THE EFFECT OF TEMPERATURE ON THE CHRONIC HYPOXIA-INDUCED
CHANGES TO pH/CO₂-SENSITIVE FICTIONAL BREATHING IN THE CANE TOAD

(BUFO MARINUS)

by

Sarah E. M. Jenkin

A thesis submitted in conformity with the requirements
for the degree of Master of Science
Graduate Department of Cell and Systems Biology
University of Toronto

© Copyright by Sarah E. M. Jenkin (2011)
The Effect of Temperature on the Chronic Hypoxia-Induced Changes to pH/CO$_2$-Sensitive Fictive Breathing in the Cane Toads (Bufo marinus)

Sarah E. M. Jenkin
Master of Science
Graduate Department of Cell and Systems Biology
University of Toronto
2011

ABSTRACT

This study examined the effects of temperature and chronic hypoxia (CH) on pH/CO$_2$-sensitive fictive breathing, and central pH/CO$_2$ chemosensitivity, in cane toads (Bufo marinus). Toads were exposed to CH (10% or 15% O$_2$) or control conditions (21% O$_2$) for 10 days at either room temperature (controls), 10°C or 30°C following which in vitro brainstem-spinal cord preparations were used to examine central pH/CO$_2$-sensitive fictive breathing (i.e., motor output from respiratory nerves which is the neural correlate of breathing). A reduction in artificial cerebral spinal fluid (aCSF) pH increased fictive breathing frequency ($f_R$) and total fictive ventilation (TFV). Cold temperature reduced and hot temperature increased $f_R$ and TFV under control conditions. CH attenuated fictive breathing independently of temperature. Additional experiments in which the aCSF temperature was varied indicate that the effects of temperature acclimation result from neural plastic changes within respiratory control centres in the brain.
ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Dr. Stephen Reid, without whom this thesis project would not be possible. Dr. Reid, thank you for your support and guidance throughout the course of this project. I also wish to thank Dr. Ken Welch and Dr. Joanne Nash for their input, suggestions and support throughout the entire process. Thank you all for reading drafts, and pointing out the flaws of my work in the privacy of committee meetings and emails. I would also like to thank Dr. Maydianne Andrade and Dr. Rongmin Zhao for supporting me in the final stage of my thesis, and special thanks to Ian Buglass for keeping me on track and helping me with everything university related.

To my lab mates, thank you for assisting me at the various stages of my thesis. Thank you to Sukaina Jaffer and Andrew Peters for being there at conferences, and thank you to Anthony Soluri for providing an entertaining lab environment over the summer.

To my family and friends, thank you for listening to me vent, and for cheering with me when things were going well. It is great to have such amazing, talented people in my life that can listen about craniotomies at the dinner table. Your support is greatly appreciated.

To Mummy and Poppy, thank you for your help with the statistical analysis, for reading countless edits and for allowing me to present in the kitchen numerous times. I am ever grateful for your early morning drop-offs, your late night pick-ups, your encouragement, and your love. I wouldn’t have been able to finish without you two supporting me throughout all the challenges.

To Jacob, thank you for being there. You have helped me see that I am capable, smart and accomplished. I wouldn’t have been able to finish without your help. Thank you for coming in on the weekends and telling me it would all be okay, and for the daily video chats to make sure I was still breathing. You support me fully and completely, and I only hope I can do the same for you.

Finally to Wyeth Consumer Healthcare Inc. Thank you for manufacturing and providing Advil Extra-Strength Liquid Gels to the Canadian Market. I wouldn’t have been able to finish without you!
### TABLE OF CONTENTS

**ABSTRACT** ................................................................................................................................. II

**LIST OF FIGURES** ....................................................................................................................... VII

**LIST OF ABBREVIATIONS** ........................................................................................................... X

**CHAPTER ONE: GENERAL INTRODUCTION** .............................................................................. 1

1.1. Breathing in animals .............................................................................................................. 2
1.2. Breathing in amphibians ......................................................................................................... 3
1.3. Amphibian breathing patterns ............................................................................................... 5
1.4. Control of breathing ............................................................................................................. 7
   1.4.1. Central processes ........................................................................................................... 7
   1.4.2. Olfactory Chemoreceptors .......................................................................................... 10
   1.4.3. Pulmonary stretch receptors ....................................................................................... 11
   1.4.4. Peripheral (arterial) \(O_2/CO_2/pH\)-sensitive chemoreceptors .................................. 12
   1.4.5. Central pH/\(CO_2\)-sensitive chemoreceptors ............................................................ 13
1.5. In vitro brainstem-spinal cord preparations ......................................................................... 15
1.6. Cane toad natural history .................................................................................................... 17
1.7. Exposure to various environmental temperatures and/or chronic hypoxia ..................... 19
1.8. Previous hypoxia studies ..................................................................................................... 22
1.9. Previous temperature studies ............................................................................................. 23
1.10. Hypothesis and Goals ......................................................................................................... 27

**CHAPTER TWO: MATERIALS & METHODS** ............................................................................ 29

2.1. Experimental Animals ......................................................................................................... 30
2.2. Exposure to chronic hypoxia and cold/hot temperatures ................................................... 30
   2.2.1. Exposure to chronic hypoxia ....................................................................................... 30
   2.2.2. Exposure to cold and hot temperatures ...................................................................... 31
2.3. In vitro brainstem-spinal cord preparation ......................................................................... 32
2.4. Experimental protocol ......................................................................................................... 35
2.4.1. Effects of chronic hypoxia and cold/hot acclimation .................................................. 35
2.4.2. Effects of acutely altering aCSF pH on preparations taken from cold and hot
acclimated toads .................................................................................................................. 35
2.5. Data and statistical analysis ................................................................................................. 36

CHAPTER THREE: RESULTS .............................................................................................. 38
3.1. Overview ............................................................................................................................... 39
3.2. Cold temperature .................................................................................................................. 39
  3.2.1. Fictive breathing frequency ........................................................................................... 39
  3.2.2. Fictive breaths per episode and fictive episodes per minute ......................................... 44
  3.2.3. Fictive breath duration and integrated fictive breath area ........................................... 49
  3.2.4. Total fictive ventilation ................................................................................................. 49
  3.2.5. Viability/vitality of the preparation over time ................................................................. 56
3.3. Hot temperature .................................................................................................................... 61
  3.3.1. Fictive breathing frequency ........................................................................................... 61
  3.3.2. Fictive breaths per episode and fictive episodes per minute ......................................... 61
  3.3.3. Breath duration, integrated fictive breath area ............................................................... 66
  3.3.4. Total fictive ventilation ................................................................................................. 71
  3.3.5. Viability/vitality of the preparation over time ................................................................. 72
3.4 The Effects of Hot aCSF on Preparations Taken from Cold Acclimated
Toads and the Effects of Cold aCSF on Preparations Taken from Hot
Acclimated Toads .................................................................................................................. 72
  3.4.1. Fictive breathing frequency ........................................................................................... 72
  3.4.2. Fictive breaths per episode and fictive episodes per minute ......................................... 83
  3.4.3. Breath duration and integrated fictive breath area ........................................................ 88
  3.4.4. Total fictive ventilation ................................................................................................. 93

CHAPTER FOUR: GENERAL DISCUSSION .......................................................................... 96
4.1. Summary of findings ............................................................................................................ 97
4.2. Effect of temperature ........................................................................................................... 98
4.3. Effect of chronic hypoxia ..................................................................................................... 100
4.4. Combined effect of temperature and CH .......................................................................... 100
4.5. Effect of altering aCSF pH ............................................................................................... 103
4.6. THE EFFECTS OF WARM aCSF ON PREPARATIONS TAKEN FROM COLD ACCLIMATED TOADS AND THE EFFECTS OF COLD aCSF ON PREPARATIONS TAKEN FROM WARM ACCLIMATED TOADS ................................................................. 104

4.7. NEURAL PLASTICITY .......................................................................................................................... 105

4.8. IN VITRO VERSUS IN VIVO EXPERIMENTATION ................................................................. 110

4.9. VALIDATION OF THE IN VITRO PREPARATION’S VIABILITY ........................................... 111

4.10. ADDITIONAL POTENTIAL IMPROVEMENTS ........................................................................ 112

4.11. FUTURE WORK .......................................................................................................................... 113

4.12. SIGNIFICANCE OF WORK ........................................................................................................ 114

REFERENCES .................................................................................................................................. 116
LIST OF FIGURES

Figure 1.1: Phases of adult anuran amphibian lung ventilation

Figure 1.2: Comparison of vertebrate breathing patterns

Figure 1.3: A proposed mechanisms for cellular sensing of CO₂

Figure 1.4: A diagram of an adult cane toad brain

Figure 1.5: The geographical range of cane toads (*Bufo marinus*)

Figure 1.6: The effect of chronic hypoxia on fictive breathing in cane toads (*Bufo marinus*)

Figure 2.1: Hourly temperature recordings from greenhouse during the hot acclimation experiments

Figure 3.1: Electroneurogram of vagal motor output from control and CH toads under room temperature, hot and cold conditions

Figure 3.2: Effect of cold temperature and chronic hypoxia on fictive breathing frequency

Figure 3.3: Effect of cold temperature and chronic hypoxia on the number of fictive breaths per episode

Figure 3.4: Effect of cold temperature and chronic hypoxia on the number of fictive episodes per minute

Figure 3.5: Effect of cold temperature and chronic hypoxia on fictive breath duration

Figure 3.6: Effect of cold temperature and chronic hypoxia on the integrated area of fictive breaths

Figure 3.7: Effect of cold temperature and chronic hypoxia on total fictive ventilation
Figure 3.8: Comparison of fictive breathing frequency at an aCSF of 7.8 prior to, and during, the experimental protocol during the cold acclimation, and RT control, experiments

Figure 3.9: Effect of time on fictive breathing frequency during the cold acclimation experiments

Figure 3.10: Effect of hot temperature and chronic hypoxia on fictive breathing frequency

Figure 3.11: Effect of hot temperature and chronic hypoxia on the number of fictive breaths per episode

Figure 3.12: Effect of hot temperature and chronic hypoxia on the number of fictive episodes per minute

Figure 3.13: Effect of hot temperature and chronic hypoxia on fictive breath duration

Figure 3.14: Effect of hot temperature and chronic hypoxia on the integrated area of fictive breaths

Figure 3.15: Effect of hot temperature and chronic hypoxia on total fictive ventilation

Figure 3.16: Comparison of fictive breathing frequency at an aCSF of 7.8 prior to, and during, the experimental protocol during the hot acclimation, and RT control, experiments

Figure 3.17: Effect of time on fictive breathing frequency during the hot acclimation experiments

Figure 3.18A: Effect of hot aCSF on fictive breathing frequency in preparations taken from cold acclimated toads

Figure 3.18B: Effect of cold aCSF on fictive breathing frequency in preparations taken from hot acclimated toads
**Figure 3.19A**: Effect of hot aCSF on the number of fictive breaths per episode in preparations taken from cold acclimated toads

**Figure 3.19B**: Effect of cold aCSF on the number of fictive breaths per episode in preparations taken from hot acclimated toads

**Figure 3.20A**: Effect of hot aCSF on the number of fictive episodes per minute in preparations taken from cold acclimated toads

**Figure 3.20B**: Effect of cold aCSF on the number of fictive episodes per minute in preparations taken from hot acclimated toads

**Figure 3.21A**: Effect of hot aCSF on fictive breath duration in preparations taken from cold acclimated toads

**Figure 3.21B**: Effect of cold aCSF on fictive breath duration in preparations taken from hot acclimated toads

**Figure 3.22A**: Effect of hot aCSF on integrated area of fictive breath in preparations taken from cold acclimated toads

**Figure 3.22B**: Effect of cold aCSF on integrated area of fictive breath in preparations taken from hot acclimated toads

**Figure 3.23A**: Effect of hot aCSF on total fictive ventilation in preparations taken from cold acclimated toads

**Figure 3.23B**: Effect of cold aCSF on total fictive ventilation in preparations taken from hot acclimated toads
LIST OF ABBREVIATIONS

aCSF: Artificial cerebral spinal fluid
ANOVA: Analysis of variance
CA: Carbonic anhydrase
CH: Chronic hypoxia
CO₂: Carbon dioxide
CSF: Cerebral spinal fluid
CT: Cold temperature (10°C)
HT: Hot temperature (30°C)
I-neuron: Inspiratory neuron
IX: Cranial nerve IX (glossopharyngeal nerve)
N₂: Nitrogen
NI: Nucleus isthmi
O₂: Oxygen
pFRG: Parafacial respiratory group
preBötC: Pre-Bötzinger complex
pre-I neuron: Pre-inspiratory neuron
PSR: Pulmonary stretch receptor
RM: Repeated measures
RT: Room temperature (20°C)
SEM: Standard error of the mean
V: Cranial nerve V (trigeminal nerve)
VII: Cranial nerve VII (facial nerve)
X: Cranial nerve X (vagus nerve)
CHAPTER ONE

GENERAL INTRODUCTION
1.1. Breathing in animals

While there are many differences in the functional morphology of the respiratory system across vertebrate groups, the functions of the respiratory system remain the same: ensure adequate oxygen (O₂) is available to the organism, expel carbon dioxide (CO₂) to the external environment and maintain homeostasis of arterial O₂ and CO₂ levels (Wasserman, 1978; Cohn, 1983; Kinkead, 1997). The main driving force behind breathing in water breathers is to obtain O₂ (Gilmour, 2001); while in air-breathing animals, the most important stimulus to breath is the level of CO₂ in the cerebral spinal fluid (Nattie, 1999).

