SLEEP IN THE WILD: The anatomy and physiology of sleep in free-roaming blue wildebeest (*Connochaetes taurinus*)

Illke Bianca Philander

0503525N

PhD Thesis

Supervisors: Dr N. Gravett and Prof P.R. Manger

This thesis is submitted in fulfilment of the requirements for the degree of Doctor of Philosophy



UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

2018

DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the Degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.



Illke B. Philander

6 July 2018

Dedication

To my parents, Charles and Sandra Philander, thank you for all of your love and support, for always believing in me and for passing on an inquiring mind.

To my husband, Heloneide Malungo, thank you for your patience and faith in me, I am forever grateful.

Publications arising from this thesis:

Manuscripts Under Review

Philander, IB., Bhagwandin, A., Davimes, JG., Manger, PR., Gravett, N. The organization of the sleep-related neural systems in the brain of the blue wildebeest, Connochaetes taurinus. Anatomical Record, AR-17-0305

Philander, I.B., Ganswindt, A., Manger, PR., Gravett, N. Male blue wildebeest increase activity levels during the rut, but not at the expense of the time spent at rest. Animal Behaviour, ANBEH-S-17-01062

Presentations arising from this thesis:

Conference Presentations

Title: The organization of the sleep related neural systems in the brain of the blue wildebeest, *Connochaetes taurinus*.

Co-Authors: Adhil Bhagwandin, Joshua G. Davimes, Paul R. Manger, Nadine Gravett

Type: Poster Presentation

Conference: 13th meeting of the Society of Neuroscientists of Africa

Place: Entebbe, Uganda

Abstract

Comparative methods of evolutionary biology are useful tools for investigating the function/s of sleep as these still remains elusive. In the present thesis, the global aim was to investigate sleep in blue wildebeest (*Connocheates taurinus*) from a neuroanatomical, physiological and behavioural point of view. Furthermore, it also set out to understand how this species sleeps in its natural environment and the effects that natural behaviours can have on sleep, specifically investigating the influence of the rut on sleep/rest phasing and architecture in dominant breeding males of this species. Neuroanatomically, a detailed qualitative description of the organization of the somnogenic systems of the blue wildebeest is provided, along with a quantitative analysis of the pontine cholinergic and noradrenergic neurons, and the hypothalamic orexinergic neurons. From the basal forebrain to the pons, the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems for the most part, showed a corresponding nuclear organization to that reported in other mammals and more specifically the Cetartiodactyla. Furthermore, the description and distribution of the GABAergic system, which was examined through immunostaining for the calcium binding proteins calbindin, calretinin and parvalbumin, was also similar to that seen in other mammals. Physiologically, sleep in the blue wildebeest was recorded, in a naturalistic setting, by means of two different measures of sleep, polysomnography (PSG) and actigraphy (ACT). The blue wildebeest were found to be polyphasic sleepers, sleeping mostly during the dark period of the day. PSG showed that total sleep time in the blue wildebeest for a 24-hour period was 4.53 h, with 4.26 h spent in non-REM sleep and 0.28 h spent in REM sleep, the remainder of the day (19.47 h) spent in Wake. Similarly, ACT showed that the blue wildebeest spent 19.23 h Active and 4.77 h Inactive. For both animals studied, Cohen's κ showed a fair agreement between the two techniques for sleep

measurement, although differences were observed between individual days and animals. Behaviourally, the daily activity and inactivity patterns of dominant male blue wildebeest were examined by means of actigraphy for a period of three months, which included the time of the rut. It was observed that there were no changes to the sleep phasing and architecture of the blue wildebeest during the rut, but there were changes to the total time spent in a state of inactivity. The studies presented in this thesis form the baseline for further studies of the influence of the environment and natural behaviours on sleep in the blue wildebeest. More broadly, these studies establish a framework from which studies of sleep in other large mammals in their natural environment can be established and compared in a fully quantitative manner.

Acknowledgements

The following individuals and organizations were exceptional in the completion of this PhD thesis:

Firstly, I would like to express my sincere gratitude to Prof. Paul Manger for the continuous support of my PhD study, for his patience, motivation, and immense knowledge. His guidance helped me in all the time of research and writing of this thesis.

I'd also like to thank Dr Nadine Gravett for all her assistance with the field research.

Thanks to Dr Adhil Bhagwandin for the many consultations and guidance in understanding the stereology and the analysis and scoring of the polysomnography, it was greatly appreciated.

I am also grateful to Josh Davimes for his patience and assistance with all the statistical analysis needed for the thesis.

I gratefully acknowledge the funding received towards my PhD from the NRF-DAAD: A grant-holder linked bursary from 2010 – 2014 PhD fellowship.

And lastly, I would also like to say a heartfelt thank you to my Mom, Dad, Heloneide, Diane and Chrystal and all my friends and family, for always encouraging me to follow my dreams and for helping in whatever way they could during this challenging period.

Table of contents

Title page	i
Declaration	ii
Dedication	iii
Publications arising from this thesis	iv
Presentations arising from this thesis	iv
Abstract	v
Acknowledgments	vii
Table of contents	viii
List of tables	xv
List of figures	xv

CHAPTER ONE: **Introduction and chapter outline**

1.1. Introduction	1
1.2. Aims and objectives	4
1.3. Chapter outline	4

CHAPTER TWO: **Literature review**

2.1. Sleep in mammals	8

2.1.1. Defining sleep 8

2.1.2. The neurobiology of sleep 9

2.2. Physiological Measurement of Sleep 15

2.3. Factors influencing sleep 18

2.3.1. Captive vs. Wild Sleep Studies 18

2.3.2. Effect of energetic constraints on sleep 20

2.3.3. Effect of predation risk on sleep 21

2.3.4. Effect of mating on sleep 23

2.4. Experimental animal 25

CHAPTER THREE: **A preliminary description of the sleep related neural systems in the brain of the blue wildebeest, *Connochaetes taurinus*.**

3.1. Introduction 29

3.2. Materials and methods 31

3.2.1. Brain acquisition 31

3.2.2. Sectioning, staining and immunohistochemistry 31

3.2.3. Qualitative analysis 34

3.2.4. Quantitative stereological analysis 34

3.2.5. Abbreviations 36

3.3. Results 41

3.3.1. Cholinergic nuclei of the basal forebrain and pons 41

3.3.2. The catecholaminergic locus coeruleus complex 43

3.3.3. Serotonergic nuclei of the dorsal raphe complex 44

3.3.4. Orexinergic neurons of the hypothalamus 45

3.3.5. GABAergic neurons and terminal networks in sleep related nuclei 46

3.3.5.1. GABAergic neurons and terminal networks in the cholinergic nuclei 47

3.3.5.2. GABAergic neurons and terminal networks in the catecholaminergic nuclei 48

3.3.5.3. GABAergic neurons and terminal networks in the serotonergic nuclei 48

3.3.5.4. GABAergic neurons and terminal networks in the orexinergic nuclei 49

3.3.5.5. GABAergic neurons and terminal networks in the thalamic reticular nucleus 49

3.4. Discussion 72

3.4.1. Similarities in the nuclear organization of the sleep-wake nuclei across mammals 72

3.4.2. Similarities in the GABAergic systems of the sleep-wake nuclei 75

across mammals

3.4.3. What do these findings say about sleep in the blue wildebeest? 77

CHAPTER FOUR: **Sleep in two free-roaming blue wildebeest (*Connochaetes taurinus*), with observations on the concordance of polysomnographic and actigraphic techniques**

4.1. Introduction 80

4.2. Materials and methods 81

4.2.1. Experimental animals and site location 81

4.2.2. Recording period and environmental conditions 82

4.2.3. Devices: Neurologger® 83

4.2.4. Devices: Actiwatches 83

4.2.5. Surgical Procedure 84

4.2.6. Sleep Recording and Data Analysis 86

4.2.6.1. Polysomnography (PSG) analysis 86

4.2.6.2. Actigraphy (ACT) analysis 87

4.2.6.3. Concordance between polysomnography and actigraphy 88

4.3. Results 88

4.3.1. State definitions 88

4.3.2. Time spent in Wake, non-REM and REM states 89

4.3.3. Number and average duration of episodes of wake and sleep states 89

4.3.4. State transition probabilities 90

4.3.5. Actigraphy (ACT) recordings 90

4.3.6. Concordance between polysomnography (PSG) and actigraphy (ACT) 91

4.4. Discussion 108

4.4.1. Comparisons to sleep in other Artiodactyla 108

4.4.2. Comparison of Actigraphy and Polysomnography as measures of total sleep time and patterns in the blue wildebeest 112

CHAPTER FIVE: **Male blue wildebeest significantly increase activity levels during the breeding season (rut), but not at the expense of rest/sleep**

5.1. Introduction 116

5.2. Materials and methods 117

5.2.1. Experimental animals and site location 117

5.2.2. Recording period and environmental conditions 118

5.2.3. Activity and temperature data loggers 119

5.2.4. Surgical implantation and removal of actiwatches and iButtons 119

5.2.5. Organization of data for analysis 121

5.2.6. Activity analyses 122

5.2.7. Inactivity analyses 122

5.2.8. Subcutaneous temperature 123

5.2.9. Measurement of faecal androgens 123

5.2.10. Statistical Analysis 125

5.3. Results 125

5.3.1. Activity levels prior to, during, and after the rut 126

5.3.2. Timing of activity prior to, during, and after the rut 126

5.3.3. Inactivity prior to, during, and after the rut 127

5.3.4. Timing of inactivity prior to, during, and after the rut 129

5.3.5. Levels of faecal androgen metabolites prior to, during, and after the rut 130

5.3.6. Average daily subcutaneous temperature prior to, during, and after the rut 130

5.3.7. Average daily variation in subcutaneous temperature prior to, during, and after the rut 130

5.3.8. Average daily ambient air temperature prior to, during, and after the rut 131

5.3.9. Average daily ambient temperature and average daily time inactive 132

5.4. Discussion 147

5.4.1. Defining the rut 147

5.4.2. Activity levels before, during and after the rut 148

5.4.3. Inactivity levels before, during and after the rut 149

5.4.5. Sleep and the rut 150

5.4.6. Summary 151

CHAPTER SIX: **Summary and Final Discussion**

6.1. Neurobiology of sleep 153

6.2. Sleep in the wild 157

6.3. Factors influencing sleep 159

6.4. Future directions 160

REFERENCES 163

Appendix A – Ethical Clearance Certificates 192

Appendix B – Turn It In Report 196

List of tables

Table 3.1: Stereological parameters used for estimating neuron numbers in the blue wildebeest. 40

Table 3.2: Stereological results for neuron numbers, volume and area in the blue wildebeest. 60

Table 3.3. The density of neurons and terminal networks of the calcium binding proteins calbindin, calretinin and parvalbumin in relation to various sleep-wake nuclei in the brain of the blue wildebeest 69

Table 4.1: Cohen's κ showing the agreement between polysomnography and actigraphy recordings, for the individual days of recording for each blue wildebeest, for the measure of total sleep time (combined non-REM and REM compared to Inactive) and wake (Wake compared to Active). 107

List of figures

Figure 3.1: Photographs of the dorsal, ventral and lateral surfaces of the brain of the blue wildebeest. 38

Figure 3.2: Diagrammatic reconstruction of a series of coronal sections from the basal forebrain to the pons of the blue wildebeest illustrating the location of cholineacetyltransferase, tyrosine hydroxylase, magnocellular orexinergic, parvocellular orexinergic and serotonergic immunoreactive neurons. 50

Figure 3.3: Photomicrographs of the diagonal band of Broca. 56

Figure 3.4: Photomicrographs of the laterodorsal tegmental and pedunculopontine tegmental cholinergic nuclei in the pons of the blue wildebeest. 58

Figure 3.5: Photomicrographs of the locus coeruleus complex of the blue wildebeest immunostained for tyrosine hydroxylase (TH). 61

Figure 3.6: Photomicrographs in the region of the diffuse division of the locus coeruleus (A6d) and the subcoeruleus diffuse division (A7d) of the locus coeruleus complex in the pons of the blue wildebeest. 63

Figure 3.7: Photomicrographs in the region of the dorsal raphe nuclear complex in the blue wildebeest. 65

Figure 3.8: Photomicrographs in the region of the border between the main magnocellular main orexinergic cluster and the parvocellular orexinergic cluster in the hypothalamus of the blue wildebeest. 67

Figure 3.9: Photomicrographs of the thalamic reticular nucleus in the diencephalon of the blue wildebeest brain. 70

Figure 4.1: Polygraphic traces illustrating examples of the electroencephalogram (EEG) and electromyogram (EMG) in Wake, non-REM sleep and REM sleep states in the blue wildebeest. 93

Figure 4.2: Graph indicating the spectral power range characteristic of Wake, non-REM and REM sleep in the blue wildebeest. 95

Figure 4.3: Histograms depicting the total amount of time spent in each of the polysomnographically and actigraphically defined states for the 24 hour period, the light period, and the dark period. 97

Figure 4.4: Histograms depicting the average number of episodes for each of the polysomnographically and actigraphically defined states for the 24 hour period, the light period, and the dark period. 99

Figure 4.5: Histograms depicting the average episode duration for each of the polysomnographically and actigraphically defined states for the 24 hour period, the light period, and the dark period. 101

Figure 4.6: Flow diagram illustrating the occurrence of state transitions between 103

Wake, non-REM sleep and REM sleep states over the 72 h recording period analyzed.

Figure 4.7: Graph plotting the relationship between body mass and total sleep time for herbivores, including the blue wildebeest. 105

Figure 5.1: The average daily percentage maximum of neck (A) and leg activity (B), the average daily time inactive (C), the average faecal testosterone levels (D), the average daily subcutaneous temperature (E), the average daily variation in subcutaneous temperature of the blue wildebeest (F) and the average daily ambient air temperature in Dinokeng Game Reserve (G). 133

Figure 5.2: Box and whisker plots showing the distributions of group averages of daily counts of percentage maximum neck (A) and leg (B) activity, the daily time inactive (C), the duration of main rest episode (D), the number of daily rest episodes (E), the faecal androgen metabolite concentration (F), the daily subcutaneous temperature (G), the daily variation in subcutaneous temperature (H) of the blue wildebeest, and the daily ambient air temperature in Dinokeng Game Reserve (I) during the three periods, Pre-rut, Rut and Postrut. 135

Figure 5.3: The average percentage maximum of the daily timing of neck activity for each 5 minutes over a 24 hour period. Graphs A, B and C show the neck average percentage maximum for the pre-rut, rut and post-rut periods, respectively. 137

Figure 5.4: The average percentage maximum of the daily timing of leg activity for each 5 minutes over a 24 hour period. Graphs A, B and C show the leg average percentage maximum for the pre-rut, rut and post-rut periods, respectively. 139

Figure 5.5: Graphs illustrating the total counts of inactivity for any given 5 min 141

period scored over 21 days in the pre-rut (A), rut (B) and post-rut (C) for all animals.

Figure 5.6: The daily pattern of subcutaneous temperature over a 24 hour period, each 30 minute recording averaged over all animals for 21 days in the pre-rut (A), rut (B) and post-rut (C). 143

Figure 5.7: Graph showing the lack of a clear relationship between average ambient air temperature and the average hours of inactivity per day for the four male wildebeest recorded during the pre-rut, rut and post-rut. 145

CHAPTER ONE: **Introduction and chapter outline**

1.1. Introduction

Although sleep is universal across the animal kingdom, there is a large void in the understanding of sleep evolution (McNamara et al., 2008; Siegel, 2013; Barton and Capellini, 2016; Blumberg and Rattenborg, 2017; Rattenborg et al., 2017). It is still unclear whether phylogeny plays a major role or whether sleep patterns are purely an adaptive state according to environmental and biological demands (Siegel, 2009; Aulsebrook et al., 2016; Barton and Capellini, 2016 Rattenborg et al., 2017). Comparative methods of evolutionary biology are useful tools for investigating the functions of sleep as this still remains elusive (McNamara et al., 2008; Siegel, 2008, 2009, 2013; Barton and Capellini, 2016; Blumberg and Rattenborg, 2017; Rattenborg et al., 2017). Until recently, due to technological limitations, the majority of studies describing sleep in mammals have been performed under controlled and simplified laboratory conditions, with very few studies being conducted on animals in their natural environments (Capellini et al., 2010; Aulsebrook et al., 2016; Rattenborg et al., 2017). The studies that have focused on sleep in "wild" animals have been mostly undertaken in Zoological Gardens where salient factors such as the risk of predation is absent and food and water is available *ad libitum*. Furthermore, many of these studies have mainly been observational in nature thereby lacking any direct physiological measures of sleep (see McNamara et al., 2008; Lesku et al., 2009; Rattenborg et al., 2017). The assumption made by the studies from captive laboratories is that the interspecific differences in sleep observed in controlled conditions mirrors similar differences in the wild (Lesku et al., 2009). Additional gaps in our knowledge include the extent to which sleep varies in mammals that experience environmental seasonality and how sleep is restrained during breeding season or during long distance migrations (Capellini et al., 2010).

