RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHORT (%LUL)</td>
<td>4.0±1.0</td>
<td>9.0±1.0</td>
<td>6.0±1.0</td>
<td>3.0±1.0</td>
</tr>
</tbody>
</table>

100% 195% 126% 73%

B

Crural Diaphragm EMG

<table>
<thead>
<tr>
<th></th>
<th>BASE</th>
<th>PEAK</th>
<th>PLATEAU</th>
<th>RECOVERY</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMG (volts)</td>
<td>4.0±1.0</td>
<td>6.0±1.0</td>
<td>5.0±1.0</td>
<td>4.0±1.0</td>
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</tbody>
</table>

100% 131% 118% 94%
**Figure 36 (figure legend)**

Group mean crural diaphragm segmental shortening and EMG activity during sustained isocapnic hypoxia in N=13 and N=12 animals, respectively. A: Crural diaphragm shortening (CRU SHORT). Tidal segmental shortening of the crural diaphragm during BASE, room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; and RECOVERY, room air breathing after hypoxia. Y-axis expresses shortening per breath as a percentage change from baseline resting muscle length at end-expiration, %L\textsubscript{BL}. B: Crural diaphragm EMG (CRU EMG\textsubscript{DIFF}). Tidal EMG activity of crural diaphragm during periods of BASE, PEAK, PLATEAU, and RECOVERY. Y-axis shows muscle EMG activity per breath as maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. Columns show group mean values with standard error bars. Numerical labels are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on CRU SHORT and EMG\textsubscript{DIFF}, both P<0.0001. †, significant difference between individual periods at P<0.01.
Figure 37: Costal and crural diaphragm segmental shortening during room air and sustained isocapnic hypoxia

Costal vs Crural Diaphragm Shortening

Group mean comparison of costal and crural diaphragm segmental shortening during sustained isocapnic hypoxia in N=11 and N=13 animals, respectively. Tidal segmental shortening of the costal and crural diaphragm during BASE, room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; and RECOVERY, room air breathing after hypoxia. Y-axis expresses shortening per breath as a percentage change from baseline resting muscle length at end-expiration, %L_{BL}. Columns show group mean values with standard error bars. COS SHORT, costal diaphragm shortening; CRU SHORT, crural diaphragm shortening. *, significant difference between COS and CRU SHORT at P<0.05.
Group mean comparison of costal and crural diaphragm segmental EMG activity during sustained isocapnic hypoxia in N=10 and N=12 animals, respectively. Tidal segmental EMG activity of the costal and crural diaphragm during BASE, room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; and RECOVERY, room air breathing after hypoxia. Y-axis shows muscle EMG activity per breath as maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. Columns show group mean values with standard error bars. COS EMG, costal diaphragm EMG activity; CRU EMG, crural diaphragm EMG activity. *, significant difference between COS and CRU EMG at P<0.05.
Figure 39: Costal and crural diaphragm segmental shortening and EMG activity response to initial hypoxia (legend on next page)

A  Initial Hypoxia: Costal vs Crural Shortening

B  Initial Hypoxia: Costal vs Crural EMG
Figure 39 (figure legend)

Group mean costal and crural diaphragm segmental shortening and EMG activity response to initial hypoxia. A: Costal and crural diaphragm shortening (COS and CRU SHORT). Tidal segmental shortening of the costal and crural diaphragm from BASE, room air breathing before hypoxia, to PEAK, first 3 min after O$_2$ saturation (SpO$_2$) decreased to 80 ± 2%. Y-axis and numerical labels express change in muscle shortening per breath as a percentage of baseline room air breathing before hypoxia, %BASE. B: Costal and crural diaphragm EMG (COS and CRU EMG). Tidal EMG activity of costal and crural diaphragm from BASE to PEAK. Y-axis and numerical labels express change in muscle EMG activity per breath as a percentage of baseline room air breathing before hypoxia, %BASE. Symbols show group mean values with standard error bars. N=11, COS SHORT; N=13, CRU SHORT; N=10, COS EMG; N=12, CRU EMG.
Figure 40: Costal and crural diaphragm segmental shortening and EMG activity response to sustained hypoxia (legend on next page)

A  Sustained Hypoxia: Costal vs Crural Shortening

B  Sustained Hypoxia: Costal vs Crural EMG
Figure 40 (figure legend)

Group mean costal and crural diaphragm segmental shortening and EMG activity response to sustained hypoxia. A: Costal and crural diaphragm shortening (COS and CRU SHORT). Tidal segmental shortening of the costal and crural diaphragm from PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%, to PLATEAU, final 5 min at SpO₂ of 80 ± 2%. Y-axis and numerical labels express change in muscle shortening per breath as a percentage of initial peak hypoxic response, %PEAK. B: Costal and crural diaphragm EMG (COS and CRU EMG). Tidal EMG activity of costal and crural diaphragm from PEAK to PLATEAU. Y-axis and numerical labels express change in muscle EMG activity per breath as a percentage of initial peak hypoxic response, %PEAK. Symbols show group mean values with standard error bars. N=11, COS SHORT; N=13, CRU SHORT; N = 10, COS EMG; N=12, CRU EMG.
Table 2: Oxygen saturation, end tidal CO\textsubscript{2} and breathing pattern during room air and sustained isocapnic hypoxia
(legend on next page)

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
<th>Hypoxia Hypoxia</th>
<th>Hypoxia Hypoxia</th>
<th>Room Air Room Air</th>
<th>Effect Effect</th>
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<tbody>
<tr>
<td></td>
<td>Initial 5 min</td>
<td>Initial 3 min</td>
<td>Final 5 min</td>
<td>Final 5 min</td>
<td>P Value</td>
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<tr>
<td></td>
<td>BASE PEAK</td>
<td>PEAK PLATEAU</td>
<td>RECOVERY</td>
<td>Hypoxia</td>
<td></td>
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<tr>
<td>Sp\textsubscript{O2} (%)</td>
<td>92.84 ± 2.18</td>
<td>79.72 ± 3.74</td>
<td>78.48 ± 3.92</td>
<td>90.30 ± 2.85</td>
<td>&lt; 0.0001</td>
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<tr>
<td>P\textsubscript{ET}CO\textsubscript{2} (mmHg)</td>
<td>37.18 ± 0.59</td>
<td>36.63 ± 2.60</td>
<td>37.78 ± 2.27</td>
<td>35.64 ± 1.90</td>
<td>0.0250</td>
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<tr>
<td>V\textsubscript{I} (l/min)</td>
<td>7.58 ± 2.60</td>
<td>17.42 ± 4.92</td>
<td>13.79 ± 5.53</td>
<td>6.70 ± 2.32</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>V\textsubscript{T} (l)</td>
<td>0.39 ± 0.06</td>
<td>0.69 ± 0.14</td>
<td>0.51 ± 0.11</td>
<td>0.34 ± 0.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>f\textsubscript{R} (breaths/min)</td>
<td>19.68 ± 6.52</td>
<td>25.45 ± 5.65</td>
<td>27.39 ± 9.79</td>
<td>20.50 ± 7.37</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T\textsubscript{I} (s)</td>
<td>1.36 ± 0.37</td>
<td>1.18 ± 0.27</td>
<td>1.11 ± 0.37</td>
<td>1.32 ± 0.46</td>
<td>0.0004</td>
</tr>
<tr>
<td>V\textsubscript{T}/T\textsubscript{I} (l/s)</td>
<td>0.30 ± 0.08</td>
<td>0.60 ± 0.14</td>
<td>0.51 ± 0.17</td>
<td>0.28 ± 0.08</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>T\textsubscript{I}/T\textsubscript{T\textsubscript{TOT}} (ratio)</td>
<td>0.41 ± 0.05</td>
<td>0.48 ± 0.04</td>
<td>0.45 ± 0.04</td>
<td>0.40 ± 0.03</td>
<td>&lt; 0.0001</td>
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Table 2 (table legend)

SpO$_2$, pulse oximeter O$_2$ saturation; P$_{ET}$CO$_2$, partial pressure of end tidal CO$_2$; $V_t$, minute ventilation; $V_T$, tidal volume; $f_R$, respiratory rate; $T_I$, inspiratory time; $V_I/T_I$, mean inspiratory flow; $T_I/T_{TOT}$, inspiratory fraction of respiration. Values are mean ± SD for $N = 13$ canines during BASE, 5 min room air breathing before hypoxia; PEAK, first 3 min after O$_2$ saturation (SpO$_2$) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO$_2$ of 80 ± 2%; RECOVERY, 5 min room air breathing after hypoxia. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Comparisons: SpO$_2$ (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, PEAK-PLATEAU, NS); P$_{ET}$CO$_2$ (BASE-PEAK, PEAK-PLATEAU, BASE-PLATEAU, BASE-RECOVERY, NS; PLATEAU-RECOVERY, P<0.05); $V_I$, $V_T$, $V_I/T_I$, $T_I/T_{TOT}$ (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, NS); $f_R$ (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, BASE-RECOVERY, NS); $T_I$ (BASE-PLATEAU, PLATEAU-RECOVERY, P<0.01; BASE-PEAK, P<0.05; PEAK-PLATEAU, BASE-RECOVERY, NS).
Table 3: Costal and crural diaphragm segmental shortening and EMG activity during room air and sustained isocapnic hypoxia (legend on next page)

<table>
<thead>
<tr>
<th></th>
<th>Room Air</th>
<th>Hypoxia</th>
<th>Hypoxia</th>
<th>Room Air</th>
<th>Effect</th>
<th>P Value</th>
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<td>5 min</td>
<td>3 min</td>
<td>5 min</td>
<td>5 min</td>
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<td></td>
</tr>
<tr>
<td>BASE</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COS SHORT (%L&lt;sub&gt;BL&lt;/sub&gt;)</td>
<td>3.18 ± 0.59</td>
<td>6.36 ± 1.14</td>
<td>4.11 ± 0.65</td>
<td>2.58 ± 0.49</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>EMG&lt;sub&gt;DIF&lt;/sub&gt; (volts)</td>
<td>3.68 ± 0.34</td>
<td>5.30 ± 0.27</td>
<td>4.43 ± 0.37</td>
<td>3.45 ± 0.26</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>CRU SHORT (%L&lt;sub&gt;BL&lt;/sub&gt;)</td>
<td>4.43 ± 0.42</td>
<td>8.47 ± 0.99</td>
<td>5.42 ± 0.52</td>
<td>3.34 ± 0.51</td>
<td>&lt; 0.0001</td>
<td></td>
</tr>
<tr>
<td>EMG&lt;sub&gt;DIF&lt;/sub&gt; (volts)</td>
<td>5.03 ± 0.48</td>
<td>6.47 ± 0.52</td>
<td>5.93 ± 0.55</td>
<td>4.79 ± 0.52</td>
<td>&lt; 0.0001</td>
<td></td>
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</tbody>
</table>
Table 3 (table legend)

COS, costal diaphragm; CRU, crural diaphragm; SHORT, segmental shortening per breath expressed as a percentage change from baseline resting muscle length at end-expiration, %L_{BL}; EMG_{DIFF}, segmental EMG activity per breath quantified as the maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. Values are mean ± SD for N=11, COS SHORT; N=10, COS EMG_{DIFF}; N=13, CRU SHORT; N=12, CRU EMG_{DIFF} during BASE, PEAK, PLATEAU, and RECOVERY. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Other conventions as Table 2. Comparisons: COS SHORT (BASE-PEAK, PEAK-PLATEAU, P<0.01; PLATEAU-RECOVERY, P<0.05; BASE-PLATEAU, BASE-RECOVERY, P<0.05); COS EMG_{DIFF} (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, NS); CRU SHORT (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, P<0.05); CRU EMG_{DIFF} (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, NS).
Project 3: Parasternal Intercostal Function during Sustained Hypoxia in Awake Canines

Summary

Ventilatory response to sustained isocapnic hypoxia in adult humans and other mammals is characterized by a biphasic pattern. Previously, in awake canines, we demonstrated that ventilation and costal diaphragm EMG rolls-off with sustained hypoxia, however, the costal diaphragm does not function alone during inspiration. At present, very little is known about the relative contribution and function of chest wall muscles when hypoxia is prolonged. Among these muscles, the parasternal intercostals are of a prime interest, as an obligatory primary inspiratory "pump" akin to the costal diaphragm. Therefore, in eleven chronically instrumented intact awake canines, we simultaneously measured muscle length, shortening (SHORT) and EMG activity of the parasternal intercostal (PARA) along with ventilation ($V_1$) during moderate levels of sustained isocapnic hypoxia lasting 20-25 min (mean 80±2% $SpO_2$).

Phasic inspiratory SHORT and EMG activity of the PARA were observed during room air and sustained hypoxia in all animals. The time course changes in PARA SHORT qualitatively tracked the biphasic changes in $V_1$ during sustained hypoxia. Mean SHORT and EMG activity of the PARA increased significantly with initial hypoxia, then markedly declined with constant hypoxia. Upon recovery room air breathing, despite reaching statistical significance, PARA SHORT and $V_1$ visibly fell below baseline without a concomitant reduction in PARA EMG activity. Furthermore, the relative magnitude of changes in PARA SHORT and EMG response were substantially different during initial and sustained hypoxia, as well as post-hypoxic recovery room air.

During sustained hypoxia, attenuation of central neural respiratory drive extends to the primary chest wall inspiratory muscle, the parasternal intercostal, directly contributing to the biphasic changes in ventilation. As well, the neuromechanical efficiency (i.e. degree of muscle shortening for a given level of activation) of the
parasternal chest wall muscle is fundamentally altered during and after persistent exposure to hypoxia.
Introduction

In adult humans and many animal species, sustained isocapnic hypoxia for 20-60 minutes elicits a biphasic ventilatory response with initial peak ventilation followed by a decline - "roll-off" or hypoxic ventilatory decline (HVD) - to a lesser intermediate plateau (Weil and Zwilich, 1976; Easton et al., 1986; Vizek et al., 1987; Easton et al., 1988; Tatsumi et al., 1992; Long et al., 1993; Praud et al., 1993). Previously, we demonstrated that in intact awake canines, spontaneously breathing through a mask, ventilation and costal diaphragm inspiratory muscle activity rolls-off with sustained isocapnic hypoxia exhibiting a biphasic pattern (Fujimura et al., 2006). However, the diaphragm does not function alone during inspiration. Coordinated action, and interaction, of other respiratory muscles, i.e. chest wall, abdominal and neck muscles, in conjunction with the diaphragm plays an important role in bringing about effective and efficient pressure generation, and thus ventilation (De Troyer, 2002; De Troyer and Loring, 2011).

While sustained HVD may represent an adaptive response of the respiratory system to the prolonged hypoxic insult that may involve some kind of central neurologic reset or attenuation of neuromotor output to the respiratory musculature, as reflected in costal diaphragm activity (Fujimura et al., 2006), there is no apriority reason that the roll-off should be a common phenomenon among the respiratory muscles participating in ventilation. Certainly, capacity for differential activation and function of two diaphragmatic segments, namely the costal and crural, has been well-documented during respiratory and non-respiratory events (Abe et al., 1994; Easton et al., 1994, 1995). In addition, other investigators have reported differential respiratory activity patterns among the muscle layers of the anterolateral abdominal wall during expiration (Abe et al., 1996), as well as between the diaphragm and the expiratory muscles during chemically stimulated ventilation (Smith et al., 1989). Therefore, even if sustained hypoxic roll-off may be considered a widespread phenomenon affecting most, if not all, respiratory muscles, this does not necessarily infer that the relative decline in activity will be an identical event among different muscle groups.
At present, very little is known about the relative contribution and function of the chest wall muscles during sustained hypoxic breathing. Among the chest wall muscles, the parasternal intercostals are of particular interest. As a primary inspiratory muscle, the parasternal intercostals are invariably and phasically active during each inspiratory breath and function to expand the chest wall and the lungs (Decramer and De Troyer, 1984; De Troyer, 2002), while stabilizing the rib cage against the negative swings in intrathoracic pressure caused by the contracting diaphragm (D'Angelo and Sant'Ambrogio, 1975; Decramer and De Troyer, 1984). In response to respiratory stress, such as airway occlusion (van Lunteren et al., 1988), diaphragm paralysis (Katagiri et al., 1994), and hypercapnia and hypoxia (Darian et al., 1989; DiMarco et al., 1992; Suzuki et al., 1997; Nishii et al., 2008), the parasternal intercostals are actively recruited to maintain effective ventilation. In addition, with relatively little muscle spindle content and minimal postinspiratory activity, the parasternal intercostals are akin to the costal diaphragm in intact awake canines and functions primarily as an inspiratory "pump" (Easton et al., 1999a). Given the functional significance of the parasternal intercostal chest wall muscles and their important contribution to the overall act of respiration, its activity and action during persistent hypoxia are certainly warranted for study.

To the best of our knowledge, a single study in adult humans exists in the literature examining the influence of prolonged steady state hypoxia on the parasternal intercostal activity (Okabe et al., 1993) - albeit employing surface EMG techniques which only provide a superficial measurement of muscle activity. Although EMG may be a good index of neuromotor drive, and thus assessing central neural control, this indicator alone cannot reliably predict with accurate certainty the mechanical consequence of an electrically activated muscle. For instance, force-generating capacity or length change from an inspiratory intercostal muscle is altered for a given level of neural drive by several mechanical factors: muscle length-tension characteristic (Farkas et al., 1985), thoracopulmonary mechanics (De Troyer and Farkas, 1993; DiMarco et al., 2000), respiratory load (van Lunteren et al., 1988), and interaction among other respiratory muscles (Decramer and De Troyer, 1984; De Troyer and Farkas, 1990). Interpretation of muscle function based solely on EMG activity may be misleading or prone to
misinterpretation without corresponding measurements of the mechanical change and/or output. In consideration of this, attempts to provide an accurate assessment of respiratory muscle function at the level of the individual muscles requires simultaneous measurement of the mechanical correlate, i.e. length and shortening, along with the neuromotor activity, i.e. EMG.

In the present study, we elected to thoroughly examine and characterize the function of the parasternal chest wall inspiratory muscle before, during and after moderate levels of sustained isocapnic hypoxia lasting 20-25 min (mean 80±2% SpO₂) in a chronically instrumented canine model. The chronic implantation technique of sonomicrometry and electromyography facilitates precise and reliable measurements of the dynamic changes in muscle length, shortening and EMG activity in vivo, in a fully conscious, spontaneously breathing, intact animal following a complete postoperative recovery period. Using this chronic animal model, accurate intramuscular assessment of the normal physiological function of the parasternal intercostal is permitted without the confounding effects of anesthetics and/or surgical complications. Thus, in our intact awake canines, we hypothesize that the parasternal intercostal muscle, as an inspiratory agonist, will phasically shorten and activate with each inspiration during room air and sustained isocapnic hypoxia; the initial exposure to hypoxia would cause an immediate increase in parasternal shortening and EMG activity similar to the acute hypoxic response; with constant hypoxia, central attenuation of neural drive will extend to the parasternal intercostal causing shortening and EMG activity to concurrently roll-off with ventilation; and, the attenuating effects of hypoxia will persist for the parasternal intercostal following the steady state exposure to hypoxia.
Methods

Implantation of transducers and electrodes

Experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines.

Eleven mongrel canines (mean weight 29.9 kg, range 20-41 kg) had pairs of sonomicrometry ultrasound transducers and bipolar fine wire EMG electrodes implanted in the left parasternal intercostal muscles of the rib cage. This technique of chronic sonomicrometry and EMG implantation has been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). In brief, implantation of transducers and electrodes was performed under general anesthesia with thiopental sodium induction and halothane. The skin was incised and deflected over the sternum, and ultrasonic transducers were implanted between muscle fibers of the parasternal intercostal muscles of the 2nd to 4th intercostal space, ~1-3 cm lateral to the edge of the sternum. Immediately adjacent to each transducer, a fine wire stainless steel bipolar EMG electrode was attached. All implants were secured by fine synthetic non-fibrotic sutures (Prolene, Ethicon, Somerville, NJ). All implant wires were externalized by a subcutaneous skin tunnel, and the animals were allowed to fully recover. Measurements of resting and hypoxic-stimulated ventilation were made a mean of 28 days post-implantation (range 8-70 days).

Measurement techniques

All measurements of ventilation and respiratory muscle function were made with the animals awake and breathing quietly, while lying in right lateral decubitus position which placed the implanted muscles in a non-dependent position. The animals were relaxed and familiar with the location, routine and personnel of the recordings. The animals were breathing spontaneously and quietly through a snout mask, which was connected through a 2-way non-rebreathable valve to a low resistance open breath circuit (<1 cmH₂O/L/s) which incorporated a pneumotachygraph (Fleisch #2) and a piezoelectric
differential pressure transducer (Model 163PC01D36, Honeywell Microswitch) to provide measurement of inspiratory airflow. On the expiratory limb, end tidal CO\textsubscript{2} (ETCO\textsubscript{2}) was sampled and analyzed continuously by an infrared CO\textsubscript{2} analyzer (Model CD-3A, AMETEK/Thermox Instrument Division, Pittsburgh, PA), and on the inspiratory side, fractional concentration of inspired O\textsubscript{2} (F\textsubscript{I}O\textsubscript{2}) was continuously sampled and analyzed by an O\textsubscript{2} analyzer (Model S-3A/1, AMETEK/Thermox Instrument Division, Pittsburgh, PA). The inspiratory limb could be switched, without alerting the animal, from entrainment of room air to a large reservoir of pre-mixed gas of low F\textsubscript{I}O\textsubscript{2} (8-10%). In addition, supplemental pressurized sources of O\textsubscript{2} and CO\textsubscript{2} were attached to the inspiratory limb to allow the experimenter to precisely titrate F\textsubscript{I}O\textsubscript{2} and ETCO\textsubscript{2} during the study. To relate the level of hypoxia, oxygen saturation (Sp\textsubscript{O}2) was continually measured by a pulse oximeter (Model Ohmeda Biox 8700, Rexdale, ON, Canada) by attaching a light sensitive analysis probe onto a shaved tendo calcaneous on the animal's hind limb.

Dynamic measurements within the respiratory muscles of the changing distance between the sonomicrometry transducers of each pair was provided by measuring the speed of transmission of ultrasonic waves using a sonomicrometer (Model 120, Triton Technology, Sand Diego, CA) (Easton et al., 1989). The output signal of the sonomicrometer was offset, amplified and then sampled to computer. For measurements of EMG, the fine wire bipolar electrode pairs from each muscle were connected to an AC differential pre-amplifier (Model 1700, AM Systems, Everett, WA). Power line interference was abolished by careful shielding techniques and the use of differential preamplifiers with a high common mode signal rejection of 110 dB. Thereafter the signal was filtered to attenuate both movement artifact and sonomicrometry noise and to perform anti-alias filtering, using a 6-pole, low pass Bessel filter at 20 Hz-700 Hz (Model 746, LT-4, Frequency Devices Incorporated, Haverhill, MA). The EMG signals were then rectified and processed by passage through resistance-capacitance, leaky "integrators" with a time constant of 100 ms, to provide moving averages of the EMG.