The structures that enable ventilation are highly adapted to exploit the external environment. This is seen most clearly as vertebrate species transition from water breathers to air breathers. For example, fish can exhibit a variety of respiratory structures. The majority of fish are exclusive water breathers, using gills as their respiratory organ. However, some fish are also able to breathe air (Jobling, 1995). Air breathing fish can be divided into obligate and facultative air breathers. Obligate air breathers must breathe air in order to survive. Facultative air breathing fish predominantly rely on gills for water breathing and only transition to air breathing when exposed to adverse respiratory conditions in the water such as hypoxia (a lack of oxygen; Jobling, 1995). Depending upon the species, air-breathing fish can use a wide variety of air-breathing organs, such as buccal and pharyngeal cavities, swim bladders, digestive tracts and lungs (Fänge, 1983; McMahon, 1987; Clements and Long, 2010). Anuran amphibians also use a variety of respiratory organs, especially across their development from tadpole to adult. The transition from a juvenile water-breathing tadpole to an adult air-breathing toad/frog...
involves trading the use of gills and skin to lungs and skin (West and Van Vliet, 1992). In other words, breathing changes from gill and cutaneous respiration to primarily pulmonary respiration (at least for $O_2$) with significant amounts of $CO_2$ lost across the skin (depending upon the species).

1.2. Breathing in amphibians

Anuran amphibians (i.e., frogs and toads) possess a unique system for breathing as they utilize cutaneous (i.e., skin), branchial (i.e., gill) and pulmonary (i.e., lung) respiration or combinations thereof at different stages of development (Burggren and West, 1982). Upon hatching (pre-metamorphosis), aquatic tadpoles utilise cutaneous respiration for approximately 60% of gas exchange and the gills for the remaining 40%, for both $O_2$ uptake and $CO_2$ release. During metamorphosis, the lungs enlarge and develop further while the gills begin to be reabsorbed. Following metamorphosis, when the gills are no longer present, the adult anuran relies on its lungs as the primary site for approximately 90% $O_2$ uptake; however the skin still plays a role in $CO_2$ excretion (Burggren and West, 1982; Pinder and Burggren, 1986; Wells, 2007).

Unlike mammals, which utilize a negative pressure pump (i.e. the diaphragm) to inhale and exhale, air-breathing ectotherms such as lungfish and amphibians utilise a buccal force pump to inflate and deflate the lungs (Jones, 1982). The pulmonary respiratory system of amphibians consists of two internal spaces, the buccal cavity (i.e., the mouth) and the lungs, which are connected in series. The glottis (a muscular sphincter) separates and controls the passage of air between the buccal cavity and the lungs. The buccal cavity receives air from the environment via the nares (i.e., nostrils)
(Jones, 1982). During buccal oscillations (lowering and raising of the buccal floor), the nares are open, the glottis is closed, and air is moved in and out of the buccal cavity with the lowering and raising of the floor of the buccal cavity. As the buccal cavity lacks a vascularised surface, and the air is not able to pass to the lungs (due to the closed glottis), this process is not considered to be involved in gas exchange. However, it has been suggested that these buccal oscillations play a role in calling and olfaction in a variety of species (Druzisky and Brainerd, 2001; Jorgensen, 2000).

A number of studies describe the mechanical events occurring during ventilation in specific amphibian species (West and Jones, 1975; Macintyre and Toews, 1976; Jones, 1982). The process of lung ventilation in anuran amphibians is complex. It begins when the buccal cavity is filled, which is accomplished by lowering the floor of the mouth (buccal cavity), which draws air into the mouth through the open nares. At this point, the buccal cavity and the lungs are isolated from each other by the closed glottis. The air that is drawn into the buccal cavity remains contained in the lower portion of the buccal cavity. The glottis then opens, and the air held in the lungs (from the previous breath or breaths) is forced out into the upper region of the buccal cavity, over the newly inspired air in the lower portion. This expired air from the lungs flows out of the open nares into the atmosphere. The nares then close, and with the glottis still open, the air that is in the lower half of the buccal cavity is pumped into the lungs by a forceful upward contraction of the floor of the buccal cavity. The glottis then closes and completes a single lung breath. In some cases, the buccal oscillations then resume, until the next period of lung ventilation occurs (Stebbins and Cohen, 1995). See Figure 1.1.
With a “typical” breath, the pre-breath lung volume is similar to the post-breath lung volume in resting amphibians (de Jongh and Gans, 1969; Vitalis and Shelton, 1990). However, under conditions of high respiratory drive multiple breaths may occur rapidly in series, resulting in lung inflation (inflation cycle). These conditions can include hypoxic environments, in which the environmental oxygen levels are reduced from normal air levels or hypercapnic environments in which inspired CO₂ levels are elevated. As a result, the post-breath lung volume is greater than pre-breath volume (Vitalis and Shelton, 1990; Sanders and Milsom, 2001). In addition, deflation breaths can also be clustered together to produce a progressive reduction in lung volume (deflation cycle). As a result, the post-breath lung volume is less than pre-breath volume (Reid, 2006). The frequency of inflation and deflation cycles varies among anuran species. Inflation cycles tend to be associated with elevated respiratory drive, defense behaviours and preparation for vocalization. Deflation cycles often follow inflation cycles after a period of apnea (i.e., a period of no breathing) (Gargaglioni and Milsom, 2007).

1.3. Amphibian breathing patterns

In addition to the complexities of multiple gas exchange organs (skin, gills and lungs) and the complex series of events that lead to buccal oscillations, a single lung breath, lung inflation cycles and lung deflation cycles, amphibians also exhibit a variety of breathing patterns (Figure 1.2). Amphibian breathing can range from intermittent single breaths or doublets (two breaths in close succession) that may, or may not, occur at regular intervals. Intermittent, or discontinuous, breathing patterns are common in lower vertebrates, such as air-breathing fish, reptiles and amphibians, and differ greatly from the continuous rhythmic breathing patterns of birds and mammals (Milsom, 1991). The
**Figure 1.1:** Phases of anuran amphibian lung ventilation. Anuran amphibian lungs are ventilated through a sequence of events represented in images A through D. (A) Buccal depression with the nares open and glottis closed draws air into the lower half of the buccal cavity. (B) The glottis then opens allowing air under high pressure in the lungs to exit through the nares. (C) Closure of the nares and elevation of the buccal cavity forces air from the buccal cavity into the lungs. (D) The glottis then closes and the nares open and the cycle either begins again or buccal oscillations occur with no lung ventilation.
discontinuous breathing demonstrated by amphibians is likely due to their reduced metabolic demands, compared to mammals and birds (Gagaglioni and Milsom, 2007). However, if respiratory drive is elevated by external stressors, such as reductions in O₂ levels (i.e., hypoxia), or elevations in CO₂ levels (i.e., hypercapnia), breaths begin to become clustered into discrete episodes separated by periods of apnea (i.e., episodic breathing). If respiratory drive is further elevated, breathing can become continuous, and mimic the breathing pattern that is typically seen in euthermic mammals and birds. However, continuous breathing in amphibians is typically only observed during conditions of extremely high respiratory drive such as moderate to severe hypercapnia (Milsom, 1991; Gargaglioni and Milsom, 2007).

1.4. Control of breathing

1.4.1. Central processes

Numerous studies have shown that breathing in mammals is generated centrally (i.e., within the brain) by two coupled, synchronous respiratory oscillators (i.e., rhythm generators) located within the rostro-ventral medulla. These are the pre-Bötzinger complex (preBötzC) and the parafacial respiratory group (pFRG) (Onimar and Homma, 2003; Feldman and Del Negro, 2006; Wilson et al., 2006). Within the preBötzC are the inspiratory (I) neurons, which fire during inspiration. Pre-I neurons located in the pFRG fire before inspiration and are coupled with the I-neurons of the preBötzC. Due to their relationship, both the pre-I and I neurons play a critical role in respiratory rhythmogenesis (Gargaglioni and Milsom, 2007).
Figure 1.2: Comparison of vertebrate breathing traces. The breathing traces, recorded from a number of vertebrates illustrate the variation in breathing patterns (i.e. continuous and discontinuous). Discontinuous breathing (as seen in amphibians) can occur in random isolated breaths or in clusters of breaths (episodes). Figure reproduced (with permission) from Milsom, 1991.
Similar paired rhythmogenic sites have also been found in non-mammalian species. Wilson et al. (2002) discovered two rhythmogenic sites located within the ventral medullary reticular formation in anuran amphibians. One site, critical for lung ventilation, is located between the VII\textsuperscript{th} (facial) and the IX\textsuperscript{th} (glossopharyngeal) cranial nerves. A second site, required for buccal oscillation regulation, is found at the level of the X\textsuperscript{th} (vagus) cranial nerve. Interaction between these two oscillators (i.e., lung and buccal/gill oscillators) generates the overall respiratory rhythm that underlies lung ventilation and buccal oscillations (Vasilakos et al., 2006; Gargaglioni and Milsom, 2007).

Further similarity between mammalian and amphibian respiratory rhythmogenesis is demonstrated in the response of each respective pair of neural oscillators to application of opioid receptor agonists. Takeda et al. (2001) showed that preBötzC neurons are depressed by opiates while pFRG/pre-I neurons are not affected. Similar results are observed between the buccal and lung oscillators in amphibians. For example, in frogs, the lung rhythm is inhibited by opioids while the buccal rhythm remains unchanged (Vasilakos et al., 2006). This suggests that the mammalian pFRG area is homologous to the amphibian buccal oscillator and the mammalian preBötzC is homologous to the amphibian lung oscillator (Wilson et al., 2006).

Based on the unique episodic nature of amphibian ventilation, studies have focused on identifying a potential third respiratory-related oscillator that clusters breaths together into episodes (Wilson et al., 2006). Reid et al. (2000) showed that a transection made slightly caudal to the optic chiasm within \textit{in vitro} bullfrog brainstem-spinal cord preparations results in the conversion of an episodic breathing pattern to a continuous
breathing pattern. It was also suggested that the nucleus isthmus (NI) of amphibians (located within mesencephalon between the midbrain and the cerebellum) might be responsible for the clustering of breaths into episodes. However, numerous studies have revealed that the NI is not involved directly in the generation of episodic breathing but, rather, is involved in the integration of CO$_2$ chemoreceptor information which can have an indirect effect on the expression of an episodic breathing pattern (Kinkead et al., 1997; Gargaglioni and Branco, 2004).

Respiratory rhythm is generated by central respiratory centres, and the overall respiratory drive, which is the integration of all peripheral input and central processes within the brain, influences the expression of any particular breathing pattern (i.e. episodic breathing, or continuous breathing). The following sections provide further detail on the role of the peripheral (pulmonary stretch receptors, arterial chemoreceptors, olfactory chemoreceptors) and central (central chemoreceptors) influences on breathing.

1.4.2. Olfactory chemoreceptors

CO$_2$-sensitive olfactory chemoreceptors are found in the nasal epithelium of anuran amphibians and some reptiles (Coates and Ballam, 1990; Coates, 2001). These chemoreceptors inhibit breathing when stimulated by elevated environmental CO$_2$ levels (i.e., ranging from 0.4- 4% CO$_2$) (Coates, 2001). While the mechanism through which the olfactory chemoreceptors sense and respond to changes in CO$_2$ is unclear, evidence suggests that carbonic anhydrase (CA) may play a role in the CO$_2$ sensing transduction pathway (Coates, 2001; Taylor et al., 2003). The role of CA in CO$_2$ chemoreception will be discussed below. Although speculative, it is thought that these olfactory
chemoreceptors serve a defense purpose. If a predator enters a small burrow (containing an amphibian), the exhaled CO$_2$ from the predator inhibits breathing in the amphibian possibly assisting to conceal it from the predator, as respiratory movements would cease (Ballam, 1985).

1.4.3. Pulmonary stretch receptors

Amphibians possess pulmonary stretch receptors, which monitor either the rate or degree of lung inflation. Previous studies have isolated three types of pulmonary stretch receptors, classified as rate (rapidly-adapting), proportional (slowly-adapting) and rate plus proportional receptors (Taglietti and Casella, 1966; Milsom and Jones, 1977; West and Van Vliet, 1992; Gargaglioni and Milsom, 2007). Rate receptors are sensitive to the rate of lung inflation, but not to the extent of lung expansion. In contrast, proportional receptors are sensitive to the degree of lung expansion, but not the expansion rate. Rate receptors increase their discharge during lung inflation and deflation while their level of activity rapidly decreases during sustained inflation (breath holding). On the other hand, proportional receptors increase their discharge during sustained lung inflation (breath holding), but have a delayed response during lung inflation (Reid and West, 2004). Rate plus proportional receptors possess the combined properties of both rate and proportional receptors. This group makes up the most prominent type of pulmonary stretch receptor and is the type of receptor responsible for the classic Hering-Breuer inspiratory termination reflex in mammals.
Peripheral (arterial) O\textsubscript{2}/CO\textsubscript{2}/pH-sensitive chemoreceptors

Peripheral (arterial) O\textsubscript{2} and/or CO\textsubscript{2}-sensitive chemoreceptors are found in all vertebrate classes (Nattie, 2006; Taylor \textit{et al.}, 2010). These receptors provide feedback regarding the oxygen and/or carbon dioxide status of the arterial blood (Lahiri \textit{et al.}, 2001; Lahiri and Forster, 2003). In anuran amphibians, arterial chemoreceptors are located within the carotid labyrinth (considered to be a structure analogous to the mammalian/avian carotid body) and in the aortic arches. Branches of the glossopharyngeal nerve innervate the carotid labyrinth, while the aortic arch is innervated by branches of the vagus nerve (O`Regan and Majcherczyk, 1982). This signifies that both the carotid labyrinth and the aortic arch also play a role in peripheral chemoreception.

Arterial O\textsubscript{2} and CO\textsubscript{2} chemoreceptors have a complex morphology. They are composed of two cell types (Type I and Type II). Type I cells (glomus cells) are the more prominent although the two types are often found to be coupled together. Although Type I cells of the carotid body are the main O\textsubscript{2} sensors within the arterial blood, they are also sensitive to changes in arterial CO\textsubscript{2} and/or pH levels. When stimulated by reduced O\textsubscript{2} levels, elevated CO\textsubscript{2} levels or reduced pH levels in the arterial blood, the Type I cells signal afferent nerve fibres of the carotid sinus nerve which, in turn, report directly to nuclei within the brainstem (Gourine \textit{et al.}, 2005). Under normoxic normocapnic conditions, carotid (and aortic) chemoreceptors provide a baseline of afferent input to central respiratory groups, but after a stimulatory input (such as exposure to hypoxia), the level of activation of the glomus cells increases, and this causes an increase in breathing. Type II cells (sustentacular) structurally support the Type I cells, and are often found...
clumped around the Type I cells (West and Van Vliet, 1992; Gargaglioni and Milsom, 2007).