The studies aimed at revealing the evolutionary patterns of sleep through comparative analyses have all suffered one way or the other from methodological differences, as it is imperative that data from different sources are comparable (McNamara et al., 2008; Siegel, 2013; Barton and Capellini, 2016; Blumberg and Rattenborg, 2017). There has however, been a developing trend of studying sleep and behaviour of wild animals in a natural setting, owing to some technological advancements (Aulsebrook et al., 2016; Rattenborg et al., 2017). Rattenborg et al. (2008) observed that laboratory recorded brown-throated sloths (*Bradypus variegatus*) sleep on average 6 hours more per day than the same species when recorded living in their natural habitat. This large discrepancy shows the importance of recording, where possible, sleep from animals in their natural habitat. The data provided from such studies would be pivotal for future comparative sleep studies which could shed more light on the role that different environmental and biological factors may have on the evolution of sleep in mammals.

Sleep is commonly analysed using polysomnography (PSG). It is however, not always possible to measure and record PSG from animals in their natural environments, as PSG is invasive, expensive and generally should be performed in a sleep laboratory (Ancoli-Israel et al., 2003; Shambroom et al., 2012; Rattenborg et al., 2017). Additionally, these studies often require the subject animals to be euthanized after the experimental procedure to retrieve the recording devices and remove the implanted electrodes. Actigraphy (ACT) on the other hand, has been shown to be a non-invasive and cost-effective method to objectively measure sleep and assess sleep disorders in humans (Ancoli-Israel et al., 2003; Paquet et al., 2007; Shambroom et al., 2012, Rattenborg et al., 2017). Furthermore, validation studies in humans, have shown that the concordance in sleep scoring between ACT and PSG range from 83 to more than 90% (Ancoli-Israel et al., 2003; Paquet et al., 2007; Shambroom et al., 2012). Even though ACT has been used in the study of sleep in humans, limited studies have made

use of this methodology to study sleep in other mammals. It is thus a central interest of the current thesis to test the adequacy of ACT in the study of sleep in free-roaming blue wildebeest (*Connochaetes taurinus*) in their natural habitat. The use of ACT, as a reliable proxy for recording sleep, could lead to a new, minimally invasive method for the study of sleep in larger mammals in their natural environment. Moreover, ACT can be used for periods of up to 6 months, whereas PSG is currently limited to less than 10 days (Ancoli-Israel et al., 2003; Kanady et al., 2011; Shambroom et al., 2012). The use of ACT could therefore open up many questions regarding sleep in free-roaming large mammals, especially in relation to the changing environmental conditions that they face in their natural habitats, that were previously thought not to be answerable.

To gain comprehensive insight into the evolution and function of sleep, one needs to consider the ecological context, the physiological and behavioural parameters of sleep, but more importantly this has to be done in conjunction with the neuroanatomical elements of the somnogenic system. Several studies have found that the anatomy of the somnogenic system shows a predictability and that changes in the complexity of this system does not readily occur within an order but rather between orders (Manger, 2005; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2012; Calvey et al., 2013). Although, the somnogenic system is mostly conserved across mammals studied thus far, it is important to compare and provide detailed qualitative descriptions and quantitative differences, as this may account for the similarities or differences in the sleep phenomenology. Currently, there are very few studies that look at both the neuroanatomy and the physiological correlates of sleep in the same animal.

Therefore, this thesis will not only investigate the physiological and behavioural correlates of sleep, but also investigate and provide a comprehensive description of the neural systems controlling sleep in the blue wildebeest.

1.2. Aims and objectives:

Aims:

The global aim of the current thesis is to investigate sleep in free roaming blue wildebeest in their natural habitat from a neuroanatomical, physiological and behavioural point of view. The thesis furthermore also sets out to understand how factors such as the rut influence sleep phasing and architecture in this species.

Objectives:

1. Provide a full description of the sleep-wake related neuronal systems in the brain of the blue wildebeest by means of immunohistochemistry and to qualitatively and quantitatively compare the results to closely related species and orders.
2. Investigate the physiological measurable parameters of sleep and wake in free roaming blue wildebeest by means of polysomnography (PSG) and actigraphy (ACT).
3. Investigate the concordance of ACT and PSG for the study of sleep in this species.
4. Investigate the effect of the rut on rest and activity patterns in male blue wildebeest by means of ACT.

1.3. Chapter outline

CHAPTER TWO: **Literature review**

This chapter presents a summation of past and present mammalian sleep research as well as the rationale for selecting the study of sleep in the blue wildebeest for this PhD thesis.

CHAPTER THREE: **A preliminary description of the sleep related neural systems in the brain of the blue wildebeest, *Connochaetes taurinus*.**

The current study provides a detailed qualitative description of the organization of the cholinergic, catecholaminergic, serotonergic, orexinergic and GABAergic sleep-related systems in the brain of the blue wildebeest (*Connocheates taurinus)*, along with a quantitative analysis of the pontine cholinergic and noradrenergic neurons, and the hypothalamic orexinergic neurons. The aim of this study was to compare the nuclear organization of these systems to other mammalian species and specifically that reported for other Cetartiodactyla. In the brain of the blue wildebeest, from the basal forebrain to the pons, the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems for the most part, showed a corresponding nuclear organization to that reported in other mammals and more specifically the Cetartiodactyla. Furthermore, the description and distribution of the GABAergic system, which was examined through immunostaining for the calcium binding proteins calbindin, calretinin and parvalbumin, was also similar to that seen in other mammals. These findings indicates that sleep in the blue wildebeest is likely to show typically mammalian features in terms of the global brain activity of the generally recognized sleep states of mammals, but Cetartiodactyl specific features of the orexinergic system may act to lower overall daily total sleep time in relation to similar sized non-Cetartiodactyl mammals. This chapter has been submitted as a paper to *The Anatomical Record* and is under review (AR-17-0305).

CHAPTER FOUR: **Sleep in two free-roaming blue wildebeest (*Connochaetes taurinus*), with observations on the concordance of polysomnographic and actigraphic techniques**

Most studies examining sleep in mammals have been performed under controlled conditions in a laboratory or zoological facility with few studies being conducted on animals

in their natural environment. It is not always possible to record sleep polysomnographically (PSG) from animals in their natural environments, as PSG is invasive, requiring the surgical implantation of electrodes on the surface of the brain and other regions of the body. In contrast, actigraphy (ACT) has been shown to be a minimally-invasive method to objectively measure sleep. The aim of this study is two-fold, first, to measure sleep polysomnographically in free-roaming blue wildebeest (*Connochaetes taurinus*) under the most natural conditions possible, and second, to establish the degree of concordance between ACT and PSG recordings undertaken simultaneously in the free-roaming blue wildebeest. The present study examined sleep in the blue wildebeest, in a naturalistic setting, by means of two different measures of sleep, polysomnography (PSG) and actigraphy (ACT). PSG showed that total sleep time (TST) in the blue wildebeest for a 24-hour period was 4.53 h (± 0.12 h), 4.26 h (± 0.11 h) spent in non-REM sleep and 0.28 h (± 0.01 h) spent in REM sleep, with the remainder of the day of 19.47 h (± 0.12 h) spent in Wake. Similarly, ACT showed that the blue wildebeest spent 19.23 h (± 0.18 h) Active and 4.77 h (± 0.18 h) Inactive. For both animals studied, the Cohen's κ showed a fair agreement between the two techniques for sleep measurement, although differences were observed between individual days and animals.

CHAPTER FIVE: **Male blue wildebeest significantly increase activity levels during the breeding season (rut), but not at the expense of the time spent at rest**

Rest may be viewed as a state of adaptive inactivity that increases the efficiency of activity by regulating its timing and reducing energy use when activity is not beneficial. This would mean that animals can go without rest when specific demands, such as mating, favour being awake. Sexually active male blue wildebeest (bulls) are typically territorial and it has been reported that when a wildebeest bull is protecting a harem in his territory during the

mating season or rut, he neither eats nor rests. We examined the daily activity and inactivity patterns of dominant male blue wildebeest by means of actigraphy for a period of three months, which included the time of the rut. In addition, we measured faecal androgen metabolite (fAM) levels and subcutaneous temperature, both of which have variances known to relate to the mating season in other mammalian species. During the rut, the male wildebeest experienced higher levels of activity, fAM, and a greater range of body temperatures. Despite previous qualitative reports, the male blue wildebeest did rest daily during the rut, and while the amount of rest was low, it did not appear to be substantially lower than in the period prior to the rut. The amount of time spent inactive increased substantially and significantly after the rut. The timing of daily activity and inactivity patterns did not vary substantially with the rut compared to the pre-rut and post-rut periods. Throughout the recording period, the average daily ambient temperatures decreased steadily, along with the change of season, and the subcutaneous body temperature followed this pattern, although it was not as marked. It appears that in the post-rut period a substantive increase in rest occurs, potentially allowing the male wildebeest time to recover following a period of intense activity.

This chapter has been submitted as a paper to *Animal Behaviour* and the manuscript is currently under review (ANBEH-D-17-00890)

CHAPTER SIX: **Summary and Final Discussion**

This chapter summarizes the implications and significance of the results obtained in the studies comprising this thesis and highlights future directions for sleep research in the Cetartiodactyla order.

CHAPTER TWO: **Literature review**

2.1. Sleep in mammals

2.1.1. Defining sleep

Sleep is a complex combination of physiological and behavioural responses (Carskadon and Dement, 2011; Lima et al, 2005; Zepelin et al., 2005). Behaviourally, sleep is defined as sustained quiescence in a species-specific body posture accompanied by reduced responsiveness to external stimuli (Tobler, 1995; Lima et al., 2005; Zepelin et al., 2005). Rapid reversibility to wakefulness, once aroused, is another characteristic to distinguish sleep from coma and hypothermic states (Lima et al., 2005; Zepelin et al., 2005). Electrophysiologically, mammalian sleep is composed of two distinct states, namely, non-rapid-eye-movement (NREM) sleep and rapid-eye-movement (REM) sleep (Carskadon and Dement, 2011; Lima et al., 2005; Zepelin et al., 2005; Lesku, et al., 2006). NREM is generally characterized by high-amplitude and low frequency waves in the electroencephalogram (EEG) (Lesku et al., 2006; Berry et al., 2015; Kirsch et al., 2017). REM sleep is distinguished from NREM sleep when the EEG shows a desynchronized pattern of low-amplitude, mixed frequency neural activity, similar to wakefulness, rapid saccades in eye movement observed on the electro-oculogram, (EOG) (Lima et al., 2005; Zepelin et al., 2005; Lesku et al., 2006; Petersen, 2007; Berry et al., 2015; Kirsch et al., 2017) and muscle atonia, except the extraocular and intercostal muscles, observed on the electromyogram (EMG) (Lima et al., 2005; Zepelin et al., 2005; Lesku et al., 2006; Berry et al., 2015; Kirsch et al., 2017).

Sleep is not a homogenous state, with the two major components of sleep, NREM and REM states, alternating in a “sleep cycle” that is repeated one or more times during a sleep bout (Zepelin et al., 2005; Petersen, 2007; Capellini et al., 2008b; Berry et al., 2015; Kirsch et

al., 2017). Sleep architecture (i.e. total sleep times, phasing of sleep and sleep cycle length) varies extensively across mammalian species, with total sleep times ranging from 2 hours to 20 hours (Zepelin et al., 2005; Capellini et al., 2008b; Rattenborg et al., 2017). Sleep architecture also varies across the mammal's lifespan. Newborn human infants have a total sleep time of 16 to 18 hours per day in short blocks of time, with the total sleep time slowly decreasing as young adults typically sleep approximately 8 hours per night (Berry et al., 2015; Kirsch et al., 2017). The timing of daily sleep also varies, depending on the specific activity pattern of the animal, such as diurnal, nocturnal and crepuscular (Zepelin et al., 2005). Futhermore, mammals may also be classified as monophasic, biphasic or polyphasic sleepers. Monophasic sleepers tend to concentrate sleep into a distinct portion of the day. Biphasic sleepers, in addition to consolidating sleep into a single bout, tend to display "napping periods" under certain conditions when undisturbed (Tobler, 1995). In contrast, polyphasic sleepers, partition their sleep time into several bouts that may occur at any time of day (Lima et al., 2005; Zepelin et al., 2005; Capellini et al., 2008a). These distinct sleep architecture differences between species highlights the importance to compare sleep and the sleep-wake cycles across a broad range of species in order to understand the evolution of sleep.

2.1.2. The neurobiology of sleep

Great strides have been made in our understanding of the brain structures and mechanisms involved in the control of the sleep-wake cycle (Moruzzi and Magoun, 1949; Pace-Shott and Hobson, 2002; Jones, 2003; Siegel, 2004; Rosenwasser, 2009; Lu and Zee, 2010; Brown et al., 2012; Schwartz and Kilduff, 2015; Van Dort et al., 2015; Luppi et al., 2016). The earliest evidence of the arousal systems was demonstrated in the experiments by Moruzzi and Magoun in 1949, which showed that the stimulation of the brainstem reticular

formation produced cortical activation by displaying a generalized desynchronization of the EEG activity. It also showed that the descending networks of the reticular formation, projecting to the spinal cord, which stimulates sensory-motor activation, displayed as high EMG activity (Moruzzi and Magoun, 1949; Jones, 2003; Rosenwasser, 2009; Lu and Zee, 2010). Several neural circuits have been associated with the synchronization and desynchronization of cortical activity that distinguishes NREM sleep from wakefulness and REM sleep states (Jones, 2003; Rosenwasser, 2009; Lu and Zee, 2010; Schwartz and Kilduff, 2015). This includes the organization of the multiple interacting neurotransmitter systems in the brainstem, hypothalamus and basal forebrain and how they coordinate and converge onto common effector systems in the thalamus and cortex (Pace-Shott and Hobson, 2002; Jones, 2003; Lu and Zee, 2010; Brown et al., 2012). These neurotransmitter systems, namely the cholinergic, catecholaminergic, serotonergic, orexinergic, histaminergic and γ-Aminobutyric acid (GABA) systems, facilitate through chemically specific neuromodulation various activities in wake, REM and NREM sleep (Pace-Shott and Hobson, 2002; Jones, 2007; Lyamin et al., 2008).

The cholinergic nuclei located in the basal forebrain and hypothalamus represents one of the main arousal systems. Whereas, the cholinergic nuclei located in the pontomesencephalic tegmentum, specifically the pedunculopontine tegmental (PPT) and laterodorsal tegmental (LDT) nuclei are linked to the generation of REM sleep (Rye et al., 1984; Woolf, 1991; Pace-Shott and Hobson, 2002; Jones, 2007; Siegel, 2006; Kruger et al., 2012). The cholinergic neurons within the pontomesencephalic tegmentum have both dorsal and ventral projections through the reticular activating system. Dorsal projections from these cholinergic centres densely innervate the thalamic relay nuclei and the thalamic reticular nuclei, with neurons displaying high firing rates during wakefulness and REM sleep (Rye et al.,1984; Jones, 2003; Rosenwasser, 2009; Lu and Zee, 2010). Ventral projections from the

LDT and PPT innervate the basal forebrain, where areas such as the medial septum and diagonal band of Broca send signals to the cerebral cortex (Rye et al.,1984; Lu and Zee 2010). The basal forebrain has high activity during wake and REM sleep thereby contributing to the EEG desynchronization (Woolf, 1991; Pace-Shott and Hobson, 2002; Jones, 2003; Siegel, 2006; Lu and Zee, 2010; Kruger et al., 2012).

The catecholaminergic system, of which the major catecholamines utilized by the nervous system are dopamine (DA), noradrenaline (NA) and adrenaline, is widely distributed throughout the brain (Smeets and Gonzalez, 2000). The catecholaminergic neurons are involved in behavioural states, including vigilance and attention and have been implicated in stress-related arousal (Smeets and Gonzalez, 2000; Lu and Zee, 2010). Noradrenergic neurons are mostly concentrated in the locus coeruleus (LC), in the dorsolateral pontine region, and projects to the thalamus, hypothalamus as well as the cortex (Manger et al., 2003; Siegel, 2004; Rosenwasser, 2009; Lu and Zee, 2010). In contrast to the cholinergic system, the noradrenergic neurons in the LC discharge most rapidly during wakefulness. They are slower during NREM sleep and have little activity during REM sleep (Smeets and Gonzalez, 2000; Jones, 2003; Schwartz and Roth 2008; Lu and Zee, 2010).

The serotonergic system, the cells of which are found in the brainstem, is distributed throughout the raphe nuclei - a midline system extending from the midbrain to the medulla. The main portion of this system is the midbrain dorsal raphe complex (DR), from where ascending projections innervate the forebrain and cortex (Törk, 1990; Fuller, 1996; Jones 2003; Siegel, 2004). Serotonin plays an important regulatory role in terms of food intake, hormone secretion, stress responses, pain, and immune responses amongst other functions (Törk, 1990; Fuller, 1996; Ursin, 2002; Jones 2003; Siegel, 2004). In the sleep-wake cycle, the release of serotonin is greatest during waking, less during NREM sleep and minimal during REM sleep (Törk, 1990; Fuller, 1996; Jones 2003; Siegel, 2004). According to Jones

(2003), serotonin is also involved in the regulation of various behavioural states, where stimulation of the midbrain raphe nuclei leads to a behavioural inhibition that results in the suspension of movement and eating during waking.