Using computer software for data acquisition (DataSponge, Bioscience Analysis Software, Calgary, AB, Canada), all signals were monitored in real time on the computer.
display and simultaneously collected at 100 Hz to hard disk on a microcomputer equipped with a single board A/D system (Model MIO-16-H-9, National Instruments, Galveston, TX).

**Experimental protocol**

The details of the sustained hypoxia technique have been described fully in a previous publication (Easton et al., 1986). To evaluate ventilation and respiratory muscle function during sustained isocapnic hypoxia, the study sequence was as follows: 1) a control period of room air, resting, baseline breathing of 6-8 min; 2) an abrupt step decrease in F\textsubscript{I}O\textsubscript{2}, which lowered SpO\textsubscript{2} to \(<80\) in 1-2 min; 3) maintenance of SpO\textsubscript{2} at 80 ± 2% for 20-25 min with constant ETCO\textsubscript{2} equal to control levels; and 4) a recovery period of room air breathing of 5-7 min. During sustained hypoxia, the target SpO\textsubscript{2} was 80%, and SpO\textsubscript{2} variability was restricted by the operator to the range 78-82%, a sustained desaturation that we abbreviate as 80 ± 2%. During the abrupt introduction of hypoxia, supplemental CO\textsubscript{2} was added via the pressurized source to the inspired limb of the breathing circuit to bring ETCO\textsubscript{2} back to a level approximating ETCO\textsubscript{2} during the preceding room air period. We did not attempt to anticipate and exclude any minor dips in ETCO\textsubscript{2} at the beginning of hypoxic hyperventilation because complete prevention of any transient fall in ETCO\textsubscript{2}, at the onset of hypoxia, would have entailed a slight risk of transient overcorrection and spurious increase in inspired minute ventilation during initial hypoxic exposure. After SpO\textsubscript{2} had been stabilized at \(\leq 82\%\), it was maintained at that level through adjustments in F\textsubscript{I}O\textsubscript{2} as necessary, utilizing the pressurized O\textsubscript{2} source on the inspiratory limb.

**Analysis of breathing pattern and muscle activity**

After acquisition and storage on disk, data were analyzed using software programs written by one author (PAE), and adapted to this project by the 2\textsuperscript{nd} author (MJ). These programs provide interactive visual display of the signals during analysis to allow careful examination of each breath. All signals were manually reviewed for movement
artifacts, transient signal disconnections, apneas and sighs, all of which were deleted from individual breath-by-breath analysis.

The flow signal was evaluated for respiratory timing and digitally integrated; minute ventilation ($V_I$), tidal volume ($V_T$), respiratory frequency ($f_R$), inspiratory time ($T_i$), mean inspiratory flow ($V_T/T_i$), and inspiratory fraction of respiration ($T_i/T_{TOT}$) were calculated breath-by-breath. The software also calculated whole breath, or “tidal” breath, values of respiratory muscle shortening and EMG activity. These calculations have been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). Briefly, the timing of inspiration was determined from the airflow tracing, and the onset and termination of individual muscle shortening and EMG activity for each breath was measured relative to the beginning and end of inspiratory airflow. Using the sonomicrometry length data from each implanted muscle, the computer calculated for each breath the baseline end-expiratory resting length of the muscle, titled $L_{BL}$, and the shortening (SHORT) for each breath expressed as a percentage change from resting length, entitled $\%L_{BL}$. Moving average EMG activity from each muscle was quantified arbitrarily per breath as the maximum difference in volts between baseline and the peak height of the moving average signal, expressed as $\text{EMG}_{\text{DIFF}}$. ETCO$_2$ and SpO$_2$ were also calculated breath-by-breath based on respiratory timing. Individual ETCO$_2$ values were converted for altitude to sea level ($\text{Patm} = 760 \text{ mmHg}$).

Measurement of all variables was continuous throughout the protocol, but for clarity of presentation, variables are summarized over corresponding periods of each study (Easton et al., 1986; Easton et al., 1988). Mean values of $V_I$ and components of breathing pattern, SpO$_2$, ETCO$_2$, $L_{BL}$, SHORT, and $\text{EMG}_{\text{DIFF}}$ were calculated from the continuous measurements during the following periods: BASE, PEAK, PLATEAU, and RECOVERY. Period BASE was the final 5 min of resting ventilation with normal SpO$_2$ before the introduction of hypoxia. Periods PEAK and PLATEAU were from opposite ends of the 20-25 min period of sustained hypoxia at $80 \pm 2\%$ SpO$_2$: PEAK represented the first 3 min of hypoxic ventilation, immediately after SpO$_2$ had descended to a level of $\leq 82\%$, while PLATEAU represented the last 5 min of hypoxic ventilation, preceding the
relief of hypoxia. Period RECOVERY represented the first 5 minutes of room air breathing after hypoxia was abruptly relieved.

Statistical analysis

After calculation, mean values were exported for review to spreadsheet software (Microsoft Excel, Microsoft, Redmond, WA), to graphic software to output figures (CorelDRAW X6, Corel Corporation, Ottawa, ON), and to PC version of SAS for statistical analysis (SAS Version 9.1, SAS Institute, NC). Mean values for parameters of breathing pattern and muscle length, shortening, and EMG activity were tested across the four periods (BASE, PEAK, PLATEAU, RECOVERY) by two-way analysis of variance with repeated measure on a single factor (Keppel, 1982; Stelle and Torrie, 1980). Multiple comparison testing of the mean values of the individual periods was performed using Tukey’s test (Steele and Torrie, 1980). A P-value of less than 0.05 was considered statistically significant.
Results

We report measurements for ventilation and breathing pattern in N=11 healthy awake canines with chronic implantation of sonomicrometry transducers and bipolar fine wire EMG electrodes. Although the implants are long-lasting, durability of implanted transducers and electrodes was not universal. Therefore, muscle length and EMG measurements from the parasternal intercostal muscle are available in N=8 and N=10 animals, respectively.

Ventilation and breathing pattern

Group mean values for ventilation and breathing pattern during room air and sustained isocapnic hypoxia are summarized in Table 4. For the group, sustained isocapnic hypoxic condition was well attained and managed throughout the entirely of the 20-25 min experimental duration (SpO$_2$: BASE-PEAK, P<0.01 and PEAK-PLATEAU, NS; P$_{ET}$CO$_2$: BASE-PEAK-PLATEAU, all NS). Upon relief of sustained hypoxia, SpO$_2$ returned from PLATEAU towards BASE and P$_{ET}$CO$_2$ fell modestly compared to PLATEAU (SpO$_2$: PLATEAU-RECOVERY, P<0.01; P$_{ET}$CO$_2$: PLATEAU-RECOVERY, P<0.05). The mean response of $V_t$, $V_T$ and $V_T/T_I$ during sustained isocapnic hypoxia exhibited a biphasic pattern, increasing significantly from BASE to PEAK with initial hypoxia ($V_t$: 7.77 ± 2.64 to 17.84 ± 5.05 l/min; $V_T$: 0.39 ± 0.06 to 0.69 ± 0.16 l; and $V_T/T_I$: 0.31 ± 0.08 to 0.60 ± 0.14 l/s; all P<0.01), and then declined or rolled-off to an intermediate PLATEAU with constant hypoxia ($V_t$: 14.09 ± 5.62 l/min; $V_T$: 0.52 ± 0.12 l; and $V_T/T_I$: 0.51 ± 0.17 l/s; all P<0.01). Although $f_R$ significantly increased from BASE to PEAK ($f_R$: 20.35 ± 6.68 to 26.02 ± 5.65 breaths/min, P<0.01), ventilation fell despite minimal changes in respiratory rate or $T_I$ during steady-state hypoxia ($f_R$ and $T_I$: PEAK-PLATEAU, NS). With RECOVERY room air breathing, $V_t$, $V_T$ and $V_T/T_I$, with the exception of $f_R$, fell below resting BASE levels, however, this change was not statistically significant (RECOVERY-BASE, all NS).
Representative tracings of the changes in ventilatory and parasternal intercostal (PARA) muscle parameters for two animals subjected to the sustained isocapnic hypoxia protocol are illustrated in Figure 41 (A: uni-phasic airflow and B: bi-phasic airflow). In both animals, dynamic changes in PARA muscle length and integrated moving average EMG (Mavg EMG) activity qualitatively followed a biphasic pattern similar to that of airflow during sustained isocapnic hypoxia. Accordingly, PARA phasically shortened and was electrically active with each inspiratory breath during initial room air breathing at BASE. With initial hypoxia representing period PEAK, the rate and magnitude of PARA SHORT and Mavg EMG activity markedly increased in association with an increase in airflow and fR. However, the extent of PARA SHORT and Mavg EMG activity at initial hypoxia was not sustained, resulting in a notable decline in both muscle parameters in concert with inspiratory airflow during final hypoxia corresponding to PLATEAU. During recovery final room air breathing, amount of PARA SHORT and Mavg EMG activity during inspiration fell below or remained comparable to baseline initial room air levels. All animals (11 of 11) without exception exhibited invariable phasic inspiratory SHORT and Mavg EMG activity of the PARA before, during and after sustained isocapnic hypoxia.

Time course: parasternal shortening and ventilation

The typical time course of PARA SHORT and V1 with a period of sustained isocapnic hypoxia is shown for a single animal in Figure 42. The record of SpO₂ demonstrates that when F1O₂ was lowered abruptly, SpO₂ dropped precipitously to reach target levels of 78-82% in ~1-2 min. For this animal, SpO₂ dropped to 80.5% (mean/20 sec) during the first ~1.3 min of hypoxia, and thereafter was maintained at 80±2% for 20 min, utilizing the dynamic adjustment of F1O₂, until stepwise reintroduction of room air. During recovery room air breathing, SpO₂ was quickly restored within the first ~20 sec, and thereafter steadily inclined to reach pre-hypoxic room air levels. Time course changes in PARA SHORT tracked a qualitatively similar biphasic pattern as that of V1.
As a result, PARA SHORT of ~1.82 %L_{BL} at room air baseline increased nearly two fold, reaching peak shortening of ~3.35 %L_{BL} as SpO_{2} fell, however, this magnitude of PARA SHORT was not sustained. Despite the lowered constant SpO_{2} of 80±2%, PARA SHORT progressively declined, or rolled-off, to an intermediate plateau with some scatter about a new PARA SHORT ~27% above initial room air values, corresponding to less than 1/2 of the initial PARA SHORT response. Upon reintroduction of room air, PARA SHORT fell quickly below original room air values within the first minute followed by a steady return back to baseline. P_{ET}CO_{2}, estimating alveolar partial pressure of CO_{2}, dipped slightly, and unavoidably, with the initial hypoxic response (~0.5 mmHg), but was quickly titrated back to and then maintained at baseline room air P_{ET}CO_{2} levels (~37 mmHg). With recovery room air breathing, P_{ET}CO_{2} naturally settled backed to resting baseline value following a minor dip in P_{ET}CO_{2} that occurred immediately upon restoration of room air.

**Group mean: parasternal shortening and EMG activity**

The group mean changes in PARA SHORT and EMG activity during room air and sustained isocapnic hypoxia of a similar duration are illustrated in Figure 43 and 44 and summarized in Table 5. From baseline resting levels of contraction, PARA SHORT increased significantly from 2.00 ± 0.58 to 4.14 ± 2.00 %L_{BL} with initial hypoxia at PEAK (P<0.01), corresponding to 204% of initial BASE room air value. The effects of persistent hypoxia on the contraction of the PARA is shown at PLATEAU, where mean PARA SHORT declined to an intermediate value of 2.82 ± 1.24 %L_{BL} or 138% of initial original BASE value. Thus, with sustained isocapnic hypoxia, there was a very significant reduction in PARA contraction from PEAK to PLATEAU (P<0.05). Despite PARA SHORT persisting 38% above initial baseline levels with constant hypoxia, PARA SHORT at PLATEAU did not reach a statistical difference when compared to starting room air BASE (BASE-PLATEAU, NS). Upon RECOVERY room air breathing, PARA SHORT fell to 1.68 ± 0.78 %L_{BL} or 82% of original BASE value, however, this was not significantly different when compared to BASE (BASE-RECOVERY, NS).
Mechanical shortening of the PARA was associated with qualitatively similar biphasic changes in the neural drive to the muscle, as measured by simultaneous recordings of EMG activity. From room air resting baseline, PARA EMG activity increased significantly from BASE value of 3.26 ± 1.35 volts to PEAK value of 5.27 ± 1.92 volts (P<0.01). Thereafter, PARA EMG activity significantly decreased (P<0.05) to reach a new steady-state PLATEAU value of 4.47 ± 1.71 volts with constant hypoxia. Accordingly, PARA EMG activity increased to 169% of BASE during initial PEAK hypoxia, and then decreased to 142% during sustained PLATEAU hypoxia remaining significantly above initial room air BASE values (BASE-PLATEAU, P<0.01). With RECOVERY room air breathing, PARA EMG activity of 3.23 ± 1.60 volts or 98% of initial BASE activity was comparable to original room air values (BASE-RECOVERY, NS).

Of particular note, breath-by-breath mean "tidal" EMG changes generally track, but are not equal to, the significant changes in SHORT during PEAK and PLATEAU hypoxia as well as post-hypoxic RECOVERY (Figure 43 and 44). Specifically, as a percentage of initial baseline, increase in PARA SHORT was relatively greater than the increase in PARA EMG with initial hypoxia (204% and 169%); persistent hypoxia caused a proportionally greater decline in PARA SHORT (66%) with a lesser fall in EMG activity (27%); and recovery room air elicited a reduction in PARA SHORT relative to baseline (18%) without much decline in EMG activity (2%).

The aforementioned changes in PARA SHORT and EMG activity occurred without remarkable or statistically significant changes in the end-expiratory resting baseline length of the muscle (Table 5, PARA L_{BL}: BASE-PEAK-PLATEAU-RECOVERY, all NS). However, there was a non-significant trend for PARA baseline length to nominally decrease with sustained hypoxia, which did not fully return back to initial room air baseline muscle length at BASE, despite restoring room air breathing during RECOVERY. Pertaining to this trend, PARA L_{BL} decreased slightly from BASE to PEAK (0.04 mm or ~0.4% of BASE L_{BL}), and then a further modest reduction from PEAK to PLATEAU (0.05 mm or ~0.6% of BASE L_{BL}). Thus, PARA L_{BL} in total
decreased 0.09 mm or ~1.0% of BASE L_{BL} throughout the 20-25 min duration of sustained hypoxia. With RECOVERY room air breathing, PARA L_{BL} compared to initial BASE room air remained smaller in length (0.05 mm or ~0.5% BASE L_{BL}).
Discussion

Summary of results

The present study demonstrates that in intact, awake, spontaneously breathing canines, the parasternal intercostal muscle exhibited phasic inspiratory shortening and EMG activity during room air and sustained hypoxia. Time course changes in parasternal intercostal shortening tracked the biphasic changes in ventilation, with an initial increase followed by a roll-off to an intermediate plateau. Changes in parasternal intercostal shortening was accompanied by qualitatively similar biphasic changes in EMG activity. Upon restoration of room air, ventilation and parasternal intercostal shortening, despite reaching statistical significance, visibly fell below baseline without a concomitant reduction in parasternal intercostal EMG activity. The relative magnitude of the changes in parasternal intercostal shortening and EMG activity were fundamentally different with initial and sustained hypoxia, as well as post-hypoxic room air recovery.

Ventilation and breathing pattern

Traditionally, it has been reported that the characteristic biphasic ventilatory response to sustained hypoxia does not occur in mask and tracheal-unloaded breathing awake canines (Cao et al., 1992; Cao et al., 1993). In direct contrast to the existing literature, in our previous work involving the awake canine model (Fujimura et al., 2006), we observed a significant roll-off in ventilation with sustained isocapnic hypoxia that exhibited the typical biphasic ventilatory pattern as that of adult humans and many other animal species. In agreement with this previous work, all awake canines (11 of 11) in this study, with an intact upper airway and breathing through a snout mask, ventilation consistently expressed a bimodal pattern during moderate levels of sustained isocapnic hypoxia lasting 20-25 min. Initial increase in \( V_I \) was mediated by changes in both \( V_T \) and \( f_R \), while the subsequent decline in \( V_I \) was brought about by a reduction in \( V_T \) without a fall in \( f_R \). With recovery room air, despite reaching statistical significance, \( V_I \) visibly fell below baseline with similar reductions in \( V_T \), while \( f_R \) remained comparable to baseline.
Such characteristic changes in $V_I$, $V_T$ and $f_R$ observed in this study are qualitatively in agreement with a range of studies previously reported in anesthetized and awake, spontaneously breathing, adult and newborn mammals during sustained hypoxia (Bureau et al., 1984; LaFramboise and Woodrum, 1985; Easton et al., 1986; Vizek et al., 1987; Eden and Hanson, 1987; Rigatto et al., 1988; Easton and Anthonisen, 1988b; Long et al., 1989). This implies that in awake canines, as in other mammals, ventilation evidently follows a biphasic time course pattern significantly rolling off with constant hypoxia that is primarily due to a volume effect rather than changes in respiratory rate. Furthermore, the attenuating effect of hypoxia appears to persist upon return to room air, as reflected by a visible reduction in ventilation during the period of recovery.

Other breathing pattern parameters: $V_T/T_i$, $T_i$ and $T_i/T_{TOT}$ were less comparable to earlier studies, likely owing to an inspiratory "off-switch" mechanism or factors affecting expiratory duration that considerably various across different mammalian species (Easton et al., 1986; LaFramboise et al., 1985; Martin et al., 1998). Nevertheless, $V_T/T_i$, $T_i$ and $T_i/T_{TOT}$ are in direct agreement with our previous work in awake canines (Fujimura et al., 2006). Of note, $T_i$ remained minimally altered with persistent hypoxia and $V_T/T_i$ paralleled the changes in $V_T$ following a biphasic pattern with sustained hypoxia. Steady or minimally altered $T_i$ during ventilatory roll-off suggests that changes in the inspiratory "off-switch" mechanism are not likely responsible for the decline in volume output. Whereas the significant decline in $V_T/T_i$ with sustained hypoxia likely suggests that the overall drive to the respiratory muscles has been reduced in a temporal manner (Akiyama and Kawakami, 1999), however, $V_T/T_i$ cannot differentiate which muscle or muscles groups would be impacted with hypoxia, nor can it suggest to what degree or capacity. Hence, direct measurement of muscle function (i.e. its length change, shortening and/or EMG activity) is an absolute requirement for accurately assessing and characterizing the mechanical and electrical behavior of the respiratory muscles during resting and chemically stimulated ventilation, such as hypoxia. With the consistency of the biphasic ventilatory response expressed in these chronically instrumented awake canines, the function of the primary inspiratory chest wall muscle, the parasternal intercostal, during sustained hypoxia can be adequately studied, employing direct intramuscular
measurement techniques of sonomicrometry and electromyography without the confounding effects of anesthetics and/or postoperative complications.

**Phasic parasternal shortening and EMG activity**

Previous studies in anaesthetized and awake animals have revealed that the parasternal intercostals are invariably active and shorten with each inspiration during quiet breathing (De Troyer and Farkas, 1990; DiMarco et al., 1990; van Lunteren et al., 1988). In these intact animal studies, phasic shortening and activation of the parasternal intercostal generally started at or just after the onset of inspiratory flow, reached peak near the end of inspiration, and then promptly returned back to end-expiratory baseline levels during expiration (Decramer and De Troyer, 1984; De Troyer and Farkas, 1993). Such characteristic pattern of phasic parasternal muscle shortening and activation were consistently observed in all our animals during baseline room air breathing (Figure 41A and 41B: Initial Room Air). Similarly, phasic shortening and activation of the parasternal intercostal has been reported during acute steady-state and progressive isocapnic hypoxia, with peak shortening and EMG activity increasing proportionately with the degree of hypoxic stimulus (Darian et al., 1989; Nishii et al., 2008; Suzuki et al., 1997). In direct agreement with the aforementioned acute hypoxia studies, our results demonstrate that peak shortening and EMG activity of the parasternal intercostal invariably increases upon initial exposure to hypoxia (Figure 41A and 41B: Initial Hypoxia). On the other hand, we further extend the work of the acute hypoxia studies to demonstrate that the phasic inspiratory shortening and EMG activity of the parasternal intercostal, although to a lesser extent, persists and continues throughout the entire duration of constant hypoxia lasting 20-25 min and upon post-hypoxic recovery room air breathing (Figure 41A and 41B: Final Hypoxia). Thus, our results further reinforce the notion that the parasternal intercostals are a true agonist that invariably and actively shortens in phase with each inspiratory breath, even during and after extended periods of hypoxic hyperventilation in the setting of a significant ventilatory decline.
Dynamic changes in parasternal intercostal shortening closely paralleled that of the biphasic time course changes in ventilation. Accordingly, peak parasternal intercostal shortening and ventilation were reached at around the same time after initial exposure to hypoxia, and then progressively declined together to reach an intermediate plateau around the 15-20 min mark of sustained hypoxia. With recovery room air breathing, parasternal intercostal shortening and ventilation fell below baseline levels, and then progressively recovered within 5 min. The characteristic bimodal pattern of parasternal intercostal inspiratory contraction, as well as the accompanying post-hypoxic decline with recovery room air breathing, were consistently observed in all canines with accompanying muscle length changes.

To the best of our knowledge, there has been no previous reports examining the mechanical action of the parasternal intercostals in response to sustained hypoxia, nor its temporal time course pattern, in any anesthetized or unanesthetized mammals. On the other hand, our prior knowledge of the parasternal intercostals during sustained hypoxia previous to this investigation rested on a single study employing surface EMG recording techniques in adult humans with or without obstructive sleep apnea (Okabe et al., 1993). In this study, Okabe and colleagues reported a biphasic inspiratory intercostal muscle EMG activity, corresponding to the 2\textsuperscript{nd} intercostal space of the parasternal intercostals, along with ventilation during sustained isocapnic hypoxia. Although changes in EMG activity as an index of neural drive generally reflect the mechanical consequence of an inspiratory intercostal chest wall muscle (DiMarco et al., 1992; van Lunteren and Cherniack, 1986), such a relationship cannot be automatically expected since the force-generating capacity or length change of an inspiratory intercostal muscle for a given neural drive may be affected by several mechanical factors, including muscle length-tension characteristics (Farkas et al., 1985), respiratory load (van Lunteren et al., 1988), changes in lung volume and position (DiMarco et al., 2000; De Troyer and Farkas, 1993), topological location on the rib cage (Greer and Stein, 1989), as well as the mechanical
arrangement and interaction with other respiratory muscles (Decramer and De Troyer, 1984; De Troyer and Farkas, 1990).