1.4.5. Central pH/CO\textsubscript{2}-sensitive chemoreceptors

Central respiratory-related pH/CO\textsubscript{2} chemoreceptors are located on the ventral surface of the medulla and are directly exposed to cerebrospinal fluid (Smatresk and Smits, 1991; Nattie and Li, 2006). Studies have shown that there are indeed a number of central chemosensitive sites within the medulla and that they all contribute to the overall ventilatory response (Coates et al., 1993). Unlike peripheral chemoreceptors, central chemoreceptors are only sensitive to changes in pH/CO\textsubscript{2} levels, not O\textsubscript{2} levels.

In all aqueous solutions, changes in the partial pressure of CO\textsubscript{2} (pCO\textsubscript{2}), and indeed CO\textsubscript{2} concentrations as a whole, can bring about changes in pH (and vice versa) via the following reaction:

\[ H_2O + CO_2 \leftrightarrow H_2CO_3 \leftrightarrow H^+ + HCO_3^- \]

The reversible hydration of CO\textsubscript{2} into a proton (H\textsuperscript{+}) and a bicarbonate ion (HCO\textsubscript{3}\textsuperscript{-}) can be accelerated by the actions of the enzyme carbonic anhydrase (CA). CO\textsubscript{2} within the blood enters the cerebrospinal fluid and diffuses into the central chemoreceptor cells (Figure 1.3). Carbonic anhydrase within the chemoreceptor cell catalyses the hydration of CO\textsubscript{2} into a bicarbonate ion (HCO\textsubscript{3}\textsuperscript{-}) and a proton. H\textsuperscript{+} ions within the cell accumulate and initiates ion exchange processes that result in an increase in intracellular Ca\textsuperscript{2+}. This rise in Ca\textsuperscript{2+} triggers the release of neurotransmitters from the cell, which signals respiratory
Figure 1.3: A proposed model for the signal transduction pathway within central pH/CO₂ chemoreceptor cells that is responsible for CO₂ sensing. This process involves CO₂ entering the cerebral spinal fluid from blood (A). The CO₂ then diffuses into the central chemoreceptor cell (B). Within the cell, carbonic anhydrase catalyses the hydration of CO₂ into a bicarbonate ion and a proton (H⁺) (C). The increase in H⁺ within the cell leads to several ion exchange processes, which, in turn, lead to an increase in intracellular calcium (Ca^{2+}) (D). This increase in calcium triggers the release of neurotransmitters (E), which signal the respiratory control centres to increase breathing (F).
control centres to increase breathing (Buckler and Vaughan-Jones, 1993; Peers and Buckler, 1995).

1.5. In vitro brainstem-spinal cord preparations

Previous studies conducted with in vitro isolated brainstem-spinal cord preparations have demonstrated their importance in studying the central control of breathing in amphibians. This preparation not only includes the functioning brainstem and spinal cord but it can (and usually does) also contain the midbrain (Figure 1.4), which is an important area for respiratory control in these animals, and other vertebrates. Briefly, the brainstem-spinal cord (with the midbrain attached) is removed from the animal and superfused in vitro with artificial cerebral spinal fluid (aCSF) gassed with various levels of O₂ and CO₂. The activity of respiratory-related cranial nerves (i.e. nerves that control respiratory muscles) allow for the measurement of fictive breathing (i.e., motor output from these respiratory nerves that is used as an index of breathing). In anuran amphibians, fictive breathing is recorded from either the trigeminal and/or hypoglossal nerves, which stimulate the raising and lowering of the floor of the buccal cavity or the vagus nerve which controls the opening and closing of the glottis (Sakakibara 1984a,b). This preparation allows for the investigation of the central control of breathing by isolating the central nervous system, and thus having a preparation devoid of any peripheral input (Reid and Milsom, 1998).
Figure 1.4: An illustration of the side (A) and the dorsal (B) view of a toad brain showing cranial nerves V (trigeminal), VII (facial), IX (glossopharyngeal) and X (vagus). The location of the nucleus isthmi (NI) is shown between the cerebellum and the optic lobes.
While the in vitro brainstem-spinal cord preparation technique was originally used to study the control of breathing in mammals (Suzue, 1984), it has been adapted for use on a number of animal species including fish (Bongianni et al., 2006), amphibians (Kinkead et al., 1994; McLean et al., 1995a, b; Reid and Milsom, 1998; Torgerson et al., 1998; Hedrick and Morales, 1999; Gheshmy et al., 2006; McAneney and Reid, 2007), reptiles (Keifer and Houk, 1989; Douse and Mitchell, 1990; Johnson et al., 1998) and other mammalian species (Ballanyi et al., 1999; Zimmer and Milsom, 2004), including marsupials (Fong et al., 2009). These in vitro brainstem-spinal cord studies have confirmed that central pH/CO$_2$-sensing chemoreceptors increase fictive breathing in response to low cerebral spinal fluid pH, which is indicative of high blood/CSF CO$_2$ levels (Kinkead et al., 1994; Lahiri and Forster, 2003; Gheshmy et al., 2006). While the in vitro preparation has been used in a variety of species, ectotherms are ideally suited for this type of preparation given that they have lower tissue metabolic rates and greater hypoxia tolerance than, for example, mammals. As a result, these preparations, from amphibians, remain viable for substantially longer periods of time compared to those from mammals (Morales and Hedrick, 2002; Reid and Milsom, 1998). Preparations from cane toads can routinely remain active for 20 hours or more.

1.6. Cane toad natural history

The natural geographical range of cane toads lies approximately between 27°N in Texas and 30°S in Brazil and Argentina (Figure 1.5). However, the geographical distribution of cane toads has expanded due to human influence as cane toads were released in the Caribbean islands, the British Virgin Islands and Jamaica. Florida also has an invasive population of cane toads due to their accidental release. Hawaii and Australia
Figure 1.5: Global distribution of the cane toad (*B. marinus*). The diagonal lines within the Americas represent the natural distribution of the species. The horizontal lines in Australia, Florida and south Asia represent the human influenced expansion of the species.
are faced with a cane toad pest problem, where cane toads were imported as a predator to a species of beetle destroying sugar cane crops. Given their extensive geographical range, it is no surprise that cane toads can be exposed to a wide range of environmental temperatures (Slade and Moritz, 1998).

1.7. Exposure to various environmental temperatures and/or chronic hypoxia

Cane toads are ectothermic and, as such, cannot utilise metabolically produced heat to regulate their body temperature to compensate for changes in environmental temperature. Instead, they conform to the external temperature and utilise behavioural adaptations to survive harsh environmental conditions (Seebacher, 2009). Adult cane toads have a critical thermal maximum of approximately 40°C, and a critical thermal minimum for adult cane toads has been estimated to be between 6°C and 12°C. As a result, they are able to withstand and survive in a wide range of temperatures (Lever, 2001).

Temperature plays a critical role in all physiological processes (Brattstrom, 1979; Seebacher, 2005). Elevating body temperature increases the rate of biochemical reactions, which in turn, increases the rate of physiological and biological processes within the body. As with other ectothermic species, the metabolic rate of amphibians is dependent upon temperature; a decrease in temperature leads to a decrease in metabolism and vice versa. However, temperature can influence metabolism of different amphibian species to differing degrees. The effect of temperature on a process can be quantified by the use of a $Q_{10}$ factor – which expresses the change in the rate of a process with a change in temperature of ten-degrees (Bickler and Buck, 2007). The $Q_{10}$ of metabolic rate for
some amphibians is very high while very low for others. For example, the Q_{10} (20°C – 30°C) of oxygen uptake is 1.6 for the African clawed frog (Xenopus laevis) and 2.7 for the common green frog (Rana erythraea) (Rome et al., 1992). While temperature has a profound effect on physiological processes, it can be very difficult to study in the whole animal. This is primarily due to the fact that whole body changes are the result of the integration of a myriad of changes at the molecular, cellular and tissue level. As temperature can influence each level differently, an integrative approach is critical to understand how temperature influences an organism (Rome et al., 1992).

Seeking shelter from the adverse external conditions is a common behaviour for many animals that inhabit environments with varying temperatures (Stewart and Pough, 1983). For example, to avoid desiccation during the hot and dry summer months, amphibians will seek shelter sites (rock crevices, hollow tree trucks, dense vegetation, burrows) that provide protection from desiccation when access to moist environments is limited (Hoffman and Katz, 1989; Schwarzkopf and Alford, 1996). During periods spent in a burrow, cane toads enter a state of reduced metabolism called aestivation.

Aestivation is defined as a state of inactivity and metabolic reduction in response to high temperatures and reduced water availability (Pinder et al., 1992). In the same way, amphibians respond to seasonally cold temperatures by hibernating or overwintering in shelters (Lemckert, 2004; Yu and Guo, 2010). This strategy minimises the impact of cold temperatures on the animal, and conserves energy when food supply is diminished (Boutilier et al., 1997). The reduced metabolism seen within amphibians during cold exposure does not appear to decrease during hibernation more than would be expected based on the reduction in temperature alone and, as such, the dormant state
induced by cold temperature is likely a simple temperature-induced reduction in metabolic rate (Pinder et al., 1992). While limited work has been done on cane toad overwintering behaviour in the wild, their global distribution does suggest that they would be exposed to lower temperatures seasonally. This is supported by the observation that cane toads can survive external temperatures as low as 6°C.

While dwelling in their shelters to avoid harsh environmental conditions, anuran amphibians can experience hypoxic microenvironments (reduced O₂ levels). Although little data has been recorded on the gas levels of amphibian burrows and shelters, studies have reported O₂ levels to range from 10 – 16% in mammalian burrows (Schaefer and Sadlier, 1979, Boggs et al., 1984). Boland (2004) found that cane toads often exploit pre-existing mammalian burrows, or bury themselves in leaf litter to avoid the surface environment. Given the similarities between the burrows of amphibians and mammals, it is likely that amphibians are also exposed to similar conditions of hypoxia during their aestivation and/or overwintering period.

Hypoxia tolerance is demonstrated in a variety of species across the vertebrate lineage; however, compared to birds and mammals who are usually only exposed to chronically hypoxic conditions at high altitude or, in some cases, in underground burrows, lower vertebrates (i.e., fish, amphibians and reptiles) are often faced with persistent hypoxic environments. According to Bickler and Buck (2007), a species must be able to maintain depressed metabolism, be tolerant to increased metabolic by-products, and avoid injury following re-oxygenation to withstand long-term hypoxia. Highly anoxia-tolerant species (i.e., turtles and carp) use extensive and extreme physiological and behavioural mechanisms to survive in an environment with a complete
lack of oxygen. Hypoxia tolerant species utilise similar mechanisms (often to a lesser
degree than anoxia-tolerant species) to withstand the reduced oxygen in their
environment. For example, amphibians are known to reduce their metabolism while
burrowing in hypoxic environments, so that low levels of oxidative phosphorylation meet
ATP demands (Bickler and Buck, 2007). Amphibians also show increased tolerance to
increased cellular calcium (Ca^{2+}), which is a critical factor leading to anoxia/hypoxia-
induced death in mammals (Hedrick, 2005).

1.8. Previous hypoxia studies

Previous work on breathing in mammals has demonstrated that exposure to long-
term (i.e., chronic) hypoxia results in a phenomenon called ventilatory acclimation to
hypoxia (VAH) (Dempsey and Forster, 1982). VAH manifests as an increase in resting
ventilation, an increase in the sensitivity of the respiratory system to subsequent bouts of
acute hypoxia, and a change in the CO_2 set-point for ventilation. This means there is a
downward shift in the CO_2 level required in the arterial blood at which breathing is
turned on and off. Mammalian species such as humans, rats and goats often require
approximately 7-10 days of hypoxic exposure for VAH to develop and VAH can persist
for a number of weeks upon re-exposure of mammalian species to normoxic conditions
(Reid and Powell, 2005).

A previous study from this laboratory (McAneney et al., 2006) examined whether
amphibians exhibit mammalian-like VAH following exposure to chronic hypoxia in vivo.
Results from that study showed that following acclimatisation to chronic hypoxia (CH;
10% O_2) for 10 days, the acute hypoxic ventilatory response, but not resting ventilation of
cane toads was blunted in comparison to normoxic controls. These results indicate that exposure to chronic hypoxia in amphibians does not lead to mammalian-like VAH, as resting ventilation was unchanged throughout the study and the hypoxic ventilatory response was blunted as opposed to augmented (as is seen in mammals).

Following that study, McAneney and Reid (2007) investigated the effects of CH on central respiratory-related pH/CO₂ chemoreceptors of cane toads by exposing toads, in vivo, to hypoxic conditions (10% O₂) for 10 days followed by experiments using in vitro brainstem-spinal cord preparations to examine the effect of CH on pH/CO₂ chemoreceptor function (i.e., pH/CO₂-sensitive fictive breathing). This study demonstrated that, compared to normoxic toads, toads exposed to CH had a reduced fictive breathing frequency and an overall depressed level of fictive breathing (Figure 1.6).

These previous studies on toads (Bufo marinus) demonstrate that exposure to CH decreases central (brain) pH/CO₂-sensitive fictive breathing, as measured using isolated in vitro brainstem-spinal cord preparations. However, the above studies have been performed on CH animals housed at room temperature. While investigating the impact of hypoxia experienced while burrowing, one must also take into account the effect of relevant environmental temperatures.