Orexin (hypocretin) is a neuropeptide synthesized predominantly in the posterior and lateral hypothalamus (Siegel, 2004; Ohno and Sakurai, 2008; Lu and Zee, 2010). Orexin is suggested to play important roles in appetite, metabolism, neuroendocrine and autonomic regulation, as well as in, addiction, reward, sexual behavior, and motor activity (Ferguson and Samson, 2003; Sakurai, 2007; Ohno and Sakurai, 2008; Rosenwasser, 2009). Orexinergic neurons also play an important role in the maintenance of wakefulness by projecting and binding to its receptors, Ox1 and Ox2. These receptors are found dispersed throughout the central nervous system, including the cerebral cortex, forebrain, thalamus, and brainstem arousal nuclei (Siegel, 2004; Ohno and Sakurai, 2008; Lu and Zee, 2010). Orexin also stimulates somatic motor neurons and the sympathetic nervous system to maintain a waking state (Lyamin et al., 2008; Schwartz and Roth, 2008; Ohno and Sakurai, 2008; Rosenwasser, 2009; Lu and Zee, 2010).

Histaminergic (HA) neurons also play a role in the maintenance of the wake state since it is known that a lesion of the posterior hypothalamus produces a comatose-like continuous sleeping state (Siegel, 2004; und Halbach and Dermietzel, 2006; Schwartz and Kilduff, 2015). HA neurons are predominantly located in the posterior hypothalamus, specifically at the site of the tuberomamillary nucleus and project widely throughout the brain, including to the wake-promoting neuronal populations in the brainstem and to the cerebral cortex (Siegel, 2004; und Halbach and Dermietzel, 2006; Schwartz and Kilduff, 2015). Additionally, HA neurons in the tuberomamillary nucleus are directly inhibited by GABAergic neurons (Siegel, 2004).

Populations of γ-Aminobutyric acid (GABAergic) neurons involved in the sleep-wake cycle are located within the ventrolateral preoptic area (vLPO) and median preoptic nucleus (MnPO) (Saper et al., 2001; Gritti et al., 2003; Siegel, 2004; Rosenwasser, 2009; Lu and Zee, 2010). GABAergic neurons induce sleep by inhibiting cells that are involved in arousal functions and are reported to be more active during NREM sleep than they are during REM sleep and waking (Saper et al., 2001; Gritti et al., 2003; Siegel, 2004; Rosenwasser, 2009; Lu and Zee, 2010). Calcium-binding proteins, such as parvalbumin, calretinin and calbindin are useful for identifying and distinguishing the different subgroups of GABAergic neurons (Gritti et al., 2003).

Comparative analysis is a powerful tool for studying evolutionary questions, such as how sleep and brains co-evolved (McNamara et al., 2008; Siegel, 2013; Barton and Capellini, 2016; Blumberg and Rattenborg, 2017; Rattenborg et al., 2017). The sleep-wake related neuronal systems have demonstrated some evolutionary trends, and while these systems are quite similar across species for the most part, differences do occur (Manger, 2005). These differences have been shown to be not only species-specific and in other cases it has been shown to be order-specific (Manger, 2005; Maseko et al., 2007; Dell et al., 2010; Kruger et al., 2012; Calvey et al., 2013). Comprehensive species-level phylogenies are very important for various comparative studies with more closely related species exhibiting more similar biological traits (Agnarsson and May-Collado, 2008, Capellini et al., 2010; Barton and Capellini, 2016; Blumberg and Rattenborg, 2017; Rattenborg et al., 2017). This is shown in the case of the Cetartiodactyla, previously thought to be two distinct orders, namely Cetacea (whales and dolphins) and Artiodactyla (even toed ungulates) (O'Leary, 1999; Price et al., 2005; Dell et al., 2012). Through various phylogenetic studies it was discovered that cetaceans and artiodactyls share a common Condylarthran ancestor (O'Leary, 1999; Price et al., 2005; Dell et al., 2012). This mammalian superorder, Cetartiodactyla, contains nearly 300

extant species including many with extensive commercial importance (e.g. cow, pig, and sheep) and of conservation and aesthetic value (e.g. antelopes, giraffe, dolphins, etc.) (Agnarsson and May-Collado, 2008). Within the Cetartiodactyla superorder, in the bovidae family, is the blue wildebeest (*Connocheates taurinus)*.

To date, very few studies have examined the neuroanatomy of the blue wildebeest, especially in the context of the neural systems involved in the generation, maintenance and modulation of sleep. Moreover, limited neuroanatomical studies have been performed in the Cetartiodactyla order, and in most cases, there is a lack of a comprehensive qualitative and quantitative description of the sleep-wake neuronal systems (Tillet and Thibault, 1989; Østergaard et al., 1992; Kitahama et al., 1994; Tillet, 1994; Leshin et al., 1995a,b, 1996; Tillet and Kitahama, 1998; Ferreira et al., 2001; Iqbal et al., 2001; Bux et al., 2010; Ettrup et al., 2010; Dell et al., 2012, 2016a,b,c; Mahady et al., 2017; Davimes et al., 2017). While these studies have revealed that the neural systems involved in the sleep-wake cycle are, for the most part, typically mammalian, certain Cetartiodactyla specific features have been observed (Manger, 2005). For example, the neuronal somal sizes of the LDT and PPT nuclei have been observed to be homogenous in most mammals studied to date, however in the giraffe (*Giraffa camelopardalis*) (Bux et al., 2010), river hippopotamus (*Hippopotamus amphibius*) (Dell et al., 2016c), the Göttingen minipig (*Sus scrofa domesticus*) (Mahady et al., 2017) and the Arabian Oryx (*Oryx leucoryx*) (Davimes et al., 2017), there are size heterogeneities. Furthermore, the orexinergic system within the hypothalamus of the harbour porpoise (*Phocoena phocoena*), minke whale (*Balaenoptera acutorostrata*), river hippopotamus, and the Arabian Oryx (Dell et al., 2012, 2016b, c; Davimes et al., 2017) exhibit a medially located parvocellular cluster not observed in most other mammals (except African elephant (*Loxodonta africana*), Maseko et al., 2013). It has also been shown quantitatively that the number of orexinergic neurons found in the hypothalamus of the

harbour porpoise exceeds those found in the hypothalamus of the giraffe; however, these neurons are larger in the giraffe (Dell et al., 2012). By investigating the differences and similarities of the anatomical structures in the somnogenic system within a specific order, this can allow for insight into the changes observed in the physiological and behavioral parameters of sleep.

2.2. Physiological Measurement of Sleep

Polysomnography (PSG), the gold standard for the study of sleep, is a multi-parametric recording method of sleep and is used as a diagnostic tool in sleep medicine (Ancoli-Israel et al., 2003; de Souza et al., 2003; Shambroom et al., 2012; Rattenborg et al., 2017). Most sleep laboratories use technology and scoring rules from the American Academy of Sleep Medicine (AASM) manual for the scoring of sleep and associated events, which is routinely updated (Berry et al., 2015; Kirsch et al., 2017). PSG is typically performed at night in a controlled laboratory setting and monitors brain activity (EEG), eye movements (EOG, electrooculogram), muscle activity (EMG, electromyogram) and heart rhythms (ECG, electrocardiogram). These parameters are then used in the assignation of the various sleep states. In humans, sleep is usually scored using different stages: stage W (wakefulness), stage N1 (Non-REM 1), stage N2 (Non-REM 2), stage N3 (Non-REM 3) and stage R (REM) (Berry et al., 2015; Gonzalez, 2016; Kirsch et al., 2017). Stage W is typically associated with alpha waves, characterised by low amplitude, high frequency EEG (8-13 Hz); stage N1 associated with theta waves, characterised by low amplitude mixed frequency (4-7 Hz) waves; stage N2 associated with theta waves (4-7 Hz) interspersed with occasional bursts of fast waves also known as K-complexes (13-16 Hz); stage N3 associated with delta waves (0.5-3 Hz) with high amplitude and stage R associated with low amplitude mixed frequency theta activity EEG that resembles the wake EEG (Berry et al., 2015; Gonzalez, 2016; Kirsch

et al., 2017). The EMG, EOG and ECG are typically used to ensure that the assignation of sleep states, based on the EEG, are correct. During a wake episode, the EMG has a high voltage and frequency and varies with the degree of head and neck movement and the EOG shows variability with eye movements. During NREM sleep the EMG voltage decreases and stabilizes, and eye movements are limited. There are two phases of REM sleep namely, phasic and tonic, where the former contains bursts of rapid eye movements, respiratory variability and brief EMG activations, which are occasionally seen as muscle twitches (Berry et al., 2015; Kirsch et al., 2017). Tonic REM sleep is the portion of REM sleep where there is more consistent limited motor activity with few eye movements (Berry et al., 2015; Kirsch et al., 2017). ECG is typically stable during periods of waking and NREM sleep and becomes more erratic during REM sleep. PSG thus provides detailed information on sleep staging and latencies as well as sleep quotas and architecture (Ancoli-Israel et al., 2003; de Souza et al., 2003; Shambroom et al., 2012; Rattenborg et al., 2017). Albeit considered the gold standard methodology, PSG does have several limitations regarding studies over long periods of time. Some of these limitations include *inter alia* more naturalistic studies as well as the high cost of this rather laboratory-dependent procedure (de Souza et al., 2003; Kanady et al., 2011, Sadeh, 2011).

Recently, there have been improvements in the technological constraints of measuring sleep in more naturalistic environments (Rattenborg et al., 2008; Lesku et al. 2011; Lyamin et al., 2012; Voirin et al., 2014; Aulsebrook et al., 2016; Rattenborg et al., 2017). A data logger has been developed for wireless recording of EEG activity in animals that records and stores the signal to a memory chip mounted on the animal, which allows sleep to be recorded over distances spanning thousands of kilometres (Lesku et al., 2011; Rattenborg et al., 2017). Although these data loggers used for EEG sleep studies in more natural environments are

relatively light (less than 2 g without a power supply), there is a limitation on the recording period due to the size of the battery (Rattenborg et al., 2017).

A possible alternative to PSG, specifically regarding more longitudinal and naturalistic studies, is Actigraphy (ACT). ACT is a non-invasive method used to measure gross motor movement and monitor rest-activity cycles in humans and typically involves the subject wearing an actigraph devise on their non-dominant wrist for a specified period. The specific actigraph device used in all experiments associated with this thesis was the Philips Respironics Actiwatch Spectrum. Each actiwatch contains a piezoaccelerometer connected to a microchip that sums and records the number of acceleration events for each minute. Each actiwatch weighs approximately 25 g and has approximate dimensions of 35 x 35 x 12 mm. These devices can record and store acceleration events for up to 6 months (Ancoli-Israel et al., 2003; Kanady et al., 2011; Shambroom et al., 2012). Some studies have shown that periods of arm immobility correlate very strongly with sleep and can serve as a proxy for EEG or video identification of sleep (Hurelbrink et al., 2005; Chae et al., 2009). It has also been shown that sleep versus wake states during 24 hours can successfully be approximated by wrist actigraphy and this property has been used in the monitoring of insomnia, circadian sleep/wake disturbances and periodic limb disorders (Sadeh and Acebo, 2002; Ancoli-Isreal et al., 2003).

The advantages of using ACT over PSG is that it is more cost-effective, less invasive, easy to use in ambulatory settings, utilizes automatic scoring algorithms and most importantly it permits the study subject to stay in their natural sleep environment and retain full mobility which may render measured data more reliable. Validation studies have furthermore also shown that the concordance of ACT and PSG in scoring sleep in humans ranges from 83 to 90% (de Souza et al., 2003; Shambroom et al., 2012; Kanady et al., 2011) and in specific domains of sleep research and sleep medicine the American Sleep Disorders

Association (ASDA) have established that ACT is a reliable and valid sleep assessment tool. Over the past two decades ACT has also grown in popularity as an assessment tool in sleep research as it has been shown that the relative growth rate in the number of ACT publications is exceeding that of PSG publications (ACT-PSG ratio of 1:10 in 1991 compared to a ratio of 1:4 in 2009) (Sadeh, 2011).

Even though ACT has been used in the study of sleep in humans for the past 40 years (Sadeh, 2011), limited studies to date have made use of this methodology to study sleep in other mammals. It is thus a central interest of this thesis to test the adequacy of ACT as a suitable alternative to PSG in the study of sleep in free-roaming blue wildebeest in their natural habitat.

2.3. Factors influencing sleep

Animals in the wild are faced with numerous demands, such as foraging for food, mating strategies and defending against competitors and predators, which limit the amount of time available for sleep (Capellini et al., 2010; Siegel, 2013; Rattenborg et al., 2017). The notion is that, as with any biological process, the functions of sleep can only be fully understood when examined in the ecological context in which they evolved (Rattenborg et al., 2017). Comparative studies of mammalian sleep have started to reveal fundamental links between ecology and sleep architecture (McNamara et al., 2008; Capellini et al., 2010; Siegel, 2013; Aulsebrook et al., 2016; Barton and Capellini, 2016; Blumberg and Rattenborg, 2017; Rattenborg et al., 2017). Some of the factors considered to have an impact on sleep architecture and emphasize the importance of studying sleep in the animal's natural environment are discussed below.

2.3.1. Captive vs. Wild Sleep Studies

The ideal is to conduct experiments in natural habitats that are usually characterized by a rich variety of temperatures, light, sound levels and that these factors are taken into account when observing the important interactions within- and between-species (Capellini et al., 2010; Rattenborg et al., 2017). While in complete contrast, the laboratory, which is mostly used for experiments on mammals, is viewed as a seemingly simple environment wherein the various demands faced by animals in the wild are removed. In the laboratory, many environmental variables can be controlled to allow for the interpretation of the resulting data (Calisi and Bentley, 2009). According to Capellini et al. (2010) studying sleep in the laboratory raises two potential challenges. The first challenge is that different laboratory conditions and procedures might influence sleep times and that these simplified laboratory environments may not capture all the salient features of sleep (Lima et al., 2005). There has, however, been various factors identified that may influence the amalgamation of data from different studies. Some of these factors include *inter alia* the different laboratory housing conditions, the amount of time over which sleep is recorded, *ad libitum* feeding conditions, photoperiod, ambient temperature and whether the experimental animals were restrained during the recording period or not (Capellini et al., 2010). It has also been shown that circadian rhythms, immune function and reproductive physiology are influenced by captivity (Calisi and Bentley, 2009; Daan, 2011; Voirin et al., 2014).

The second challenge relates to the fact that sleep times in the laboratory setting does not necessarily reflect the sleep time in the wild (Capellini et al., 2010). Mice and hamsters are the dominant subjects of circadian rhythms research and these rodents are often used specifically due to their precise expression of endogenously generated rhythms of nocturnal activity and diurnal rest (Daan, 2011). The assumption is that this pattern reflects their behaviour in nature. Recent studies however show that the activity in both species in the field is to a large extent diurnal rather than nocturnal (Daan, 2011). A study by Rattenborg et

al. (2008) of the brown-throated three-toed sloths (*Bradypus variegatus*) found that in the rainforest the sloths slept 6 h less than the ones in captivity. Although the reasons for more sleep in captive sloths remain unclear, Voirin et al., (2014) proposes that they do not face the risk of predation as do their wild counterparts, and therefore they are free to sleep for longer. Capellini et al. (2010) suggests that sleep in the laboratory is an estimate of the optimal sleep needed for a species. Whereas sleep times in the wild may more closely represent the minimal sleep requirement of an individual. This occurrence again highlights the relevance and importance to study the variation in timing and duration of sleep within and between individuals of a particular species and more importantly to take into consideration the various ecological and species-specific factors when measuring sleep.

2.3.2. Effect of energetic constraints on sleep

A commonly cited theory on sleep function is one that suggests that sleep conserves energy, even more so than the energy conserved during quiet wake (Schmidt, 2014). The hypothesis is that grazing animals may need to spend more time awake to eat because of their low caloric food (Siegel, 2005). There does appear to be a correlation between body mass and total sleep time across mammals (Siegel, 2005; Gravett et al., 2017), and it seems to become more apparent when only the herbivores are considered (Siegel, 2005). Capellini et al. (2008b), further suggests that smaller species tends to have a higher total sleep time than larger species and are more likely to be polyphasic sleepers. Capellini et al. (2008b) suggests that sleep is partitioned into multiple bouts which in the case of smaller animals allows for them to forage more frequently than larger animals, mainly due to their higher mass-specific metabolic rates and limited fat reserves. Sleep is also often negatively correlated with the trophic position of the animal (Joiner, 2016). Herbivores generally spend more time than

carnivores in search of food. This behaviour in herbivores may suggest that they forfeit sleep and risk predation in favour of foraging.

Blue wildebeests are grazers with a preference for feeding on short, lush green grass and therefore have seasonal movements during the dry season, in search of better grazing (Estes, 1997; Skinner and Chimimba, 2005). A study by Selebatso et al. (2017) on the adaptive activity patterns of the blue wildebeest to environmental variability showed that the wildebeest did not display greater activity at night during the dry season when compared to the wet season. Instead activity during the wet season was highest at night thereby suggesting a strategy of conserving body stores over the dry season. Selebatso et al. (2017) suggests that the wildebeest chooses to conserve energy by reducing activity in forage limited dry seasons and chooses to maximise foraging in the wet season in order to accumulate enough body reserves. This indicates that although foraging plays an important role on the activity/inactivity of the animal, the seasonal variation also has an influence on the levels of activity and inactivity of the animal. This is particularly evident in the Arabian oryx that survives in the desert and exhibits seasonal flexibility in their temporal niche switching of 24h activity patterns at a seasonal level. They exhibit distinct nocturnal/crepuscular activity during summer and diurnal activity during winter (Davimes et al., 2016).