Thus, despite previous observation for a biphasic EMG activity response of the parasternal intercostals during sustained hypoxia in adult humans, the mechanical consequence of these primary inspiratory muscles with persistent hypoxia remained until the present study essentially unknown and unexamined. Here we extend the findings of the aforementioned surface EMG study to demonstrate that the parasternal intercostal inspiratory contraction in awake canines exhibits a biphasic time course pattern in response to the changes in electrical activity.

**Parasternal inspiratory shortening**

Quantitative analysis of the degree of parasternal intercostal shortening with respect to the individual time periods revealed a clear biphasic contractile pattern. The initial augmentation of parasternal shortening of 204% baseline in response to acute hypoxia in our study are compatible with the findings in anesthetized canines exposed to 5 min of hypoxia at an FIO2 level of 15-10% (Suzuki et al., 1997). With 20-25 min of sustained hypoxia, there was a marked reduction in parasternal muscle shortening, causing more than half of the initial contractile output from these chest wall inspiratory muscles to be lost with constant hypoxia. While during recovery room air, parasternal intercostal shortening fell modestly below baseline, however, this effect did not endure, as parasternal shortening in most animals, after a transient decline, quickly returned back to comparable baseline levels within 5 min (individual examination not reported). Whether subsequent exposure to hypoxia on parasternal shortening would reveal a persistent suppression effect and a time dependent recovery, as that of ventilation reported in adult humans (Easton et al., 1988), remains uncertain. Nevertheless, it is interesting to observe a quick recovery of the parasternal intercostal shortening to baseline levels following a sustained period of hypoxia.
Currently, no studies exist in the literature that have directly examined the consequence of sustained hypoxia on the contractile output of the inspiratory chest wall muscles, therefore our results remain to be compared and evaluated against future studies employing either the direct measurement techniques of mechanical length change and/or some other measurement relating to the contractile output.

**Parasternal inspiratory EMG activity**

The biphasic changes in muscle shortening was accompanied by qualitatively similar changes in the electrical activation of the parasternal intercostal muscle. In the present study (Figure 41; Figure 44), as well as in the works by Nishii et al. (2008) (Fig. 2) and Darian et al. (1989) (Table 1), hypoxia elicited an increase in the peak and rate-of-rise of the parasternal intercostal EMG activity in association with the degree of hypoxic stimulus as indicated by low FiO$_2$/SpO$_2$. The immediate augmentation of parasternal EMG activity of 169% with initial hypoxia observed in our animals are commensurate with these acute hypoxia studies (Darian et al., 1989; Nishii et al., 2008). However, the increase in EMG activity was not sustained in our animals, as prolonged hypoxia caused nearly 1/4 of the initial increase in neural drive to the parasternal intercostal to diminish with hypoxia lasting 20-25 min. These results, in general, are in qualitative agreement with the aforementioned studies by Okabe et al. (1993), examining the chest wall muscle activity during sustained hypoxia in adult humans, however, the relative decline in EMG activity observed in our canines was much less compared to the former study using surface electrodes. Whether this difference is ascribed to the recording technique and/or real species differences in the degree and magnitude of the decline in parasternal intercostal muscle activity remains uncertain. Nevertheless, significant reduction in parasternal EMG activity with sustained hypoxia may be a result of some kind of central neurologic reset or centrally mediated attenuation of neural respiratory drive with constant hypoxia affecting the primary chest wall inspiratory muscle. With post-hypoxic room air breathing, parasternal intercostal inspiratory EMG activity was comparable to baseline activity, suggesting that if an inhibitory effect of hypoxia had been present, it
may have manifested only to a small extent and/or was quickly resolved, such that mean activity over a 5 min recovery period was not much different compared to baseline.

**Attenuation of central drive in other muscles**

Attenuation of central drive, as noted in the present study, is not unprecedented for the parasternal intercostals as our lab and other investigators have previously reported a biphasic EMG activity response in the costal diaphragm during prolonged exposure to hypoxia (Fujimura et al., 2006; McEvoy et al., 1996; van Lunteren et al., 1989; Vizek and Bonora, 1998). In comparison, the relative magnitude of the parasternal intercostal EMG roll-off observed in our awake canines is visibly less compared to the costal diaphragm EMG of our previous investigation in the same species (Fujimura et al., 2006). Specifically, in our former study, costal diaphragm EMG activity after an initial peak response dropped relatively by half, ending at an intermediate plateau 122% of baseline activity (see Figure 32 in Project 1: *Costal diaphragm EMG activity during room air and sustained isocapnic hypoxia*). By contrast, the parasternal intercostal EMG in our animals after reaching peak activity proportionally dropped by a quarter, remaining 42% above original room air baseline levels (Figure 44). These results in awake canines suggests that the effects of central attenuation may not be an identical event between different respiratory muscle groups. As to whether such differential EMG changes would actually translate mechanically into differences in contractile or force output remains at present unknown, and thus prompts further investigation.

**Differences in shortening and EMG activity**

Although in these experiments, parasternal intercostal EMG generally tracked the biphasic changes in shortening, the relative change in these two important physiologic parameters assessing muscle function were certainly not identical during and after sustained hypoxia (Figure 43 and 44). In particular, initial hypoxia elicited a two fold increase in parasternal shortening from room air baseline, however, EMG activity increased to a lesser extent or 1.69 fold. Changes in shortening and EMG activity were
striking with constant 20-25 min of hypoxia, with parasternal shortening declining roughly by 1/2 of the initial increase, whereas parasternal EMG activity only fell by a 1/4 of the initial response. Similarly, changes in shortening were also distinct from EMG activity following sustained hypoxia, as shortening fell relatively below initial baseline levels during recovery room air breathing despite EMG remaining comparable to baseline.

These results emphasize the important fact that, although the relationship of EMG and shortening of the parasternal intercostal are generally well-correlated during resting and stimulated breathing (DiMarco et al., 1992; van Lunteren and Cherniack, 1986), EMG activity as an index of neuromotor drive alone cannot with accurate certainty predict the mechanical consequence of an electrically activated muscle. Consequently, having measurements of length change not only reinforces the presumption of normal EMG activity by concurrent evidence that each muscle is actually shortening as expected (Easton et al., 1993), but permits the resulting mechanical changes to be precisely quantitated and evaluated against changes in EMG activity. Thus, accurate assessment of respiratory muscle function at the level of the individual muscles relies upon having both measurements of the neuromotor output, i.e. EMG activity, as well as its corresponding mechanical correlate, i.e. length change or shortening.

**Determinants of parasternal contractile shortening**

Such notable differences in the relative degree of parasternal shortening and EMG activity may be ascribed to the fact that length change or shortening of the respiratory muscles are critically dependent on other mechanical factors beyond changes in neuromotor drive. Previous studies have demonstrated that the force-generating capacity, or length change, of the parasternals for a given neural activation depends on the muscle's pre-contractile length (Farkas et al., 1985), and is altered with changes in thoracopulmonary mechanics (De Troyer and Farkas, 1993; DiMarco et al., 2000). More importantly, mechanical interaction of the parasternal intercostals with other chest wall respiratory muscles has been shown to play an important role in determining the length change of the parasternals (Decramer and De Troyer, 1984; De Troyer and Farkas, 1989;
DiMarco et al., 1992). From a muscle interaction standpoint, the relatively large parasternal shortening response compared to EMG with initial hypoxia likely represents some kind of amplification or enhancement of the neuromechanical efficiency (i.e. muscle shortening for a given level of activation) of the parasternal intercostal muscle that may be brought about by significant facilitatory and synergistic actions of other chest wall respiratory muscles. While the proportionately larger decline in the parasternal shortening relative to EMG activity during the sustained hypoxic roll-off may represent a significant loss of the mechanical interaction that may have been present during initial hypoxia. Moreover, the relatively greater parasternal EMG activity observed compared to muscle shortening during post-hypoxic room air recovery breathing reflects a reduction in the neuromechanical efficiency of this primary chest wall inspiratory muscle.

At present, little is known about the relative activity and action of other chest wall respiratory muscles, including the interosseous intercostals and the triangularis sterni muscle, during sustained hypoxia. Certainly further studies are warranted to investigate the effects of sustained hypoxia on the temporal dynamic of other inspiratory and expiratory chest wall muscles, and its potential interaction with the parasternal intercostals.

Adequacy of experimental technique

Accurate assessment of muscle function requires both the characterization of the mechanical output and the electrical activity of the muscle. In this regard, the success of this study critically depended on having the appropriate technique to precisely and reliably record muscle length and EMG activity directly from the muscle of interest. Employing our direct measurement technique of sonomicrometry and electromyography, we were able to faithfully and continuously obtain measurements of muscle length and shortening from a specific region of the parasternal intercostal at any given interspace, within a selection of motor units, with an accuracy surpassing \(1/100\) mm with concomitant EMG activity. We were careful to place each implant in the medial fibers of the parasternal intercostals throughout the \(2^{nd}-4^{th}\) interspace to evaluate the function of
this particular parasternal intercostal muscle region. Such uniformity was important because muscle length change and activity across the medial, middle and lateral regions of the parasternal intercostals has been shown to be inhomogeneous during spontaneous ventilation in anesthetized canines (De Troyer and Legrand, 1995). And thus, consistent placement of the implants served to control for this innate physiologic variability. However, despite our best efforts, the durability of our implanted sonomicrometry transducers and fine wire EMG electrodes were not universal, resulting in the loss of some muscle length and EMG measurements (9 of 11 and 10 of 11 animals, respectively). If our loss of sample size in these muscle parameters had any selective disadvantage in this study, it might have slightly reduced the power of our statistical analysis to determine a significant difference between the individual periods of room air and sustained hypoxia. However, this was not a problem, since statistical significance between the individual periods for both muscle parameters were met at our chosen alpha level of 0.05 and below. Thus, despite the small loss of sample size, our experimental technique allowed for an accurate evaluation of muscle function with respect to its central neural control as well as its mechanical output.

**Conclusion**

Our study lends further support to the notion that the parasternal intercostal is a true inspiratory agonist that phasically activates and contracts during room air and sustained hypoxia. The time course changes in parasternal intercostal shortening and EMG activity during sustained hypoxia mirrored the biphasic changes in ventilation, with mechanical output changing as a consequence of neuromotor drive. Post-hypoxic room air recovery was associated with a transient reduction in parasternal intercostal muscle contraction compared to baseline, without a concomitant fall in neuromotor drive to the muscle. Moreover, the neuromechanical efficiency of the parasternal intercostal was fundamentally altered with initial and sustained hypoxia, as well as post-hypoxic room air recovery, suggesting a significant respiratory muscle interaction effect altering the function of the parasternal intercostal muscle. Finally, during sustained hypoxia, central
attenuation extends to the primary chest wall inspiratory muscle, the parasternal intercostal, which directly contributes to the biphasic changes in ventilation.
Acknowledgements

This study was supported by grants from the Canadian Institutes of Health Research. Expert technical assistance was provided by Ms. Leslie Jacques. Excellent research and laboratory support from colleague Ms. Jenny V. Jagers and Dr. Tetsunori Ikegami. The provision of all suture materials by Ethicon Sutures Ltd., a Johnson & Johnson Company, is gratefully acknowledged.
Figure 41: Parasternal intercostal length and EMG activity during room air and sustained isocapnic hypoxia (legend on next 2 pages)
Figure 41 (figure legend)

Representative tracing of airflow, parasternal intercostal length and parasternal intercostal integrated moving average EMG activity during room air and sustained isocapnic hypoxia for two subjects (A: uni-phasic airflow and B: bi-phasic airflow). Traces shown are breaths during initial room air breathing before hypoxia; initial hypoxia once O$_2$ saturation reached 80 ± 2%; final hypoxia preceding return to room air; and final room air breathing after hypoxia. Each tracing captures 10 sec worth of data with start time (min:sec) and SpO$_2$ (%) noted at the bottom-left and top-right, respectively. Horizontal bars on the bottom-right denotes a time span of 1 sec. PARA Length, parasternal length; PARA Mavg EMG, parasternal moving average EMG; L$_{BL}$, baseline resting muscle length at end-expiration; SpO$_2$, pulse oximeter O$_2$ saturation. Note: upward deflection of airflow marks inspiratory airflow, or inspiration, where as downward deflection marks expiratory airflow, or expiration; downward deflection of PARA Length reflects muscle shortening and upward deflection of PARA Mavg EMG signal represents muscle activation.
Figure 42: Parasternal intercostal shortening and ventilation during room air and sustained isocapnic hypoxia

Time course of parasternal intercostal muscle shortening and ventilation in response to sustained isocapnic hypoxia in a representative animal. Closed diamond, open circle (lined), open circle (unlined), and open triangle represent mean values/20 sec for parasternal intercostal shortening (PARA SHORT), minute ventilation ($V_1$), O$_2$ saturation (SpO$_2$), and partial pressure of end tidal CO$_2$ (PETCO$_2$), respectively.
Figure 43: Parasternal intercostal shortening during room air and sustained isocapnic hypoxia

Group mean parasternal intercostal shortening during room air and sustained isocapnic hypoxia in eight animals (N=8). Columns show group mean values with standard error bars. Parasternal intercostal shortening (PARA SHORT). Tidal shortening of the parasternal intercostal during BASE, 5 min room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; RECOVERY, 5 min room air breathing after hypoxia. Y-axis expresses shortening per breath as a percentage change from baseline resting muscle length at end expiration, %L_BL. Numerical labels are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on PARA SHORT, both P<0.0001. ♦ and †, significant difference between individual periods at P<0.01 and P<0.05, respectively.
Group mean parasternal intercostal EMG activity during room air and sustained isocapnic hypoxia in ten animals (N=10). Columns show group mean values with standard error bars. Parasternal intercostal EMG (PARA EMG$_{DIFF}$). Tidal EMG activity of the parasternal intercostal during BASE, PEAK, PLATEAU, and RECOVERY. Y-axis expresses EMG activity per breath as the maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. Numerical labels are means expressed as a percent of baseline room air ventilation (%BASE). Other conventions as Figure 43. Significance of the overall effects of hypoxia on PARA EMG$_{DIFF}$, both P<0.0001. ‡ and †, significant difference between individual periods at P<0.01 and P<0.05, respectively.
Table 4: Oxygen saturation, end tidal CO$_2$ and breathing pattern during room air and sustained isocapnic hypoxia

(legend on next page)

<table>
<thead>
<tr>
<th></th>
<th>Room Air Initial 5 min</th>
<th>Hypoxia Initial 3 min</th>
<th>Hypoxia Final 5 min</th>
<th>Room Air Final 5 min</th>
<th>Effect P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SpO$_2$ (%)</strong></td>
<td>BASE 93.00 ± 2.34</td>
<td>PEAK 80.39 ± 3.34</td>
<td>PLATEAU 79.13 ± 3.91</td>
<td>RECOVERY 90.40 ± 3.01</td>
<td>Hypoxia &lt; 0.0001</td>
</tr>
<tr>
<td><strong>P$_{ET}$CO$_2$ (mmHg)</strong></td>
<td>37.27 ± 1.42</td>
<td>36.49 ± 2.33</td>
<td>37.59 ± 2.01</td>
<td>35.66 ± 2.40</td>
<td>0.0405</td>
</tr>
<tr>
<td><strong>V$_1$ (l/min)</strong></td>
<td>7.77 ± 2.64</td>
<td>17.84 ± 5.05</td>
<td>14.09 ± 5.62</td>
<td>7.03 ± 2.38</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>V$_T$ (l)</strong></td>
<td>0.39 ± 0.06</td>
<td>0.69 ± 0.16</td>
<td>0.52 ± 0.12</td>
<td>0.34 ± 0.07</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>f$_R$ (breaths/min)</strong></td>
<td>20.35 ± 6.68</td>
<td>26.02 ± 5.65</td>
<td>27.66 ± 9.51</td>
<td>21.25 ± 7.66</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>T$_1$ (s)</strong></td>
<td>1.36 ± 0.39</td>
<td>1.18 ± 0.27</td>
<td>1.10 ± 0.35</td>
<td>1.30 ± 0.48</td>
<td>0.0043</td>
</tr>
<tr>
<td><strong>V$_T$/T$_1$ (l/s)</strong></td>
<td>0.31 ± 0.08</td>
<td>0.60 ± 0.14</td>
<td>0.51 ± 0.17</td>
<td>0.29 ± 0.08</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td><strong>T$<em>f$/T$</em>{TOT}$ (ratio)</strong></td>
<td>0.42 ± 0.04</td>
<td>0.48 ± 0.03</td>
<td>0.46 ± 0.04</td>
<td>0.40 ± 0.04</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>
Table 4 (table legend)

SpO₂, pulse oximeter O₂ saturation; P<sub>ET</sub>CO₂, partial pressure of end tidal CO₂; V<sub>I</sub>, minute ventilation; V<sub>T</sub>, tidal volume; f<sub>R</sub>, respiratory rate; T<sub>I</sub>, inspiratory time; V<sub>I</sub>/T<sub>I</sub>, mean inspiratory flow; T<sub>I</sub>/T<sub>TOT</sub>, inspiratory fraction of respiration. Values are means ± SD for N=11 canines during BASE, 5 min room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; RECOVERY, 5 min room air breathing after hypoxia. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Comparisons: SpO₂ (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, PEAK-PLATEAU, NS); P<sub>ET</sub>CO₂ (BASE-PEAK, PEAK-PLATEAU, BASE-PLATEAU, BASE-RECOVERY, NS; PLATEAU-RECOVERY, P<0.05); V<sub>I</sub>, V<sub>T</sub>, V<sub>I</sub>/T<sub>I</sub> (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, NS); f<sub>R</sub> (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, BASE-RECOVERY, NS); T<sub>I</sub>: (BASE-PLATEAU, P<0.01; PLATEAU-RECOVERY, P<0.05; BASE-PEAK, PEAK-PLATEAU, BASE-RECOVERY, NS); T<sub>I</sub>/T<sub>TOT</sub>: (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, P<0.05; BASE-RECOVERY, NS).
Table 5: Parasternal intercostal shortening, EMG activity and resting length during room air and sustained isocapnic hypoxia (legend on next page)

<table>
<thead>
<tr>
<th>PARA</th>
<th>Room Air</th>
<th>Hypoxia</th>
<th>Hypoxia</th>
<th>Room Air</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Initial</td>
<td>Final</td>
<td>Final</td>
<td>P Value</td>
</tr>
<tr>
<td></td>
<td>5 min</td>
<td>3 min</td>
<td>5 min</td>
<td>5 min</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>BASE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SHORT (%L&lt;sub&gt;BL&lt;/sub&gt;)</td>
<td>2.00 ± 0.58</td>
<td>4.14 ± 2.00</td>
<td>2.82 ± 1.24</td>
<td>1.68 ± 0.78</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>EMG&lt;sub&gt;DIF&lt;/sub&gt; (volts)</td>
<td>3.26 ± 1.35</td>
<td>5.27 ± 1.92</td>
<td>4.47 ± 1.71</td>
<td>3.23 ± 1.60</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Length (L&lt;sub&gt;BL&lt;/sub&gt;, mm)</td>
<td>9.37 ± 2.68</td>
<td>9.33 ± 2.68</td>
<td>9.28 ± 2.71</td>
<td>9.32 ± 2.72</td>
<td>0.0886</td>
</tr>
</tbody>
</table>
Table 5 (table legend)

PARA, parasternal intercostal; SHORT, shortening per breath expressed as a percentage change from baseline resting muscle length at end-expiration, %L_{BL}; EMG_Diff, EMG activity per breath quantified as the maximum difference between baseline and peak height of the integrated moving average EMG signal in volts; Length (L_{BL}), end-expiratory baseline resting muscle length in mm. Values are means ± SD for N=8, PARA SHORT and L_{BL}; N=10, PARA EMG_Diff during BASE, PEAK, PLATEAU, and RECOVERY. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Other conventions as Table 4. Comparisons: PARA SHORT (BASE-PEAK, P<0.01; PEAK-PLATEAU, PLATEAU-RECOVERY, P<0.05; BASE-PLATEAU, BASE-RECOVERY, NS); PARA EMG_Diff (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, P<0.05; BASE-RECOVERY, NS); PARA Length (BASE-PEAK-PLATEAU-RECOVERY, all NS).
Project 4: Abdominal Muscle Action during Sustained Hypoxia in Awake Canines

Summary

Classic studies suggest that expiratory neuronal activity is inhibited by hypoxia, and the activity of the expiratory muscles during hypoxia is inconsistent and varies across species and experimental conditions. Recently, we demonstrated in awake canines that during sustained hypoxia, costal diaphragm and parasternal chest wall inspiratory muscle EMG activity rolls off with ventilation. To date, activity and function of the abdominal expiratory muscles during sustained hypoxia is unknown, in any awake, non-anesthetized adult mammal. In the present study, continuous direct measurements of the respiratory changes in length and electrical activity of the transversus abdominis (TA) were made along with ventilation (\(V_I\)) in twelve intact awake canines, before, during and after sustained isocapnic hypoxia lasting 20-25 min (mean \(\text{SpO}_2\) 80±2%).

Phasic expiratory shortening (SHORT) and EMG activity of the TA was observed during quiet room air and sustained hypoxic breathing in all animals. In particular, quiet expiration was associated with minimal activity which was invariably phasic in most and intermittently phasic in few animals. Time course response of the TA SHORT followed a biphasic pattern similar to that of ventilation during sustained hypoxia, but the magnitude and the rate of decline was much more prominent in the TA. As a group, initial acute hypoxia elicited a marked increase in mean tidal expiratory TA SHORT and EMG activity, which was subsequently followed by significant decline to near baseline room air levels during sustained hypoxia. Upon recovery, TA SHORT and EMG visibly fell below baseline room air values, with animals exhibiting an intermittent or complete quiescence of EMG activity.

We conclude in intact awake canines that: 1) quiet expiration is mostly an active process which certainly includes a passive component, as revealed by EMG intermittence; 2) contrary to classic studies, expiratory muscles are markedly recruited with hypoxia, however, significant inhibition does occur if hypoxia is sustained; and, 3)
inhibitory effects of hypoxia appears to persist even upon restoration of normoxic breathing. Temporal dynamic of the expiratory activity exhibiting both an excitatory and inhibitory response to hypoxia may account for much of the discordance in expiratory activity with hypoxia reported in the past.
Introduction

Expiration constitutes an essential part of respiration that functions to move air out of lungs and to facilitate breathing. As work of breathing increases there is a proportional increase in the expiratory activity to the effectors of respiration to facilitate expiration. This is especially true with hypercapnic stimulated breathing (Gilmartin et al., 1987; Leevers and Road, 1994; Abe et al., 1996), presence of respiratory load (Martin and De Troyer, 1982; Leevers and Road, 1989; De Troyer et al., 1989), and changes in posture when acting to disadvantage the diaphragm and rib cage inspiratory muscles (De Troyer and Ninane, 1987; Estenne et al., 1988; Farkas and Schroeder, 1990). Such enhanced expiratory activation is recognized to cause recruitment of the expiratory muscles which are well suited to assist expiratory airflow, maintain end-expiratory lung volume, and to share the work of breathing with the inspiratory muscles (De Troyer, 1983; De Troyer and Loring, 1986). Despite this critically important role, our fundamental understanding of the expiratory activity response to hypoxia has been inconsistent and equivocal, muddled with numerous studies that have led to considerable discordance and variability.