1.9. Previous temperature studies

Temperature has a great impact on physiological processes, especially within amphibian systems, as they derive their body heat from the environment (Stebbins and Cohen, 1997). Morales and Hedrick (2002) investigated the effect of temperature on
**Figure 1.6**: Fictive breathing frequency (fictive breaths·min⁻¹) as a function of artificial cerebrospinal fluid (aCSF) pH in chronically hypoxic (CH; closed circles) and normoxic control (open circles) cane toads (*Bufo marinus*). The data are plotted as mean values ± SEM. Letters (a, b, and c) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates a significant difference between CHC and controls. Figure modified from McAneney and Reid (2007).
fictive breathing in the bullfrog (*Rana catesbeiana*). Within this experiment, *in vitro* brainstem spinal cord preparations were exposed to 2% CO$_2$ and a range of temperatures (10 - 30°C). Results showed that fictive breathing activity was present in all toads exposed to temperatures between 15-25°C; however was absent when temperatures were below 15 or above 25°C. This suggests that there is a limited temperature range in which fictive breathing is active in the bullfrog. Another study (Reid and West, unpublished) demonstrated that fictive breathing could be abolished in a similar *in vitro* preparation by lowering the temperature of the aCSF to approximately 5°C for a 2 hour period. However, upon return to an aCSF temperature of 20°C, normal fictive breathing was restored.

Branco *et al.* (1993) investigated the effect of temperature and central chemoreceptor drive on ventilation in the toad (*Bufo paracnemis*). Surgical exposure of the 4$^{th}$ ventricle within the animal allowed for a flow-through perfusion of aCSF to the central chemoreceptor region. Ventilation of the animal under varied temperature and aCSF pH levels was measured using a pneumotachograph – an air-tight mask applied to the animal’s nostrils. Animals were held at 15, 25 and 35°C. A decrease in the aCSF pH produced hyperventilation at all test temperatures, indicating the effect of lowering pH on central chemoreceptor activity is maintained at different temperatures. In addition, the slope of the ventilatory response curve was greatly increased at 35°C (increased central chemoreceptor sensitivity) and decreased at 15°C (reduced central chemoreceptor sensitivity).

Kruhoffer *et al.* (1987) also investigated the effects of temperature and hypoxia on breathing in *Bufo paracnemis*. The toads were kept in a sealed chamber and exposed to a
specific temperature (either 15, 25 or 32°C) for 12 hours prior to and throughout the experiment, and then exposed to various hypoxia levels (5%, 10% and 15% O₂) throughout data collection. Results showed that cold temperature reduced, and hot temperature increased, ventilation at all oxygen levels and the ventilation trends were enhanced by hypoxia (i.e. hypoxic hot toads had further increased ventilation).

Branco and Wood (1993) investigated the effect of temperature on the central chemosensitive areas involved in the ventilatory drive of alligators (Alligator mississippiensis). Drilling a hole through the skull to expose the cephalic portion of the fourth ventricle allowed for the administration and removal of aCSF throughout the experiment. Changes in ventilation were recorded using a pneumotachograph. Branco and Wood (1993) found that changes in aCSF pH altered breathing in these alligators. However, these experiments were conducted at 15, 25, and 35°C, and the results indicated that as temperature was lowered to 15°C, the effect of changing aCSF pH on ventilation was abolished, and that changes in aCSF pH had a greater effect at higher temperatures. The results from this study demonstrate that, in alligators, central chemoreceptor sensitivity is greatly influenced by changes in temperature.

In some reptiles, CO₂-sensitive intrapulmonary chemoreceptors (IPC) are an important respiratory control system. These chemoreceptors, which are not found in other animal groups, decrease their firing rate as CO₂ levels increase. When the animal inspires, the reduced CO₂ level in the inspired air causes an increase in IPC firing rate. This increase in activity serves as an inspiratory-termination reflex in much the same way as pulmonary stretch receptors do in other animals. The activity of these IPC is temperature dependent. Douse and Mitchell (1988) investigated whether the decrease in
the reptilian ventilatory response that occurs during exposure to low temperature is due to an effect of temperature on these CO₂-sensitive intrapulmonary chemoreceptors. Decreasing body temperature of yellow banded tegu lizards (*Tupinambis nigropunctatus*) from 30°C to 20°C, resulted in a decrease in discharge frequency of the IPCs. These results indicate that acute changes in temperature have a significant effect on the discharge pattern of IPC, offering insight into the effect of temperature on the activity of chemoreceptors.

1.10. Hypothesis and Goals

Most air-breathing animals are not normally exposed to low levels of inspired oxygen (hypoxia). However, some species of anurans (frogs and toads) face such conditions while overwintering (underwater or in terrestrial burrows) or during aestivation (in moist, cool burrows) during the summer months (Storey, 2002). While a variety of studies have examined the respiratory systems of amphibians and other vertebrates (Gregg, 1960; Belkin, 1969; Tankersley *et al*., 1996), relatively few studies have examined the effects of long-term (chronic) hypoxia (CH) on amphibian breathing and respiratory control systems.

While varying oxygen levels are a pertinent stressor that anurans are faced with during hibernation and aestivation, other concurrent environmentally relevant stressors, such as temperature, also act on the animal. Building upon the foundation of previous studies of fictive breathing in cane toads, the aim of my thesis is to examine the role of environmentally relevant temperature conditions, (i.e., lower temperatures when
overwintering, or higher temperatures when aestivating) on the chronic hypoxic-induced attenuation of fictive breathing in anuran *in vitro* brainstem-spinal cord preparations.

Given the effects of changing the temperature of the cerebrospinal fluid on the fictive breathing response of other ectotherms (reptiles and amphibians) and based on the direct effect that temperature has on the physiological processes of amphibians, I hypothesise that the fictive breathing response in toads exposed to both chronic hypoxia and normoxic conditions will be mediated by temperature. Therefore if temperature influences central pH/CO₂ sensitivity, then hot temperatures will increase chemosensitivity and cold temperatures will decrease chemosensitivity, which will directly affect the fictive breathing response. Given that hot temperatures increase and cold temperatures decrease fictive breathing (Morales and Hedrick, 2002) and that CH causes an attenuation of both breathing *in vivo* (McAneney *et al.*, 2006) and fictive breathing *in vitro* (McAneney and Reid, 2007), I predict that exposure to CH under cold temperatures would decrease fictive breathing compared to cold temperature normoxic preparations. In addition, I predict that exposure to CH under warm temperatures would reduce the fictive breathing response compared to hot normoxic preparations.
CHAPTER TWO

MATERIALS AND METHODS
2.1. Experimental animals

Adult cane toads (*Bufo marinus*, N = 66; 155 ± 4 g) were obtained from a commercial supplier (Boreal Scientific, St. Catherines, Ontario) and house in fibreglass tanks at the University of Toronto Scarborough. The animals were held at room temperature (20°C) on a 12L:12D photoperiod cycle. Toads were supplied with both terrestrial and aquatic environments. They were fed live crickets or wax worms once a week. Holding conditions and experimental protocols met the approval of the University of Toronto Animal Care Committee and conformed to the guidelines established by the Canadian Council for Animal Care.

2.2. Exposure to chronic hypoxia and cold/hot temperatures

2.2.1. Exposure to chronic hypoxia

Cane toads were exposed to chronically hypoxic (CH) conditions for 10 days. To establish hypoxic conditions, inspired O$_2$ levels were maintained at 10% or 15% O$_2$ (depending upon the experiment; see below) using a ProOx110 control unit (Biospherix, NY, USA). An O$_2$ electrode within the chamber actively measured O$_2$ levels. When the amount of O$_2$ deviated from the set point (10 or 15% O$_2$), the ProOx unit delivered a small amount of N$_2$ into the chamber to stabilise the O$_2$ level at 10 or 15%. The level of 10% O$_2$ (CH) was selected from previous hypoxic studies (Gheshmy *et al.*, 2006; McAneney and Reid, 2007). The level of 15% O$_2$ was selected for the hot acclimation experiments given that the combination of 30°C and 10% O$_2$ produced significant mortality. The hypoxic chamber was maintained at room temperature (20°C) and exposed to a 12L:12D cycle. Normoxic cane toads were exposed to the same temperature and
light conditions, but were exposed to normal oxygen levels (78.09% N₂, 20.95% O₂, 0.003% CO₂, 0.96% other gases). These animals were used as controls.

2.2.2. Exposure to cold and hot temperatures

Cold acclimation was accomplished by maintaining cane toads within the acclimation chamber in a cold room. The cold room was maintained at a constant temperature of 10°C. Chronically hypoxic and control animals were maintained, as described above, under these conditions of cold acclimation.

In order to maintain an environmentally relevant hot temperature, toads were placed in a greenhouse at the University of Toronto Scarborough, during the summer months. Exposure to varying levels of sunlight fluctuated the hourly temperature, but still maintained an average temperature of roughly 30°C (Figure 2.1).

The following environmental conditions as described above were used:

1. Room temperature (20°C); chronic normoxia (21% O₂); n = 8
2. Room temperature (20°C); chronic hypoxia (10% O₂); n = 8
3. Room temperature (20°C); chronic hypoxia (15% O₂); n = 6
4. Cold temperature (5°C); chronic normoxia (21% O₂); n = 8
5. Cold temperature (5°C); chronic hypoxia (10% O₂); n = 8
6. Hot temperature (30°C); chronic normoxia (21% O₂); n = 8
7. Hot temperature (30°C); chronic hypoxia (15% O₂); n = 8
Figure 2.1: The recorded hourly fluctuations in temperature of toads exposed to hot temperature conditions for 10 days. The closed circles represent the hourly temperatures of hot acclimated toads exposed to room temperature aCSF. The open circles represent the hourly temperatures of the hot acclimated toads exposed to cold temperature aCSF (see below for details on this second series of experiments).
2.3. *In vitro* brainstem-spinal cord preparation

Cane toads were anaesthetized by emersion in a solution of 3-aminobenzoic acid ethyl ester (MS222) buffered to pH 7.0 with sodium bicarbonate. Animals were kept in the anaesthetic until the toe-pin and eye-blink reflexes were abolished. Using ronguer forceps, the maxillary, scapular and pro-otic bones of the animal were broken. Using bone shears, the cranial case was removed from the animal and placed into a dissecting dish. The brain was exposed and superfused with ice-cold oxygenated artificial cerebral spinal fluid (aCSF) (in mmol/L; NaCl, 103; KCl, 4.05; MgCl\(_2\), 1.38; glucose, 10; NaHCO\(_3\), 29.2; CaCl\(_2\), 2.45; Sigma Chemicals; pH 7.8, i.e., normocapnic CSF; Taylor *et al*., 2003a,b; Gheshmy *et al*., 2006). Cranial nerves were cut close to their exit from the skull. The preparation was removed from the brain case and placed in a Sylgard-coated dissecting dish and secured with insect pins. The dura matter surrounding the brain and nerves was removed, and the nerves were cut to provide a clean surface for recording. The preparation was then transferred and pinned to a stainless steel mesh of a flow-through chamber. The preparation was continuously superfused with oxygenated aCSF using peristaltic pumps that delivered and removed the aCSF from the chamber. The aCSF was recycled throughout the experiment. The preparation was maintained at pH 7.8 and room temperature for 60 min before commencing the experiment.

Suction electrodes were made from thin-walled capillary glass (1 mm diameter) and pulled to a fine tip using a vertical pipette puller (Kopf model 720; Tujunga, CA). The tips were smoothed using a grinding stone. A suction electrode, held in place with a micro-manipulator, was positioned near the end of the vagus nerve root that was carefully aspirated into the suction electrode. Care was taken to ensure a tight seal was created.
between the nerve and the electrode. Motor output activity from the vagus nerve was recorded in all preparations. In the intact animal, the laryngeal branch of the vagus nerve innervates the glottis, which opens and closes with each breath for lung ventilation (Sakakibara, 1984a,b; Kogo et al., 1997). Since the brainstem-spinal cord preparations lack afferent input and breathing is an inherently rhythmic process generated in the brainstem, all spontaneous rhythmic activity recorded from the vagus nerve was assumed to represent motor output to the respiratory muscles. This nerve activity is the neural correlate of breathing and is termed fictive breathing (Kinkead et al., 1994; Reid and Milsom, 1998; Gheshmy et al., 2006; McAneney and Reid, 2007).

Nerve activity from the suction electrodes was amplified 10X and filtered (30 Hz, high pass; 1 kHz, low pass) using a DAM50 AC amplifier (World Precision Instruments; Sarasota, FL, USA) or amplified 1000X using a CP511 AC amplifier (Grass Instruments; Warwick, RI, USA). The DAM50 AC output was then further amplified by 100X using a second AC amplifier (IS08A, WPI). The amplified, filtered nerve signal was sent to a moving averager (CWE MA821/RSP; CWE Inc., Ardmore, PA, USA; time constant = 200 ms) for integration and to an audio monitor (AM Systems Model 3300; Carlsborg, WA, USA). The amplified/filtered and integrated traces were monitored and stored using a data acquisition system (Biopac Systems, MP150; Goleta, CA, USA). The sampling rate of analogue to digital conversion was 2000 Hz. The aCSF pH was altered by gassing the solution with different CO₂ levels (0-5% CO₂; balance O₂%). The CO₂ and O₂ levels were controlled using digital mass flow controllers (Smart-Trak 100, Sierra Instruments; Monterey, CA, USA). A pH electrode (Mettler Toledo) was used to monitor the aCSF pH.
level. The electrode was calibrated with standard buffers (pH 4.0, 7.0 and 10.0) and placed within the aCSF solution.

2.4. Experimental protocol

2.4.1. Effects of chronic hypoxia and cold/hot acclimation

Following the observation of stable levels of fictive breathing and the 1 hour stabilisation period at an aCSF pH of 7.8, each preparation was exposed to varying levels of aCSF pH [7.4 (hypercapnic), 7.6, 7.8 (normocapnic), 8.0, 8.2 (hypocapnic)]. Each pH level was maintained for 1 hour to allow for the pH to stabilise at the novel level. The different aCSF pH levels were delivered in random order. The pH range used was consistent with previous studies on amphibian brainstem-spinal cord preparations (Gheshmy et al., 2006; McAneney and Reid, 2007). Regardless of the conditions under which the toad was subjected (room temperature, hot or cold), the in vitro experiments (in this series) were performed at room temperature (approximately 20˚C).