2.3.3. Effect of predation risk on sleep

A factor hypothesized to have the most influence on sleep phasing is predation risk. However, the nature of its influences is still contentious (Lima et al., 2005; Capellini et al., 2010; Acerbi and Nunn, 2011). It is believed that animals faced with a greater risk of predation will exhibit shorter sleep bouts with periods of arousal occurring more frequently. There is also a possible reallocation of sleep to a different time of the day for these animals (Lima et al., 2005; Acerbi and Nunn, 2011). Some have argued that sleep is an adaptation and

protective mechanism from predators thereby making them less conspicuous and enforcing immobility at times of the day when it is either dangerous or unproductive to be awake. This supports the immobilization hypothesis (Meddis, 1975; Zepelin et al., 2005; Capellini et al., 2010). Voirin et al. (2014) however, argue against this notion, indicating that although three-toed sloths remain still and cryptic while awake, they do not need to sleep to accomplish this immobility. This difference may ring true for sloths as this is their nature, but in larger mammals it is not as simple. Given that sleep is defined as a reduced responsiveness to external stimuli, a sleeping animal is therefore less aware of potential threats than an animal that is only quietly resting (Zepelin et al., 2005; Capellini et al., 2010).

The behavior and interaction between predator and prey has also proved to be important. Acerbi and Nunn (2011) developed an evolutionary model demonstrating that predator behaviour can have a strong influence on prey sleep patterns, including whether prey are monophasic or polyphasic. Lima et al. (2005) suggests that prey facing generalist predators are most likely to show major changes in the timing of their sleep patterns, i.e. sleeping when the predators are active so as to reduce risk. Whereas, a predator that specializes on a given prey species will most likely match any change in the prey's activity pattern (Lima et al., 2005).

Attention has also been directed to the predatory environment and the group size of the prey and its effect on sleep architecture (Allison and Van Twyver, 1972; Allison and Cicchetti, 1976). Animals sleeping in exposed sites and exposed to an increased risk of predation are believed to exhibit more fragmented sleep patterns, reduced sleep durations, more frequent arousals, lighter forms of NREM sleep and a disproportionate decrease in REM sleep (Tobler, 1989; Ball, 1992; Lima et al., 2005; Lesku et al., 2006; Capellini et al., 2008a, b). Conversely, a study performed on two sloth species in the wild found that although predation pressure influenced the timing of sleep, it did not influence the amount of

time spent asleep, which is contrary to what would be expected (Voirin et al., 2014). The group size of the prey is also suspected to influence sleep as the expectation that an animal's predation risk is decreased due to the benefits of collective vigilance, dilution and confusion effects and thus predicting an increase in an individual's sleep duration (Capellini et al., 2010).

2.3.4. Effect of mating on sleep

A mating system describes how males and females pair when choosing a mate (Krasnec et al., 2012). Common mating systems include monogamy (one male mates with one female exclusively); polyandry (one female gets exclusive mating rights with multiple males); and polygyny (one male gets exclusive mating rights with multiple females) (Beletsky and Orians, 1994; Byers et al., 1994; Estes, 1997; Krasnec et al., 2012). Polygyny can further be described as harem defence polygyny, in which males control access to females directly by taking advantage of social gatherings of females or by controlling resources (Beletsky and Orians, 1994). This mating system is commonly seen in mammals like pinnipeds (seals and walruses) and ungulates. In the case of the pronghorn (*Antilocapra americana*) the mate-sampling strategy of females forces males into many energetically taxing acts of harem defence (Byers et al., 1994). The males that parent the greatest number of offspring are typically those that were able to defend a harem for many successive days during the rut (Byers et al., 1994; Byers et al., 2010).

Moreover, sexual selection has led to the evolution of costly morphological, physiological and behavioural traits (Andersson, 1994; Lesku et al., 2012). The androgen testosterone influences various aspects of male reproductive physiology. Before and during the mating period, males will have increased levels of testosterone, which is associated with the aggressive behaviours associated with courtship, such as intensified calling, herding,

fighting and reproduction (Estes, 1997; Muller and Wrangham, 2004). With the onset of the mating season, testosterone levels rise modestly to support basic reproductive functions, such as spermatogenesis and courtship behaviour (Muller and Wrangham, 2004). During periods of heightened male aggression, testosterone levels increase further to a maximum physiological level (Muller and Wrangham, 2004). The conventional way of assaying sexual hormone secretion is to measure the hormone levels in blood samples. However, given the practical difficulties in free-roaming animals as well as the physical and chemical restraint for getting repeated blood samples that could in turn affect the animal's behaviour, an alternative is required (Ganswindt et al., 2002; Hodges et al., 2010). By monitoring the sexual hormone concentrations in faeces, the seasonal fluctuation of faecal androgen concentrations can be determined and the relationship between reproductive behaviours and faecal androgens can be analysed (Ganswindt et al., 2002; Hodges et al., 2010).

The strenuous activity associated with the mating period has been linked to an increase in body temperature (Kortner and Geiser, 1994). This increase in body temperature has been seen in the antechinus (*Antechinus stuartii*) during the mating period, where males showed a markedly elevated activity level (Kortner and Geiser, 1994). During the mating season the average daily body temperature was increased because animals maintained high body temperatures for longer time periods and because the resting body temperature was also elevated (Kortner and Geiser, 1994). The elevated resting body temperature was also reported in bull dromedary camels during the rut, but showed that the bulls would lower their body temperature early in the day to allow for the increase during the day with the strenuous activity (Grigg et al., 2009).

Sexual selection is fundamental to all species survival and the same can be said about sleep. Some have stated that sleep might simply be viewed as an adaptive state due to the varying ecological demands for wakefulness (Lesku et al., 2012; Siegel, 2012). The majority

of studies on the adaptive state or plastic response to sleep have been carried out on wild birds (Capellini et al., 2010). Laboratory studies have shown that birds sleep less around the time of their seasonal migration when they have to travel large distance with little opportunity for sleep (Rattenborg et al., 2004; Capellini et al., 2010). Lesku et al. (2012) showed that male pectoral sandpipers (*Calidris melanotos*) can maintain high neurobehavioral functioning even though the time spent sleeping during a 3-week period of intense male-male competition for access to fertile females is greatly reduced. There have been limited studies to assess whether mammals adjust their sleep patterns in response to mating, as can be seen in the adaptation made by birds to their sleep patterns.

The majority of artiodactyls are polygynous and are known to practice the two forms of polygyny, female defense polygyny, and resource defense polygyny (Beletsky and Orians, 1994; Byers et al., 1994; Estes, 1997; Krasnec et al., 2012). Mating season in the blue wildebeest, also known as the rut, lasts three weeks and normally occurs immediately after the rainy season when the animals can feed on lush healthy grasses. Leading up to the rut, increased testosterone production stimulates sperm production, resulting in increased calling, herding, and fighting amongst males (Estes, 1997). It has been reported that the males do not sleep or eat while there are sexually active females in the vicinity, and are constantly mating with or herding together as many females as possible (Estes, 1997). Therefore, sexually active territorial blue wildebeest bulls during the rut appear to be potential candidates that may exhibit a plastic response to sleep by substantially lowering, or even eliminating, the time spent at rest during the mating season or rut (Estes, 1997).

2.4. Experimental Animal

The animal investigated in the studies presented in this thesis is the blue wildebeest (*Connocheates taurinus*). Blue wildebeest are gregarious mammals, a distinctive, high-

shouldered antelope with a long broad muzzle and cow-like horns. Blue wildebeest typically form herds ranging from 20 to thousands of individuals (Estes, 1997; Skinner and Chimimba, 2005). Adults are dark grey in colour with a silvery sheen, with juveniles and females being browner in colour. Males are territorial and have an average shoulder height of 147 cm, and body mass range of 230 – 295 kg, whereas the females have a mean shoulder height of 135 cm and body mass range of 164 - 215 kg (Estes, 1997; Skinner and Chimimba, 2005). Both sexes have unridged horns, although males have longer and thicker horns (Estes, 1997; Skinner and Chimimba, 2005). The blue wildebeest is known to form some of the largest migratory herds of all antelopes. They are typically associated with savannah woodland habitats and are distributed throughout the northern and southern sub-regions of Africa (Skinner and Chimimba, 2005). Seasonal movements take place during the dry season when they are in search of better grazing. Blue wildebeests are grazers, with a preference for feeding on areas of short green grassland.

The blue wildebeest's social organization consists of territorial males, female herds and bachelor groups (Skinner and Chimimba, 2005). The primary predator of these artiodactyls is the lion, *Panthera leo* (Funston et al., 1998; Fryxell et al., 2007; Owen-Smith, 2008; Hayward et al., 2011; Thaker et al., 2010).

The timing of calving and rutting in the blue wildebeest is influenced by the climate in such a way that both occur under favourable conditions (Estes, 1997). These activities are highly synchronised and lasts for about three weeks (Estes, 1997; Skinner and Chimimba, 2005). The rut comes after the rains when the animals are in their best condition, thus ensuring conception (Estes, 1997). Sexual activity during this period is very marked and affects the territorial bull's physical wellbeing with very little time for grazing and resting (Estes, 1997; Skinner and Chimimba, 2005). Before and during the rut, males show a greatly increased hormone and sperm production, which leads to intensified calling, herding and

fighting (Estes, 1997; Skinner and Chimimba, 2005). The gestation period of the blue wildebeest is about 250 days.

Ungulates are a diverse group of mammals and consists of two main orders, namely: Cetartiodactyla (even-toed ungulates) and Perissodactyla (odd-toed ungulates). Ungulates comprise most of the world's largest herbivores. They are indigenous to almost every continent and have been capable of adapting to extreme environments ranging from harsh desert habitats to subarctic forests. Ungulates are an interesting group to study as the members of this group show great diversity in their body sizes and morphologies, phenotypes and lifestyles. Despite the nearly 300 species in this group and the great diversity exhibited by this order, very few studies have examined sleep phenomenology in these animals. Sleep has been recorded electrophysiologically for the cow (Ruckebusch et al., 1970; Ruckebusch, 1972; Hanninen et al., 2008), sheep (Ruckebusch, 1972; Bell and Itabisashi, 1973), pig (Ruckebusch, 1972; Campbell and Tobler, 1984; Robert and Dellaire, 1986), goat (Klemm, 1966; Bell and Itabisashi, 1973), horse (Ruckebusch, 1972), and donkey (Ruckebusch, 1972). Behavioural investigations of sleep or activity have been made in deer (Bubenik, 1960), okapi (Immelman and Gebbing, 1962), giraffe (Immelman and Gebbing, 1962; Tobler and Schwierin, 1996), black wildebeest (Von Richter, 1972; Vrahimis and Kok, 1993), and blue wildebeest (Talbot and Talbot, 1963; Berry, 1997; Selebatso et al., 2017). These studies, however, only represent a small percentage of ungulate species and points to the need for further investigations of sleep within this anatomically and ecologically diverse group. The studies that have examined ungulate sleep have either been conducted under controlled laboratory conditions or in Zoological gardens where it has been purely observational in nature. The data available for total sleep times for the different species examined thus far have furthermore also been obtained using different methodologies, i.e. electrophysiological

recordings *vs* behavioural observations, different ages, sexes and number of the animals and no standardization with regard to the length of recording.

This literature review has highlighted the need and the converging trend towards integrating the physiological, behavioural and neuroanatomical features of sleep in the continuing quest to understand the function and evolution of sleep. The evidence has shown that laboratory conditions and certain ecological factors, such as predation risk, sexual selection and foraging for food are likely important constraints on the duration of sleep and may also affect the architecture of sleep. This leads to the question whether sleep in mammals is merely an adaptive state that exhibits some form of plasticity. If this is indeed the case then a further question that arises is the degree to which these animals exhibit this kind of flexibility. The investigations into sleep in free roaming blue wildebeest in their natural habitat from a neuroanatomical, physiological and behavioural point of view, thereby contribute to a greater understanding of the function and evolution of sleep.

CHAPTER THREE: **A preliminary description of the sleep related neural systems in the brain of the blue wildebeest, *Connochaetes taurinus*.**

3.1. Introduction

The neural structures and mechanisms involved in the sleep-wake cycle facilitate, through chemically specific neuromodulation, various activities in arousal (Wake), rapid eye movement (REM) and non-REM (NREM) sleep (Pace-Shott and Hobson, 2002; Jones, 2003; Lyamin et al., 2008). The cholinergic nuclei located in the basal forebrain and hypothalamus represent one of the main arousal systems, whereas the cholinergic nuclei located in the pontomesencephalic tegmentum have been linked to the generation of REM sleep (Woolf, 1991; Pace-Shott and Hobson, 2002; Jones, 2003; Siegel, 2006; Kruger et al., 2012). The noradrenergic neurons in the locus coeruleus complex discharge rapidly during wake, slowly during NREM sleep, and show minimal activity during REM sleep (Jones, 2003; Schwartz and Roth, 2008). Similarly, the release of serotonin from dorsal raphe neurons is greatest during wake, decreased during NREM sleep and minimal during REM sleep (Törk, 1990; Jones, 2003). Orexin (hypocretin), a hypothalamic neuromodulatory peptide, plays an important role in the maintenance of wakefulness (Lu and Zee, 2010). GABAergic neurons are known to be involved in the sleep-wake cycle as regulators that affect the activity of the cholinergic, catecholaminergic, serotonergic and orexinergic neuronal groups (Gritti et al., 2003; Siegel, 2004; Bhagwandin et al., 2013). Calcium binding proteins, such as parvalbumin, calretinin and calbindin are useful for identifying and distinguishing the different subgroups of GABAergic neurons (Gritti et al., 2003).

The mammalian superorder Cetartiodactyla contains nearly 300 extant species, many with socio-economic (cow, pig, sheep) and conservation value (antelopes, giraffe, cetaceans)

(Agnarsson and May-Collado, 2008). The blue wildebeest (*Connochaetes taurinus*) is a member of the speciose Bovidae family within the Cetartiodactyla. Blue wildebeest are a distinctive, high-shouldered antelope with a long broad muzzle and cow-like horns, which typically form gregarious herds ranging from 20 to thousands of individuals (Smithers, 1966; Dorst and Dandelot, 1973; Estes, 1997; Skinner and Chimimba, 2005). The blue wildebeest are known to form some of the largest migratory herds of all mammals, and are typically associated with savannah woodland habitats throughout the northern and southern sub-regions of Africa (Estes, 1997; Skinner and Chimimba, 2005).

To date, only a few neuroanatomical studies have been conducted on the neural systems involved in the generation, maintenance and modulation of sleep in the order Cetartiodactyla (Tillet and Thibault, 1989; Østergaard et al., 1992; Kitahama et al., 1994; Tillet, 1994; Leshin et al., 1995a,b, 1996; Tillet and Kitahama, 1998; Ferreira et al., 2001; Iqbal et al., 2001; Bux et al., 2010; Ettrup et al., 2010; Dell et al, 2012, 2016a,b,c; Mahady et al., 2017; Davimes et al., 2017). While these studies have revealed that the neural systems involved in sleep are, for the most part, typically mammalian, certain Cetartiodactyl specific features have been observed (Manger, 2005). The neurons of the cholinergic laterodorsal tegmental (LDT) nucleus are larger than those of the pedunculopontine tegmental (PPT) nucleus in the giraffe (Bux et al., 2010) and the river hippopotamus (Dell et al., 2016c), but they were similar in size in the harbour porpoise and minke whale (Dell et al., 2016a,b), whilst the converse was true for the Göttingen minipig (Mahady et al., 2017) and the Arabian oryx (Davimes et al., 2017). The orexinergic neurons of the Cetartiodactyl hypothalamus exhibit a medially located parvocellular cluster not observed in most other mammals (except African elephant, Maseko et al., 2013), but that has been observed in the giraffe, harbour porpoise, minke whale, river hippopotamus, and Arabian oryx (Dell et al., 2012, 2016b,c; Davimes et al., 2017). In order to broaden our basis for comparison, the current study provides a detailed qualitative

description of the organization of the cholinergic, catecholaminergic, serotonergic, orexinergic and GABAergic sleep-related systems in the brain of the blue wildebeest, along with a quantitative analysis of the pontine cholinergic and noradrenergic neurons, and the hypothalamic orexinergic neurons.

3.2. Material and Methods

3.2.1. Brain acquisition

Two adult male blue wildebeest (average body mass 202 kg, average brain mass of 390.1 g) were captured from a wild population in the Dinokeng Game Reserve, South Africa, and were overdosed using sodium pentobarbital (200 mg/kg i.v.) under permission from the relevant governmental authorities. Both animals were handled according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (clearance number 2008/36/1), which parallel those of the National Institute of Health (NIH) for the care and use of animals in scientific experimentation. The entire head of these animals were perfusion fixed *via* the common carotid arteries with 0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4, 4°C) (Manger et al., 2009). The brains were then removed from the skulls (Fig. 3.1) and post-fixed for 48 h in 4% paraformaldehyde in 0.1 M PB at 4°C. The brains were then transferred to a 30% sucrose in 0.1 M PB solution at 4°C until equilibrated (approximately 7 days), and then placed in an antifreeze solution for the same length of time. The brains were then stored in the antifreeze solution at -20°C until immunohistochemical processing.