Classic studies suggest that expiratory neuronal activity is inhibited by hypoxia (Nesland et al., 1966; St. John and Wang 1977). Such inhibitory influence of hypoxia on expiratory activity has been directly examined and supported at the level of the motorneurons in cats. In particular, Sears et al. (1982) in the Journal of Nature published their remarkable findings of a distinct 'inspiratory shift' of the neural motor output with hypoxia to cause reciprocal inhibition of the expiratory nerve activity. Likewise, Fregosi and colleagues reported a similar effect of hypoxia to inhibit the expiratory activity of the abdominal and internal intercostal nerves (Fregosi et al., 1987; Fregosi et al., 1989). However, such findings are directly contested by a reported increase in expiratory nerve activity with hypoxia in the same species (Ledlie et al., 1983; Fregosi et al., 1990). In addition, expiratory activity has been assessed at the level of the effector muscles in humans and animals. However, this too has resulted in inconsistent reports, with considerable variability spanning across individual species and experimental conditions.
Accordingly, expiratory muscle activity during hypoxia has been reported to cause either excitation (Kelsen et al., 1977; Matsumoto, 1987; Takasaki et al., 1989; Smith et al., 1990; Yasuma et al., 1993; Praud et al., 1993; Fregosi, 1994; Mateika et al., 1996), inhibition (Bouverot and Fitzgerald, 1969; van Lunteren et al., 1989; Brice et al., 1990), or no change (Bishop and Bachofen, 1973).

How can we reconcile such incongruence in the expiratory activity response to hypoxia? Based on past evidence, logical reasoning and intuition suggests that expiratory activity with hypoxia may not be as definitive as one might have previously thought. Rather it is our contention and hypothesis that expiratory activity may in fact progress and change over time to significantly alter the respiratory neuromotor output occurring with hypoxia. To this effect, in adult humans and other mammals, isocapnic hypoxia sustained 20-60 minutes elicits a biphasic ventilatory response, with initial peak ventilation followed by a decline ("roll-off") to an intermediate plateau (Weil and Zwillich, 1976; Easton et al., 1986; Vizek et al., 1987; Easton et al., 1988; Tatsumi et al., 1992; Long et al., 1993; Praud et al., 1993). In accordance with this, we previously demonstrated in awake canines that during sustained isocapnic hypoxia the primary inspiratory muscles rolls off with ventilation exhibiting a biphasic EMG activity pattern (Fujimura et al., 2006; Ikegami et al., 2008; Ji et al., 2011). On the other hand, only a few studies exist in the literature which have attempted to examine the temporal effect of steady-state hypoxia on the activity of expiratory nerves and muscles in adult mammals (Smith et al., 1989; Brown et al., 1992; Izuka and Fregosi 2007). However, these studies did not control for arterial CO₂ during hypoxia, making it difficult to single out the effects of hypoxia on expiratory activity. Additionally in some of these studies, the animals were not in their 'intact' physiologic state or their methods involved the use of state altering anaesthetics known to impair ventilation (Hickey and Severinghaus, 1981; Pavlin and Hornbein, 1986). Thus to date, activity and action of the abdominal expiratory muscles during sustained isocapnic hypoxia and its time course effects on expiratory activity is unknown, in any awake, non-anesthetized, adult mammals.
The objective of the present study was to carefully inspect and characterize the mechanical and neural activation of the primary expiratory abdominal muscle, the transversus abdominis, before, during and after sustained exposure to mild-moderate levels of hypoxia lasting 20-25 min (mean SpO₂ 80±2%). Our specific goal was to eradicate any potential confounding variables that may have impacted earlier studies to permit an adequate assessment of the normal physiologic function of the transversus abdominis during hypoxia. Accordingly, we elected to undertake the present investigation employing a chronically instrumented, intact, consciously breathing, awake canine model, free of any state altering anaesthetics and/or acute surgical interventions. Continuous recordings of the respiratory length change and electrical activity of the muscles were made utilizing the direct measurement technique of sonomicrometry and electromyography allowing for in vivo intramuscular assessment of the mechanical and electrical activity of the transversus abdominis, during room air and hypoxic stimulated ventilation. To single out the time dependent influence of sustained hypoxia on the activity of the transversus abdominis without the confounding influence of varying levels of arterial CO₂, steady-state isocapnic conditions were maintained at room air levels for the entire period of constant exposure to hypoxia.

We hypothesize in our intact awake canines that: 1) the transversus abdominis would phasically shorten with expiratory activity during quiet room air breathing; 2) hypoxia would cause a marked increase in the expiratory activity of the transversus abdominis, as reflected by an enhanced shortening and EMG activity, however, if prolonged, expiratory activity would be met by a significant central inhibition resulting in a dramatic loss of the transversus abdominis shortening and EMG activity with sustained hypoxia; and, 3) inhibiting effects of hypoxia would persist causing transversus abdominis shortening and EMG activity to fall below baseline room air levels upon recovery room air breathing. We further hypothesize that the discordance in expiratory activity response to hypoxia may be accounted for by this temporal variance in expiratory activity when confounded by other factors modulating this normal dynamic profile.
Methods

*Implantation of transducers and electrodes*

The study and the experimental procedures were approved by the University of Calgary Animal Care Committee and were in accordance with national guidelines.

Twelve mongrel canines (mean weight 29.4 ± 5.52 kg; range 20-41 kg) had pairs of sonomicrometry transducers and bipolar fine wire EMG electrodes implanted in the left transversus abdominis muscle of the abdominal wall. This technique of chronic sonomicrometry and EMG implantation has been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). In brief, implantation of transducers and electrodes was performed under general anesthesia with thiopental sodium induction and halothane. The left transversus abdominis was exposed through a mid-abdominal incision, and ultrasonic transducers were implanted between muscle fibers midway between the inferior costal margin and the iliac crest, in the plane of the anterior axillary line. Immediately adjacent to each transducer, a fine wire stainless steel bipolar EMG electrode was attached. All implants were secured by fine, synthetic, non-fibrotic sutures (Prolene, Ethicon, Somerville, NJ) and were externalized by a subcutaneous skin tunnel. Animals were allowed to fully recover prior to the measurements of resting and hypoxic-stimulated ventilation.

*Measurement techniques*

All measurements of ventilation and respiratory muscle function were made with the animals awake and breathing quietly, while lying in right lateral decubitus position which placed the implanted muscles in a non-dependent position. The animals were relaxed and familiar with the location, routine and personnel of the recordings. The animals were breathing spontaneously and quietly through a snout mask, which was connected through a 2-way non-rebreathable valve to a low resistance open breath circuit (<1 cmH\textsubscript{2}O/L/s) which incorporated a pneumotachograph (Fleisch #2) and a piezoelectric
differential pressure transducer (Model 163PC01D36, Honeywell Microswitch) to provide measurement of inspiratory airflow. On the expiratory limb, end tidal CO$_2$ (ETCO$_2$) was sampled and analyzed continuously by an infrared CO$_2$ analyzer (Model CD-3A, AMETEK/Thermox Instrument Division, Pittsburgh, PA), and on the inspiratory side, fractional concentration of inspired O$_2$ (F$_{I}O_2$) was continuously sampled and analyzed by an O$_2$ analyzer (Model S-3A/1, AMETEK/Thermox Instrument Division, Pittsburgh, PA). The inspiratory limb could be switched, without alerting the animal, from entrainment of room air to a large reservoir of pre-mixed gas of low F$_{I}O_2$ (8-10%). In addition, supplemental pressurized sources of O$_2$ and CO$_2$ were attached to the inspiratory limb to allow the experimenter to precisely titrate F$_{I}O_2$ and ETCO$_2$ during the study. To relate the level of hypoxia, oxygen saturation (SpO$_2$) was continually measured by a pulse oximeter (Model Ohmeda Biox 8700, Rexdale, ON, Canada) by attaching a light sensitive analysis probe onto a shaved tendo calcaneous on the animal's hind limb.

Dynamic measurements within the respiratory muscles of the changing distance between the sonomicrometry transducers of each pair was provided by measuring the speed of transmission of ultrasonic waves using a sonomicrometer (Model 120, Triton Technology, Sand Diego, CA) (Easton et al., 1989). The output signal of the sonomicrometer was offset, amplified and then sampled to computer. For measurements of EMG, the fine wire bipolar electrode pairs from each muscle were connected to an AC differential pre-amplifier (Model 1700, AM Systems, Everett, WA). Power line interference was abolished by careful shielding techniques and the use of differential preamplifiers with a high common mode signal rejection of 110 dB. Thereafter the signal was filtered to attenuate both movement artifact and sonomicrometry noise and to perform anti-alias filtering, using a 6-pole, low pass Bessel filter at 20 Hz-700 Hz (Model 746, LT-4, Frequency Devices Incorporated, Haverhill, MA). The EMG signals were then rectified and processed by passage through resistance-capacitance, leaky "integrators" with a time constant of 100 ms, to provide moving averages of the EMG.

Using computer software for data acquisition (DataSponge, Bioscience Analysis Software, Calgary, AB, Canada), all signals were monitored in real time on the computer.
display and simultaneously collected at 100 Hz to hard disk on a microcomputer equipped with a single board A/D system (Model MIO-16-H-9, National Instruments, Galveston, TX).

Experimental protocol

The details of the sustained hypoxia technique have been described fully in a previous publication (Easton et al., 1986). To evaluate ventilation and respiratory muscle function during sustained isocapnic hypoxia, the study sequence was as follows: 1) a control period of room air, resting, baseline breathing of 6-8 min; 2) an abrupt step decrease in F\textsubscript{I}O\textsubscript{2}, which lowered Sp\textsubscript{O}2 to \(\leq\)80 in 1-2 min; 3) maintenance of Sp\textsubscript{O}2 at 80 ± 2\% for 20-25 min with constant ETCO\textsubscript{2} equal to control levels; and 4) a recovery period of room air breathing of 5-7 min. During sustained hypoxia, the target Sp\textsubscript{O}2 was 80\%, and Sp\textsubscript{O}2 variability was restricted by the operator to the range 78-82\%, a sustained desaturation that we abbreviate as 80 ± 2\%. During the abrupt introduction of hypoxia, supplemental CO\textsubscript{2} was added via the pressurized source to the inspired limb of the breathing circuit to bring ETCO\textsubscript{2} back to a level approximating ETCO\textsubscript{2} during the preceding room air period. We did not attempt to anticipate and exclude any minor dips in ETCO\textsubscript{2} at the beginning of hypoxic hyperventilation because complete prevention of any transient fall in ETCO\textsubscript{2}, at the onset of hypoxia, would have entailed a slight risk of transient overcorrection and spurious increase in inspired minute ventilation during initial hypoxic exposure. After Sp\textsubscript{O}2 had been stabilized at \(\leq\)82\%, it was maintained at that level through adjustments in F\textsubscript{I}O\textsubscript{2} as necessary, utilizing the pressurized O\textsubscript{2} source on the inspiratory limb.

Analysis of breathing pattern and muscle activity

After acquisition and storage on disk, data were analyzed using software programs written by one author (PAE), and adapted to this project by the 2\textsuperscript{nd} author (MJ). These programs provide interactive visual display of the signals during analysis to allow careful examination of each breath. All signals were manually reviewed for movement
artifacts, transient signal disconnections, apneas and sighs, all of which were deleted from individual breath-by-breath analysis.

The flow signal was evaluated for respiratory timing and digitally integrated; minute ventilation ($V_I$), tidal volume ($V_T$), respiratory frequency ($f_R$), inspiratory time ($T_I$), expiratory time ($T_E$), mean inspiratory flow ($V_T/T_I$), and inspiratory fraction of respiration ($T_I/T_{TOT}$) were calculated breath-by-breath. The software also calculated whole breath, or “tidal” breath, values of respiratory muscle shortening and EMG activity. These calculations have been described in detail elsewhere (Easton et al., 1989; Katagiri et al., 1994). Briefly, the timing of expiration was determined from the airflow tracing, and the onset and termination of individual muscle shortening and EMG activity for each breath was measured relative to the beginning and end of expiratory airflow. Using the sonomicrometry length data from each implanted muscle, the computer calculated for each breath the baseline end-inspiratory resting length of the muscle, titled $L_{BL}$, and the shortening (SHORT) for each breath expressed as a percentage change from resting length, entitled $\%L_{BL}$. Moving average EMG activity from each muscle was quantified arbitrarily per breath as the maximum difference in volts between baseline and the peak height of the moving average signal, expressed as $EMG_{DIFF}$. $ETCO_2$ and $SpO_2$ were also calculated breath-by-breath based on respiratory timing. Individual $ETCO_2$ values were converted for altitude to sea level ($Patm = 760 \text{ mmHg}$).

Measurement of all variables was continuous throughout the protocol, but for clarity of presentation, variables are summarized over corresponding periods of each study (Easton et al., 1986; Easton et al., 1988). Mean values of $V_I$ and components of breathing pattern, $SpO_2$, $ETCO_2$, $L_{BL}$, SHORT, and $EMG_{DIFF}$ were calculated from the continuous measurements during the following periods: BASE, PEAK, PLATEAU, and RECOVERY. Period BASE was the final 5 min of resting ventilation with normal $SpO_2$ before the introduction of hypoxia. Periods PEAK and PLATEAU were from opposite ends of the 20-25 min period of sustained hypoxia at $80 \pm 2\% \ SpO_2$: PEAK represented the first 3 min of hypoxic ventilation, immediately after $SpO_2$ had descended to a level of $\leq 82\%$, while PLATEAU represented the last 5 min of hypoxic ventilation, preceding the
relief of hypoxia. Period RECOVERY represented the first 5 minutes of room air breathing after hypoxia was abruptly relieved.

**Statistical analysis**

After calculation, mean values were exported for review to spreadsheet software (Microsoft Excel, Microsoft, Redmond, WA), to graphic software to output figures (CorelDRAW X6, Corel Corporation, Ottawa, ON), and to PC version of SAS for statistical analysis (SAS Version 9.1, SAS Institute, NC). Mean values for parameters of breathing pattern and muscle length, SHORT and EMG activity were tested across the four periods (BASE, PEAK, PLATEAU, RECOVERY) by two-way analysis of variance with repeated measure on a single factor (Keppel, 1982; Steele and Torrie, 1980). Multiple comparison testing of the mean values of the individual periods was performed using Tukey’s test (Steele and Torrie, 1980). *P*-value of less than 0.05 was considered statistically significant.
Results

The study was conducted in twelve healthy, awake canines (mean weight 29.4 ± 5.52 kg; range 20-41 kg) with chronic implantation of sonomicrometry transducers and bipolar fine wire EMG electrodes. On average the animals recovered post surgically for 27 days (range 8-70 days) prior to the study. Although the implants are long-lasting, durability of implanted transducers and electrodes was not universal. Thus, muscle length and EMG recordings from the transversus abdominis muscle are available in N=11 and N=12 animals, respectively.

Oxygen and carbon dioxide control

The sustained isocapnic hypoxic condition was successfully attained for each animal throughout the study (Table 6). Mean SpO₂ decreased significantly from BASE to PEAK to reach target SpO₂ levels (~80%) of the study (92.91 ± 2.26 to 79.74 ± 3.91%, P<0.01), and then remained virtually unchanged from PEAK to PLATEAU (79.74 ± 3.91 to 78.85 ± 3.86%, NS). Mean P_{ET}CO₂ was maintained isocapnic with respect to BASE (37.92 ± 2.42 mmHg) throughout PEAK and PLATEAU (37.13 ± 3.73 and 38.56 ± 4.10 mmHg, respectively; BASE-PEAK-PLATEAU, all NS). With RECOVERY, SpO₂ increased (78.85 ± 3.86 to 90.19 ± 2.95%, P<0.01) and P_{ET}CO₂ decreased (38.56 ± 4.10 to 35.48 ± 4.20 mmHg, P<0.05) significantly from PLATEAU upon restoration of room air.

Ventilation and breathing pattern

Group mean values for ventilation and breathing pattern during room air and sustained isocapnic hypoxia are summarized in Table 6. Steady-state isocapnic hypoxia for 20-25 min had significant effects on ventilation and components of breathing pattern. V_I, V_T and V_T/T_I increased significantly from BASE to PEAK (V_I: 7.50 ± 2.69 to 17.33 ± 5.13 l/min; V_T: 0.39 ± 0.06 to 0.69 ± 0.15 l; and V_T/T_I: 0.30 ± 0.08 to 0.59 ± 0.14 l/s; all P<0.01), then declined, or "rolled off", to an intermediate PLATEAU value (V_I: 13.54 ±
5.69 l/min; V_T: 0.51 ± 0.11 l; and V_T/T_I: 0.49 ± 0.17 l/s; PEAK-PLATEAU, all P<0.01). Ventilation fell despite minimal changes in f_R during PEAK to PLATEAU (25.33 ± 5.88 to 26.60 ± 9.78 breaths/min, NS). Similarly, T_T/TTOT increased from BASE to PEAK during initial acute hypoxia (0.41 ± 0.05 to 0.48 ± 0.03 ratio, P<0.01) with a significant reduction T_E and T_I (2.06 ± 0.79 to 1.34 ± 0.36 s and 1.38 ± 0.38 to 1.20 ± 0.27 s, respectively; BASE-PEAK, P<0.01). With sustained hypoxia, however, T_T/TTOT significantly decreased from PEAK to PLATEAU (0.48 ± 0.03 to 0.45 ±0.04 ratio; PEAK-PLATEAU, P<0.05) in concert with a nominal increase in T_E and decrease in T_I that did not meet statistical significance because of the variability between animals (T_E: 1.34 ± 0.36 to 1.46 ± 0.63 s; T_I: 1.20 ± 0.27 to 1.14 ±0.36 s; PEAK-PLATEAU, NS). Upon RECOVERY, V_I, V_T and V_T/T_I visibly fell below original BASE values, however, this was not statistically significant; all other ventilatory parameters (f_R, T_E, T_I, T_T/TTOT) returned promptly back to or near BASE values.

**Transversus abdominis length change and EMG activity**

Raw tracings of the changes in transversus abdominis (TA) length and integrated moving average EMG activity with inspiratory airflow (uni-phasic) from a single representative animal subjected to sustained isocapnic hypoxia is shown in Figure 45 (A–D) - with each panel representing a period within BASE, PEAK, PLATEAU, and RECOVERY. During initial room air at BASE (Figure 45A), TA phasically shortened and was invariably active with each expiratory breath, as demarcated by periods of zero inspiratory airflow (flat line). Such invariable phasic EMG activity was observed in the majority of animals (10 of 12) during quiet room air expiration, with the exception of two animals exhibiting an intermittent phasic EMG activity of the TA (as characterized by the presence of EMG inactivity in the midst of phasic muscle activation). Regardless of the invariable or intermittent EMG pattern, TA phasically shortened with each and every expiratory breath, albeit to a lesser degree during periods of EMG quiescence.

With initial hypoxia corresponding to PEAK (Figure 45B), phasic TA expiratory SHORT and EMG activity markedly increased more than two fold from BASE room air
levels in concert with an increase in inspiratory airflow and \( f_R \) (from 3 to 4 breaths per trace). However, such magnitude of expiratory SHORT and EMG activity noted during initial hypoxia did not persist, as final hypoxia representing the period PLATEAU (Figure 45C) was associated with a dramatic loss of the expiratory SHORT and EMG activity of the TA, with a lesser decline in inspiratory airflow and minimal alteration in \( f_R \) (still 4 breaths per trace). All animals (12 of 12) without exception showed invariable phasic expiratory EMG activity of the TA during the entire period of constant hypoxia lasting 20-25 min.

Upon restoring final room air at RECOVERY (Figure 45D), phasic TA expiratory SHORT and EMG activity conjointly returned or slightly fell below initial room air levels at BASE with inspiratory airflow and \( f_R \). Following sustained hypoxia, expiratory EMG activity in the TA became silent in three animals (3 of 12) and intermittently phasic in four (4 of 12), while the remaining animals (5 of 12) continued to show invariable phasic activation of the TA. Nonetheless, TA phasically and invariably shortened with each expiratory breath, even in the animals with complete quiescence of EMG activity.

**Time course of transversus abdominis shortening and ventilation**

The typical time course of TA expiratory SHORT and ventilation before, during and after sustained isocapnic hypoxia for a single animal is illustrated in Figure 46. The record of SpO\(_2\) demonstrates that when low \( F_{O_2} \) gas was entertained after 5 min of initial room air, resting, baseline breathing, SpO\(_2\) precipitately dropped to 81.7% (mean/20 sec) within the first minute, and thereafter was sustained at 80 ± 2% for the remainder of the hypoxic period of 20 minutes, prior to the step reintroduction of room air. Upon room air recovery breathing, SpO\(_2\) steadily increased to reach baseline pre-hypoxic levels within 5 min. \( P_{ET}CO_2 \), estimating alveolar partial pressure of CO\(_2\), in the beginning dipped unavoidably with the initial burst of hypoxic hyperventilation, but then was quickly titrated back to and then maintained at levels isocapnic to baseline (~38 mmHg) for the remainder of the steady-state hypoxic period. With recovery room air breathing, \( P_{ET}CO_2 \) initially dipped below steady-state levels prior to settling back to resting baseline values.
The time course changes in TA expiratory SHORT exhibited a prominent biphasic pattern following that of ventilation during 20 min of sustained isocapnic hypoxia, however, TA SHORT visibly showed a greater magnitude and rate of roll-off when compared to the temporal changes in V₁. In this animal, as SpO₂ fell, TA expiratory SHORT increased substantially from baseline resting levels of contraction (more than three fold) with the initial hypoxic hyperventilation followed by a precipitous and profound loss of TA SHORT (more than a third of the initial response) which reached a steady level within ~5-10 min. Thus despite the constant lowered SpO₂ of 80 ± 2%, TA SHORT after exhibiting a peak response fell rather quickly towards, and approaching, initial room air levels with some scatter about a new steady-state. Conversely, although V₁ initially increased in a similar time frame with the TA, subsequent roll-off in V₁ was relative less dramatic and developed in a much more progressive manner compared to that of the TA. As a result, V₁ after reaching peak ventilation declined to reach a new steady-state intermediate plateau level above baseline around the 10-15 min mark of sustained isocapnic hypoxia. Upon reintroduction of room air, TA SHORT and V₁ fell abruptly below initial room air levels, then steadily returned to near baseline values by the end of the recovery room air period.

As a group, all animals with available length measurements (11 of 11) exhibited the characteristic biphasic time course pattern in TA expiratory SHORT during sustained isocapnic hypoxia with modest degrees of variability in the magnitude and the rate of the roll-off. Likewise, all animals (12 of 12) showed a qualitatively similar biphasic time course pattern in expiratory EMG activity with that of the changes in muscle SHORT and ventilation during sustained isocapnic hypoxia (individual examination not reported).