2.4.2. Effects of acutely altering aCSF pH on preparations taken from cold and hot acclimated toads

To further investigate if the changes in fictive breathing observed in the normoxic control protocol preparations was simply due to a $Q_{10}$ effect or if the temperature acclimation had caused a fundamental change within central respiratory control systems, two new additional groups of toads were utilised. These toads were exposed to 10 days of either cold (10°C) (n=6) or hot (30°C) temperatures (n=6). The experimental protocol was conducted in the same way as described above with one exception. Instead of the preparation being bathed and maintained at room temperature, the preparation was
superfused with either a cooled (10°C) or heated (30°C) aCSF. That is, after frogs were subjected to chronic exposure to cold temperatures brainstem preparations were placed in hot aCSF and vice versa.

Following the observation of stable levels of fictive breathing and the 1 hour stabilisation period at an aCSF pH of 7.8, each preparation was exposed to the varying levels of aCSF pH described above. For all toads acclimated to the cold temperatures for 10 days (N=6), the aCSF was heated to 30°C using a hot plate for the duration of the experiment. Each pH was maintained for 1 hour. For all the toads acclimated to the hot temperature for 10 days (N=6), the aCSF was cooled to 10°C using an ice bath for the duration of the experiment.

2.5. Data and statistical analysis

A stable 10 minute section of data at each pH level was analysed to determine mean values for fictive breathing frequency (breaths·min\(^{-1}\)), fictive breaths per episode, fictive episodes per minute, breath duration (seconds), integrated fictive breath area (an approximation of breath volume (volts/seconds\(^2\)) and total fictive ventilation (volts·sec·min\(^{-1}\)). The data for the integrated fictive breath area was normalised across all trials for a given individual toad. This was done by selecting the highest mean value of breath area across each toad, and taking a ratio of the areas within each toad across pH levels. The total fictive ventilation index for each trace was calculated as the product of the average fictive breathing frequency and the average integrated breath area. Fictive breaths were less than 1 second in duration, and fictive breaths in a given episode were defined as occurring within two seconds of each other (Kinkead et al., 1994; Reid and
A concern with the *in vitro* brainstem-spinal cord preparation technique is the potential for the brain to have reduced vitality throughout the duration of the experiment. In other words, the motor output recorded from the brain could weaken independently of an effect of aCSF pH over time. As described above, each preparation was exposed to five different pH levels (7.4, 7.6, 7.8, 8.0 and 8.2) in random order and the data were plotted in sequence of increasing pH level. To ensure that time had no effect on the vitality of the fictive breathing response, the data were also plotted sequentially in order of exposure.

Additional steps were taken to confirm the vitality of the preparations. This involved comparing the fictive breathing frequency during the 1-hour stabilisation period (pH 7.8) prior to data collection, with the fictive breathing frequency at pH 7.8 during the data collection protocol. This allowed for a direct comparison between two different exposure times at the same pH level.

All statistical analyses were performed using commercial software (SigmaStat 8.0). Data are reported as the mean ± one standard error of the mean (SEM). A one-way repeated measures analysis of variance (RM-ANOVA) followed by a Holm-Sidak test was used to compare the effects of pH on fictive breathing within each group. The Holm-Sidak post hoc test allowed for follow up multiple comparisons to be completed. The effect of temperature or chronic hypoxia and pH between groups was analysed using a two-way ANOVA. When normality was not passed, the appropriate non-parametric tests were used. The comparison between the fictive breathing frequency at pH 7.8 prior to the
start of the protocol and during the protocol was analyzed using a paired t-test. The limit of significance was 5% (p<0.05).
CHAPTER THREE

RESULTS
3.1. Overview

Figure 3.1 illustrates fictive breathing recorded at an aCSF pH level of 7.4 in brainstem-spinal cord preparations taken from control and chronically hypoxic animals at room, hot and cold temperatures. Chronic hypoxia reduced fictive breathing frequency in all temperature conditions (compare Fig. 3.1A and B; 3.1C and D; 3.1E and F). Exposure to cold temperature reduced fictive breathing compared to control conditions (compare Fig. 3.1A with E). Hot temperature increased fictive breathing to almost continuous levels (compare Fig. 3.1A with C).

3.2. Cold temperature

3.2.1. Fictive breathing frequency

In preparations taken from toads exposed to both room temperature normoxic conditions and room temperature (RT) chronically hypoxic conditions, fictive breathing frequency increased as aCSF pH was lowered (Figure 3.2A; control and CH, \( p<0.001 \)). In both cold temperature (CT) normoxic and cold temperature chronically hypoxic groups, fictive breathing frequency was not significantly changed by aCSF pH (Figure 3.2B; control, \( p=0.343 \); CH, \( p=0.825 \)).

Exposure to chronic hypoxia significantly decreased fictive breathing frequency at all aCSF pH levels compared to control groups in the room temperature group (Figure 3.2A; \( p<0.001 \)). However, there was no significant decrease in fictive breathing frequency in the chronically hypoxic groups compared to control groups in the cold acclimated group, with the exception of the value at pH 7.6 (Figure 3.2B; \( p=0.041 \)). Cold temperature significantly reduced fictive breathing frequency in the normoxic control
**Figure 3.1**: Fictive breathing (motor output recorded from the vagus nerve) recorded from *in vitro* brainstem-spinal cord preparations (at pH 7.4) taken from (A) room temperature normoxic (B) room temperature chronically hypoxic (C) hot temperature normoxic (D) hot temperature chronically hypoxic (E) cold temperature normoxic and (F) cold temperature chronically hypoxic. In all cases the upper trace represents the raw electroneurogram (eng X) recorded from the vagus nerve root while the lower trace (∫eng X) represents the integrated trace.
**Figure 3.2:** Fictive breathing frequency (fictive breaths per minute; breaths•min⁻¹) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8), (B) cold temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

Fictive Breathing Frequency (breaths/min)

B. Cold Temperature

Fictive Breathing Frequency (breaths/min)
groups (compare open circles in Figures 3.2A and 3.2B; \( p < 0.001 \)). Cold temperature significantly reduced fictive breathing frequency in the chronically hypoxic groups at pH levels of 7.6 (\( p = 0.007 \)) and 7.4 (\( p < 0.001 \)) (Compare closed circles in Figures 3.2A and 3.2B).

3.2.2. Fictive breaths per episode and fictive episodes per minute

Fictive breathing frequency is the product of fictive breaths per episode and episodes per minute. Changes in aCSF pH had no effect on the number of fictive breaths per episode in any of the experimental groups (Figures 3.3A and 3.3B; RT normoxic, \( p = 0.727 \); RT CH, \( p = 0.087 \); CT normoxic, \( p = 0.826 \); CT CH, \( p = 0.302 \)). In the room temperature acclimated groups (Figure 3.3A), CH significantly increased the number of breaths per episode at pH levels of 7.8 (\( p < 0.001 \)) and 8.2 (\( p = 0.034 \)). CH did not significantly reduce breaths per episode in cold conditions (Figure 3.3B; \( p = 0.664 \)). Cold temperature did not alter the number of breaths per episode in the normoxic control groups (compare open circles in Figures 3.3A and 3.3B; \( p = 0.824 \)). In the CH groups, cold temperature did not significantly reduce breaths per episode, with the exception of the value at pH 7.8 (compare closed circles in Figures 3.3A and 3.3B; \( p = 0.009 \)).

In preparations taken from both normoxic and chronically hypoxic toads exposed to room temperature (Figure 3.4A), the number of fictive episodes per minute increased as aCSF pH decreased (\( p < 0.001 \)). However, there was no significant change in the number of fictive episodes per minute, as aCSF was altered, in either the normoxic control (\( p = 0.356 \)) or chronically hypoxic (\( p = 0.884 \)) groups acclimated to cold temperatures (Figure 3.4B). The number of fictive episodes per minute was significantly reduced in the RT CH group compared to the RT normoxic group at all aCSF pH levels.
**Figure 3.3:** Fictive breaths per episode as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) room temperature normoxic control toads (n=8) and chronically hypoxic toads (n=8), (B) cold temperature normoxic control toads (n=8) and chronically hypoxic toads (n=8). The data are plotted as the mean values ± SEM. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronically hypoxic groups across the different temperatures.
Figure 3.4: Fictive episodes per minute as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8), (B) cold temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

B. Cold Temperature

Fictive Episodes per Minute

aCSF pH
(Figure 3.4A; \( p < 0.001 \)). In the cold acclimated toads, the number of fictive episodes per minute was not significantly decreased in the chronically hypoxic groups (Figure 3.4B) compared to control groups, except at a pH of 7.4 (\( p = 0.036 \)). Exposure to cold temperature significantly decreased the number of fictive episodes per minute in the normoxic groups (\( p < 0.001 \)). In the chronically hypoxic groups, exposure to cold temperature significantly decreased the number of fictive episodes per minute at pH 7.4 (\( p < 0.001 \)) and pH 7.6 (\( p = 0.033 \)).

### 3.2.3. Fictive breath duration and integrated fictive breath area

Without exception, neither changes in aCSF pH, acclimation to cold temperature nor exposure to CH had any significant effect on breath duration (Figure 3.5) or the integrated area of the fictive breaths (Figure 3.6).

### 3.2.4. Total fictive ventilation

The total fictive ventilation index is the product of fictive breathing frequency and the integrated fictive breath area. In other words, it is an index of the overall level of fictive breathing. The total fictive ventilation index increased significantly as aCSF pH was reduced to 7.4 in the RT normoxic group (Figure 3.7A; \( p = 0.010 \)). On the other hand, there was no significant effect of altering aCSF pH on the total ventilation index in the RT CH group (Figure 3.7A; \( p = 0.130 \)), the CT normoxic group (Figure 3.7B; \( p = 0.516 \)) or the CT CH group (Figure 3.7B; \( p = 0.867 \)). CH significantly reduced the total fictive ventilation index in the room temperature group (Figure 3.7A) at pH 8.0 and lower (\( p = 0.002 \)). CH did not significantly reduce the total fictive ventilation index in the cold
Figure 3.5: Fictive breath duration (s) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8), (B) cold temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

B. Cold Temperature
**Figure 3.6**: The compared ratio of integrated fictive breath area (V•s²) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (N=8) and chronically hypoxic toads (closed circles) (n=8), (B) cold temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

B. Cold Temperature
**Figure 3.7:** Total fictive ventilation (V•s•min$^{-1}$) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8), (B) cold temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

B. Cold Temperature

Total Fictive Ventilation Index (volts sec/min)

aCSF pH

Control

CH

#
temperature acclimated group ($p=0.839$). Cold temperature acclimation significantly reduced the total fictive ventilation index in the normoxic control groups at pH 8.0 and lower ($p<0.001$) and in the CH groups at pH 7.4 ($p=0.040$).

3.2.5. Viability/ vitality of the preparation over time

There was no significant different between fictive breathing frequency at pH 7.8 prior to the start of the protocol (stabilisation period) and the frequency at pH 7.8 during the protocol in the RT normoxic ($p=0.781$) and RT CH ($p=0.938$) groups (Figure 3.8). There was also no significant difference between fictive breathing frequency at pH 7.8 prior to the start of the protocol (stabilisation period) and the frequency at pH 7.8 during the protocol in the CT control ($p=0.781$) and CH ($p=0.831$) groups (Figure 3.8).

As an additional measure to ensure there was also no effect of time on the viability of the preparation, the pH levels were organized sequentially. In other words, rather than plot the data as a function of the actual pH value, the data are plotted as a function of the order of the pH changes that occurred during the data collection. The sequentially ordered data did not show any time-dependent effect on the fictive breathing frequency in the RT normoxic ($p=0.245$) and RT CH ($p=0.811$), and CT normoxic ($p=0.069$) and CT CH ($p=0.76$) groups (Figure 3.9).
**Figure 3.8:** Comparison of fictive breathing frequency during the first exposure to aCSF pH 7.8 (i.e., during the 1-hour stabilisation period) and exposure to aCSF pH 7.8 during the data collection for the room temperature control, room temperature chronically hypoxic, cold temperature control and cold temperature chronically hypoxic groups. Data are plotted as mean values ± SEM. Black bars represent the values recorded at pH 7.8 during the experimental protocol. Gray bars represent the values recorded at pH 7.8 prior to the experimental protocol (during the stabilisation period).
Figure 3.9: Fictive breathing frequency (breaths•min⁻¹) as a function of the sequential order of exposure to the various levels of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) room temperature normoxic control toads (n=8) and chronic hypoxic toads (n=8), (B) cold temperature normoxic control toads (n=8) and chronic hypoxic toads (n=8). The data are plotted as mean values ± SEM. There was no significant difference amongst the sequential order of pH levels in of the groups.
3.3. Hot temperature

3.3.1. Fictive breathing frequency

In both the RT normoxic and RT CH groups, fictive breathing frequency increased as aCSF pH was lowered (Figure 3.10A; control and CH, $p<0.001$). In the hot temperature (HT) normoxic group, fictive breathing frequency increased as aCSF pH was lowered (Figure 3.10B; $p<0.001$). There was no effect of altered aCSF pH within the HT CH group (Figure 3.10B; $p=0.332$). Exposure to chronic hypoxia significantly decreased fictive breathing frequency at all aCSF pH levels, compared to the controls, in both the room temperature (Figure 3.10A; $p<0.001$) and hot temperature groups (Figure 3.10B; $p<0.001$). Hot temperature significantly increased fictive breathing frequency in both the normoxic groups at all pH levels ($p<0.001$) and the chronically hypoxic groups ($p=0.043$) with the exception of pH 7.4.