3.2.2. Sectioning, staining and immunohistochemistry

Prior to sectioning, both brains underwent MR imaging to reveal the general anatomy of the brain of the blue wildebeest (see Manger et al., 2010, 2012; Maseko et al., 2011, 2012

for MRI protocol). As both specimens showed a similar anatomy (i.e. there was no obvious gross morphological variance between the two specimens), we selected a single specimen to section for subsequent staining. The basal forebrain, diencephalon and pons were dissected from the remainder of the brain as a single block of tissue. This block of tissue was placed in 30% sucrose in 0.1 M PB at 4°C until it had equilibrated. The tissue was then frozen in crushed dry ice and mounted onto an aluminium stage that was attached to a sliding microtome modified to allow the sectioning of large tissue blocks. The entire tissue block was sectioned in the coronal plane, with a section thickness of 50 μm. A 1-in-20 series of sections was taken and the first ten series were stained for Nissl substance, myelin, choline acetyltransferase (ChAT), tyrosine hydroxylase (TH), dopamine-ß-hydroxylase (DBH), serotonin (5HT), orexin (OxA), parvalbumin (PV), calbindin (CB) and calretinin (CR), while the remaining series were placed back in antifreeze solution and returned to the freezer for future use. Sections used for the Nissl series were mounted on 0.5% gelatine coated glass slides and stained with 1% cresyl violet. Myelin sections were stored in 5% formalin at 4°C for two weeks and then mounted on 1.5% gelatine coated glass slides and subsequently stained with a modified silver stain to reveal myelin sheaths (Gallyas, 1979).

The sections used for immunohistochemistry were initially treated for 30 min with an endogenous peroxidase inhibitor (49.2% methanol: 49.2% 0.1M PB: 1.6% of 30% H_2O_2), followed by three 10 min rinses in 0.1 M PB. The sections were then pre-incubated at room temperature for 2 h in a blocking buffer solution containing 3% normal serum (normal rabbit serum, NRS, Chemicon, for ChAT sections, normal horse serum, NHS, Chemicon for DBH sections, and normal goat serum, NGS, Chemicon, for the remaining sections), 2% bovine serum albumin (BSA, Sigma) and 0.25% Triton X-100 (Merck) in 0.1M PB. The sections were then placed in a primary antibody solution (blocking buffer with correctly diluted primary antibody) and incubated at 4°C for 72 hours under gentle shaking. To reveal

cholinergic neurons, anti-choline acetyltransferase (ChAT) (AB144P, Chemicon, raised in goat) at a dilution of 1:3000 was used. To reveal putative catecholaminergic neurons, anti-tyrosine hydroxylase (TH) (AB151, Chemicon, raised in rabbit) was used at a dilution of 1:3000 and anti-dopamine-ß-hydroxylase (DBH) (MAB308, Merck-Millipore, raised in mouse) was used at a dilution of 1:4000. To reveal serotonergic neurons, anti-serotonin (5-HT) (AB938, Chemicon, raised in rabbit) at a dilution of 1:5000 was used. To reveal orexinergic neurons, anti-orexin-A (OxA) (AB3704, Merck-Millipore, raised in rabbit) at a dilution of 1:3000 was used. To reveal calbindin, calretinin and parvalbumin containing neurons and terminal networks, we used anti-calbindin (CB) (CB38a, Swant, raised in rabbit), anti-calretinin (CR) (7699/3H, Swant, raised in rabbit) and anti-parvalbumin (PV) (PV28, Swant, raised in rabbit), all at a dilution of 1:10000. This was followed by three 10 min rinses in 0.1M PB, after which the sections were incubated in a secondary antibody solution for 2 h at room temperature. The secondary antibody solution contained a 1:1000 dilution of biotinylated anti-goat IgG (BA-5000, Vector labs, for ChAT sections), biotinylated anti-mouse (BA-2001, Vector labs, for DBH sections), or biotinylated anti-rabbit IgG (BA-1000, Vector Labs, for the remaining sections) in a solution containing 3% NGS/NRS and 2% BSA in 0.1M PB. This was followed by three 10 min rinses in 0.1M PB after which the sections were incubated in AB solution (Vector Labs) for 1 h. After three further 10 min rinses in 0.1M PB, the sections were placed in a solution of 0.05% diaminobenzidine in 0.1M PB for five minutes (2 ml/section), followed by the addition of 3 µl of 30% H_2O_2 to each 1 ml of solution in which each section was immersed. Chromatic precipitation of the sections was monitored visually under a low power stereomicroscope. This process was allowed to continue until the background staining of the sections was appropriate enough to assist with architectonic reconstruction without obscuring any immunopositive neurons. The precipitation process was stopped by immersing the sections in

0.1M PB and then rinsing them twice more in 0.1M PB. To check for non-specific staining from the immunohistochemistry protocol, we omitted the primary antibody and the secondary antibody in selected sections, which produced no evident staining. The immunohistochemically stained sections were mounted on 0.5% gelatine coated slides and left to dry overnight. The sections were then dehydrated in graded series of alcohols, cleared in xylene and cover slipped with Depex.

3.2.3. Qualitative analysis

The sections were viewed with a low power stereomicroscope, and the architectonic borders of the sections traced according to the Nissl and myelin stained sections using a camera lucida. The immunostained sections were then matched to the drawings and the immunopositive neurons marked for ChAT, TH, 5-HT and OxA. The drawings were then scanned and redrawn using the Canvas 8 (Deneba) drawing program. The immunopositive parvalbumin (PV), calbindin (CB) and calretinin (CR) neurons and the distribution of their terminal networks, due to their often very high number and density, were matched to these drawings under the microscope and the relative densities of immunopositive neurons and terminal networks noted and photographed using high magnification light microscopy.

3.2.4. Quantitative stereological analysis

For the quantitative estimates of the number of orexinergic immunopositive neurons (OxA) in the hypothalamus, cholinergic immunopositive neurons (ChAT) in the laterodorsal tegmental nucleus (LDT) and pedunculopontine tegmental nucleus (PPT), as well as the noradrenergic immunopositive neurons (TH) in the locus coeruleus complex, we employed an unbiased designed based systematic random sampling stereological protocol. We used a MicroBrightfield (MBF) (Colchester, Vermont, USA) system with three plane motorised

stage, Zeiss.Z2 vario axioimager and StereoInvestigator software (MBF, version 11.08.1; 64-bit). Initially, pilot studies for the LDT, PPT, locus coeruleus, and hypothalamus were conducted to optimize sampling parameters (Table 3.1). Counting frames and grid sizes were optimized to achieve a mean coefficient of error of 10% or less (Gunderson and Jensen, 1987; 1999), and a guard zone of 2 μm was set so as to not include errors due to sectioning artifacts (West et al., 1991). Section thickness was measured at every sampling site, and the number of immunopositive neurons was counted in accordance with the principles of the optical fractionator method (West et al., 1991). The 'optical fractionator' method was used to stereologically estimate the number of ChAT+ neurons in the LDT and PPT as well as the number of TH+ neurons in the locus coeruleus and the OxA+ neurons in the hypothalamus using the following formula:

$$N = \sum Q- \bullet F1 \bullet F2 \bullet F3$$

N is the total estimated neuronal number, ∑Q- is the number of neurons counted, F1 = 1/section sampling fraction (SSF) = 1/ # sampled sections /total # of sections through reference space, F2 = 1/ area sampling fraction (ASF) = 1/area disector frame/ area of sampling step, F3 = 1/thickness sampling fraction (TSF) = 1/disector height (um)/ post-processing sections thickness (um) (Table 3.1).

The 'nucleator' probe was used to estimate the mean volume and cross-sectional area of the immunopositive neurons (Gundersen, 1988). To measure the somal volume and area of the PPT, LDT and orexin neurons, we exhaustively counted the respective immunopositive cells in the regions of interest. It should be noted that the hypothalamic parvocellular and magnocellular orexinergic positive cells of the current study are intermingled parcellations with no distinct anatomical features that separate them. To avoid observer bias in determining the neuronal size of these orexinergic sub-parcellations we exhaustively measured the parvo/magno neurons as one anonymous group. Once the results were generated by the

'nucleator' probe, we assigned those with smaller sizes as belonging to the parvocelluar cluster and those with larger volumes as belonging to the magnocelluar cluster. Stereological values generated for somal volume and area of orexinergic parvocellular and magnocellular groups, as well as LDT and PPT groups, were first tested for normality and then subject to two-sample analysis to test for statistical significance. The estimated stereological results obtained in the current study should be interpreted with the caveat that serial coronal sections from the brain of one individual was utilized due to the rarity and absolute large size of the tissue sample. With this caveat in mind, we used strict criteria, e.g. only neurons with complete cell bodies were counted, and obeyed all commonly known stereological rules.

3.2.5. Abbreviations

III – oculomotor nucleus; **Vmot** – motor nucleus of trigeminal nerve; **Vsens** – sensory nucleus of trigeminal nerve; **3V** – third ventricle; **4V** – fourth ventricle; **5n** – trigeminal nerve; **A6d** – diffuse portion of locus coeruleus; **A7d** – nucleus subcoeruleus, diffuse portion; **A7sc** – nucleus subcoeruleus, compact portion; **A8** – retrorubral nucleus; **A9l** – substantia nigra, lateral; **A9m** – substantia nigra, medial; **A9pc** – substantia nigra, pars compacta; **A9v** – substantia nigra, ventral, pars reticulata; **A10** – ventral tegmental area; **A10c** – ventral tegmental area, central; **A10d** – ventral tegmental area, dorsal; **A10dc** – ventral tegmental area, dorsal caudal; **A11** – caudal diencephalic group; **A12** – tuberal cell group; **A13** – zona incerta cell group; **A14** – rostral periventricular nucleus; **A15d** – anterior hypothalamic group, dorsal division; **A15v** – anterior hypothalamic group, ventral division; **Arc** – hypothalamic arcuate nucleus; **B9** – supralemniscal serotonergic nucleus; **C** – caudate nucleus; **ca** – cerebral aqueduct; **CLi** – caudal linear nucleus; **Diag.B** – diagonal band of Broca; **DRc** – dorsal raphe, caudal division; **DRd** – dorsal raphe, dorsal division; **DRif** – dorsal raphe, interfascicular division; **DRl** – dorsal raphe, lateral division; **DRp** – dorsal

raphe, peripheral division; **DRv** – dorsal raphe, ventral division; **DT** – dorsal thalamus; **f** – fornix; **fr** – fasciculus retroflexus; **GC** – central gray matter; **GiCRt** – gigantocellular reticular nucleus; **GP** – globus pallidus; **Hyp.d** – dorsal hypothalamic cholinergic nucleus; **Hyp.l** – lateral hypothalamic cholinergic nucleus; **Hyp.v** – ventral hypothalamic cholinergic nucleus; **IC** – inferior colliculus; **ic** – internal capsule; **IP** – interpeduncular nucleus; **Is.Call/TOL** – islands of Calleja/olfactory tubercle; **LDT** – laterodorsal tegmental nucleus; **ll** – lateral lemniscus; **LOT** – lateral olfactory tract; **LV** – lateral ventricle; **Mc** – main magnocellular cluster of orexinergic neurons; **mcp** – middle cerebellar peduncle; **mlf** – medial longitudinal fasciculus; **MnR** – median raphe nucleus; **N.Acc** – nucleus accumbens; **N.Bas** – nucleus basalis; **OT** – optic tract; **OTc** – optic tract cluster of orexinergic neurons; **P** – putamen nucleus; **PBg** – parabigeminal nucleus; **PC** – cerebral peduncle; **Pc** – parvocellular orexinergic cluster; **PCRt** – parvicellular reticular nucleus; **PIR** – piriform cortex; **PPT** – pedunculopontine tegmental nucleus; **R** – thalamic reticular nucleus; **Rmg** – raphe magnus nucleus; **RtTg** – reticulotegmental nucleus of the pons; **SC** – superior colliculus; **scp** – superior cerebellar peduncle; **Sep.L** – lateral septal nucleus; **Sep.M** – medial septal nucleus; **SON** – supraoptic nucleus; **STN** – subthalamic nucleus; **VPO** – ventral pontine nucleus; **xscp** – decussation of the superior cerebellar peduncle; **zi** – zona incerta; **Zic** – zona incerta cluster of orexinergic neurons

Figure 3.1: Photographs of the dorsal, ventral and lateral surfaces of the brain of the blue wildebeest (*Connochaetes taurinus*). Scale bar = 2 cm.

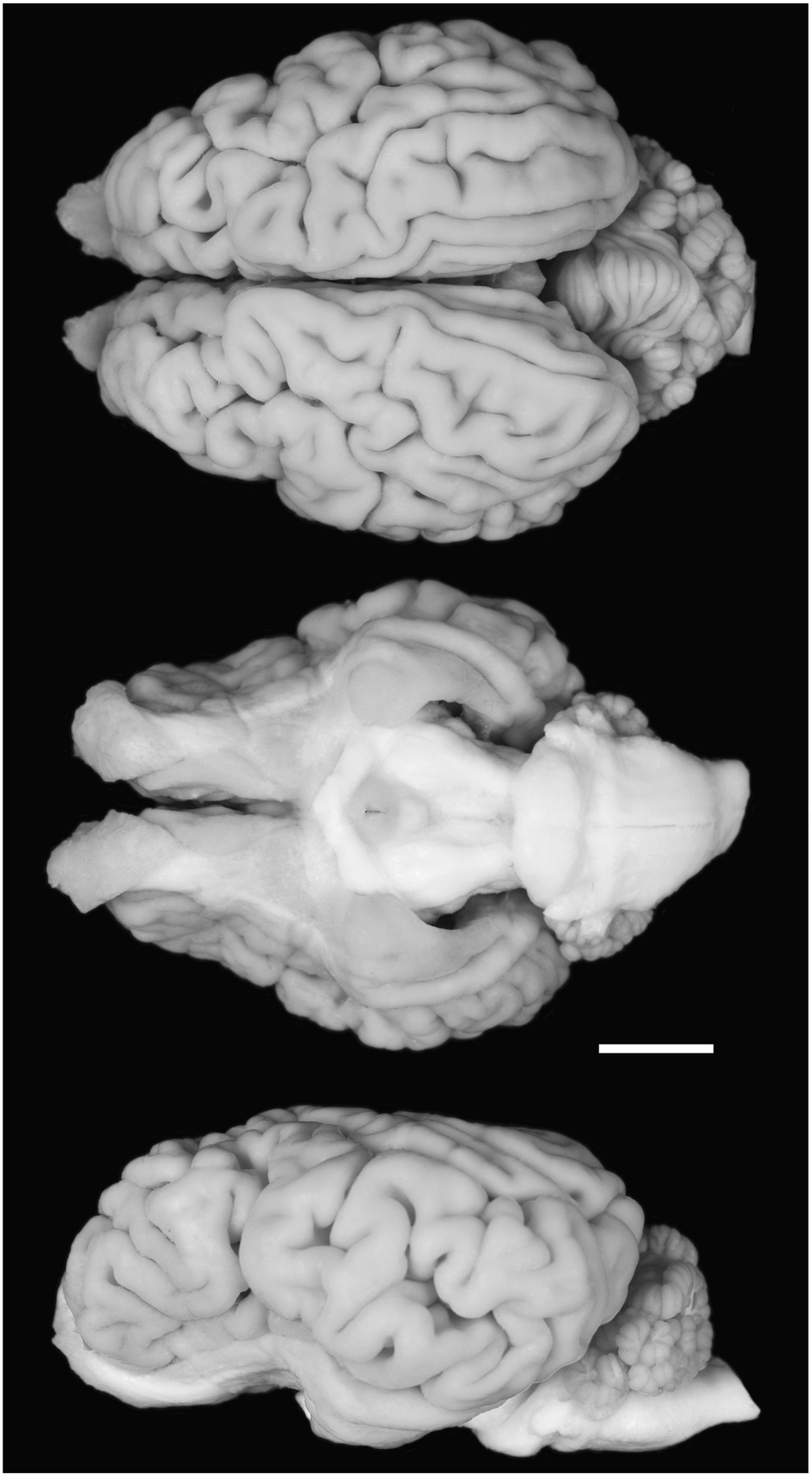


Table 3.1: Stereological parameters used for estimating neuron numbers in the blue wildebeest. LDT – laterodorsal tegmental nucleus, PPT – pedunculopontine tegmental nucleus, LC – locus coeruleus complex, ChAT+ - neurons immunopositive for choline acetyltransferase, TH+ - neurons immunopositive for tyrosine hydroxylase, OxA+ neurons immunopositive for orexin .

Nucleus examined	Counting frame size (µm)	Sampling grid size (µm)	Disector height (µm)	Cut thickness (µm)	Measured mounted thickness (µm)	Vertical guard zones (top and bottom, µm)	Section Interval	Number of sections	Number of sampling sites	Coefficient of error (Gundersen M=0)	Coefficient Error (Gundersen M=1)	1st Estimated CE (Schmitz-Hof)	2nd Estimated CE (Schmitz-Hof)
LDT (ChAT+)	400 x 400	400 x 400	16	50	20.5	2.0	20	3	106	0.20	0.15	0.22	0.15
PPT (ChAT+)	400 x 400	400 x 400	16	50	22.1	2.0	20	5	764	0.12	0.06	0.07	0.06
LC (TH+)	400 x 400	400 x 400	15	50	21.4	2.5	20	6	1249	0.10	0.07	0.08	0.06
Hypothalamus (OxA+)	400 x 400	400 x 400	14	50	18.5	2.0	20	7	2005	0.10	0.07	0.08	0.06

3.3. Results

In this study we assessed the neural systems involved in the regulation, modulation and coordination of the sleep-wake cycle in the blue wildebeest. The cholinergic, putative catecholaminergic, serotonergic and orexinergic systems were revealed in the basal forebrain, diencephalon, midbrain and pons of the wildebeest by means of immunohistochemical techniques (Fig. 3.2). In addition, we examined the distribution of neurons and terminal networks of the GABAergic system related to these specific nuclei. The results showed the nuclear organization of these neuronal regions was consistent with that reported in other mammals, and specifically other Cetartiodactyla.