**Transversus abdominis mean shortening and EMG activity**

The group mean tidal changes in TA expiratory SHORT during room air and sustained isocapnic hypoxia is illustrated in Figure 47 and summarized in Table 7. From baseline resting levels of contraction, mean TA expiratory SHORT significantly
increased from $2.41 \pm 0.69$ to $5.86 \pm 2.57 \% L_{BL}$ with initial hypoxia at PEAK ($P<0.01$), corresponding to 248% of initial BASE room air value. However, such magnitude of contraction with initial hypoxia was short-lasting as sustained hypoxia was equally met by a considerable loss of the contractile output of the TA muscle. Specifically, tidal TA expiratory SHORT from PEAK significantly declined ($P<0.01$) with sustained isocapnic hypoxia falling down to near BASE room air levels at PLATEAU ($2.74 \pm 0.62 \% L_{BL}$ or 117% of BASE). Thus, after the significant roll-off, TA expiratory SHORT remained 17% above the initial baseline level with constant hypoxia and was not significantly different when compared to starting room air BASE values (BASE-PLATEAU, NS). Moreover, although expiratory SHORT of the TA visibly dropped in RECOVERY to values below the original BASE, mean RECOVERY value of $1.91 \pm 0.87 \% L_{BL}$ or 79% of BASE were not different statistically from starting BASE levels (BASE-RECOVERY, NS).

The corresponding tidal changes in group mean TA expiratory EMG activity during room air and sustained isocapnic hypoxia is shown in Figure 48 and summarized in Table 7. Expiratory EMG activity of the TA during room air resting baseline was very minimal in this study group of animals (less than 0.6 volts). With initial hypoxia, mean TA expiratory EMG activity exhibited a very large significant increase from BASE value of $0.58 \pm 0.14$ volts to PEAK hypoxic value of $2.71 \pm 0.80$ volts ($P<0.01$), a 469% increase of starting BASE room air activity. The striking effect of constant hypoxia on the expiratory activity of the TA is undoubtedly shown at PLATEAU, where mean tidal EMG activity was remarkably inhibited down to near BASE room air levels ($0.88 \pm 0.38$ volts or 154% of BASE). Accordingly there was a very significant decrease in expiratory TA EMG activity from PEAK to PLATEAU ($P<0.01$) with sustained isocapnic hypoxia, and this was not statistically different when compared to original BASE activity prior to hypoxia (BASE-PLATEAU, NS). With RECOVERY room air, expiratory EMG activity of the TA of $0.44 \pm 0.16$ volts or 78% of BASE visibly fell below initial BASE activity, however, this did not reach statistical significance (BASE-RECOVERY, NS).
These large changes in tidal expiratory SHORT and EMG activity of the TA during sustained isocapnic hypoxia occurred without statistically significant changes in baseline end-inspiratory resting length of the muscle (Table 7; TA L_{BL}: BASE-PEAK-PLATEAU-RECOVERY, all NS). However, it is worth noting that TA L_{BL} exhibited a modest trend to decrease with initial (BASE to PEAK: 0.15 mm or ~1.18% of BASE L_{BL}) and constant (PEAK to PLATEAU: 0.13 mm or ~1.27% of BASE L_{BL}) hypoxia to cause a total reduction in TA L_{BL} of 0.28 mm or ~2.45% of BASE L_{BL} throughout the sustained hypoxia. During RECOVERY room air, TA L_{BL} did not return to initial room air BASE levels and remained smaller in length (0.33 mm or ~2.38% of BASE L_{BL}).
Discussion

Summary of results

The present study demonstrates that in intact, awake canines, in the right lateral decubitus position, expiratory abdominal muscle, the transversus abdominis, phasically shortens during resting room air ventilation regardless of the invariable or intermittent phasic EMG activity pattern of the muscle. Initial acute hypoxia causes a prominent recruitment of the transversus abdominis muscle, as reflected by an increase in expiratory shortening and EMG activity, however, such enhanced expiratory activity was short-lasting. With sustained hypoxia, expiratory shortening and EMG activity of the transversus abdominis significantly fell down to near baseline room air levels, reflecting a marked inhibition of expiratory activity with constant exposure to hypoxia. Regardless of the time dependent nature of expiratory abdominal activity, the transversus abdominis invariably and phasically shortened with EMG activity during the entire period of hypoxic ventilation. Upon recovery room air, transversus abdominis shortening and EMG activity visibly fell below baseline control levels, with a greater number of animals showing an intermittent EMG activity pattern, while some even exhibited complete EMG quiescence following sustained hypoxia.

Breathing pattern during sustained hypoxia

Demonstrated in the study, all animals exhibited the characteristic biphasic pattern of ventilation during sustained isocapnic hypoxia, with an initial increase in minute ventilation followed by a decline, or roll-off, to an intermediate plateau above baseline room air levels. In accordance with earlier sustained hypoxia studies in adult mammals, $V_I$ increased in parallel with $V_T$ and $f_R$ while the subsequent roll-off was associated with a fall in $V_T$ with minimal alterations in rate (Bureau et al., 1984; LaFramboise and Woodrum, 1985; Easton et al., 1986; Vizek et al., 1987; Eden and Hanson, 1987; Rigatto et al., 1988; Easton and Anthonisen, 1988b; Long et al., 1989; Fujimura et al., 2006; Ikegami et al., 2008). Although we do not have measurements of
inspiratory muscle activity, the biphasic changes in $V_T/T_I$ observed in the present study, as an index of inspiratory drive (Akiyama and Kawakami, 1999), suggests that the neuromotor output to the inspiratory muscles would decline in a similar manner with ventilation. In this regard, inspiratory drive as indexed by EMG activity from the parasternal intercostals as well as the costal diaphragm has been previously reported to roll-off during sustained hypoxia in both humans and animals (van Lunteren et al., 1989; Okabe et al., 1993; McEvoy et al., 1996; Vizek and Bonora, 1998; Fujimura et al., 2006; Ji et al., 2011).

Here we extend our previous work in awake canines to demonstrate that $T_E$ also decreases in parallel with $T_I$ in response to sustained hypoxia (Fujimura et al., 2006; Ji et al., 2011). However, such findings directly contrast with earlier reports where hypoxia did not alter the $T_E$ or $T_I$ response in awake rats (Walker et al., 1985; Coles et al., 2002). Although the exact reasons are unclear, this certainly reflects a species difference in the respiratory timing response to hypoxia between canines and rats. In addition, our results also indicate that changes in ventilation were accompanied by biphasic changes in the inspiratory fraction of respiration, $T_I/T_{TOT}$. Specifically, initial increase in $T_I/T_{TOT}$ (41 to 48%) was brought about by a reduction in $T_{TOT}$ (or increase in $f_R$) accompanied by a relatively greater $T_I$ when compared to $T_E$, while the subsequent decline in $T_I/T_{TOT}$ (48% to 45%) occurred without significant changes in $T_{TOT}$ with a proportionately larger $T_E$ compared to $T_I$. Such changes in $T_I/T_{TOT}$, serving as a dimensionless index of effective respiratory timing, have previously been attributed to the changes in the timing of the inspiratory "off-switch" mechanism and/or vagal afferents (Bradley et al., 1975), and thus may account for the biphasic changes in $T_I/T_{TOT}$ noted in the current study. Furthermore, our observation for a persistent inhibitory influence of hypoxia resulting in an incomplete recovery of the ventilatory parameters ($V_I$, $V_T$ and $V_T/T_I$) are in general agreement and extend the support for time dependent recovery of ventilation following sustained hypoxia previously reported in adult humans (Easton et al., 1988).
Abdominal muscle activity with room air

Invariable phasic activation of the transversus abdominis during quiet room air breathing has been consistently reported in a number of awake and lightly anesthetized animals in a variety postures: sitting, standing, prone, and supine (Smith et al., 1990; Arnold et al., 1988; De Troyer et al., 1989; Gilmartin et al., 1987; Brice et al., 1990; Smith et al., 1993). In accordance with previous reports, our results for a phasic expiratory activation of the transversus abdominis in our animals during quiet room air breathing are emphatic and further reinforce the notion that the transversus abdominis is the primary expiratory abdominal muscle in canines and that quiet spontaneous expiration is an active, rather than passive, process (Gilmartin et al., 1987; De Troyer, 1989). In contrast, activity of the transversus abdominis during quiet breathing in adult humans has produced mixed results. For instance, nominal phasic transversus abdominis activity has been reported using fine wire electrodes in supine adult humans (Abe et al., 1996, 1999), suggesting an active role for this primary abdominal muscle during quiet expiration in the supine posture. While on the other hand, other investigators have been unable to detect the activity of the transversus abdominis employing concentric needle electrodes, and have suggested that normal adults do not utilize the transversus abdominis when breathing quietly in the supine or seated posture (Estenne et al., 1988; De Troyer et al., 1990). However, when adopting the standing posture, during hypercapnic stimulated breathing, or in the presence of lung disease, the transversus abdominis, as well as other expiratory muscles; namely the: triangularis sterni, external oblique and internal oblique, have been previously shown to play a role in facilitating respiration (Estenne et al., 1988; Ninane et al., 1992; Ninane et al., 1993, Abe et al., 1996). Although the exact technical and/or physiologic reasoning for the earlier discrepancy in adult humans are unclear, the available evidence with fine-wire EMG suggests that the transversus abdominis in humans, as with that of canines and other mammals, likely plays an important role in facilitating quiet expiration during eupnoea, even in the supine posture.

In the present study, we did not observe in any subject a complete silence or inactivity of the transversus abdominis during quiet room air ventilation (prior to the
introduction of hypoxia), but two animals did exhibit intermittent phasic activation of the transversus abdominis. Previous accounts of intermittent expiratory muscle activity during quiet breathing has been limited to the external oblique and triangularis sterni muscles in humans and canines (De Troyer et al., 1989; Gilmartin et al., 1987; Estenne et al., 1988). To the best of our knowledge, with the exception of patients with respiratory disease (Ninane et al., 1992; Ninane et al., 1993; Estenne et al., 1998), ours is the first study reporting the intermittent nature of the transversus abdominis activation in awake canines while breathing quietly at rest. Given that changes in body position have been shown to profoundly influence abdominal muscle activity in spontaneously breathing canines and humans (De Troyer et al., 1989; Gilmartin et al., 1987; Abe et al., 1999; Estenne et al., 1988), intermittent patterns noted in the present study may be a unique consequence of the lateral decubitus positioning. Nevertheless, we have little reason to doubt the validity of our recorded EMG from these two animals, as the intermittent activity became invariably phasic with hypoxia, and then reverted back to the intermittent pattern upon restoration of room air breathing after sustained hypoxia. Additionally, respiratory length change of the transversus abdominis during quiet expiration when accompanied by phasic EMG activity was visibly greater when compared to the length changes occurring during the period of EMG quiescence in these two animals (individual examination not reported), and thus further reinforces the validity of the intermittent EMG pattern recorded in these two animals.

Despite the invariable or intermittent nature of the transversus abdominis EMG activity during room air ventilation, the extent of this electrical activation was very small for the entire group of animals (mean 0.58 volts or 22% of maximal activity with sustained hypoxia). Such minimal EMG activity of the transversus abdominis observed in this study, by and large, agrees with the magnitude of the EMG activity previously reported from the same muscle in supine adult humans by Abe and colleagues (1996, 1999). Moreover, although quantitative comparisons of EMG activity are difficult to compare accurately across different studies due to species differences as well as the variation in the normalization methods involved, similar levels of activity in the transversus abdominis, i.e. roughly 20% of maximal activity, during quiet expiration has
been previously reported in anesthetized and awake canines, when EMG is expressed as a percentage of maximal activity detected with an expiratory threshold loading of 25 cm H$_2$O positive end-expiratory pressure (Farkas and De Troyer, 1989; Gilmartin et al., 1987) or with hypercapnic stimulated rebreathing at alveolar PCO$_2$ of 59 Torr (Yasuma et al., 1993).

Given these results, we can reasonably conclude that spontaneous quiet expiration in awake canines, although an active process, requires very little activity of the primary expiratory abdominal muscle, the transversus abdominis. Such interpretation would be probable, if much of expiration is driven by the passive elastic recoil forces of the respiratory system following inspiration, and/or the relative contribution of other expiratory muscles may be accounting for a greater extent during quiet breathing therefore requiring minimal activation of the transversus abdominis. In this regard, the other ventrolateral abdominal wall muscles; the rectus abdominis, external oblique, and internal oblique, are likely not the major contributors, as they exhibit very minimal activation during room air in awake canines (De Troyer et al., 1989). On the other hand, the major chest wall expiratory muscle, the triangularis sterni, may be contributing to a greater extent, as this muscle has been shown to be consistently active in anesthetized canines (De Troyer and Ninane, 1986). Taken together, quiet expiration in awake canines may be partly accounted for by the passive recoil characteristics of the respiratory system and/or the active contribution of the expiratory chest wall muscles, and thus requiring minimal activity and participation of the transversus abdominis at rest.

**Abdominal muscle shortening with room air**

Regardless of the phasic or intermittent pattern of the muscle activity, transversus abdominis invariably shortened with each expiratory breath. Transversus abdominis expiratory shortening of 2.41% from baseline pre-contraction length at end inspiration ($\%L_{BL}$) in this study is quantitatively similar with the total tidal length changes previously reported in tracheostomized awake canines in a similar lateral decubitus position (2.5 - 3.0 $\%L_{RL}$) (Leevers and Road, 1993b; Leevers and Road, 1994). However,
tidal expiratory length change of the transversus abdominis in supine anesthetized canines of 7.9 - 12.7 %L_{FRC/REL} (Leevers and Road, 1989; Ninane et al., 1988; Arnold et al., 1988) is considerably greater than the expiratory shortening observed in this group of conscious canines. Although we cannot easily reconcile for this difference, the greater expiratory shortening may be an artifact of the confounding effects of anesthesia causing an inhibition of respiratory muscle tone (hence a reduction in FRC) (Hickey and Severinghaus, 1981; Pavlin and Hornbein, 1986), an increase in diaphragm resting length (Fitting et al., 1987), loss of post-inspiratory inspiratory activity (Fitting et al., 1987), and/or potential lengthening of the transversus abdominis resting length (Leevers and Road, 1995a). For instance, loss of normal/ambient system tension coupled with a mechanically advantaged diaphragm secondary to an increased pre-contraction resting length could lead to a greater inspiratory contraction and lengthening of the transversus abdominis during inspiration, and thus resulting in a greater tidal length change during expiration. As well, expiratory shortening in the absence of diaphragm post-inspiratory activity during early expiration (acting to impede or retard expiratory length change) may also account for the greater tidal shortening of the transversus abdominis in the anesthetized state. In addition, there is evidence to suggest that transversus abdominis may be operating at a longer length (~5%) when anesthetized compared to the awake state (Leevers and Road, 1995a). Thus, to the extent that the transversus abdominis in anesthetized canines have been previously reported to be operating at ~74% L_{o} (optimal length) (Farkas, 1992), the same muscle in awake canines would presumably be operating at a shorter length with a mechanical disadvantage compared to the anesthetized state. Consequently greater tidal expiratory shortening may result from a longer baseline resting muscle length. Alternatively, we cannot discount the significant effects of anesthesia causing a mismatch in ventilation and perfusion leading to hypercapnia and hypoxia (Hickey and Severinghaus, 1981; Pavlin and Hornbein, 1986), which would presumably via the peripheral and central chemoreflex loop augment the neural drive to produce a greater shortening response of the transversus abdominis.
Discordance in expiratory activity with hypoxia

Influence of hypoxia on expiratory activity has been a subject of great interest, along with considerable disagreement, over the past several decades.

Classic experiments include examinations of the effects of hypoxia at the level of the medullary respiratory neurons in cats with intact peripheral chemoreceptors. In 1966, Nesland and colleagues, in decerebrate spontaneously breathing cats, demonstrated that hypoxia depressed the activity of the medullary expiratory neurons, whereas the inspiratory units exhibited considerable variability resulting in no significant change in their discharge frequency despite an increase in $V_T$. Such an inhibitory based influence of hypoxia on the population of medullary expiratory neurons has been also reported by St. John and Wang in decerebrate, paralyzed, artificially ventilated cats (St. John and Wang, 1977). Accordingly, although limited to a few studies in cats without an intact neuroaxis, classic studies suggest an inhibition of expiratory neuronal activity with hypoxia.

Expiratory activity has also been examined at the level of the individual motorneurons. In 1982, Sears and colleagues published in the journal of *Nature* their notable findings for a distinct 'inspiratory shift', or reciprocal inhibition, of the internal intercostal (expiratory) motorneuron activity, while the phrenic neurogram increased in response to isocapnic hypoxia in anesthetized mechanically ventilated cats. Similar findings for an expiratory activity inhibition with hypoxia has been confirmed in the abdominal and internal intercostal nerves by Fregosi and colleagues (1987, 1989) through a series of careful experiments in vagotomized, artificially ventilated, decerebrate cats. However, such inhibitory influence of hypoxia on expiratory activity has been directly contested in the vagus nerve intact, artificially ventilated, anesthetized canines (Ledlie et al., 1983) and decerebrate cats (Fregosi et al., 1990), where investigators reported an increase in abdominal expiratory nerve activity with hypoxia.

Others have tried to determine effects of hypoxia on expiratory activity by examining the effector muscles involved in expiration. In anesthetized and awake,
spontaneously breathing animals, as well as in conscious adult humans, hypoxia is associated with an increase in the expiratory activity of the chest wall (internal intercostal and triangularis sterni) (Matsumoto, 1987; Smith et al., 1990) and abdominal (external and internal oblique, transversus abdominis) muscles (Fregosi, 1994; Mateika et al., 1996; Kelsen et al., 1977; Takasaki et al., 1989; Yasuma et al., 1993; Praud et al., 1993). Such findings, however, directly contrast with other investigators reporting an inhibitory consequence of hypoxia on the expiratory activity of the triangularis sterni and transversus abdominis, as well as the tonic activity of the internal intercostals, in anesthetized and awake animals (van Lunteren et al., 1989; Brice et al., 1990; Bouverot and Fitzgerald, 1969). In further comparisons, Bishop and Bachofen (1973) reported in dial-anesthetized cats that hypoxia did not modify the activity of the abdominal expiratory muscles with or without continuous positive pressure breathing, whereas the activity of the diaphragm increased irrespectively with hypoxia.

Looking at the breadth of literature on this topic, expiratory activity during hypoxia has resulted in mixed reports to cause either excitation, inhibition, or no change. Hence, despite the widespread and collective efforts of past investigators to carefully and methodically characterize the expiratory activity response to hypoxia at several output levels (i.e. neuronal, neural and muscular), the results thus far have been equivocal with considerable variability across individual species and experimental conditions.

**Reconciliation of expiratory activity with hypoxia**

How can we attempt to reconcile such incongruent reports of the past in the view of the current study? Our examination of the temporal response to sustained isocapnic hypoxia in intact awake canines illustrates that the abdominal expiratory muscle activity follows a distinct biphasic pattern, as shown by the changes in the transversus abdominis EMG (Figures 45 and 48). In this time domain, hypoxia without the confounding influence of CO₂ caused an immediate increase in the transversus abdominis EMG activity within minutes, which was followed by a significant attenuation or roll-off in expiratory activity down to, or near, baseline room air levels. This bimodal activity
pattern of the transversus abdominis was observed in all intact awake canines of our study, and directly supports the thesis of a distinct peripheral chemoreceptor excitatory and central inhibitory influence of hypoxia (via the accumulation of net inhibitory neuromodulators), which has been widely attributed to explain the biphasic changes in ventilation (Woodrum et al., 1981; Eden and Hanson, 1987; Neubauer et al., 1990; Bisgard and Neubauer, 1995; Mortola, 1996) and respiratory neural output (Brown et al., 1992; Vizek et al., 1987) during sustained hypoxia.

Evidence supporting a peripheral chemoreceptor excitatory effect of hypoxia on expiratory activity has been demonstrated in transient hypoxia and isolated carotid body perfusion studies in animals (Smith et al., 1990; Smith et al., 1993; Matsumoto, 1987). Whereas the evidence in support of a central inhibitory effect of hypoxia on expiratory activity has been shown in animals devoid of peripheral chemoreceptor afferents (Chae et al., 1992; St. John and Wang, 1977; Brice et al., 1990; Fregosi et al., 1987). Based on these earlier studies, it is our contention and hypothesis that the biphasic expiratory activity response during sustained hypoxia, as observed in the present study, is a direct physiologic consequence of the dual and opposing influences of hypoxia acting out simultaneously to affect the motor output of the transversus abdominis. Thus accordingly, the initial excitation of the transversus abdominis with acute hypoxia would be mediated by the peripheral chemoreceptors, while the sustained hypoxic roll-off in transversus abdominis activity would be the result of a central inhibition that develops over time to presumably override the constant peripheral chemoreceptor stimuli, as previously suggested for ventilation (Mortola, 1996).

In this framework, published reports for an increase in expiratory activity with hypoxia are consistent with our data showing an immediate augmentation of transversus abdominis EMG activity with hypoxia. Moreover, significant attenuation of the transversus abdominis EMG activity with sustained hypoxia extends support for an inhibitory influence of hypoxia on expiratory activity. However, in contrast to former studies, where expiratory activity from baseline hyperoxic/normoxic levels was frankly inhibited and abolished with hypoxia (Sears et al., 1982; Fregosi et al., 1987; van
Lunteren et al., 1989; Brice et al., 1990), our data in awake canines illustrates an attenuation that manifests over a peak hypoxic response to cause expiratory activity to fall down to near baseline activity levels. Despite this qualitative difference, we envision that the outcome of the former studies could have possibly occurred if the balance of peripheral excitation and central inhibition were to have been somehow altered in such a way to favor a central inhibitory influence of hypoxia compared to a peripheral excitation. For instance, a minimal or lesser degree of peripheral excitation or enhanced central inhibition would tilt the dual and opposing influences of hypoxia to favor a direct expiratory inhibition with or without an initial increase in expiratory activity during constant hypoxia. Similarly, previous studies reporting no changes in expiratory activity with hypoxia may also be accounted for a similar mechanism, whereby the opposing peripheral excitation and central inhibition may be acting equally or not acting at all. The former could be an instance where peripheral excitation by hypoxia is counterbalanced by a developing hyperventilation induced hypocapnia, which acts centrally to reduce respiratory output. On the other hand, the latter instance could result from an absence of peripheral excitation with hypoxia, when coupled with a central inhibition that did not have sufficient time to develop and become expressed.