3.3.2. Fictive breaths per episode and fictive episodes per minute

The number of fictive breaths per episode was not affected by changes in aCSF pH in the RT normoxic group (Figure 3.11A; $p=0.727$), but significantly increased as aCSF pH was reduced in the RT CH group albeit with a biphasic response (Figure 3.11A; $p=0.003$). The number of fictive breaths per episode was not affected by changes in aCSF pH in the HT normoxic ($p=0.757$) or HT CH ($p=0.991$) groups (Figure 3.11B). Exposure to chronic hypoxia significantly increased the number of breaths per episode in the room temperature group (Figure 3.11A) at aCSF pH levels of 7.4 ($p<0.001$) and pH 8.0 ($p=0.002$). Chronic hypoxia had no effect on the number of fictive breaths per episode within the hot temperature groups (Figure 3.11B; $p>0.050$). Exposure to hot temperature
**Figure 3.10:** Fictive breathing frequency (breaths•min$^{-1}$) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=6), (B) hot temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

Fictive Breathing Frequency (breaths/min)

aCSF pH

B. Hot Temperature

Fictive Breathing Frequency (breaths/min)

aCSF pH
**Figure 3.11**: Fictive breaths per episode as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=6), (B) hot temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
had no effect on the number of fictive breaths per episode in the normoxic groups ($p=0.814$). Within the chronically hypoxic groups, exposure to hot temperature caused an increase in the number of fictive breaths per episode at pH levels 7.4 ($p=0.008$) and pH 8.0 ($p=0.033$).

In both the normoxic and the chronically hypoxic room temperature groups, the number of fictive episodes per minute increased as aCSF pH was reduced (Figure 3.12A; $p<0.001$). In the HT normoxic group, the number of fictive episodes per minute significantly increased as aCSF pH was reduced (Figure 3.12B; $p<0.001$); however there was no effect of changing aCSF pH in the HT CH group ($p=0.172$). Exposure to chronic hypoxia significantly reduced the number of fictive episodes per minute in both room temperature (Figure 3.12A; $p<0.001$) and hot temperature groups (Figure 3.12B; $p<0.001$). Hot temperature significantly increased the number of fictive episodes per minute in the normoxic groups from pH 7.4 to 7.8 ($p<0.001$). In the CH group, hot temperatures significantly increased the number of fictive episodes per minute at pH 7.4 ($p=0.013$), pH 8.0 ($p=0.007$) and pH 8.2 ($p=0.008$).

### 3.3.3. Breath duration, integrated fictive breath area

Fictive breath duration was not significantly affected by changes in aCSF pH in any group (Figures 3.13A and 3.13B; RT normoxic, $p=0.385$; RT CH, $p=0.424$; HT normoxic, $p=0.664$; HT CH, $p=0.715$). Breath duration in the room temperature chronically hypoxic group was significantly reduced at pH levels of 7.6 ($p=0.047$) and higher compared to the room temperature normoxic group. Exposure to chronic hypoxia did not significantly reduce breath duration in the hot temperature group.
**Figure 3.12:** Fictive episodes per minute as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=6), (B) hot temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
**Figure 3.13**: Fictive breath duration (s) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=6), (B) hot temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
(Figure 3.13B; \( p=0.921 \)). Hot temperature acclimation did not increase breath duration in the normoxic groups (Figure 3.13A; \( p=0.254 \)). The same is true for the effect of hot temperature on chronically hypoxic preparations, with the exception of pH 8.2 in which case breath duration was elevated by the hot temperature (\( p<0.001 \)).

Without exception, neither changes in aCSF pH, acclimation to hot temperature nor exposure to CH had any significant effect on the integrated area of the fictive breaths (Figure 3.14A, B).

3.3.4. Total fictive ventilation

The total fictive ventilation index increased significantly as aCSF pH was reduced in the RT normoxic group (Figure 3.15A; \( p=0.010 \)) and in the RT CH group (Figure 3.15B) albeit only at pH 7.4 in the CH group (\( p=0.023 \)). The total fictive ventilation index increased significantly as a function of aCSF pH in the HT normoxic group (\( p<0.001 \)); there was no effect of altering aCSF pH in the HT CH group (\( p=0.218 \)). CH significantly reduced the total fictive ventilation index in both the room temperature group at pH levels of 7.4, 7.6 and 8.0 (Figure 3.15A; \( p=0.002 \)) and in hot temperature groups at all pH levels (Figure 3.15B; \( p<0.001 \)). Acclimation to hot temperature increased the total fictive ventilation index at pH 7.6 (\( p=0.045 \)) in the control normoxic groups, and did not significantly increase the total fictive ventilation index in the chronically hypoxic groups (\( p>0.050 \)).
3.3.5. Viability/ vitality of the preparation over time

There was no significant different between fictive breathing frequency at pH 7.8 prior to the start of the protocol (stabilisation period) and the frequency at pH 7.8 during the protocol in the control \((p=0.781)\) and CH \((p=0.938)\) groups in preparations taken from RT acclimated animals (Figure 3.16). There was also no significant different between fictive breathing frequency at pH 7.8 prior to the start of the protocol (stabilisation period) and the frequency at pH 7.8 during the protocol in the control \((p=0.781)\) and CH \((p=0.831)\) groups in HT groups (Figure 3.16).

An additional measure to ensure there was also no effect of time on the viability of the preparation, the pH levels were organized sequentially. In other words, rather than plotting the data as a function of the actual pH value, the data were plotted as a function of the order of the pH changes. The sequentially ordered data did not show any time-dependent effect on the fictive breathing frequency in the RT normoxic \((p=0.245)\) and RT CH \((p=0.713)\) and HT normoxic \((p=0.762)\) and HT CH \((p=0.982)\) groups (Figure 3.17).

3.4 The effects of hot aCSF on preparations taken from cold acclimated toads and the effects of cold aCSF on preparations taken from hot acclimated toads

3.4.1. Fictive breathing frequency

In preparations taken from cold acclimated toads bathed with RT aCSF, the fictive breathing frequency was not affected by changes in aCSF pH (Figure 3.18A; \(p=0.343\)). In preparations taken from cold acclimated toads bathed with hot \((30^\circ C)\) aCSF, there also was no effect of altering aCSF pH on the fictive breathing frequency (Figure 3.20A; \(p=0.101\)). The fictive breathing frequency of cold acclimated preparations bathed with
Figure 3.14: The compared ratio of integrative fictive breath area (V·s²) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=6), (B) hot temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
Figure 3.15: Total fictive ventilation (V·s·min⁻¹) as a function of aCSF pH in preparations taken from (A) room temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=6), (B) hot temperature normoxic control toads (open circles) (n=8) and chronically hypoxic toads (closed circles) (n=8). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. A plus sign (+) indicates significant differences between the control and chronically hypoxic groups within each temperature condition. A number sign (#) indicates a significant difference between control or chronic hypoxic groups across the different temperatures.
A. Room Temperature

B. Hot Temperature

Total Fictive Ventilation Index (volts sec/min)

aCSF pH

7.4  7.6  7.8  8.0  8.2

Control

CH

+    +    +    +    +
Figure 3.16: Comparison of fictive breathing frequency during the first exposure to aCSF pH 7.8 (i.e., during the 1-hour stabilisation period) and exposure to aCSF pH 7.8 during the data collection for the room temperature control, room temperature chronically hypoxic, hot temperature control and hot temperature chronically hypoxic groups. Data are plotted as mean values ± SEM. Black bars represent the values recorded at pH 7.8 during the experimental protocol. Gray bars represent the values recorded at pH 7.8 prior to the experimental protocol (during the stabilisation period).
**Figure 3.17**: Fictive breathing frequency (breaths•min$^{-1}$) as a function of the sequential order of exposure to the various levels of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) room temperature normoxic control toads (n=8) and chronic hypoxic toads (n=6), (B) hot temperature normoxic control toads (n=8) and chronic hypoxic toads (n=8). The data are plotted as mean values ± SEM. There was no significant difference amongst the sequential order of pH levels in of the groups.
Figure 3.18: Fictive breathing frequency (breaths•min⁻¹) as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) normoxic cold temperature-acclimated toads bathed with either RT aCSF (n=8) or hot (30˚C) aCSF (n=6), (B) normoxic hot temperature-acclimated toads bathed with either RT aCSF (n=8) or cold (10˚C) aCSF (n=6). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. No significant difference found between cold acclimated room aCSF and cold acclimated hot aCSF, and hot acclimated room aCSF and hot acclimated cold aCSF.
RT aCSF was the same as that of cold acclimated preparations bathed with hot aCSF ($p=0.095$). Fictive breathing frequency increased in preparations taken from hot acclimated toads regardless of whether they were bathed with RT aCSF ($p<0.001$) or cold ($10^\circ C$) aCSF ($p<0.001$). Fictive breathing was the same in preparations taken from hot acclimated toads regardless of whether they were bathed in RT or cold aCSF ($p=0.095$).

### 3.4.2. Fictive breaths per episode and fictive episodes per min

In preparations taken from cold acclimated toads, reducing aCSF pH had no effect on the number of fictive breaths per episode regardless of whether the preparations were bathed in RT ($p=0.594$) or hot ($p=0.444$) aCSF. The number of fictive breaths per episode in these two groups were similar across all aCSF pH levels ($p=0.102$). (Figure 3.19A)

In preparations taken from hot acclimated toads, a reduction in aCSF pH had no effect on the number of fictive breaths per episode in the preparations bathed with RT aCSF ($p=0.757$), or in the preparations bathed with cold aCSF ($p=0.444$). The number of fictive breaths per episode in these two groups were significantly different at pH 7.4 ($p=0.046$) and pH 7.6 ($p=0.024$) (Figure 3.19B).

In preparations taken from cold acclimated toads, reducing aCSF pH had no effect on the number of episodes per minute regardless of whether the preparations were bathed in RT ($p=0.356$) or hot ($p=0.109$) aCSF. The number of episodes per minute fictive breaths per episode in these two groups were identical across all aCSF pH levels ($p=0.692$) (Figure 3.20A).
**Figure 3.19:** Fictive breaths per episode as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken (A) normoxic cold temperature control aCSF toads (n=8) and normoxic cold temperature hot aCSF toads (n=6), (B) normoxic hot temperature control aCSF toads (n=8) and normoxic hot temperature cold aCSF toads (n=6). The data are plotted as mean values ± SEM. no significant difference found with aCSF pH or between cold acclimated room aCSF and cold acclimated hot aCSF, and hot acclimated room aCSF and hot acclimated cold aCSF.
Figure 3.20: Fictive episodes per minute as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) normoxic cold temperature control aCSF toads (n=8) and normoxic cold temperature hot aCSF toads (n=6), (B) normoxic hot temperature control aCSF toads (n=8) and normoxic hot temperature cold aCSF toads (n=6). The data are plotted as mean values ± SEM. No significant difference found with aCSF pH or between cold acclimated room aCSF and cold acclimated hot aCSF, and hot acclimated room aCSF and hot acclimated cold aCSF.
In preparations take from hot acclimated toads, a reduction in aCSF pH caused an increase in the number of fictive episodes per minute in the preparations bathed with RT aCSF \((p<0.001)\), but not in the preparations bathed with cold aCSF \((p=0.615)\). The number of episodes per minute fictive breaths per episode in these two groups were identical across all aCSF pH levels \((p=0.054)\). (Figure 3.20B).

3.4.3. Breath duration and integrated fictive breath area

In preparations taken from cold acclimated animals, breath duration was not affected by changes in aCSF pH regardless of whether the preparations were bathed with RT \((p=0.512)\) or hot \((p=0.833)\) aCSF (Figure 3.21A). Breath duration was identical in these two groups (i.e., cold acclimated RT aCSF and cold acclimated warm aCSF; \(p=0.779\)).

In preparations taken from hot acclimated animals, breath duration was not affected by changes in aCSF pH regardless of whether the preparations were bathed with RT \((p=0.664)\) or cold \((p=0.681)\) aCSF (Figure 3.21B). Breath duration was identical in these two groups (i.e., hot acclimated RT aCSF and hot acclimated warm aCSF; \(p=0.914\)).

In preparations taken from cold acclimated animals, integrated breath area was not affected by changes in aCSF pH regardless of whether the preparations were bathed with RT \((p=0.895)\) or hot \((p=0.673)\) aCSF (Figure 3.22A). Integrated breath area was identical in these two groups (i.e., cold acclimated RT aCSF and cold acclimated hot aCSF; \(p=0.843\)).
Figure 3.21: Fictive breath duration (s) as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) normoxic cold temperature control aCSF toads (n=8) and normoxic cold temperature hot aCSF toads (n=6), (B) normoxic hot temperature control aCSF toads (n=8) and normoxic hot temperature cold aCSF toads (n=6). The data are plotted as mean values ± SEM. no significant difference found with aCSF pH or between cold acclimated room aCSF and cold acclimated hot aCSF, and hot acclimated room aCSF and hot acclimated cold aCSF.
A. Cold Temperature

Fictive Breath Duration (sec)

7.4  7.6  7.8  8.0  8.2
aCSF pH

Room aCSF
Hot aCSF

B. Hot Temperature

Fictive Breath Duration (sec)

7.4  7.6  7.8  8.0  8.2
aCSF pH

Room aCSF
Cold aCSF
**Figure 3.22**: The integrated fictive breath area ($V \cdot s^2$) as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) normoxic cold temperature control aCSF toads ($n=8$) and normoxic cold temperature hot aCSF toads ($n=6$), (B) normoxic hot temperature control aCSF toads ($n=8$) and normoxic hot temperature cold aCSF toads ($n=6$). The data are plotted as mean values ± SEM. no significant difference found with aCSF pH or between cold acclimated room aCSF and cold acclimated hot aCSF, and hot acclimated room aCSF and hot acclimated cold aCSF.
In preparations taken from hot acclimated animals, integrated breath area was not affected by changes in aCSF pH regardless of whether the preparations were bathed with RT ($p=0.493$) or CT ($p=0.713$) aCSF (Figure 3.22B). Integrated breath area was identical in these two groups (i.e., hot acclimated RT aCSF and hot acclimated cold aCSF; $p=0.936$).