3.3.1. Cholinergic nuclei of the basal forebrain and pons

Within the basal forebrain, four cholinergic nuclei, those with neurons immunopositive to the choline acetyltransferase antibody (ChAT+) were identified: the islands of Calleja/olfactory tubercle, medial septal nucleus, the diagonal band of Broca and the nucleus basalis (Fig. 3.2A-C). The islands of Calleja and olfactory tubercle were located in the most ventral aspect of the cerebral hemisphere, ventral to the overlying nucleus accumbens and rostral to the level of the anterior commissure. The islands of Calleja, which are embedded in the olfactory tubercle, formed distinct clusters of ChAT+ neurons. The ovoid shaped, multipolar and bipolar ChAT+ neurons in this region were distributed in a moderate density throughout and showed no specific dendritic orientation. The medial septal nucleus was identified by the moderate to high density of ChAT+ neurons located in the septal nuclear complex in the rostral half of the medial wall of the cerebral hemisphere. The ChAT+ neurons in the medial septal nucleus were mostly bipolar and ovoid shaped with a predominately dorsoventral dendritic orientation. In the ventromedial corner of the cerebral hemisphere, rostral to the hypothalamus, the diagonal band of Broca was identified. The

ChAT+ neurons were distributed in a high to moderate density throughout the diagonal band, and were mostly multipolar showing no specific dendritic orientation (Fig. 3.3A). The nucleus basalis was located rostral to the hypothalamus, ventromedial to the globus pallidus, and dorsal to the olfactory tubercle. A moderate density of ovoid, multipolar and bipolar ChAT+ neurons, showing no specific dendritic orientation, was observed in the nucleus basalis.

Within the pontomesencephalon we identified the pedunculopontine tegmental nucleus (PPT) and the laterodorsal tegmental nucleus (LDT) (Figs. 3.2N-P, 3.4A). The PPT was located in the dorsal aspect of the pontine tegmentum immediately inferior to the inferior colliculus and rostral to the trigeminal motor nucleus. The ChAT+ neurons of the PPT were found in a high to moderate density, exhibited a variety of somal shapes, a mixture of multipolar and bipolar types, and showed no specific dendritic orientation. Stereological analysis revealed (Table 3.2) that there were approximately 16 605.4 - 16 659.48 ChAT+ neurons in the PPT. The mean somal volume and surface area of these ChAT+ neurons were approximately 1 320.78 μm^3 (s.d. ± 838.50) and 135.62 μm^2 (s.d. ± 58.28), respectively. A high to moderately dense cluster of ChAT+ neurons in the ventrolateral aspect of the periaqueductal and periventricular grey matter immediately caudal to the oculomotor nucleus were designated as the LDT nucleus (Figs. 3.2O-P, 3.4A). The ChAT+ neurons of the LDT exhibited ovoid shaped soma, a combination of unipolar and bipolar types, and showed no specific dendritic orientation. Stereological analysis (Table 3.2) estimated that the number of ChAT+ neurons in the LDT was 2 411.16 – 2445.5. The average estimated somal volume was 772.82 μm^3 (s.d. ± 507.24), and a mean surface area of 95.47 μm^2 (s.d. ± 40.41). The ChAT+ neurons in the LDT were statistically significantly smaller than the neurons found in the PPT (Mann-Whitney, $p<0.001$).

3.3.2. The catecholaminergic locus coeruleus complex

Within the pontine region, the locus coeruleus complex was identified as a large number of tyrosine hydroxylase immunopositive (TH+) and dopamine-ß-hydroxylase immunopositive (DBH+) neurons (Fig. 3.5) that could be subdivided into four distinct nuclei: the subcoeruleus compact nucleus (A7sc), subcoeruleus diffuse nucleus (A7d), locus coeruleus diffuse nucleus (A6d), and the fifth arcuate nucleus (A5) (Fig. 3.2M-R). Neither an A6c (the compact division of the locus coeruleus) nor an A4 (the dorsal medial division of locus coeruleus) nucleus were identified in the blue wildebeest. The A7sc nucleus was located in the dorsolateral region of the pontine tegmentum adjacent to the ventrolateral border of the periventricular grey matter. The A7sc nucleus exhibited densely packed TH+/DBH+ neurons that were mostly ovoid shaped, bipolar and exhibited a mostly mediolateral dendritic orientation. Ventrolateral to the A7sc and anterior to the trigeminal motor nucleus, TH+/DBH+ neurons within the pontine tegmentum were assigned to the A7d nucleus. These TH+/DBH+ neurons exhibited a moderate to low density, had ovoid shaped soma, were either bipolar or multipolar type, and showed mostly dorsoventral dendritic orientation (Figs. 3.5, 3.6A). Some of the TH+/DBH+ neurons of the A7d were located around the superior cerebellar peduncle. A low density of TH+/DBH+ neurons located in the ventrolateral aspect of the periventricular grey matter was assigned to the A6d nucleus (Figs. 3.5, 3.6A). The TH+/DBH+ neurons in the A6d nucleus were ovoid, mostly bipolar, and showed no specific dendritic orientation. The A5 nucleus was located within the ventral pontine tegmentum lateral to the superior olivary nuclear complex. The TH+/DBH+ neurons of the A5 nucleus were found in a low density, were ovoid, multipolar and the dendrites showed no specific orientation. Stereological analysis of the locus coeruleus complex estimated that there were 14 227.92 – 14 844.66 neurons in this complex (Table 3.2). These

neurons had an average somal volume of 1204.56 μm^3 (s.d. ± 781.41) and a mean somal surface area of 128.35 μm^2 (s.d. ± 52.61).

3.3.3. Serotonergic nuclei of the dorsal raphe complex

The dorsal raphe nuclear complex was comprised of 6 distinct nuclei: interfascicular (DRif), ventral (DRv), dorsal (DRd), peripheral (DRp), lateral (DRl), and caudal (DRc) nuclei. This nuclear complex extended from the level of the oculomotor nerve nucleus to the level of the trigeminal motor nerve nucleus, mostly within the periaqueductal and periventricular grey matter and close to the midline (Fig. 3.2L-P). The DRif nucleus was defined as a moderate to high density nucleus within the most ventral medial aspect of the periventricular grey matter between the two medial longitudinal fasciculi. These DRif serotonergic immunopositive (5HT+) neurons were ovoid in shape, a mixture of bipolar and multipolar types, and showed no specific dendritic orientation. Immediately dorsal to the DRif, a high density of similar neurons, but with dendrites oriented in a mostly dorsoventral plane, were assigned to the DRv nucleus. The DRd nucleus was observed immediately dorsal to the DRv nucleus and ventral to the inferior border of the cerebral aqueduct (Fig. 3.7A). A high density of 5HT+ neurons was found within the DRd, and these neurons were similar to those observed in the DRv. In the ventrolateral aspect of the periaqueductal grey matter, located lateral to the DRd and DRv nuclei, a moderate density of 5HT+ neurons were assigned to the DRp nucleus (Fig. 3.7A). Some of the 5HT+ neurons assigned to this nucleus were also found in the adjacent dorsolateral midbrain tegmentum. The 5HT+ neurons in the DRp nucleus were larger than those of the DRif, DRv and DRd, exhibited various somal shapes, were mostly multipolar and showed no specific dendritic orientation. Positioned dorsolateral to the DRd nucleus and adjacent to the ventrolateral border of the cerebral aqueduct, a moderate density group of 5HT+ neurons was assigned to the DRl nucleus. These

neurons were larger than those of the DRd, showing a similar morphology to those of the DRp (Fig. 3.7A). As the DRl nucleus is followed caudally, where the cerebral aqueduct opens into the fourth ventricle, a low density arc of 5HT+ neurons were observed across the dorsal midline of the periventricular grey matter and were assigned to the DRc nucleus. The morphology of the 5HT+ neurons in the DRc nucleus were similar to those of the DRl and DRp. The DRc nucleus appears to be formed by the caudal coalescences of the two lateralised clusters of the DRl.

3.3.4. Orexinergic neurons of the hypothalamus

Orexin-A immunopositive (OxA+) neurons were found in the hypothalamus and immediately adjacent areas. The OxA+ neurons could be classified into two distinct neuronal types based on somal size, specifically the parvocellular and magnocellular OxA+ groups (Fig. 3.2D-G). The magnocellular OxA+ group could be divided into three distinct clusters: a main magnocellular orexinergic cluster (Mc) in the perifornical and lateral hypothalamic region; a magnocellular cluster associated with the zona incerta (Zic); and a magnocellular cluster associated with the optic tract (OTc). The parvocellular OxA+ neurons, found in the medial hypothalamus, formed a topographically continuous cluster.

The magnocellular OxA+ neurons were found throughout the perifornical and lateral aspects of the hypothalamus. There was a moderate to highly dense Mc of OxA+ neurons which was found in the lateral hypothalamus and perifornical region (Fig. 3.8A). The OxA+ neurons in the Mc exhibited ovoid shaped soma, were a combination of bipolar and multipolar types, and showed no specific dendritic orientation. A cluster of OxA+ neurons associated with the zona incerta (Zic) was located at the mid-level of the hypothalamus, extending laterally from the dorsolateral border of the Mc, investing into the medial most aspects of the zona incerta, similar to the A13 catecholaminergic nucleus of the

hypothalamus. The morphology of these neurons was similar to that of the OxA+ neurons of the Mc, but the dendrites were oriented in a mediolateral direction, and the soma were more elongated. Ventrally, at more rostral levels within the hypothalamus, a small number of OxA+ neurons were located in the ventrolateral region of the hypothalamus adjacent to the optic tract, forming the optic tract cluster (OTc). The morphology of these OxA+ neurons was similar to that of the Mc OxA+ neurons.

The parvocellular OxA+ cluster (Pc) was restricted to the medial zone of the hypothalamus, between the wall of the third ventricle and the fornix, but with the highest density of cells being located paraventricularly. The Pc OxA+ neurons were found in a moderate density along the wall of the third ventricle and there were no distinct topological discontinuities (Fig. 3.8A), which precludes dividing this neuronal cluster into smaller units. The somal bodies were ovoid to round in shape, mostly bipolar, and were markedly smaller than the magnocellular OxA+ neurons.

Stereological analysis of the orexinergic neurons in the hypothalamus revealed an estimate of approximately 11 716.58 – 11 790.76 neurons, with an average somal volume and an average surface area of 2 414.61 μm^3 (s.d. ± 1351.21) and 203.97 μm^2 (s.d. ± 70.42) respectively (Table 3.2). The magnocellular OxA+ neurons had an average surface area of 309.83 μm^2 (s.d. ± 64.89) and an average somal volume of 3 983.51 μm^3 (s.d. ± 1 479.93), while the parvocellular OxA+ neurons had an average surface area of 156.21 μm^2 (s.d. ± 30.35) and an average somal volume of 1 566.98 μm^3 (s.d. ± 436.05).

3.3.5. GABAergic neurons and terminal networks in sleep-related nuclei

In the blue wildebeest the GABAergic neurons and terminal networks, specifically the subtypes containing the calcium-binding proteins calbindin (CB), calretinin (CR) and parvalbumin (PV), were immunolabelled. The density of the immunopositive (CB+, CR+

and PV+) neurons and terminal networks were described in relation to the specific sleep-wake nuclei associated with the cholinergic, catecholaminergic, serotonergic and orexinergic systems (Table 3.3).

3.3.5.1. GABAergic neurons and terminal networks in the cholinergic nuclei

In the islands of Calleja/olfactory tubercle, a low to moderate density of CB+ neurons was observed, associated with a low density CB+ terminal network. A moderate density of CR+ and PV+ neurons and terminal networks was also found in this nucleus, although the PV+ terminal network showed a slightly higher density than the CR+ terminal network. In the medial septal nucleus, the density of CB+ neurons and terminal networks was absent to low, while the density of CR+ neurons and terminal networks was high, and the density of PV+ neurons and terminal networks was moderate to high. A moderate to high density of both PV+ and CR+ neurons and terminal networks was found in the diagonal band of Broca, but a low to moderate density of CB+ neurons and a moderate density of CB+ terminal networks was observed (Fig. 3.3). In the nucleus basalis both the CB+ neurons and terminal networks showed a high density compared to the moderate density of both PV+ and CR+ neurons and terminal networks.

In the pontomesencephalic region, in both the LDT and PPT nuclei, a low density of CB+ neurons and terminal networks was observed (Fig. 3.4). In both nuclei there was a low to moderate density of CR+ neurons and in LDT the CR+ terminal networks were observed in a low to moderate density, while in PPT the CR+ terminal networks were in a moderate to high density. The PV+ neurons and terminal networks were found in a low to moderate density in both the LDT and PPT.

3.3.5.2. GABAergic neurons and terminal networks in the catecholaminergic nuclei

A low to moderate density of CB+ neurons and a moderate density of CB+ terminal networks were observed in the A7sc nucleus. The density of the CR+ neurons in the A7sc was low to moderate, while the terminal networks showed a moderate to high density in this nucleus. The PV+ neurons and terminal networks displayed a low to moderate density in both the A7sc nucleus and the A7d nucleus. Within the A7d nucleus a very low density of CB+ neurons, but a moderate density of CB+ terminal networks was observed. A moderate density of CR+ neurons and a moderate to high density of CR+ terminal networks was found in the A7d nucleus. Within the A6d nucleus, the CB+ neurons were sparse, but the density of CR+ neurons was low to moderate. The CB+ and CR+ terminal network densities were moderate in density in the A6d nucleus. The PV+ neurons and terminal networks exhibited low to moderate densities in the A6d nucleus (Fig. 3.6).

3.3.5.3. GABAergic neurons and terminal networks in the serotonergic nuclei

A moderate density of both CB+ neurons and terminal networks were found throughout the dorsal raphe nuclear complex (Fig. 3.7), with the exception of the DRc, which showed low CB+ neuron and terminal network densities. In the DRv, DRd and DRp nuclei of the dorsal raphe complex, the CR+ neurons were low to moderate in density with a moderate density of CR+ terminal networks. The DRl and DRc showed low density of CR+ neurons and low to moderate density of CR+ terminal networks. A moderate density of CR+ neurons was observed in the DRif along with a moderate to high density of CR+ terminal networks. A very low density of PV+ neurons and terminal networks was observed in all six nuclei of the dorsal raphe nuclear complex.

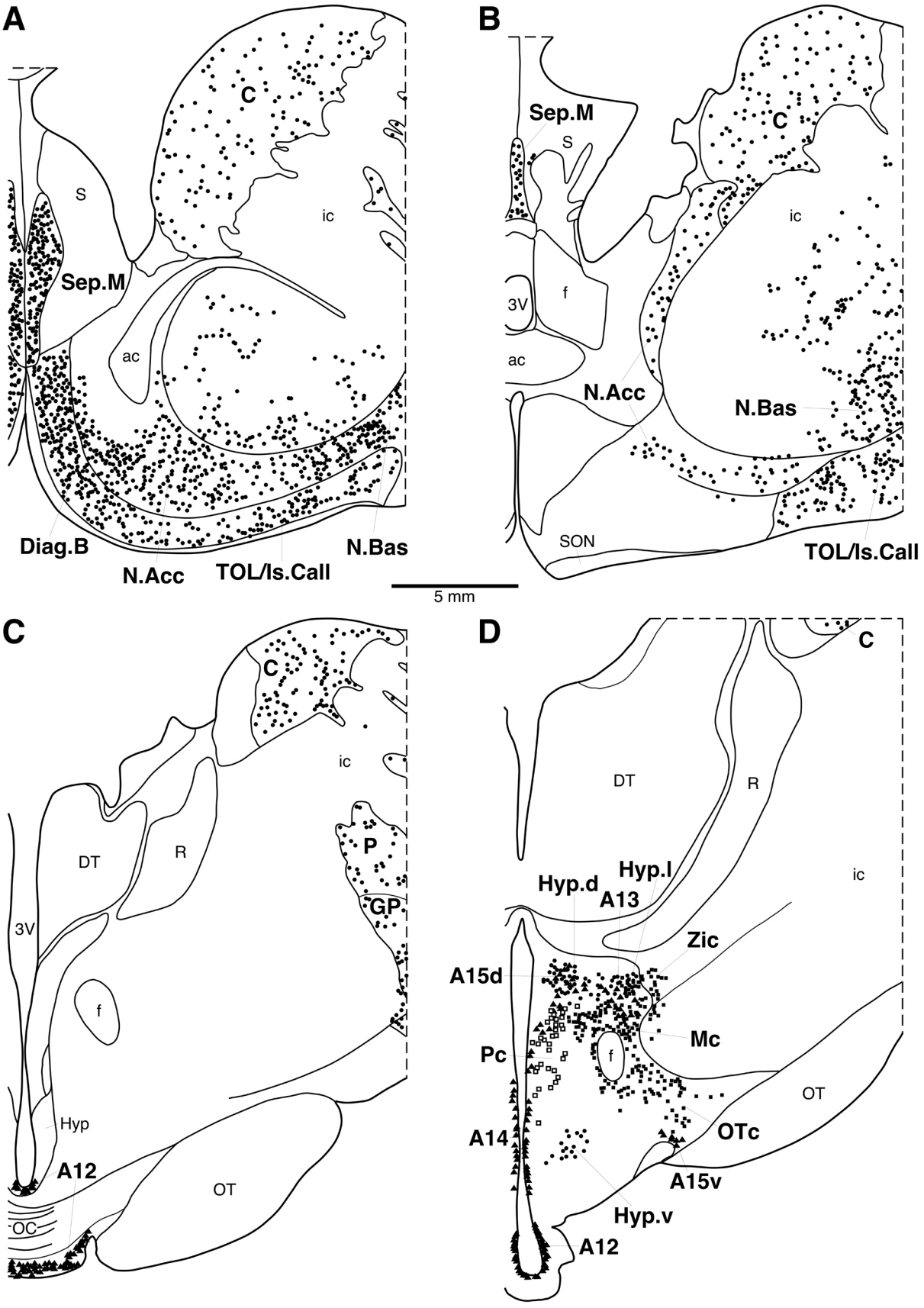
3.3.5.4. GABAergic neurons and terminal networks in the orexinergic nuclei