Factors that influence expiratory activity

Although the exact reasoning and cause accounting for the considerable degree of variance and discordance reported in the past may remain unknown, it is worth mentioning and pointing out that expiratory motor activity is highly influenced and modulated by a number of factors. These factors include but are not limited to the presence of vagal and segmental reflex pathways (Bajic et al., 1992; Iizuka, 2011; Marek et al., 2008; Newsom Davis, 1970; Sears, 1964); contribution of peripheral chemoreceptors (Chae et al., 1992; St. John and Wang, 1977; Brice et al., 1990; Fregosi et al., 1987); type and degree of anesthesia (Fregosi et al., 1987; Fregosi and Bartlett, 1989; Warner and Warner, 1995; Freund et al., 1964; Warner et al., 1992); influence of arterial/cerebral hypocapnia (Smith et al., 1989; Mateika et al., 1996; Praud et al., 1993); changes in posture (Farkas and Schroeder, 1990; De Troyer and Ninane, 1987; Estenne et
al., 1988); and/or type of surgical intervention (decerebration, laparotomy or superficial incision) (Iizuka, 2011; Leevers and Road, 1995a; Farkas and De Troyer, 1989). Thus, any one or combination of the above factors may have potentially obscured the expiratory neural output response by altering the peripheral excitatory and central inhibitory influence of hypoxia with respect to both timing and magnitude. Additionally, the severity of hypoxia, the species differences, as well as the differential recruitment pattern among different expiratory effectors may have further contributed to the variability of expiratory activity response to hypoxia (Smith et al., 1989; Abe et al., 1996; Mortola, 1996). The present study also illustrates the important influence of time in the accurate assessment of expiratory activity with hypoxia. Thus taken together, expiratory neuromotor output during hypoxia appears to be determined by a complex central integration mechanism involving peripheral excitation and central inhibition that underlies a temporal dynamic that is highly influenced by both experimental and physiological factors.

Abdominal muscle activity with sustained hypoxia

Despite the considerable discordance and large variability of the literature, what is certainly clear from the present study is that in intact awake canines (representing the integrated respiratory system response in its 'natural' physiologic state), without the confounding influence of anesthetics, surgical interventions and/or alterations in arterial/brain CO₂, temporal response of expiratory abdominal activity during sustained isocapnic hypoxia is distinctly biphasic in pattern with an initial augmentation followed by a significant roll-off.

Evidence in support of the bimodal expiratory activity pattern and the time dependent influence of steady-state hypoxia has been previously reported in awake canines and ponies during poikilocapnic hypoxia (Smith et al., 1989; Brown et al., 1992), and more recently in anesthetized, vagotomized, artificially ventilated rats during isocapnic hypoxia (Izuka and Fregosi, 2007). Our study extends the findings of the previous poikilocapnic steady-state hypoxia studies to demonstrate that hypoxia alone
without the confounding influence of CO$_2$, i.e. isocapnic condition, elicits a roll-off in the expiratory muscle activity during sustained hypoxia in an intact awake mammal. Moreover, in the isocapnic condition, expiratory muscle activity in the transversus abdominis in our study never fell below baseline activity even though hypoxia was held constant for 20-25 min. Such findings are in direct contrast with the previous reports in awake ponies and canines, where the abdominal and chest wall expiratory muscles exhibited a biphasic expiratory activity falling below baseline control levels with poikilocapnic hypoxia (Smith et al., 1989; Brown et al., 1992). Considering the previous poikilocapnic studies in light of our current results, we conclude that the biphasic expiratory activity response is critically dependent on the influence of hypoxia over time, and that the development of hypocapnia secondary to hypoxic hyperventilation profoundly modulates this bimodal activity pattern to induce a greater rate and/or magnitude of expiratory activity roll-off in large mammals.

In addition, although not representing the 'intact' physiologic state of a spontaneously breathing conscious animal, biphasic expiratory abdominal activity response during isocapnic steady-state hypoxia has been recently reported in vagotomized, paralyzed and mechanically ventilated anesthetized rats (Izuka and Fregosi, 2007). In the context of the anesthetized rat study, the biphasic expiratory abdominal activity response to sustained isocapnic hypoxia, without the inhibitory influence of arterial/brain hypocapnia, may be species specific and/or related to body size. For instance, in anesthetized rats (small animal), expiratory abdominal activity after reaching peak within one minute precipitously fell down to or near baseline normoxic control levels by four minutes of constant hypoxia. This is in contrast to the moderately slower biphasic expiratory abdominal activity response profile expressed in our awake canines (medium sized animal), where peak expiratory EMG activity generally reached peak within several minutes followed by a more temperate roll-off reaching steady-state levels around the 5-10 min mark of sustained hypoxic exposure. While in awake ponies (larger bodied animal), even with the concurrent development of hypocapnia acting to suppress respiratory activity centrally, the expiratory abdominal EMG activity rolled-off with a much slower time profile than our awake canines - taking more than twice as long to
reach steady-state levels below normoxic control levels (Brown et al., 1992). Such findings are consistent with the notion that the biphasic ventilatory response to sustained hypoxia with respect to time and magnitude may be very much species specific and/or associated with body size (see discussion in Project 1: *Species variation in ventilation during sustained hypoxia*).

**Abdominal muscle shortening with sustained hypoxia**

Simultaneous measurements of muscle length change and EMG activity as provided in this study permitted an adequate and accurate assessment of expiratory abdominal muscle function with respect to the changes in its mechanical output in relation to its neuromotor drive, i.e. neuromechanical association. To the best of our knowledge, our is the first study to report on the actual mechanical contraction and shortening from any expiratory muscle during hypoxia, as well as its post hypoxic recovery response in any mammal. Our results clearly demonstrate that the tidal expiratory shortening of the transversus abdominis with initial acute hypoxia significantly increased in conjunction with an increase in the phasic EMG activation of muscle (Figure 47 and 48). However, the relative change in neural drive did not result in an equal change in the contractile output of the transversus abdominis. More specifically, the expiratory EMG activity increased nearly 4.7 fold from baseline activity to achieve a much lesser 2.5 fold increase in the tidal shortening of transversus abdominis. Such disproportionality of EMG and shortening (469% vs. 248% BASE) may reflect the greater degree work, or energy requirement, associated with the active recruitment and contraction of the transversus abdominis during initial hypoxia when compared to the passive characteristics of quiet expiration at rest.

As noted previously, quiet expiration in our awake canines require very minimal activity of the transversus abdominis (as indexed by EMG), likely owing to the passive recoil characteristics of the respiratory system, and presumably involves very little work or energy expenditure. In direct contrast, active recruitment and contraction of the transversus abdominis during initial hypoxia likely entails more work or energy.
requirement, as reflected in EMG activity, to facilitate expiratory airflow. This may arise given the greater caudal displacement of the diaphragm and the abdominal contents as inspiratory drive increases to augment ventilation. In such a case, the transversus abdominis would have to forcefully contract against the preload of the abdominal contents and the diaphragm to increase expiratory airflow and to effectively decrease $T_E$. Thus, considering the minimal activity of the transversus at baseline in direct comparison to the active and forceful contraction of the transversus abdominis during augmented ventilation, it is not surprising that we noted a relatively large increase in EMG activity (469% BASE) with initial peak hypoxia that did not correspond mechanically to similar changes in tidal shortening (248% BASE). Alternatively, the greater activity with initial hypoxia may simply reflect a relative magnitude of EMG change from the very minimally active transversus abdominis (less than 0.6 volts) without any mechanical impedance or preload imposed on the muscle itself. Additionally, it is important to cautiously mention that our suggestion of greater work or energy expenditure based on the relative changes in EMG activity associated with initial hypoxic ventilation is only implied in a comparative manner to the very minimal activity requirements of the transversus abdominis during quiet expiration. Therefore, rather than interpreting our relative data as the transversus abdominis being an inefficient muscle, associated with a large EMG activity that proportionately results in roughly half of the mechanical output, during hyperventilation, we would like to contend that the relatively large EMG increase compared to shortening is a direct consequence of the minimally active and presumably efficient transversus abdominis during quiet expiration, and reflects the true physiological change in the relative degree of recruitment of the primary expiratory abdominal muscle and its corresponding mechanical output with acute hypoxia.

On the other hand, sustained isocapnic hypoxia resulted in a dramatic loss of the transversus abdominis contribution to hypoxic ventilation, as illustrated by a significant decline in tidal expiratory shortening down to near base levels with constant hypoxia. Specifically, tidal expiratory shortening in response to EMG activity fell by more than 3/4 from its initial peak response to nearly abolish the early transversus abdominis shortening response to hypoxic ventilation (Figure 47 and 48). Of note, the marked
decline in expiratory shortening and EMG activity was nearly equivalent falling ~90% and ~86% of the initial peak response, respectively. Such proportional roll-offs in shortening and EMG activity suggests that the changes in the contractile output of the transversus abdominis with sustained hypoxia likely resulted as a direct consequence of neural activation without considerable changes in muscle mechanics, i.e. changes in muscle preload, interaction with other muscles and/or changes in operating length in relation to $L_o$ (optimal length). In addition, the relative disassociation of greater expiratory EMG activity compared to shortening as noted during initial hypoxia was still evident, although to a much lesser extent, after 20-25 min of hypoxia (EMG vs. SHORT: 154% and 117% BASE). Such results likely reflect an expiratory strategy that relies more on the passive recoil characteristics of the respiratory system and/or contribution of other expiratory muscles akin to quiet room air expiration.

Attenuation of respiratory neural drive and its consequent mechanical output during sustained hypoxia is not unprecedented in intact awake canines. Previously, we demonstrated in a similar chronically instrumented awake canine model that tidal inspiratory shortening of the parasternal intercostal and ventilation significantly rolled-off with sustained isocapnic hypoxia, reflecting a central attenuation mechanism affecting the neural output to the chest wall inspiratory muscles (Ji et al., 2011). Albeit only having EMG measurements, in another study we reported a significant decline in the costal diaphragm EMG activity during sustained hypoxic ventilation in awake canines (Fujimura et al., 2006). Here we extend the previous work to demonstrate that sustained hypoxic roll-off is not a unique phenomenon to the inspiratory muscles, and that central attenuation, likely acting on the medullary respiratory neurons, extends to the transversus abdominis expiratory abdominal muscle.

**Differential muscle roll-off with sustained hypoxia**

In addition, our results when compared to our previous studies further illustrate that the magnitude and timing of the roll-off observed in the transversus abdominis expiratory activity is distinct and unique from that of the primary inspiratory muscles (see
Figure 27 in Project 1: *Ventilation and costal diaphragm EMG activity during room air and sustained isocapnic hypoxia*; see Figure 42 in Project 3: *Parasternal intercostal shortening and ventilation during room air and sustained isocapnic hypoxia*). Specifically, the costal diaphragm EMG activity and parasternal intercostal shortening gradually rolled-off to reach an intermediate plateau above baseline activity levels within 15-20 min of sustained hypoxia. Conversely, the transversus abdominis shortening declined to a greater extent much more quickly, falling to near baseline activity levels by the 5-10 min mark of sustained hypoxic exposure (Figure 46). Taken together, our findings clearly illustrates a distinct sustained hypoxic mediated central neuromotor activity and attenuation response descending to the primary inspiratory and expiratory muscle groups. Although the exact central mechanism is unclear, allowances for a differential grade of neuromotor output with sustained hypoxia could reflect the distinctiveness and/or sensitivity of the medullary inspiratory and expiratory neurons to the inhibiting influence of hypoxia. Sustained hypoxia, alternatively, could potentially act through the modulating influence of the vagal and segmental afferent pathways to mediate a distinct roll-off in the primary inspiratory and expiratory muscle groups. Certainly further studies are warranted to elucidate the central mechanism(s) involved in producing the differential grade of respiratory neuromotor attenuation as well as its impact on the respiratory muscles subserving ventilation.

**Abdominal muscles with recovery room air**

With post hypoxic recovery room air breathing, transversus abdominis tidal expiratory shortening visibly fell below baseline room air control levels coupled with an equivalent decline in the EMG activity (SHORT and EMG: 79% and 78% BASE; Figure 47 and 48). Such persistent inhibitory influence could be further gauged by the expiratory EMG activity pattern noted during recovery, where EMG activity became more intermittent or was completely abolished following sustained hypoxia (individual examination not reported). Interestingly, even in the subgroup of animals (3 of 12) where expiratory activity became totally silent, the transversus abdominal shortened invariably with each expiratory breath. In these animals exhibiting EMG quiescence, expiratory
length change of the transversus abdominis likely occurred in a passive manner driven by the elastic recoil of the respiratory system following inspiration and/or active contribution of chest wall expiratory muscles. This strongly suggests that total/tidal expiratory shortening of the transversus abdominis in awake canines indeed constitutes a passive and active component (i.e. inspiratory lengthening and expiratory shortening), as previously suggested in other canine studies (Arnold et al., 1988; Ninane et al., 1988; Farkas et al., 1993; Leevers and Road, 1993a). Our results of a visible decline in the respiratory EMG activity of the transversus abdominis following sustained isocapnic hypoxia are consistent with our prior findings in awake canines on the activity of the primary inspiratory muscles (Fujimura et al., 2006; Ji et al., 2011), and suggest an inhibitory influence that persists following the constant exposure to hypoxia that affects the neuromotor and mechanical output of the transversus abdominis. The exact timing of complete restoration of expiratory activity back to baseline room air levels is currently unknown, as the period of recovery in the present study was limited to five minutes.

**Abdominal muscle contribution to hypoxic and quiet ventilation**

Overall, the mechanical consequence of the primary expiratory abdominal muscle, the transversus abdominis, has been suggested to facilitate expiratory airflow, maintain end-expiratory lung volume, and assist in the inspiratory action of the diaphragm to share the work of breathing between the inspiratory and expiratory muscles (De Troyer, 1983; De Troyer and Loring, 1986).

In the context of improving the efficiency/effectiveness of the respiratory system, our findings of a marked recruitment of the transversus abdominis during initial hypoxia could result in a cephalad displacement of the diaphragm to enhance its force-generating capacity at end-expiration and/or facilitate early inspiration through a passive decent of the muscle following expiration. Such an assist in the inspiratory action of the transversus abdominis in order to improve the mechanical output of the diaphragm would greatly enhance the capacity of the respiratory system to augment ventilation in an effort to restore normoxic blood gas levels with acute hypoxia. However, such enhancement of the
respiratory system through the recruitment of the abdominal muscles would not come without an incurred cost, i.e. energy in the form of ATP coupled with oxygen consumption. Accordingly, our findings of a dramatic loss of the transversus abdominis when hypoxia is sustained, albeit at a significant loss of the system efficiency/effectiveness, may in fact act in a manner to save or conserve energy during the sustained period of low oxygenation. Nevertheless, further studies are warranted to determine whether the transversus abdominis does indeed impart a facilitatory inspiratory action on the diaphragm during initial acute hypoxia, as well as its mechanical consequence on the respiratory system during the significant sustained hypoxic roll-off.

Our data also indicates that the mechanical action of the transversus abdominis may in fact be present even during quiet room air breathing, as indicated by the greater expiratory airflow and tidal expiratory shortening in the presence of phasic expiratory activation versus when the muscle is inactive. Such presumptions, at least in awake canines, and likely extending to humans, go directly against the traditional view that quiet expiration is a passive process without much contribution from the expiratory muscles.

**Evaluation of experimental technique**

Previously, in tracheostomized awake canines in the lateral decubitus position (similar posture employed in this study) (Leevers and Road, 1993a; Leevers and Road, 1993b; Leevers and Road, 1995a; Leevers and Road, 1994), assessment of the passive and active components of expiratory shortening of the transversus abdominis were made via the identification of an inflection point in the length change of the muscle immediately prior to the start of phasic expiratory EMG activity (as the muscle shortened from its end-inspiratory length). By defining this point as the resting baseline length of the expiratory muscles ($L_R$), previous investigators were able to quantitate the extent of the passive (length change before EMG activity) and active (length change after EMG activity) components of expiratory shortening in a phasically activated muscle. However, in the present study, our attempt to similarly identify such an inflection point in our respiratory length traces of the transversus abdominis was for the most part unsuccessful,
as in our upper airway intact awake animals, phasic expiratory EMG activity coincided with or started prior to the expiratory length changes occurring following end-inspiration (Figure 45). This would suggest that in our animals both passive and active length change components are acting in concert during early expiration, and thus by our definition would be considered as active expiratory shortening, i.e. tidal expiratory shortening accompanied by EMG activation.

Given the ambiguity and difficulty in differentiating the passive and active components of the expiratory length change of the transversus abdominis in intact awake canines, in the present study, we define baseline resting length (L_{BL}) of the expiratory muscle as the point coinciding with the end of inspiration, and then expressing tidal expiratory muscle shortening as a percentage change from this baseline length, i.e. \%L_{BL}. With this scheme, the expiratory shortening reported in this study would then be comparable to the total/tidal shortening, including the passive and active length change components, as previously reported in awake and anesthetized canines (Leevers and Road, 1993a; Leevers and Road, 1994; Arnold et al., 1988; Ninane et al., 1988; Farkas et al., 1993). Our method of reporting total/tidal expiratory shortening of the expiratory muscle based on end-inspiratory baseline length is an unbiased and reliable approach to quantify the mechanical output of the expiratory muscles (which presumably consists of both the passive and active length change components). However, if expiratory activity and length change occurs immediately prior to end inspiration, then our method of calculating tidal expiratory shortening would have slightly underestimate the true shortening response of the muscle. Nonetheless, such an underestimation would have occurred consistently throughout the sustained hypoxic trial for each animal, and hence would not have significantly altered or biased the results of the present study.

**Conclusion**

Our results demonstrate that quiet expiration is a predominately active process in awake canines which certainly includes a passive component. Time course changes in the transversus abdominis expiratory shortening and EMG activity during sustained hypoxia
qualitatively tracked the biphasic changes in ventilation, but with greater magnitude and rate of roll-off occurring in the transversus abdominis. Such temporal variance in expiratory activity when compounded by other confounding factors modulating this normal biphasic activity response may account for much of the past discordance in expiratory activity response to hypoxia. Contrary to classic studies, in intact awake canines, expiratory muscles are markedly recruited with hypoxia, however, significant inhibition of expiratory muscles does occur if hypoxia is sustained. Inhibitory effects of hypoxia appears to persist even upon restoration of normoxic breathing. During sustained hypoxia, significant loss of the primary expiratory abdominal muscle, the transversus abdominis, would have a considerable mechanical consequence to impact expiratory airflow as well as the overall efficiency of the respiratory apparatus/system.
Acknowledgements

This study was supported by grants from the Canadian Institutes of Health Research. Expert technical assistance was provided by Ms. Leslie Jacques. Excellent research and laboratory support from colleague Ms. Jenny V. Jagers. The provision of all suture materials by Ethicon Sutures Ltd., a Johnson & Johnson Company, is gratefully acknowledged.
Figure 45: Transversus abdominis length and EMG activity during room air and sustained isocapnic hypoxia (legend on next page)
Figure 45 (figure legend)

Representative tracing of inspiratory airflow, transversus abdominis length (TA Length) and transversus abdominis integrated moving average EMG activity (TA Mavg EMG) during room air and sustained isocapnic hypoxia. Traces shown are breaths during A) initial room air breathing before hypoxia (BASE); B) initial hypoxia once O₂ saturation (SpO₂) reached 80 ± 2% (PEAK); C) final hypoxia preceding return to room air (PLATEAU); and D) final room air breathing after hypoxia (RECOVERY). Each tracing captures 11 sec worth of data with corresponding SpO₂ (%) noted at top-right. Horizontal bars on the bottom-right denotes a time span of 1 sec. L_BL indicates baseline resting muscle length at end-inspiration. Note: upward deflection of airflow marks inspiratory airflow, or inspiration; downward deflection of TA Length reflect muscle shortening; upward deflection of TA Mavg EMG signal represent muscle activation.
Figure 46: Transversus abdominis shortening and ventilation during room air and sustained isocapnic hypoxia

Typical ventilatory and transversus abdominis muscle shortening response to sustained isocapnic hypoxia in a representative animal. Open circle (dashed), open triangle (dashed), closed diamond (lined), and open circle (lined) represent mean values/20 sec for pulse oximeter O₂ saturation (SpO₂), partial pressure of end tidal CO₂ (PₑTCO₂), transversus abdominis expiratory shortening (TA SHORT), and minute ventilation (V₁), respectively.
Figure 47: Transversus abdominis shortening during room air and sustained isocapnic hypoxia

Transversus Abdominis Shortening

Group mean transversus abdominis shortening during sustained isocapnic hypoxia (20-25 min) in eleven animals (N=11). Transversus abdominis shortening (TA SHORT). Tidal expiratory shortening of transversus abdominis during BASE, PEAK, PLATEAU, and RECOVERY. Y-axis shows muscle shortening per breath as percent change from baseline resting muscle length at end inspiration, %LBL. Columns show group mean values with standard error bars. Numerical labels are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on TA SHORT, P<0.0001. ††, significant difference between individual periods at P<0.01.
Figure 48: Transversus abdominis EMG activity during room air and sustained isocapnic hypoxia

Transversus Abdominis EMG

Group mean transversus abdominis EMG activity during sustained isocapnic hypoxia (20-25 min) in twelve animals (N=12). Transversus abdominis EMG (TA EMG\text{DIFF}). Tidal expiratory EMG activity of the transversus abdominis during periods of BASE, PEAK, PLATEAU, and RECOVERY. Y-axis shows muscle EMG activity per breath as maximum difference between baseline and peak height of the integrated moving average EMG signal in volts. Columns show group mean values with standard error bars. Numerical labels are means expressed as a percent of baseline room air ventilation (%BASE). Significance of the overall effects of hypoxia on TA EMG\text{DIFF}, P<0.0001. †, significant difference between individual periods at P<0.01.
### Table 6: Oxygen saturation, end tidal CO₂ and breathing pattern during room air and sustained isocapnic hypoxia
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<tr>
<td>SpO₂ (%)</td>
<td>92.91 ± 2.26</td>
<td>79.74 ± 3.91</td>
<td>78.85 ± 3.86</td>
<td>90.19 ± 2.95</td>
<td>&lt; 0.0001</td>
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<tr>
<td>P&lt;sub&gt;ET&lt;/sub&gt;CO₂ (mmHg)</td>
<td>37.92 ± 2.42</td>
<td>37.13 ± 3.73</td>
<td>38.56 ± 4.10</td>
<td>35.48 ± 4.20</td>
<td>0.0490</td>
</tr>
<tr>
<td>V₁ (l/min)</td>
<td>7.50 ± 2.69</td>
<td>17.33 ± 5.13</td>
<td>13.54 ± 5.69</td>
<td>6.81 ± 2.38</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Vₜ (l)</td>
<td>0.39 ± 0.06</td>
<td>0.69 ± 0.15</td>
<td>0.51 ± 0.11</td>
<td>0.34 ± 0.07</td>
<td>&lt; 0.0001</td>
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<tr>
<td>fₚ (breaths/min)</td>
<td>19.65 ± 6.81</td>
<td>25.33 ± 5.88</td>
<td>26.60 ± 9.78</td>
<td>20.55 ± 7.70</td>
<td>&lt; 0.0001</td>
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<td>T₁ (s)</td>
<td>1.38 ± 0.38</td>
<td>1.20 ± 0.27</td>
<td>1.14 ± 0.36</td>
<td>1.34 ± 0.48</td>
<td>0.0021</td>
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<tr>
<td>Tₑ (s)</td>
<td>2.06 ± 0.79</td>
<td>1.34 ± 0.36</td>
<td>1.46 ± 0.63</td>
<td>2.07 ± 0.91</td>
<td>&lt; 0.0001</td>
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<td>Vₜ/T₁(l/s)</td>
<td>0.30 ± 0.08</td>
<td>0.59 ± 0.14</td>
<td>0.49 ± 0.17</td>
<td>0.28 ± 0.08</td>
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<td>Tₑ/TₜOT (ratio)</td>
<td>0.41 ± 0.05</td>
<td>0.48 ± 0.03</td>
<td>0.45 ± 0.04</td>
<td>0.40 ± 0.03</td>
<td>&lt; 0.0001</td>
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Table 6 (table legend)