3.4.4. Total fictive ventilation

In preparations taken from cold temperature acclimated animals, changes in aCSF pH had no effect on total fictive ventilation (Figure 3.23A) in those preparations bathed with RT aCSF ($p=0.516$) or HT aCSF ($p=0.161$); the values in the two groups were identical ($p=0.228$). In preparations taken from hot acclimated animals and bathed with RT aCSF, reductions in aCSF pH caused an increase in total fictive ventilation (Figure 3.23B; $p<0.001$); there was no effect of reducing pH in those preparations bathed with cold aCSF ($p=0.395$). The values in the RT aCSF and cold aCSF bathed preparations were identical ($p=0.138$).
**Figure 3.23**: Total fictive ventilation (V·s·min⁻¹) as a function of artificial cerebrospinal fluid (aCSF) pH in preparations taken from (A) normoxic cold temperature control aCSF toads (n=8) and normoxic cold temperature hot aCSF toads (n=6), (B) normoxic hot temperature control aCSF toads (n=8) and normoxic hot temperature cold aCSF toads (n=6). The data are plotted as mean values ± SEM. Letters (a, and b) indicate a significant difference amongst pH levels in any one group. No significant difference found between cold acclimated room aCSF and cold acclimated hot aCSF, and hot acclimated room aCSF and hot acclimated cold aCSF.
CHAPTER FOUR

GENERAL DISCUSSION
4.1. Summary of findings

The major findings of this study are:

1) In the control, normoxic groups, total fictive ventilation increased as aCSF pH was lowered. This increase was due to an increase in fictive breathing frequency, and not due to an increase in the integrated area of the fictive breaths. In turn, the increase in fictive breathing frequency was caused by an increase in the number of fictive episodes per minute rather than a change in the number of fictive breaths per episode.

2) At room temperature (RT), exposure to chronic hypoxia (CH) caused a reduction in total fictive ventilation. This occurred at both 10% O₂ (i.e., in the RT series that accompanied the cold acclimation experiments) and 15% O₂ (i.e., in the RT series that accompanied the hot acclimation experiments). In both cases, the CH-induced decrease in total fictive breathing was caused by a reduction in fictive breathing frequency that was, in turn, due to decreases in both the number of fictive episodes per minute and fictive breaths per episode.

3) In the cold temperature acclimated groups (control and CH), changing aCSF pH had no effect on fictive breathing.

4) In the hot acclimated group, lowering aCSF pH caused an increase in total fictive ventilation, fictive breathing frequency and the number of fictive episodes per minute in the control (normoxic) group but not in the CH group.
5) Exposure to cold temperature caused a reduction in fictive breathing in both the control and CH groups although the cold-induced decrease was limited to the lower aCSF pH levels in the CH group.

6) Exposure to hot temperature increased total fictive breathing, fictive breathing frequency, the number of fictive breaths per episode and the number of fictive episodes per minute albeit sometimes at different pH levels for the different variables.

7) Superfusing preparations from cold acclimated toads with hot aCSF did not reverse the cold temperature-induced reduction in fictive breathing.

8) Superfusing preparations from hot acclimated toads with cold aCSF did not reverse the hot temperature-induced increase in fictive breathing.

4.2. Effect of temperature

The results of this thesis illustrate that, within normoxic preparations, chronic exposure to hot and cold temperatures alters fictive breathing. Exposure of cane toads to hot (30°C) temperature for 10 days prior to the *in vitro* recording of fictive breathing resulted in an increase in the fictive breathing response compared to toads exposed to room temperature (20°C). In contrast, cane toads exposed to cold (10°C) temperature for 10 days prior to the *in vitro* recording of fictive breathing exhibited a decrease in fictive breathing. Preparations taken from toads exposed to hot temperature were highly sensitive to changes in aCSF pH/CO$_2$ levels while preparations taken from toads exposed to cold temperature had reduced sensitivity to changes in aCSF pH/CO$_2$ levels. These results suggest a temperature dependency of pH/CO$_2$-sensitive fictive breathing. This
temperature dependency could occur anywhere in the brain, from the sensing of CO$_2$/pH by the central chemoreceptors to integration of the chemosensory information by central respiratory centres, to the motor output recorded from the vagus nerve root.

Previous studies, in a variety of species, have investigated the effect of exposing the brain to various temperatures on breathing and the breathing response to altered levels of inspired O$_2$ and CO$_2$. Cherniack et al. (1979) cooled the brains of anesthetised cats using a water-circulated surgically-implanted thermode that was in contact with the brain. Cooling of chemosensitive areas located on the ventral surface of the medulla resulted in a decrease in tidal volume (the volume of air taken into the lungs in one breath) and breathing frequency. Using this technique to lower the medullary surface temperature from 37 to 20°C produced a progressive increase in the amount of CO$_2$ required to initiate respiration in anesthetised cats. That study indicates that the function of chemosensitive areas responsible for CO$_2$-sensitive breathing is indeed sensitive to changes in temperature.

Morales and Hedrick (2002) reported that fictive breathing measured in vitro from a brainstem-spinal cord preparation taken from the American bullfrog (*Rana catesbeiana*) exposed to 10°C was nearly absent. This could be explained by cold-temperature induced interference with synaptic transmission to respiratory motor neurons. However, in their experiment, exposure to 30°C also resulted in an absence in fictive breathing. This high-temperature induced reduction in fictive breathing is in contrast to the increase in fictive breathing reported in this thesis. While the two studies used different species, this is likely not the cause for the difference at higher temperatures. Instead, it seems Morales
and Hedrick (2002) only had two active preparations exposed to 30°C, so a rigid statistical analysis of their hot temperature exposure data would not be possible.

4.3. Effect of chronic hypoxia

The effects of chronic hypoxia in the room temperature groups are consistent with observations from previous studies in which exposure to CH caused a reduction in fictive breathing and an attenuation, or abolition, of pH/CO₂ sensitivity. These changes were observed following chronic exposure to both 10% O₂ (i.e., in the RT CH group that accompanied the cold acclimation experiments) and 15% O₂ (i.e., in the RT CH group that accompanied the hot acclimation experiments). These results are consistent with work conducted previously in this laboratory by McAneney and Reid (2007), as they observed, that exposure of cane toads to chronic hypoxia significantly reduced both fictive breathing as well as the magnitude of changes in fictive breathing that accompanied alterations in aCSF pH. With a few exceptions, the results indicate that exposure to CH reduced fictive breathing regardless of whether the preparations were taken from toads that were exposed to room temperature, hot temperature or cold temperature.

4.4. Combined effect of temperature and CH

Interestingly, exposure to chronic hypoxia reduced fictive breathing independently of temperature. CH and cold temperature acclimation were found to have the same effect on fictive breathing (i.e., CH and cold together give the same reduction as cold alone or CH alone). In contrast, hot temperature acclimation and CH do not have the same effect. Indeed, exposure to CH in the hot temperature acclimation group led to the same
reduction in fictive breathing as exposure to CH in the RT acclimation group. This suggests that CH has a more powerful influence on fictive breathing than hot temperature, as exposure to hot temperature did not influence (i.e., reverse) the CH-induced attenuation of fictive breathing. It is important to note that while there was a statistically significant increase in the fictive breathing response of the hot acclimated CH toads and room temperature CH toads, there was likely no physiological difference between the two groups based off of the trends of both groups (see Figure 3.10A, B).

Exposure to acute hypoxia is known to lower body temperature in a variety of mammals, and some ectotherms (Branco et al., 2000). Recently, it has been thought that this decrease in body temperature is due to alterations in the neurophysiological control of body temperature (i.e., it is likely produced by a downward resetting of the thermoregulatory set point) (Branco et al., 2000). Given that the hot temperatures in this study were experimentally induced and that the toads could not behaviourally remove themselves from these conditions, it is unlikely that the toads would have been able to regulate their body temperature. As a result, the reduced breathing response seen within the hot temperature chronically hypoxic toads is likely not due to the fact that the body temperature of the toads was in fact lowered due to the exposure to hypoxia. In other words, hypoxia-induced hypothermia was almost certainly not occurring in the hot temperature acclimated toads. It is, however, more probable that the CH-induced reduction in fictive breathing is due to a reduction in central chemoreceptor sensitivity/function/activity following exposure to chronic hypoxia and that CH has a greater influence on the function of chemoreceptors than the effect of hot temperature.
According to McAneney and Reid (2007) a decrease in central pH/CO$_2$ chemoreceptor sensitivity (i.e., as assessed by measuring pH/CO$_2$-sensitive fictive breathing) is likely due to influences or inputs arising from the rostral mid-brain and synapsing with respiratory groups in the medulla. This conclusion arises from the observation that a midbrain transection in vitro reversed the CH-induced reduction in fictive breathing observed by McAneney and Reid (2007). Any influence from the midbrain (that mediates the CH-induced reduction in fictive breathing) may be so powerful that the effect of hot temperature was not able to overcome it and maintain a hot temperature-induced state of elevated fictive breathing. It is also possible that the CH-induced reduction in fictive breathing in the hot acclimated toads was due to a reduction in metabolism arising from the reduction in available oxygen under the hypoxic conditions. In this scenario, it is possible that a hypoxia-induced reduction in metabolism was greater than any hot temperature-induced increase in metabolic rate.

Previous studies have suggested that central pH/CO$_2$ chemoreceptors communicate via gap junctions (Solomon and Dean, 2002; Dean et al., 2002). Exposure to hypoxia, seems to repress gap junction communication between cells (Zeevi-Levin et al., 2005), and this reduction in cell-cell communication may have led to the reduction in fictive breathing. Again, gap junction communication may have been more sensitive to CH exposure rather than hot temperature exposure (Dean et al., 2002). It is, however, not as surprising that fictive breathing in the preparations taken from the cold temperature chronically hypoxic toads did not decrease further than fictive breathing from the preparations taken from control (normoxic) chronically hypoxic toads. This is because
there is, presumably, to a limited scope for reducing metabolism and breathing frequency short of death.

4.5. Effect of altering aCSF pH

Total fictive ventilation (an index of overall fictive breathing in the *in vitro* brainstem-spinal cord preparation) increased as the aCSF pH was lowered (by increasing the CO₂ level) in the control preparations. This was due to an increase in fictive breathing frequency rather than the integrated area of fictive breaths (an index of breath amplitude or volume). The increase in fictive breathing frequency was mostly due to an increase in the number of fictive episodes per minute rather than the number of fictive breaths per episode. Within the room temperature CH (15% O₂) acclimated toads, the number of fictive breaths per episode was influenced by changes in aCSF pH. The other components of fictive breathing (i.e., breath duration and amplitude) analyzed in this study did not change in response to acidification/alkalinisation of the aCSF pH. It is important to note, however, that while there was still a statistically significant increase in fictive breathing as aCSF was lowered in the CH groups, the slope and the response is reduced compared to the controls.

The effects seen due to reduced aCSF pH described above are consistent with what has been seen in previous studies within this laboratory. Gheshmy *et al.* (2006) and McAneney and Reid (2007) found that fictive breathing frequency increased with decreased aCSF pH. This increase was due to an increase in fictive episodes per minute, as fictive breaths per episode were not influenced by changes in aCSF pH. In addition, both previous studies found that total fictive ventilation increased with a decrease in
aCSF pH. This increase was due to an increase in fictive breathing frequency and not due to an increase in the integrated area of the fictive breaths. These results are consistent with the observations in my thesis, with the exception of the room temperature CH 15% O2 exposed toads, in which the number of breaths per episode was influenced by aCSF pH. In addition, neither study found any effect of aCSF pH on breath duration or integrated breath area (Gheshmy et al., 2006; McAneney and Reid, 2007). Interestingly, Srivaratharajah et al. (2008) reported slightly different results, as within control toads, breath duration and the integrated area of fictive breaths was increased with a decrease in aCSF pH in control groups.

4.6. The effects of warm aCSF on preparations taken from cold acclimated toads and the effects of cold aCSF on preparations taken from warm acclimated toads

In the first two series of experiments (cold and hot acclimation with the associated RT controls), CH caused a reduction in fictive breathing in the RT control groups. This occurred when the level of CH was set at 10% O2 (in the cold acclimation experiments) and 15% O2 (in the hot temperature acclimation experiments). In addition, CH in the hot acclimated group also caused a reduction in fictive breathing. CH did not cause a further reduction in fictive breathing, compared to cold temperature alone in the cold acclimated group. A third series of experiments was performed in order to determine if the changes in fictive breathing observed in the normoxic control preparations was a “superficial” effect of temperature (i.e., just a Q10 effect) or if the temperature acclimation had caused a fundamental change within central respiratory control systems. These experiments consisted of bathing the brainstem spinal cord preparations taken from cold acclimated toads with hot aCSF and bathing preparations taken from hot acclimated toads with cold
aCSF. If the effects of cold and hot acclimation on fictive breathing were simply a $Q_{10}$ effect, then I predicted that warm aCSF would reverse the cold acclimation-induced reduction in fictive breathing and that cold aCSF would reverse the hot acclimation-induced increase in fictive breathing.

Bathing the brainstem preparations of toads acclimated to hot temperatures with cold aCSF did not result in a reduction of fictive breathing. Fictive breathing was identical in the hot acclimated groups regardless of whether the preparations were superfused with RT or cold aCSF. Similarly, in preparations taken from cold-acclimated toads, fictive breathing was the same regardless of whether the aCSF was at room temperature or warmed to 30°C. Given that the temperature acclimation-induced changes in fictive breathing were not reversed by the reciprocal changes in aCSF temperature, these results suggest that a plastic change has occurred within central respiratory control centres. Plasticity in this case is defined as a persistent change in a neural control system (morphological and/or functional) based on prior experience (Mitchell and Johnson, 2003).

4.7. Neural plasticity

Neural plasticity such as that described above indicates that cane toads are likely able to adapt to new (hot and cold) environments (Ghalambor et al., 2007; Young et al., 1989). Such plasticity is critical when considering an animal’s ability to survive environmental changes, especially with respect to climate change. Seebacher and Franklin (2010) showed that cane toads function very well within hot environments. They investigated the plasticity of oxygen transport and metabolism during exercise and at rest
in cane toads at different temperatures and found that hot acclimated toads have a greater cardiac scope at hot temperatures compared to non-acclimated toads. While work done within this thesis supports the notion that cane toads are indeed able to adapt to hot temperature under normoxic conditions and exposure to chronic hypoxia (15% O₂), there was high mortality of cane toads exposed to hot temperature at 10% O₂. As a result of this, the hot temperature acclimation experiments at 10% O₂ were abandoned and replaced with the hot temperature acclimation at 15% O₂. This could illustrate potential ecological significance in shelter site selection in the wild when exposed to hot temperatures (i.e., toads might avoid shelter sites with lowered hypoxia (10% O₂) levels when exposed to hot temperatures. Although the majority of ecological work done on cane toads has focused on their ability to survive hot climates (Leonard, 1933; Sutherst, 1996; Seebacher and Franklin, 2010), the work of my thesis illustrates that cane toads are able to modify their ventilation upon exposure to cold temperature, and likely be able to withstand exposure to cold environments as well. This has implications on the geographical distribution models of cane toads (Sutherst et al., 1996; Kearney et al., 2008), which have mostly been focused on warm temperature regions.