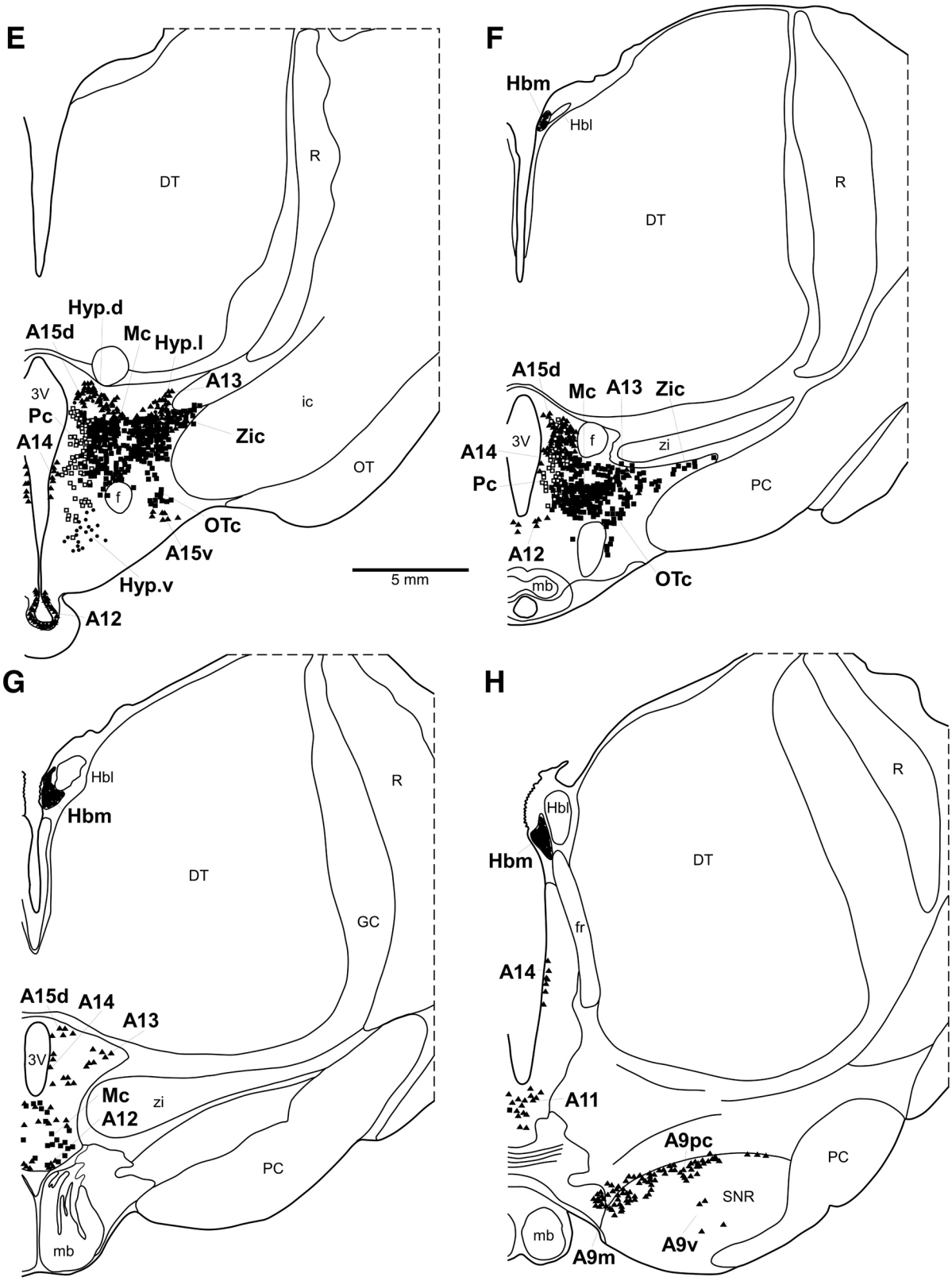
In the Mc a low to moderate density of CB+ neurons and a low density of CB+ terminal networks was observed (Fig. 3.8). The densities of CR+ neurons and terminal networks within the Mc both showed a moderate density, while the PV+ structures were in a very low density. In the OTc a moderate to high density of both CB+ and CR+ neurons and terminal networks was observed, but only a very low density of PV+ neurons and terminal networks was present. Within the Zic, the density of CB+ neurons was low to moderate, with a moderate density of CB+ terminal networks, and a very low density of PV+ neurons and terminal networks. A moderate density of CR+ neurons and terminal networks was observed in the Zic. A moderate density of CB+ neurons and a low to moderate density of CB+ terminal networks was observed in the Pc. CR+ neurons in the Pc displayed a low density, while there was a moderate density of CR+ terminal networks. The PV+ neurons and terminal networks exhibited a very low density in the Pc.

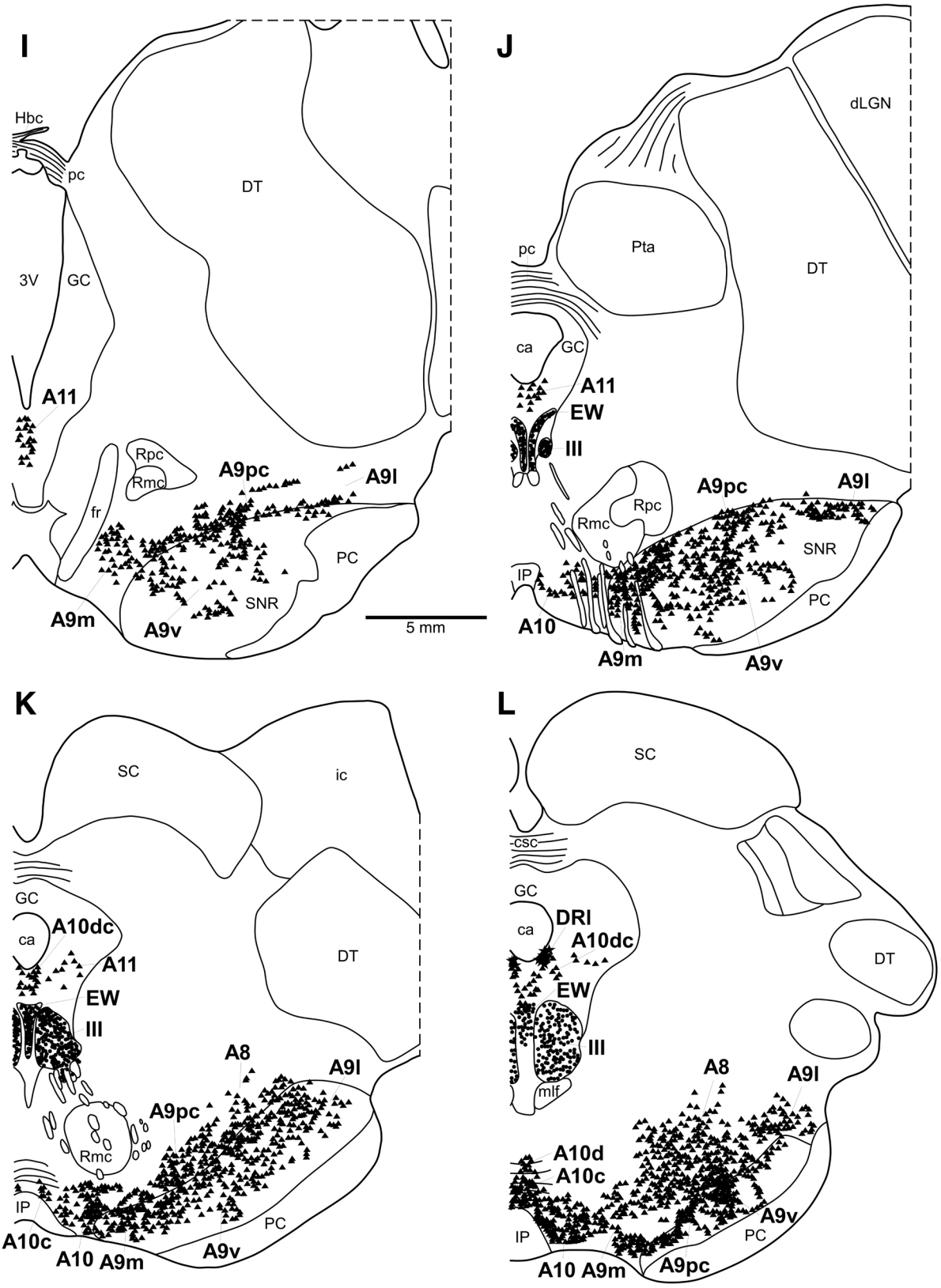
3.3.5.5. GABAergic neurons and terminal networks in the thalamic reticular nucleus

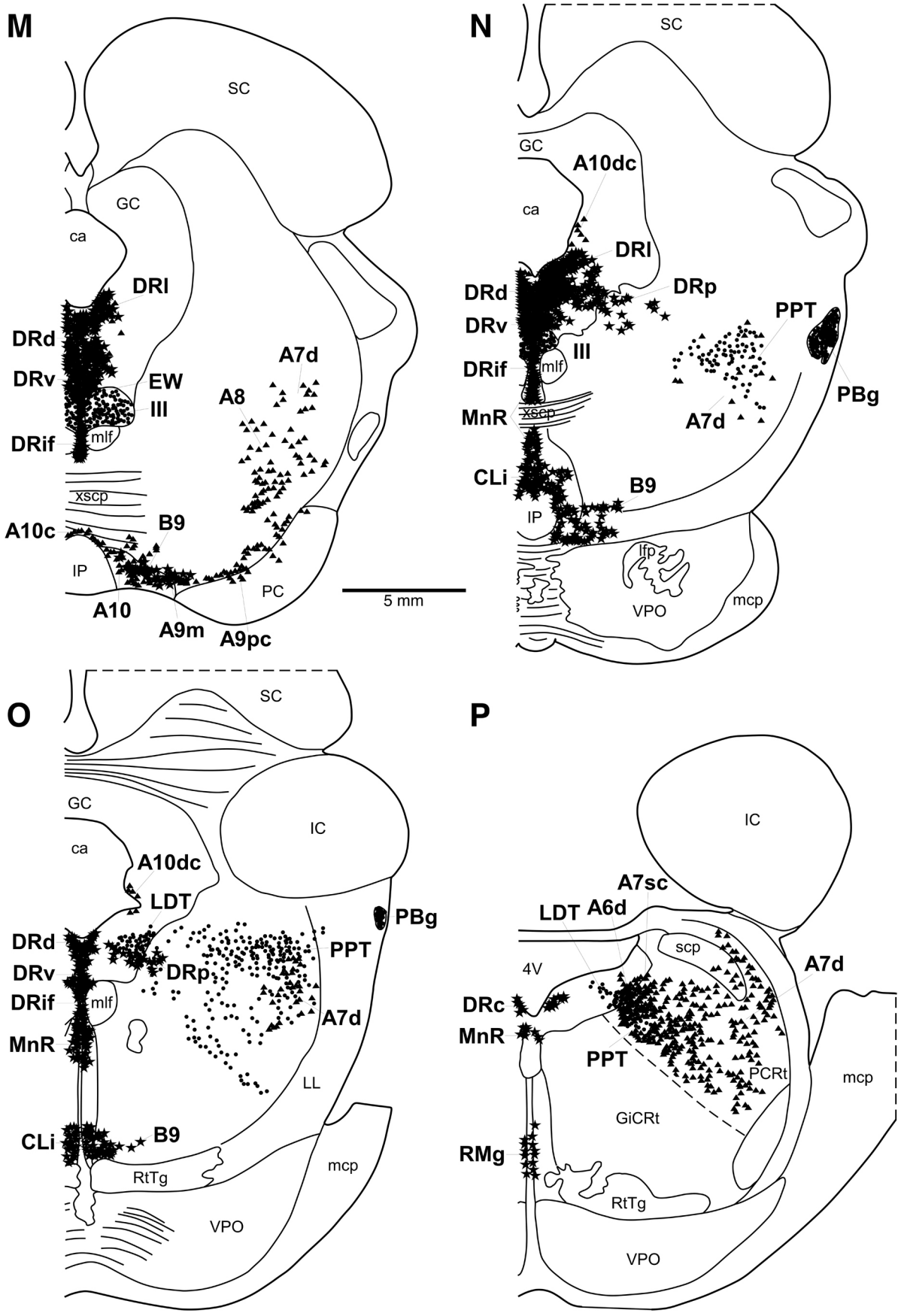
The location of the thalamic reticular nucleus in the brain of the blue wildebeest was typical in location and appearance of that of other mammals (Fig. 3.9), being found mostly lateral and anterior to the nuclear mass of the dorsal thalamus. A low density of PV+ neurons were found throughout the reticular nucleus, and these neurons were triangular in shape, multipolar, but had no specific dendritic orientation (Fig. 3.9B). Occasional CB+ bipolar neurons were found in the reticular nucleus (Fig. 3.9C), and a higher number of CR+ bipolar neurons were observed (Fig. 3.9D), but for both the CB+ and CR+ neurons, the intensity of the immunostaining was less than that observed for the PV+ neurons.

Figure 3.2: Diagrammatic reconstruction of a series of coronal sections from the basal forebrain to the pons of the blue wildebeest illustrating the location of choline acetyltransferase (ChAT, closed circles), tyrosine hydroxylase (TH, closed triangles) magnocellular orexinergic (OxA, closed squares), parvocellular orexinergic (OxA, open squares) and serotonergic (5HT, closed stars) immunoreactive neurons. Each symbol represents a single neuron. The outlines of the architectonic regions were drawn using Nissl and myelin stain and immunoreactive neurons marked on the drawings. Figurine **A** represents the most rostral section, **R** the most caudal. Each figurine is approximately 2000 μm apart. See list for abbreviations.