SpO₂, pulse oximeter O₂ saturation; P<sub>ET</sub>CO₂, partial pressure of end tidal CO₂; V<sub>r</sub>, minute ventilation; V<sub>T</sub>, tidal volume; f<sub>r</sub>, respiratory rate; T<sub>Ι</sub>, inspiratory time; T<sub>E</sub>, expiratory time; V<sub>T</sub>/T<sub>Ι</sub>, mean inspiratory flow; T<sub>Ι</sub>/T<sub>TOT</sub>, inspiratory fraction of respiration. Values are mean ± SD for N=12 canines during BASE, 5 min room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; PLATEAU, final 5 min at SpO₂ of 80 ± 2%; RECOVERY, 5 min room air breathing after hypoxia. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Comparisons: SpO₂ (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, PEAK-PLATEAU, NS); P<sub>ET</sub>CO₂ (BASE-PEAK, PEAK-PLATEAU, BASE-PLATEAU, BASE-RECOVERY, NS; PLATEAU-RECOVERY, P<0.05); V<sub>r</sub>, V<sub>T</sub>, V<sub>T</sub>/T<sub>Ι</sub> (BASE-PEAK, PEAK-PLATEAU, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; BASE-RECOVERY, NS); f<sub>r</sub> (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, BASE-RECOVERY, NS); T<sub>Ι</sub> (BASE-PEAK, BASE-PLATEAU, PLATEAU-RECOVERY, P<0.05; PEAK-PLATEAU, BASE-RECOVERY, NS); T<sub>E</sub> (BASE-PEAK, BASE-PLATEAU, PLATEAU-RECOVERY, P<0.01; PEAK-PLATEAU, BASE-RECOVERY, NS); T<sub>Ι</sub>/T<sub>TOT</sub> (BASE-PEAK, PLATEAU-RECOVERY, BASE-PLATEAU, P<0.01; PEAK-PLATEAU, P<0.05; BASE-RECOVERY, NS).
Table 7: Transversus abdominis shortening, EMG activity and resting length during room air and sustained isocapnic hypoxia
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<td>SHORT (%L_BASE)</td>
<td>2.41 ± 0.69</td>
<td>5.86 ± 2.57</td>
<td>2.74 ± 0.62</td>
<td>1.91 ± 0.87</td>
<td>&lt; 0.0001</td>
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<tr>
<td>EMG_DIFF (volts)</td>
<td>0.58 ± 0.14</td>
<td>2.71 ± 0.80</td>
<td>0.88 ± 0.34</td>
<td>0.44 ± 0.16</td>
<td>&lt; 0.0001</td>
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<tr>
<td>Length (L_BASE, mm)</td>
<td>11.27 ± 3.59</td>
<td>11.12 ± 3.51</td>
<td>10.99 ± 3.57</td>
<td>10.94 ± 3.31</td>
<td>0.0757</td>
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Table 7 (table legend)

TA, transversus abdominis; SHORT, shortening per breath expressed as a percentage change from baseline resting muscle length at end-inspiration, %L_{BL}; EMG_{DIFF}, EMG activity per breath quantified as the maximum difference between baseline and peak height of the integrated moving average EMG signal in volts; Length (L_{BL}), baseline end-inspiratory resting muscle length in mm. Values are mean ± SD for N=11, TA SHORT and L_{BL}; N=12, TA EMG_{DIFF} during BASE, PEAK, PLATEAU, and RECOVERY. Right column reports the significance of the overall effects of hypoxia; NS, non-significant. Other conventions as Table 6. Comparisons: TA SHORT (BASE-PEAK, PEAK-PLATEAU, P<0.01; PLATEAU-RECOVERY, BASE-PLATEAU, BASE-RECOVERY, NS); TA EMG_{DIFF} (BASE-PEAK, PEAK-PLATEAU, P<0.01; PLATEAU-RECOVERY, BASE-PLATEAU, BASE-RECOVERY, NS); TA Length (BASE-PEAK-PLATEAU-RECOVERY, all NS).
Conclusion

The investigative and developmental work undertaken within this thesis research project pertaining to the central theme of sustained hypoxia, and the resultant new findings and insights embodied within this treatise, are direct evidence of original scholarship. This concluding section highlights the original contribution to research and knowledge that have culminated from this thesis work.

Claims for Original Research

1. The first successful chronic implantation of sonomicrometry transducers and fine wire bipolar EMG electrodes for the study of respiratory muscle function during sustained hypoxia in any intact mammal

2. The first direct, intramuscular, simultaneous measurement of length and EMG activity of the primary inspiratory and expiratory muscles, namely the costal and crural diaphragm, parasternal intercostal, and transversus abdominis, during and immediately following sustained hypoxia

3. Evidence that typical mammalian biphasic ventilatory response to sustained hypoxia is clearly expressed in spontaneously breathing, awake canines, with an intact upper airway. Our results directly contest the current literature understanding that canines do not exhibit a biphasic ventilatory decline or roll-off during sustained hypoxia

4. The first report demonstrating the biphasic segmental contraction and neural activation pattern of the costal and crural diaphragm in response to sustained hypoxia. Specifically, this work confirmed, by direct measurement, that both diaphragm segments roll-off during sustained hypoxia, and thus contributing to the changes in ventilation
5. Direct evidence that costal and crural diaphragm can function as separate muscles exhibiting distinct differential segmental activity in response to initial and sustained hypoxia, as well as post-hypoxic room air recovery. The prominent neuromechanical disassociation of the crural segment compared to the costal, may reflect dynamic alterations in crural segmental length to enhance/preserve the pre-contraction resting length of costal diaphragm during sustained hypoxia.

6. Demonstration that the parasternal chest wall inspiratory muscle concurrently rolls-off with ventilation during sustained hypoxia. Biphasic contractile shortening and EMG activity response of the parasternal intercostal with persistent hypoxia indicates an attenuation of central drive extending to the primary chest wall inspiratory muscle.

7. Evidence that neuromechanical efficiency, as assessed by the relative relationship of muscle shortening and EMG activity, of the parasternal chest wall inspiratory muscle is fundamentally altered during sustained hypoxia, with an enhancement/amplification of mechanical efficiency of the parasternal with initial hypoxia which is subsequently lost with constant hypoxia.

8. Direct assessment of mechanical action along with neural activation of the primary abdominal expiratory muscle, the transversus abdominis, during sustained hypoxia. Contrary to classic studies, in intact awake animals representative of a normal physiologic state, we reveal that expiratory activity is markedly recruited with hypoxia, however, if sustained, hypoxia causes a significant inhibition of expiratory activity.

9. Evidence that hypoxia elicits both an excitation and inhibition of expiratory activity indicating that much of the past discordance of expiratory activity with hypoxia; inhibition, excitation or no change may be largely accounted for by this dual and opposing influence of hypoxia, when coupled with other confounding factors modulating this natural integrated expiratory activity response.
10. Evidence of differential central attenuation of neural drive affecting the primary inspiratory and expiratory muscles during sustained hypoxia. Specifically, relative roll-off in muscle shortening/EMG activity was noted to be distinct for the costal and crural diaphragm, parasternal intercostal, and transversus abdominis.

11. The first report of the persistent inhibitory effects of hypoxia affecting the neuromotor output and mechanical activity of the primary inspiratory and expiratory muscles, and its associated effects on ventilation, upon restoration of recovery room air breathing immediately following sustained hypoxia.

12. The relative relationship between muscle shortening and EMG activity of the primary respiratory muscles was assessed in this work during and after sustained hypoxia. We found that relative change in EMG activity generally correlates with mechanical output, however, the two important muscle parameters assessing muscle function were certainly not equivalent or identical. These results suggest that precise and optimal assessment of individual respiratory muscle function necessitates a measurement of neural/electrical activation along with another direct measurement of the corresponding mechanical change, such as muscle length.

13. The in-house development of a brand new data acquisition software permitting continuous, high-precision, real-time, data recording and storage of biological signals for review and analysis on state-of-the-art operating system (Window 7/8 and beyond) and computing platform (32/64-bit). The new data acquisition program developed by the candidate was employed to carry out bulk of the data analysis presented in the current thesis, and has been actively utilized in the lab for ongoing, as well as new, respiratory physiology research in humans and animals.

14. The first hand experimental and data acquisition system setup for sustained hypoxia research in awake canines; as well as for ongoing novel, clinically relevant, physiology research in patients with respiratory disease. In the latter research, the
candidate's data acquisition program allowed for the first successful continuous recording and examination of 12 KHz+ raw parasternal EMG activity from severe COPD patients using fine wire EMG electrodes. This high resolution raw EMG signal recording permits for the evaluation of parasternal muscle activity at the fundamental level of individual motor units (this work is summarized in the final section of this thesis, Concurrent and Future Studies: Beta Agonists)
Concurrent and Future Studies

Graduate work in a research laboratory involving both chronic animal and human physiology studies has been a fruitful endeavor. During the course of this graduate training, the candidate's research contribution has been greatly expanded beyond this thesis work to include additional studies involving humans and animals in collaboration with current members of the research lab, including clinical fellows and graduate students. In addition, the new data acquisition software, DataSponge™-7, has been actively employed to carry out our latest clinical respiratory physiology investigations involving severe COPD patients, and to support the data review and analysis of concurrent projects occurring in the lab. Thus far, this collective effort has led to ten published abstracts and international conference presentations (either as first or second author), with a similar number of manuscripts underway at various stages of preparation and publication. Some of this research is highlighted in this section in conclusion of this thesis.

Sustained Hypoxia

Our interest in sustained hypoxia and its effects on respiratory muscle control and function continues. The latest research in the chronically instrumented canine preparation during sustained hypoxia has involved two investigative studies exploring the effects of persistent hypoxia on diaphragm postinspiratory inspiratory activity (PIIA) and pre-contraction baseline resting length, respectively. These new lines of physiologic inquiries and investigations have directly stemmed from the present thesis work on the costal and crural diaphragm, and has resulted in two abstracts and conference presentations (Ji et al., 2013, Ji et al., 2014a). The preliminary findings resulting from the PIIA work in awake canines are summarized below.
Diaphragm postinspiratory inspiratory activity (PIIA) with sustained hypoxia in awake canines

It is well-recognized that each breath consists of three distinct phases: inspiration, postinspiration and expiration. Postinspiration, or early expiration, is an active neural inspiratory event that is discrete and separate from the initial inspiratory activity, and thought to be independently mediated by the central respiratory controller. From a functional perspective, postinspiratory activity has been interpreted to cause braking of expiratory airflow during early-expiration via certain inspiratory muscles. However, postinspiratory activity, i.e. "expiratory braking" or lengthening (eccentric) contraction, may not be universal and may be undesirable in some muscles.

Since little is known about postinspiratory inspiratory (PIIA) of the diaphragm during hypoxia, especially when sustained, we investigated the impact of sustained hypoxia on the PIIA of the costal and crural segments of the diaphragm in chronically instrumented canines. Figure 49 (next page) illustrates the segmental length change and EMG activity of the two diaphragmatic segments in a representative animal during room air and sustained isocapnic hypoxia, corresponding to the periods of BASE, PEAK and PLAT similar to those presented throughout this thesis. For clarity of presentation the post-inspiration epoch from end inspiration extending to late/end of expiration is noted by the two vertical lines for each of the traces during BASE, PEAK and PLAT. Of note, the distinct activity profile between the two diaphragmatic segments with visibly greater EMG PIIA of the crural compared to the costal at each individual period, with corresponding segmental length change showing a slower rate of muscle relaxation/lengthening when accompanied by EMG PIIA.
Figure 49: Postinspiratory diaphragm segmental length and EMG activity during room air and sustained isocapnic hypoxia

Figure 49 (figure legend)

Representative tracing of inspiratory airflow, costal diaphragm segmental length (COS Length) and integrated moving average EMG activity (COS Mavg EMG), and crural diaphragm segmental length (CRU Length) and integrated moving average EMG activity (CRU Mavg EMG) during room air and sustained isocapnic hypoxia. Traces shown are breaths during BASE, room air breathing before hypoxia; PEAK, first 3 min after \(O_2\) saturation (\(SpO_2\)) decreased to \(80 \pm 2\%\); and PLATEAU, final 5 min at \(SpO_2\) of \(80 \pm 2\%\). Each tracing captures a single breath within each period with corresponding start time (min:sec) and \(SpO_2\) (%) noted at the bottom. \(L_{BL}\) indicates baseline resting muscle length at end-expiration. Post-inspiration is indicated between the vertical lines.
The group mean diaphragm postinspiratory segmental shortening with sustained isocapnic hypoxia is illustrated in Figure 50, normalized as an intrabreath profile (as detailed in the General Methods: Intrabreath Analysis) which allows for direct "within breath" comparison of segmental PIIA shortening of the costal and crural diaphragm, despite changes in respiratory timing and/or peak shortening activity. The two vertical lines denote the post-inspiration epoch from end inspiration to mid expiration. Within-breath shortening clearly revealed PIIA for the crural segment during BASE room air, but minimal for the costal segment. During sustained isocapnic hypoxia, PIIA of both costal and crural shortening increased significantly from BASE to PEAK (P<0.05 at 60% T\textsubscript{TOT}), and then remained elevated through PEAK to PLATEAU (NS at 60% T\textsubscript{TOT}). Moreover, crural shortening PIIA was always visibly greater than corresponding costal shortening PIIA at each individual periods of BASE, PEAK and PLATEAU.

**Figure 50: Normalized diaphragm postinspiratory segmental shortening during room air and sustained isocapnic hypoxia (legend on next page)**
Figure 50 (figure legend)

Group mean intrabreath profiles of inspiratory airflow (N=10) and costal and crural diaphragm segmental shortening (N=8 and N=10, respectively) during sustained isocapnic hypoxia. Normalized intrabreath values averaged for all respiration during BASE, room air breathing before hypoxia; PEAK, first 3 min after O₂ saturation (SpO₂) decreased to 80 ± 2%; and PLATEAU, final 5 min at SpO₂ of 80 ± 2%. Y-axis shows standardized values for shortening as percent of maximum shortening per individual breath. X-axis marks mean values for each muscle standardized per 5% of total breath time (%T_TOT). Post-inspiration is indicated between the vertical lines.

Our data indicates that hypoxia elicits a prominent PIIA shortening, i.e. lengthening (eccentric) contraction, of the diaphragm, which does not resolve when hypoxia is sustained. This occurred even in the costal diaphragm which has minimal muscle spindle content or proprioceptive feedback for protection. This deviation of normal costal diaphragm function with sustained hypoxia may be potentially strenuous leading to muscle fiber breaking and damage, if it becomes chronic. We postulate that chronic eccentric contraction of spindle poor inspiratory "pump" muscles leading to muscle damage and dysfunction may be the underlying cause of frank respiratory failure.

In this context, postmortem histological studies of the examination of muscle fibrils in a chronically hypoxic induced animal or in patients who have experienced mild-moderate repetitive or continues hypoxic respiratory failure, accompanied by prior repeated observations of PIIA EMG data from the diaphragm or other non-spindle rich inspiratory muscles such as the parasternal intercostals, would shed light on the relationship of PIIA and muscle fiber damage. We could also accompany the longitudinal EMG PIIA data recording sessions with the force generating capacity of the diaphragm via phrenic nerve stimulation to assess the contractile function of the diaphragm and its relationship with the sustained PIIA.
In addition, monitoring of PIIA and assessment of diaphragm force output in the intensive care setting may also reveal clinically significant information about the clinical state and progression of critically ill patients, as it relates to the function and dysfunction of respiratory muscles underpinning the core science of respiratory failure. EMG data, not just PIIA, could also provide differential information as to the population of patients who are or unable to be weaned from the mechanical ventilation - in other words, which patient group will recover from frank respiratory failure? which would not?

With the capacity of the latest DataSponge™7 to record high-precision, continuous, raw EMG data, we can also envision undertaking an in-depth analysis of respiratory muscle EMG activity at the fundamental level of the individual motor units (i.e. frequency characteristics and recruitment pattern), as it relates to the basic science of respiratory muscle function and dysfunction, and a stride towards the investigation of clinically relevant questions and problems. Certainly, more time, critical thinking and developmental work (instruments, software and hardware) will be required to systematically undertake the envisaged scientific and clinically relevant explorations. This is definitely a less charted area of respiratory muscle physiology with the potential to develop as a major line of research focus for an investigator.

**Beta Agonists**

Our latest line of clinical physiology interest and investigative work has been on the effects of ultra long acting beta_2_-agonist (ultra LABA), vilanterol trifenate/ fluticasone furoate (VFF or Breo Ellipta), on ventilation and respiratory muscle function in severe COPD patients. Beta_2_-agonists have been a longstanding interest in our research lab given their capability to enhance respiratory muscle function, as well as their wide use as bronchodilators in COPD.

Using the new data acquisition program, DataSponge™7, we have successfully and continuously measured raw parasternal intercostal muscle EMG activity in severe COPD patients at a very high sampling rate of 12 KHz, with great precision in a
electrically noisy hospital setting. To the best of our knowledge, this is the first time ever recording of raw parasternal EMG activity at such precision in any clinical population.

Raw parasternal EMG activity was measured before and after ultra LABA (post 2 hours, 2 puffs VFF), along with measurements of ventilation, breathing pattern parameters, dyspnea scores, snip nasal inspiratory pressure (SNIP), and isolated magnetic bilateral phrenic simulation to assess diaphragmatic contraction pressure (MagSTIM). For purpose of visual illustration, a representative subject - with airflow, moving average and raw parasternal EMG activity, and the results of our new frequency domain analysis of motor unit action potentials - is shown in Figure 51 (next page).

The collective efforts of our labor has so far resulted in clinically relevant and novel work to generate three published abstracts and conference presentations (Ji et al., 2014b; Jagers, et al., 2014; Easton et al., 2014). This line of investigation on the effects of ultra LABA on respiratory muscle and ventilation is currently ongoing with further experimentation planned in the new year.
Figure 51: Effects of ultra LABA on airflow, parasternal EMG activity and turns rate and centroid frequency parameters

Representative human subject before and after vilanterol trifenate/fluticasone furoate (VFF) on respiratory and parasternal muscle parameters. Traces show airflow and parasternal intercostal moving average EMG activity (PARA Mavg EMG), raw EMG activity (PARA Raw EMG), turns rate (PARA Turns Rate), and centroid frequency (PARA Fc), respectively. Note the marked changes in airflow and all parasternal muscle parameters following 2 hours after VFF (Post 2 hr) compared to baseline no drug (No Drug).
References


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Appendix

Software Programming

The success of this thesis critically relied upon the development and utilization of appropriate software tools to accompany the analysis of the physiologic dataset amassed over the years employing our chronically instrumented awake canine model. Among the pertinent tools, the latest in-house data acquisition and analysis software developed by the candidate, namely DataSponge™7, was extensively employed in this project as well as in concurrent and future projects occurring in the lab, and thus this section describes the work pertaining to the development of this data acquisition and analysis program.

**Data Acquisition and Analysis Software: DataSponge™7**

An essential complementary component of this thesis work involved successive iterations of dedicated and intense computer programming ultimately leading to the development of the latest data acquisition and analysis software, DataSponge™7 (Bioscience Analysis Software, Calgary, AB). This in-house, custom designed, program written by the candidate permits the real-time display and storage, as well as subsequent visualization and analysis, of respiratory/physiological waveforms, including airflow, ECG, mouth pressure, SpO₂, ETCO₂, muscle length and moving average/raw EMG activity from several individual respiratory muscles, and is fundamental to our research involving both human clinical and basic science animal work.

DataSponge™7 incorporates several new features and components unmet by predecessor versions, including: 1) hours of continuous, high-throughput, high-precision, multi-channel, real-time biological signal display and storage (12-bit, 4096 AD value, 16 channels, up to +12 KHz per channel); 2) compatibility with state-of-the-art operating systems (Windows 7/8 and beyond) and computing platforms (32/64-bit architecture); 3) capacity to inspect and process high-resolution raw EMG signals at the level of individual motor unit action potentials (MUAPs); 4) brand new graphical user interface
and design using the Aero Interface, including new toolbars, menus, buttons, etc; and 5) support for the latest line of National Instruments data acquisition drivers (NI-DAQmx 9.x) and analog-to-digital boards.

Developmental work leading to the most recent version and release of the DataSponge™7 (v7.2 released in May 2012) (Figure 52) is a culmination of several years of part-time programming accompanying the commencement of this thesis work. As the thesis project progressed and expanded over the years, so did the extent of the software development work. The key milestones towards of the deployment of the latest data acquisition and analysis software and its expanded capabilities are described in chronological order.

**New DataSponge™7 – Windows 7 AERO Interface (Official Release: 2012)**

![New DataSponge™7 - released as of May 2012](image)

**Figure 52: New DataSponge™7 - released as of May 2012**
Reworking of DataSponge2000/XP

In the beginning, the primary task of the candidate, with prior training in computer science as an undergraduate, was to perform a complete reworking of the predecessor version of data acquisition and analysis software, DataSponge2000/XP (written in C/C++ and complied using visual C++ 4.1) (Figure 53), natively running on a 32-bit Windows 2000/XP operating system (OS).

![Predecessor DataSponge2000/XP – Windows 2000/XP Interface](image)

**Figure 53: Predecessor DataSponge2000/XP - employed prior to 2012**

Note: for contextual background, prior to DataSponge2000/XP there were three other predecessor versions of DataSponge, with two of the earliest running as stand-alone programs on disk operating system (DOS) (written mostly in assembly) and the subsequent successor running on Windows 95/98 OS (written mostly in C).