While the results of the reciprocal hot/cold aCSF on hot/cold acclimated toads suggests a degree of neural plasticity with respiratory control systems, the location of this plasticity cannot be determined with the results of this thesis. However, studies have shown that the neural mechanisms involved in the control of breathing are able to change and that respiratory-related neural plasticity is quite common. Douse and Mitchell (1988) examined the effect of temperature on respiratory-related CO₂-sensitive intrapulmonary chemoreceptors (IPCs) in the yellow banded tegu lizard (Tupinambis nigropunctatus).
Acute temperature fluctuations (a decrease of 10°C) from the ideal body temperature (roughly 20°C) of the lizards resulted in a decrease in IPC output frequency. This 10°C change in body temperature exerted a significant effect on the discharge pattern of IPCs, suggesting that chemoreceptors are temperature sensitive. These authors speculated that the change in IPC neural activity may have resulted from reduced ion channel function within the brain. While this study investigated the effect of temperature on intrapulmonary chemoreceptors of lizards, it offers some insight, in general, into how ectotherm chemoreceptors are able to physiologically (i.e., decrease in IPC output) change based on exposure to different temperatures, and, as a result, become less sensitive to CO₂ stimuli.

The long-held conception that the brain is rigid and unable to change has been shown to be false (Alvarez-Buylla and Lois, 1995; Klob et al. 2003; von der Ohe et al. 2006). In a variety of mammalian species, there is substantial evidence of neural plasticity found within a number of structures in the brain. Mitchell and Johnson (2003) outline a myriad of forms of neural plasticity specifically for the motor control of breathing within mammals. Two types of synaptic plasticity have been identified using in vitro brainstem-spinal cord preparations such as the one used in this thesis. These are (1) changes in synaptic strength and (2) serotonin-dependent plasticity. Mitchell and Johnson (2003) report that synaptic strength (efficiency of synaptic transmission) is enhanced by previous activity that may arise from concurrent pre- and postsynaptic activity that alters pre-synaptic transmitter release and post-synaptic receptor function. Serotonin-dependent plasticity is critical in respiratory motor control as the increase in serotonin released into the pre-synaptic terminal initiates intracellular cascades, increasing synaptic strength.
While there is no direct evidence of the effect of temperature on this serotonergic system, it seems to be modified by an increase of pre- and postsynaptic activity. If the increase in breathing frequency seen with elevated temperature (in this thesis) occurred due to an increase in synaptic activity, then this increase in activity could, in turn, be due to increased synaptic strength.

While neural plasticity is clearly a common phenomenon, previous studies focused on exposure to different temperatures are more relevant to understanding how long-term adaptation to different temperatures can be related to the breathing responses observed in this thesis. Von der Ohe et al. (2006) investigated the effect of cold temperature on neural plasticity observed in respiratory control within hibernating ground squirrels. Ground squirrels were exposed to either 5°C or 15°C and allowed to enter hibernation for 1 to 6 days. The animals were then sacrificed and the brains were stained and sliced for neuronal comparisons. They investigated multiple brain regions (the cortex, thalamus and hypothalamus), and found that the cells exhibited a significant decrease in neuron microstructure (defined by cell body area, number of dendrites, and spine density). The neuron microstructure of hibernating ground squirrels was reduced compared to euthermic ground squirrels. This suggests that hibernation has led to a ubiquitous pattern of cell reduction. Furthermore, hibernating ground squirrels held at 5°C had further reduced neuron microstructure than those held at 15°C. As such, neuron microstructure appears to be temperature dependent. These studies show that in addition to the reduction in neuron microstructure seen during hibernation, the temperature at which hibernation takes place is also important in determining the extent of changes in neural structure that occur during hibernation.
There is also evidence of temperature-induced neural plasticity within ectotherms. Exposing a locust to high, but sublethal, temperatures has been shown to extend the temperature range in which neurons can function (measured by successful action potential generation). The changes in action potential generation were due to the effect of altered temperature suggesting the presence of temperature-sensitive neural plasticity. Wu et al. (2001) investigated the effect of heat shock (45°C) on the action potential of locust (Locusta migratoria) flight muscle. They found that after heat shock exposure, there was an increase in the temperature (by roughly 10°C) at which failure for action potential generation in the flight motor neurons occurred when compared to control locusts. Thus, after long term exposure to high, but sublethal temperature, the neurons and synapses still function when the animal is exposed to future higher (and potentially otherwise lethal) temperatures (Wu et al. 2001; Money et al. 2005). Plasticity of physiological processes can, at least in part, explain how animals are able to adapt to thermal changes in the external environment, and is an important mechanism to maintain function under the stress of varying environmental temperatures.

Plasticity of respiratory control has been demonstrated in a variety of vertebrate groups. For example, humans demonstrate respiratory plasticity. When high altitude natives transition to sea level, they reduce their ventilation, which elevates arterial CO₂ compared to sea level natives. Similarly, when sea level natives transition to high altitudes, they increase their ventilation and lower their arterial CO₂ levels (Léon-Velard et al., 1996). Interestingly, Rivera-Ch et al. (2003) found that high altitude natives that reside at sea level for several years and then transition back to high altitudes, respond to the hypoxia similarly to sea-level natives at high-altitudes. This indicated a form of
plasticity within the human breathing response. Mammals respond to chronic environmental hypoxia by increasing the sensitivity of respiratory control systems to further bouts of acute hypoxia in a classical response termed ventilatory acclimatisation to hypoxia (Dempsey and Forster, 1982; Bisgard and Neubauer, 1995; Powell et al., 1998; Teppema and Dahan, 2010). This is a well-described example of neural plasticity that arises from changes in neurotransmission in central respiratory control centres (Powell et al. 2000). Previous research (Carroll, 2003; Bavis, 2005) has also shown that during mammalian development there is a critical period in which exposure to chronic hypoxia can result in a long-term decrease in resting ventilation. However, exposure to chronic hypoxia after this period does not result in a change in resting ventilation (Carroll, 2003).

4.8. In vitro versus in vivo experimentation

The experiments in this thesis used an in vitro brainstem-spinal cord preparation. Given that this preparation is devoid of any peripheral input from respiratory control systems, such as arterial chemoreceptors, pulmonary stretch receptors or olfactory chemoreceptors, it is a highly useful tool to investigate central (brain) mechanisms involved in the control of breathing. However, its greatest strength is also a potential limitation. Feedback from both peripheral and central control systems are integrated in vivo, resulting in an overall level of respiratory drive that is manifest as motor output to respiratory muscles. Respiratory stressors (i.e., hypoxia) that cause a certain response in vivo may not result in as profound a response when investigated within a reduced in vitro preparation (Reid, 2006). Indeed, certain manipulations made in vitro may not manifest in the same manner when performed on intact animals (Gheshmy et al. 2006). However,
the value of the *in vitro* brainstem spinal cord preparation as a tool to study the central control of breathing is without question as this preparation has been used routinely for the past 20 years on a variety of species from mammals to reptiles to amphibians to fish to invertebrates.

4.9. Validation of the *in vitro* preparation’s viability

In the experiments conducted within this thesis, the preparation was allowed to stabilise for a one-hour period at an aCSF pH of 7.8 prior to changing the aCSF pH to given levels (i.e., 7.4 to 8.2) at regular intervals. The pH changes during the experimental protocol were presented in a random order. The results (variables associated with fictive breathing) are plotted as a function of aCSF pH. In order to confirm that the observed effects were due to changes in pH rather than the time since the establishment of the preparation, the data for breathing frequency were plotted as a function of time (or rather, as a function of the first, second, third, fourth and fifth periods of exposure to different pH levels). When this was done, the data averaged across individuals manifest as a straight line across the various time points. This indicates that preparations were not losing viability as the experiment progressed. This maintenance of viability was expected given the low metabolic rate and high hypoxia tolerance of amphibian brains (Reid and Milsom, 1998; Morales and Hedrick, 2002). Indeed, it has been well established that the amphibian brainstem-spinal cord preparation maintains its function for significantly longer periods of time than a mammalian preparation (Gheshmy *et al*., 2006; McAneney and Reid, 2007; Srivararatharajah *et al*., 2008).
As a further control to assess preparation viability, fictive breathing frequency during the one-hour stabilisation period at pH 7.8 was compared to that during exposure to pH 7.8 during the data collection period. Without exception, there was no significant difference between fictive breathing frequency during the initial stabilisation period of pH 7.8 and during exposure to pH 7.8 during the data collection period.

4.10. Additional potential improvements

While I was able to utilise a cold room to recreate a consistent cold temperature environment, a greenhouse at the University of Toronto Scarborough was used for the hot temperature environment. Although this environment provided ecologically relevant daily fluctuations in temperature, it was a challenge to select and maintain a specific hot temperature level. This is especially true when comparing the toads that were exposed to the hot temperature acclimation conditions with their preparations bathed with RT aCSF, and the toads exposed to hot temperature acclimation conditions with their preparations bathed with cold aCSF.

The hot temperature experimental environment also resulted in increased mortality when the toads were exposed to chronically hypoxia conditions of 10% O₂. Unfortunately, it is not clear whether the low survival rate was due to the increased temperature, the hypoxia level, a combination of the two, or an external factor within the greenhouse. However, by changing the oxygen levels from 10% to 15%, all the toads survived their 10-day exposure period. While there was a slight difference in fictive breathing frequency between the hypoxia groups at 10% O₂ and 15% O₂, it was not
significant and the overall reduction in fictive breathing compared to normoxic hot toads and room temperature conditions was conserved.

4.11. Future work

A major finding from this thesis was the evidence of neural plasticity in the respiratory control systems after exposure to chronic cold and hot temperature environments. It would be interesting to investigate where within the central respiratory control systems these changes occurred. The use of histological preparations before and after exposure to the various environmental conditions may offer some insight in structural changes in central structures, as was done in the von der Ohe et al. (2006) paper. Also, it would be interesting to see if the plasticity of the breathing response is maintained after a longer period of time post-exposure to chronic cold and hot temperature environments. Unfortunately, this type of experimentation could possibly be limited by the duration of the functioning of the brainstem-spinal cord preparation although these are very robust preparations that can function for hours. Aside from this caveat, potentially setting the aCSF pH to 7.8 (normocapnic for amphibians) and then exposing the preparation to alternate aCSF temperatures, could reveal a time-dependent plastic response. Alternatively, animals could be exposed to CH or to hot/cold acclimation temperature, then returned to control conditions for a period of days before making fictive breathing measurements with the in vitro preparation.

Previous work in the Reid Lab has investigated the effect of hypercapnia (increased inspired CO$_2$ levels) on the breathing response (both in vivo and in vitro) of cane toads. In the same way that this study utilised environmentally relevant temperatures as a
concurrent stressor to hypoxia, it would be beneficial to investigate the role of these same temperatures on the hypercapnic breathing response. A previous study from the Reid laboratory reported that exposure of cane toads to chronic hypercapnia (3.5% CO$_2$ for 9 days) at room temperature caused an increase in central pH/CO$_2$ chemoreceptor-stimulated fictive breathing measured using the *in vitro* brainstem-spinal cord preparation (Gheshmy *et al*., 2006). It would be interesting to see if the same effects of temperature acclimation found within my thesis also apply during chronic hypercapnia.

In addition, it is often true that within the burrow microenvironment, animals are exposed to both hypoxia and hypercapnia (Shams *et al*. 2005; Srivaratharajah *et al*. 2008). The effect of chronic hypoxic hypercapnia on fictive breathing of cane toads at room temperature has also been investigated previously. Srivaratharajah *et al*. (2008) utilized an *in vitro* brainstem-spinal cord preparation, and found that chronic hypoxic hypercapnia increased fictive breathing frequency but decreased the increased fictive breath area, and, as a result, there was no change in total fictive ventilation. It would be interesting to see how temperature influences this breathing response.

4.12. Significance of work

My thesis illustrates how the fictive breathing response of cane toads (*B. marinus*) is modified upon chronic exposure to environmentally relevant stressors (both temperature and hypoxia). In addition it offers insight into the physiological plasticity of respiratory control in cane toads and their ability to adapt to a range of different environments. As cane toads pose such a threat to a variety of ecological systems (especially in Australia), the results offer evidence that these anurans will not only thrive
in hot environments, but will withstand long-term exposure to cold environments as well. Animal populations have been substantially influenced by the effect of climate change (Thomas et al., 2004). The mechanisms that allow cane toads to withstand long periods of both hot and cold environmental temperature offers insight into potential physiological adaptations that could allow for survival of climate change. In addition, experimental studies on the ability of cane toads to withstand environmentally relevant stressors can help predict the effect of climate change on other anuran amphibians.

In addition to the ecological significance, this thesis specifically looks at the impact of relevant stressors on the central control of breathing. The data clearly illustrate how elevated temperature increases, and how lowered temperature decreases, the sensitivity and/or function of central CO$_2$/pH chemoreceptors under normoxic conditions. The data also show the effect of chronic hypoxia exposure on the sensitivity of CO$_2$/pH chemoreceptors, especially with respect to the HT CH toad group. This research also provides evidence for neural plasticity potential within the central respiratory control sites, demonstrating that the effect of temperature on the central control of breathing in amphibians is not superficial, but in fact alters the functioning of this control system in the long-term.

Finally, although this work was conducted on cane toads, the similarities between the neural structures involved in mammalian and amphibian central respiratory control systems control offers some insight in the conserved abilities of chemoreceptors across the vertebrate groups.
REFERENCES