Although DataSponge2000/XP was operable from a practical standpoint and had been actively employed in the lab, for many years, prior to the candidate joining the lab,
the program itself had several problems and shortcomings, including broken subroutines, bugs and programming errors, unstable throwing of exceptions, and redundant and inefficient code lacking proper documentation. For the most part, DataSponge2000/XP was unstable, which caused unexpected system crashes, and the code did not meet the established programming standards as expected by industry or from the perspective of a professional/individual with a former training in computer programming. To address this deficiency, the project was undertaken to completely rewrite the source code and header files of DataSponge2000/XP to bring it up to a professional standard, with full line-to-line documentation, resolution of errors and bugs, and code optimization to enhance program performance. In total, 112 source code and 135 header files were reworked with each source code ranging anywhere from 50 to 14000 lines of code (LOC). There was no simple approach, each subroutine and its lines of code had to be scrutinized and understood, corrected and optimized, as well as fully documented. The revised version of DataSponge2000/XP was re-written in C/C++ and complied and tested using visual C++ 7.1 (.net 2003). A representative section of source code prior to and after complete revisions are illustrated in Figure 54 and Figure 55, respectively.
BOOL CKalFile::ReadKalFile (CString szFileName)
{
    ASSERT (!szFileName.IsEmpty());
    m_szKalFileName = szFileName;
    m_bLoading = TRUE;
    ReadLastChannel();
    if (m_nLastChannel <= 0 || m_nLastChannel > MAXCHANNELS)
        return FALSE;
    ReadKalVersion();
    CString sstamp;
    CString sbackp = (" ");
    CString slocstring;
    ReadKeyAnnotations();
    ReadCommonInfo();
    ReadChannelSets();
    int zeros = 0;
    for (int d = 0; d < 3; d++)
    {
        if (m_aSetIndex[d].GetSize() == 0)
            zeros++;
    }
    if (zeros == 3)
    {
        AfxMessageBox ("There was an error reading the Channel Sets from the Calibration File. Aborting.");
        Clear();
        return FALSE;
    }
    CChannel* pNewChannel;
    CChannel* pPrevChannel;
    CString szerr;
    double minf, maxf, dFreqs[MAXCHANNELS];
    int hold = 0;
    double intercept;
    for (int i = 0; i < m_nLastChannel; i++)
    {
        hold = m_aKalChannelInfo.Add (new CChannel());
        ASSERT (hold == i);
        pNewChannel = (CChannel*) m_aKalChannelInfo[hold];
        pNewChannel->SetColorCode (ReadChannelColor(i + 1));
        pNewChannel->SetChannelName (ReadChannelName(i + 1));
        pNewChannel->SetBoardInput (ReadBoardInput(i + 1));
        pNewChannel->SetFreq (ReadFrequency(i + 1));
        dFreqs[i] = pNewChannel->GetFreq();
        pNewChannel->SetGain (ReadGain(i + 1));
        pNewChannel->SetKnabledState (ReadKnabledState(i + 1));
        pNewChannel->SetCalibrationState (ReadCalibrationState(i + 1));
        if (pNewChannel->IsCalibrated () == CAL_CaRT) // if its calibrated by points
        {
            pNewChannel->SetNumPairs (ReadNumPairs(i + 1));
            if (!ReadTriplets(i + 1, pNewChannel->m_aTriplets, pNewChannel->
                return TRUE;
            }
            
        }
    
}
Clear();
return FALSE;
}
pNewChannel->SetRCoord(ReadRCoord(i+1));
}
szlocstring = ReadCoeff(i+1);
sscanf(szlocstring, GetRafffer(0),
"%lf %lf %lf %lf %lf", &pNewChannel->m_dCoeff[i],
&pNewChannel->m_dCoeff[i+1], &pNewChannel->m_dCoeff[i+2],
&pNewChannel->m_dCoeff[i+3], &pNewChannel->m_dCoeff[i+4],
&intercept);
pNewChannel->SetIntercept(intercept);

// guidelines
int low = ReadGuideLow(i + 1), high = ReadGuideHigh(i + 1);
// guidelines either have to be both non -1 or both -1
BOOL bErr = FALSE;
if(low == -1)
{
    if(high != -1)
        bErr = TRUE;
}
else if(low != -1)
{
    if(high == -1)
        bErr = TRUE;
}
if(bErr)
{
    sserr.Format("The Guidelines for Channel-%d are invalid. Please fix this channel.", i + 1);
    AddMessageBox(sserr);
    Clear();
    return FALSE;
}

// now set the guidelines and create a chanscale if need be
pNewChannel->SetUnits(ReadUnits[i + 1]);
pNewChannel->SetGuides(low, high);
if(pNewChannel->IsCalibrated())
    pNewChannel->SetGuideState(ReadGuideState[i + 1],
    ADtoPHYS((double)low, pNewChannel), ADtoPHYS((double)high, pNewChannel));
else
    pNewChannel->SetGuideState(ReadGuideState[i + 1],
    ADtoVIN((double)low, pNewChannel), ADtoVIN((double)high, pNewChannel));
// gets is orientation
pNewChannel->SetCh SCALEOrientation(ReadScaleOrientation(i + 1));

// read in the user values, or default to 0 and 4096
pNewChannel->SetMinMax(ReadMinMax(i + 1));
if(pNewChannel->GetMinMax())
{
    int laser, huser;
    ReadUserRange(i + 1, slaser, shuser);
    pNewChannel->SetUserRange(laser, huser);
}

// read in the offset of the channel
pNewChannel->SetOffset(ReadChAmOffset(i + 1));

Figure 54: Example source code before revision of DataSponge2000/XP
// this function reads and stores the calibration file information from the KAL file
BOOL CKalFile::ReadKalFile(CString s fileName)
{
    // define and initialize variables
    CString s temp; // temporarily string
    CString szback = " "; // space character string
    CString szlocstring; // channel coefficient string
    int zeros = 0; // counter
    CChannel* pNewChannel; // new channel object
    CChannel* pPrevChannel; // previous channel object
    CString szErr; // error message string
    double minf, maxf, dFreqs[MAXCHANNELS]; // variable speed variables
    double intercept; // intercept value (calibration equation)
    int hold = 0; // exception variable

    // ensure .KAL file name to be read is not null
    ASSERT((szFileName.IsEmpty()));

    // set .KAL file name to be read in
    m_szKalFileName = szFileName;

    // set loading flag to indicate .KAL file is being read
    m_bLoading = TRUE;

    // read in last channel number from the .KAL file (equivalent to number of channels)
    ReadLastChannel();

    // number of channels cannot be less than 1 or exceed max channels (16)
    if(m_nLastChannel < 0 || m_nLastChannel > MAXCHANNELS)
    {
        return FALSE;
    }

    // read in version info
    ReadKalVersion();

    // read in the event annotations (once only)
    ReadKeyAnnotations();

    // read in common information (board info, kal notes, etc ...)
    ReadCommonInfo();

    // read in channel sets
    ReadChannelSets();

    // count number of empty channel display sets
    for(int d = 0; d < 3; d++)
    {
        if(m_nSetIndex[d].GetSize() == 0)
            zeros++;
    }

    // there must be at least one channel display set
    if(zeros == 3) {
        AfxMessageBox("There was an error reading the Channel Sets from
            the Calibration File. Aborting.");
    }
}

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Clear();
return FALSE;
}

// read in channel info for each channel
for(int i = 0; i < m_nLastChannel; i++) {

// add new channel info object to channel info array
hold = m_aKalChannelInfo.Add(new CChannel());
ASSERT(hold == i);

// get channel info object
pNewChannel = (CChannel*) m_aKalChannelInfo[hold];

// read and set channel info object with channel color, name, board input, and frequency
pNewChannel->SetColorCode(ReadChannelColor(i+1));
pNewChannel->SetChannelName(ReadChannelName(i + 1));
pNewChannel->SetBoardInput(ReadBoardInput(i + 1));
pNewChannel->SetFreq(ReadFrequency(i + 1));

// store channel frequency into array
dFreq[i] = pNewChannel->GetFreq();

// read and set channel info object with channel gain, enabled state, and calibration state
pNewChannel->SetGain(ReadGain(i + 1));
pNewChannel->SetEnabledState(ReadEnabledState(i + 1));
pNewChannel->SetCalibrationState(ReadCalibrationState(i + 1));

// if the channel is calibrated by data points
if(pNewChannel->IsCalibrated() == CAL_CAL)

    // read and set channel info object with number of pairs
    pNewChannel->SetNumPairs(ReadNumPairs(i + 1));

    // read and set channel info object with calibated points (data pairs)
    if((ReadTriplets(i + 1, pNewChannel->m_aTriplets, pNewChannel->GetNumPairs()))
        Clear();
        return FALSE;
    }

    // read and set channel info object with Rsq (regression value)
    pNewChannel->SetRsq(ReadRsq(i + 1));
}

// read channel coefficients (calibration equation) & intercept value
szLocstring = ReadCoeff(i+1);

// set channel info object with coefficients (calibration equation)
// and store intercept value
sscanf(szLocstring.GetDataBuffer(0),
    "%lf %lf %lf %lf %lf",
    &pNewChannel->m_dCoeff[4],
    &pNewChannel->m_dCoeff[3],
    &pNewChannel->m_dCoeff[2],
    &pNewChannel->m_dCoeff[1],
    &pNewChannel->m_dCoeff[0],
    Intercept);

Figure 55: Example source code after revision of DataSponge2000/XP
Development of DataSponge™7

The second major programming project involved the transition of the revised DataSponge2000/XP acquisition and analysis software to a state-of-art Windows 7 OS and computing platform supporting 32/64-bit systems. This project commenced in early 2010, and involved a period of intense programming and testing to develop the first stable version of DataSponge™7 (v7.1 released in January 2011).

The main developmental task required in porting DataSponge to a new Windows 7 environment involved the work of incorporating the latest National Instruments data acquisition drivers, NI-DAQmx 9.x, in replacement of the older drivers, Traditional NI-DAQ 7.x, which were restricted to the Windows 2000/XP OS environment. This work required the development of the subroutines responsible for the main data acquisition processing loops for real-time data storage and visual display. Since these two subroutines constitute the foundation of our data acquisition and analysis software, the critical portions of the source code are included for reference. A snippet of source code for the main data acquisition processing loop in DataSponge™7 to retrieve data from the A/D board and to write it to permanent storage on disk is shown in Figure 56. Likewise, a fragment of source code depicting the main data acquisition loop to retrieve and draw data on the screen, via invoking a drawing event, is illustrated in Figure 57. Refer to the inline documentation in the source code for a detailed line-by-line description of the code and its critical function for retrieving and processing data from the A/D board.
/* Acquire data continuously from DAQ board's circular buffer (double buffer) until user stops the data collection */

// loop until data acquisition has been completed
// (only if the data collection has been stopped/halted)
while(iStatus == 0 && !DAQstopped) {
    
    //********** NIDAQ_MX CODE check half-buffer ready status **********/
    EnterCriticalSection(&m_cs);

    // retrieve total samples per channel acquired
    Status = DAQmxGetReadTotalSampPerChanAcq(taskHandle, &totSampPerChanAcq);
    LeaveCriticalSection(&m_cs);

    //*************** NIDAQ_MX CODE half-buffer ready - process data *****************/
    // check if enough samples are available to fill the transfer buffer (i.e. samples per channel)
    if(totSampPerChanAcq - SampReadCounter >= numSampPerChan) {
        
        //*************** NIDAQ_MX CODE transfer half-buffer ************/
        // re-calculate last read position by transfer buffer (number of times around the loop with Rate/2)
        DAQmxAbsoluteOffset = (int32)transferBufferSize + numSampPerChan;
        EnterCriticalSection(&m_cs);

        // re-position the Relative To (first sample acquired) and Offset (last read position by transfer buffer)
        // (as the drawing thread may have affected the read position in the background)
        Status = DAQmxSetReadRelativeTo(taskHandle, DAQmx_Val_FirstSample);

        Status = DAQmxSetReadOffset(taskHandle, DAQmxAbsO);ffer_Offset);

        // read samples per channel into transfer buffer
        Status = DAQmxReadBinaryTail(taskHandle, numSampPerChan / Rate/2, NULL, 10.0 /* timeout */, DAQmx_Val_GroupByScanNumber, pTransferBuffer, 
            TransferBufferSize, &numSampReadCh, NULL);

        // retrieve the current read position after transferring data from circular buffer to transfer buffer
        Status = DAQmxGetReadCurrReadPos(taskHandle, &curReadPosTrans);
        LeaveCriticalSection(&m_cs);

        // total number of samples read
        totNumSampReadCh = numSampReadCh * (int32)1NumChans;
    }
}
/* Acquire data continuously from DAQ board's circular buffer (double buffer) until user stops the data collection */

// loop until data acquisition has been completed
// (only if the data collection has been stopped/halted)
while(iStatus == 0 && !iDAQStopped) {
    //********** NI_DAQ_Mx CODE check half-buffer ready status *****/
    EnterCriticalSection(&m_cs);

    // retrieve total samples per channel acquired
    Status = DAQmxGetReadTotalSampPerChanAcq(taskHandle, &totSampPerChanAcq);
    LeaveCriticalSection(&m_cs);

    //********** NI_DAQ_Mx CODE half-buffer ready - process data **********/
    // check if enough samples are available to fill the transfer buffer (i.e. samples per channel)
    if(totSampPerChanAcq - SampReadCounter >= numSampPerChan) {
        //********** NI_DAQ_Mx CODE transfer half-buffer *****/
        // re-calculate last read position by transfer buffer (number of times around the loop with Rate/2)
        DAQmxAlBuffer_Offset = (int32)transferBufferRead * numSampPerChan;
        EnterCriticalSection(&m_cs);

        // re-position the Relative To (first sample acquired) and Offset (last read position by transfer buffer)
        // (as the drawing thread may have affected the read position in the background)
        Status = DAQmxSetRelReadOffset(taskHandle, DAQmx_Rel_FirstSample);
        Status = DAQmxSetRelOffset(taskHandle, DAQmx_AlBuffer_Offset);

        // read samples per channel into transfer buffer
        Status = DAQmxReadBinary16(taskHandle, numSampPerChan /* Rate/2 */, 10.0 /* timeout */, DAQmx_Val_GroupByScanNumber, &pItTransferBuffer, TransferBufferArraySize, &numSampPerChanCh, NULL);

        // retrieve the current read position after transferring data from circular buffer to transfer buffer
        Status = DAQmxGetReadCurrReadPos(taskHandle, &currReadPosTran);
        LeaveCriticalSection(&m_cs);

        // total number of samples read
        totNumSampReadCh = numSampReadCh * (int32)iNumChans;
    }
}

Figure 56: Main data acquisition processing loop for data storage
continuously loop until data acquisition has completed
- only if the data collection has been stopped/halted by user
while(!DAQstopped) {
    EnterCriticalSection(&em_cs);
    // set the RelativeTo and Offset to read the latest sample acquired
    Status = DAQmxGetReadRelativeTo(taskHandle, DAQmx_Val_MostRecentSamp);
    Status = DAQmxSetReadOffset(taskHandle, -1);
    // read the latest sample acquired into memory buffer for each channel
    Status = DAQmxReadBinaryFile(taskHandle, 1 /* single data pt for each channel */,
                                 10.0 /* timeout */, DAQmx_Val_GroupByScanNumber, piMonBuff /* buffer */,
                                 iNumChans /* # of chs */, &numRead, NULL);
    // retrieve the current read relative position (index) after transferring data from
    // circular buffer to memory buffer
    Status = DAQmxGetReadCurrReadPos(taskHandle, &curReadPos);
    LeaveCriticalSection(&em_cs);
    // convert current read relative position (index) to absolute index (i.e. application
    index to access data in the DAT file)
    absCurReadPos = curReadPos + iNumChans;
    /*------------------draw data to screen---------------------*/
    // check to see if drawing can proceed
    // - if currently drawing: the current data points must be skipped
    // - if currently not drawing: the data points can be drawn to screen
    if(!WaitForSingleObject(gEventIsDrawing, 0) == WAIT_OBJECT_0 & !iStatus && !iDAQgetstopped) {
        // ensure that the data point to be drawn is not the last data point drawn
to the screen
        if(absOldReadPos == absCurReadPos | piMonBuff[iNumChans-1] == lastdatum) {
// if new data point is not available, then reset the drawing thread by
// calling gEventIsDrawing.SetEvent();
// - causes all threads wishing to access this event to pass
// gEventIsDrawing.SetEvent();

        } // not the last data point, proceed to drawing data to screen
        else {
            // check to see if data save signal has been received from the “save” event
            // - if save data event is received: must keep track and update the
            //   application index (g_index)
            // - if save data event not received: skip and draw data without
            //   updating the application index
        }
    }
}

302
if(::WaitForSingleObject(gEventSave, 0) == WAIT_OBJECT_0) {

    // circular buffer over flown (i.e. cursor position wraps around):
    if(absCurReadPos < absOldReadPos) {
        // read iNumChans data points
        // - update absolute temp index with the correct offset
        //   (iNumChans) adjusting for cursor position wrap around
        // - ulCount is the actual circular buffer size (should be
          same as the collect thread)
        temp += (absCurReadPos + ulCount /* freq x # of channels */) -
                  absOldReadPos;
    }
    // circular buffer not over flown: continue updating absolute index
    // with number of data points read
    else {

        // read iNumChans data points [i.e. number of channels]
        // - update absolute temp index with the correct offset
        // (iNumChans)
        temp += (absCurReadPos - absOldReadPos);

    }

    // save relative index for drawing data to screen
    // - when drawing, the relative index is converted to absolute
      index values: OnDrawCollect()
    dataObj->index = temp / iNumChans;
    EnterCriticalSection(&m_cs);

    // update application index with relative index
    g_index = temp / iNumChans;
    LeaveCriticalSection(&m_cs);

    } // if not saving data, no need to update the application index

    else

        temp = 0; // reset temp index

    // transfer data from memory buffer to a data object such that it can be
    // drawn to screen
    memcpy(dataObj->data, pMemBuff, (iNumChans) * sizeof(116));

    // set draw event data to draw data to screen by passing the data object
    // (containing the channel data)
    ::PostMessage((HWND)pParam, WM_DRAW_DATA, 0, (LPARAM)dataObj);

    } // skip current data points because data is currently being drawn to screen

    TRACE("Skipped Drawing in DrawDataThread. iStatus = %d, DAQstopped = %d
       oldIndex = %d, newIndex = %d\n", iStatus, iDAQstopped, oldIndex, newIndex);

}
Additional programming work was undertaken to achieve 32/64-bit interoperability for DataSponge™7, permitting greater addressing and storage capacity for continuous, high-speed, real-time storage of data. To this end, 32-bit variable declarations, i.e. uInt32 curReadPosTran, were replaced with their 64-bit equivalents, i.e. uInt64 curReadPosTran; as well, 32-bit type casting were reinstated with 64-bit variants, i.e. SampReadCounter += (uInt64)numSampReadCh in lieu of SampReadCounter += (uInt32)numSampReadCh, throughout the entirety of the source code. Furthermore, new icons, toolbars, images, and menus were designed and incorporated into the latest DataSponge to provide a renewed look and interface compared to its predecessors.

DataSponge™7 was written in C/C++ and compiled using Visual Studio 2010 Professional with the option for 32/64-bit system deployment. With the new NI-DAQmx drivers in place, DataSponge™7 provides for the latest driver software (NI-DAQmx 9.x) and board support from National Instruments to ensure the life and sustainability of our data acquisition and analysis software well into the future. Last but not least, DataSponge™7 being a native 64-bit program will be fully compatible with future Windows OS release (beyond Windows 7/8) as well as 64-bit computing platforms and hardware.
Extending the Capability of DataSponge™7

Another very important software development that was accomplished is the extension of DataSponge™7 to incorporate a data replay and review feature known as the display time ratio (DTR). This crucial feature of the new DataSponge permits the user to customize and manipulate the time base, i.e. sec to msec scale/resolution or vice versa, to effectively display physiologic waveforms/biological signals collected at a high sampling speed, i.e. raw EMG collected at a sampling speed of 3000-4000 Hz. In the predecessor version of DataSponge, this key feature was not available, and thus restricting or limiting the way the user could interact with the program to inspect and analyze any data collected at a high sampling speed. In order to realize this important functionality in DataSponge™7, a well-known data acquisition technique was integrated to effectively down sample fast signals by a positive or negative factor, i.e. DTR, to temporarily decimate (discard) or overwrite (overlay) data samples drawn on the screen without manipulating the actual data stored on disk (Figure 58). As such, the time base is effectively increased from a msec to sec scale to facilitate the visualization of fast sampled waveforms/signals in a breath-by-breath manner. Alternatively, this process of down sampling the fast signal can be reversed to adjust the time base from a sec to msec scale as a means to visualize fast sampled signals at a much higher fidelity and resolution. According, the display time ratio feature has extended the capabilities of datasponge7 to replay and inspect data acquired at a high sampling speed, which is critical for the purpose of raw EMG signal review and analysis of respiratory muscles.
Another distinguishing feature that sets the new DataSponge apart from its predecessors is its superior sampling performance and hours of continuous data/signal recording and playback. In DataSponge™7, an effective sampling rate of 12 KHz+ can be achieved compared to its previous versions limited at ~3000-4000 Hz, which was further restricted to a finite time period lasting several minutes. By exploiting the latest operating system environment (Windows 7 and beyond), multi-core and multi-threading processing (Intel CORE and beyond, with two or more processors), greater addressing (64-bit) and storage space (Gigabytes), and the most up-to-date National Instruments drivers and board support, DataSponge™7 with the appropriate software and hardware optimizations in place can achieve very high sampling speeds for continuous recording and playback of physiological/biological signals for numerous hours from each of the discrete channels supplied by the A/D board (16 channels for the current NIDAQ board) (Figure 59).
DataSponge™ 7 – New Feature: Superior Sampling Speed and Storage

High Throughput and Acquisition Capability & Large Storage Capacity

- 12,000 Hz+ Sampling Rate per channel (Up to 16 channels) [former: 3000-4000 Hz]
- Continuous Raw/Mavg EMG and Biological Signal Recording (Many Hours) [former: Several Minutes]
- Efficient Coding Scheme
- Acquisition Prioritization
- Processor Dedication
- Superior Hardware

Figure 59: New Feature of DataSponge™7 - Superior Sampling and Storage

Last but not least, the new DataSponge with its DTR and very high sampling data acquisition has permitted the visualization and analysis of raw EMG down to the level of individual MUAPs (Figure 60). Using this feature, we have recently exploited the new capabilities of DataSponge™7 to successfully acquire, for the first time, raw parasternal intercostal EMG activity from severe COPD patients at an incredibly fast sampling speed (12 KHz) from a finite population of motor units, via fine wire percutaneous electrodes. With such high resolution data, we have extended our raw EMG analysis in the frequency domain to the level of individual motor units employing turns counting and power spectrum analysis (refer to Concurrent and Future Studies: Beta Agonists for further details).
Beginning in 2012, all new data acquisition and analysis has been carried out employing the latest release of DataSponge™7 (v7.2 released in May 2012), as developed by the candidate. DataSponge™7 and its host of additional features has permitted a more advanced mode of studying the physiologic waveforms acquired from the individual respiratory muscles and has opened up new avenues of research.