A
C
S
ic
Sep.M
ac
Diag.B
N.Acc
TOL/ls.Call
N.Bas
B
Sep.M
S
C
ic
3V
f
ac
N.Acc
N.Bas
SON
TOL/ls.Call
5 mm
C
C
ic
DT
R
P
GP
3V
f
Hyp
A12
OT
OC
D
C
DT
R
ic
Hyp.d
Hyp.l
A13
Zic
A15d
Mc
Pc
f
OT
A14
OTc
A15v
Hyp.v
A12


E
DT
R
Hyp.d
A15d
Mc
Hyp.l
A13
3V
Pc
A14
ic
Zic
OT
f
OTc
A15v
Hyp.v
A12
5 mm
F
Hbm
Hbl
DT
R
A15d
Mc
A13
Zic
3V
f
zi
A14
Pc
PC
A12
mb
OTc
G
Hbl
Hbm
R
DT
GC
A15d
A14
A13
3V
Mc
A12
zi
PC
mb
H
Hbl
R
Hbm
DT
fr
A14
A11
A9pc
PC
SNR
mb
A9v
A9m


I
Hbc
pc
DT
3V
GC
A11
Rpc
Rmc
A9pc
A9l
fr
PC
SNR
A9m
A9v
5 mm
J
dLGN
pc
Pta
DT
ca
GC
A11
EW
III
A9pc
A9l
Rpc
Rmc
SNR
IP
PC
A10
A9m
A9v
K
SC
ic
GC
A10dc
ca
DT
A11
EW
III
A8
A9l
A9pc
Rmc
IP
PC
A10c
A10
A9m
A9v
L
SC
csc
GC
DRl
ca
A10dc
DT
EW
III
A8
A9l
mlf
A10d
A10c
A9v
IP
PC
A10
A9m
A9pc


M
SC
GC
ca
DRl
DRd
DRv
EW
III
DRif
mlf
A7d
A8
xscp
A10c
B9
IP
PC
A10
A9m
A9pc
5 mm
N
SC
GC
A10dc
ca
DRl
DRd
DRp
DRv
PPT
III
DRif
mlf
PBg
MnR
xscp
A7d
CLi
B9
IP
lfp
VPO
mcp
O
SC
GC
IC
ca
A10dc
LDT
PBg
DRd
PPT
DRv
DRp
DRif
mlf
A7d
MnR
LL
CLi
B9
RtTg
mcp
VPO
P
IC
A7sc
LDT
A6d
scp
A7d
4V
DRc
MnR
PPT
PCRt
mcp
GiCRt
RMg
RtTg
VPO

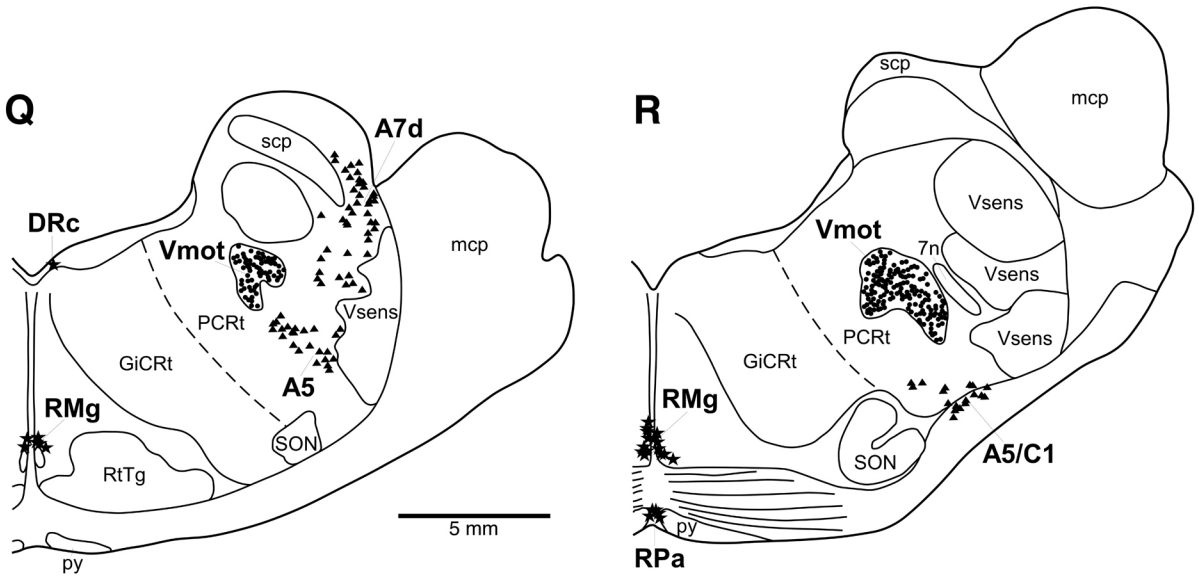

Q
A7d
scp
DRc
Vmot
mcp
PCRt
Vsens
GiCRt
A5
RMg
SON
RtTg
5 mm
py
R
scp
mcp
Vsens
Vmot
7n
Vsens
PCRt
Vsens
GiCRt
RMg
SON
A5/C1
py
RPa

Figure 3.3: Low (main image) and high (inset) magnification photomicrographs of the diagonal band of Broca (**Diag.B**) of the blue wildebeest. **(A)** Neurons immunoreactive for choline acetyltransferase showing part of the nucleus accumbens (**N.Acc**). (**B)** Parvalbumin immunoreactivity, note the presence of a low density of parvalbumin immunoreactive structures in this nucleus. **(C)** Calbindin immunoreactivity, note the low density of immunoreactive terminals and neurons. (**D)** Calretinin immunoreactivity, note the moderate density of immunoreactive terminals and neurons. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.

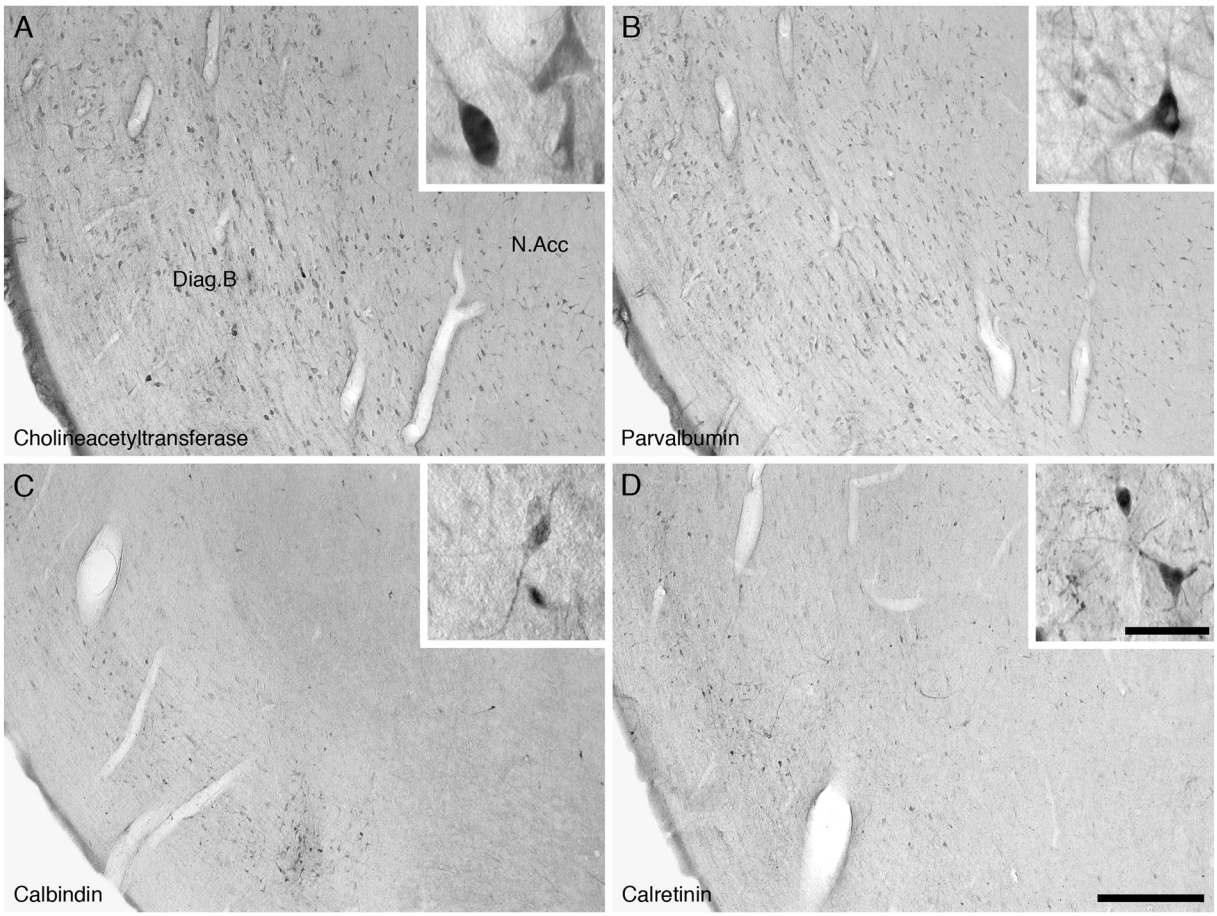

A
N.Acc
Diag.B
Cholineacetyltransferase
B
Parvalbumin
C
Calbindin
D
Calretinin

Figure 3.4: Low (main image) and high (inset) magnification photomicrographs of the laterodorsal tegmental (**LDT**) and pedunculopontine tegmental (**PPT**) cholinergic nuclei in the pons of the blue wildebeest. **(A)** Neurons immunoreactive for choline acetyltransferase showing the laterodorsal tegmental nucleus (**LDT**) and part of the pedunculopontine tegmental nucleus (**PPT**). **(B)** Parvalbumin immunoreactivity, note the low density of parvalbumin immunoreactive structures in these nuclei. **(C)** Calbindin immunoreactivity, note the absence of cells and terminals in these nuclei. (**D)** Calretinin immunoreactivity, note the presence of the occasional cell in these nuclei. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.

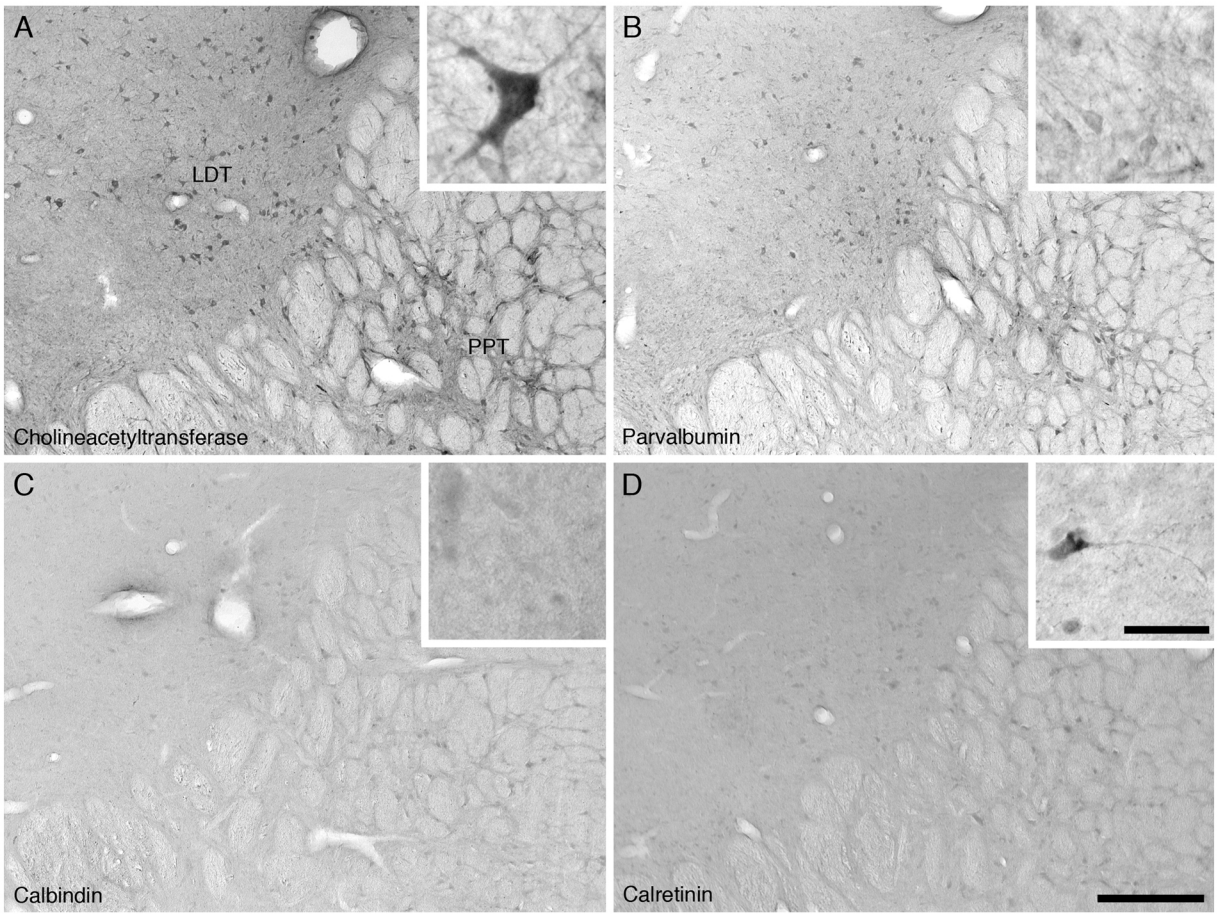

A
LDT
PPT
Cholineacetyltransferase
B
Parvalbumin
C
Calbindin
D
Calretinin

Table 3.2: Stereological results for neuron numbers, volume and area in the blue wildebeest. LDT – laterodorsal tegmental nucleus, PPT – pedunculopontine tegmental nucleus, LC – locus coeruleus complex, ChAT+ - neurons immunopositive for choline acetyltransferase, TH+ - neurons immunopositive for tyrosine hydroxylase, OxA+ - neurons immunopositive for orexin.

Nucleus examined	Estimated population on using mean section thickness	Estimated population on using mean section thickness with counts	Estimated population on using number weighted section thickness	Average estimated cell volume (μm^3)	Average estimated cell area (μm^2)
LDT (ChAT+)	2411.16	2440.62	2445.5	772.82	95.47
PPT (ChAT+)	16659.48	16605.4	16568.26	1 320.78	135.62
LC (TH+)	14227.92	14857.92	14844.66	1 204.57	128.36
Hypothalamus (OxA+) All cells	11716.58	11776.76	11790.76	2 414.61	203.97
Hypothalamus (OxA+) Magnocellular	-			3983.51	309.83
Hypothalamus (OxA+) Parvocellular	-			1566.98	156.21

Figure 3.5: Low (**A**, **B**) and high (**C**, **D**) magnification photomicrographs of the locus coeruleus complex of the blue wildebeest immunostained for tyrosine hydroxylase (**TH**) (**A**, **C**) and dopamine-ß-hydroxylase (**DBH**) (**B**, **D**). The neurons of the locus coeruleus complex, in both the diffuse division of the locus coeruleus (**A6d**) and the subcoeruleus diffuse division (**A7d**) of the blue wildebeest show clear and specific immunoreactivity for both TH and DBH, indicating that these neurons produce noradrenalin. ***** - indicates the same blood vessel in adjacent sections, **4V** – fourth ventricle, **scp** – superior cerebellar peduncle. Scale bar in **B** = 1000 µm and applies to **A** and **B**, scale bar in **D** = 250 µm and applies to **C** and **D**. In all images medial is to the left and dorsal to the top.

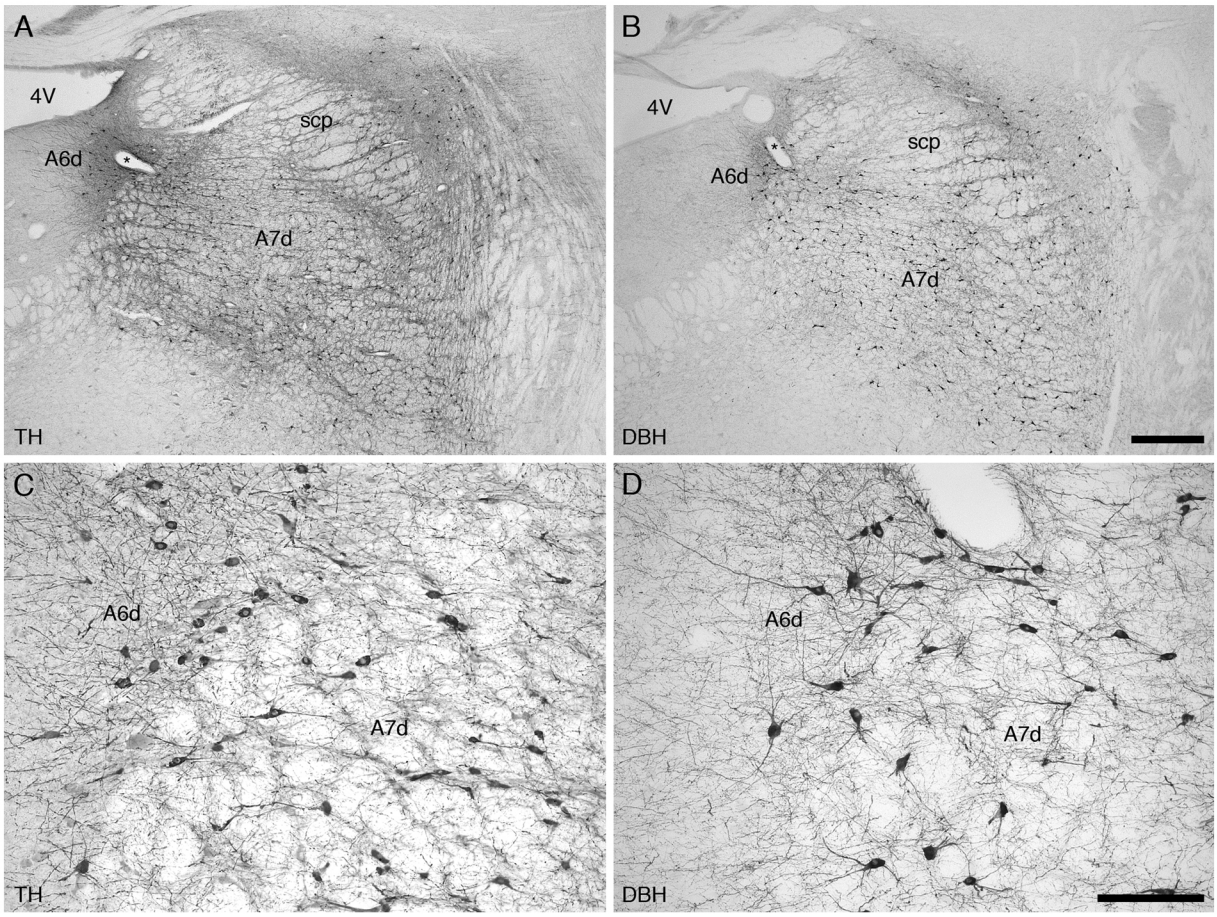

A
4V
scp
A6d
*
A7d
TH
B
4V
scp
A6d
*
A7d
DBH
C
A6d
A7d
TH
D
A6d
A7d
DBH

Figure 3.6: Low (main image) and high (inset) magnification photomicrographs in the region of the diffuse division of the locus coeruleus (**A6d**) and the subcoeruleus diffuse division (**A7d**) of the locus coeruleus complex in the pons of the blue wildebeest. **(A)** Neurons immunoreactive for tyrosine hydroxylase showing the diffuse portions of both the locus coeruleus (**A6d**) and subcoeruleus (**A7d**). Note the absence of parvalbumin **(B)**, calbindin **(C)** and very low density of calretinin **(D)** immunoreactive structures in these nuclei. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.

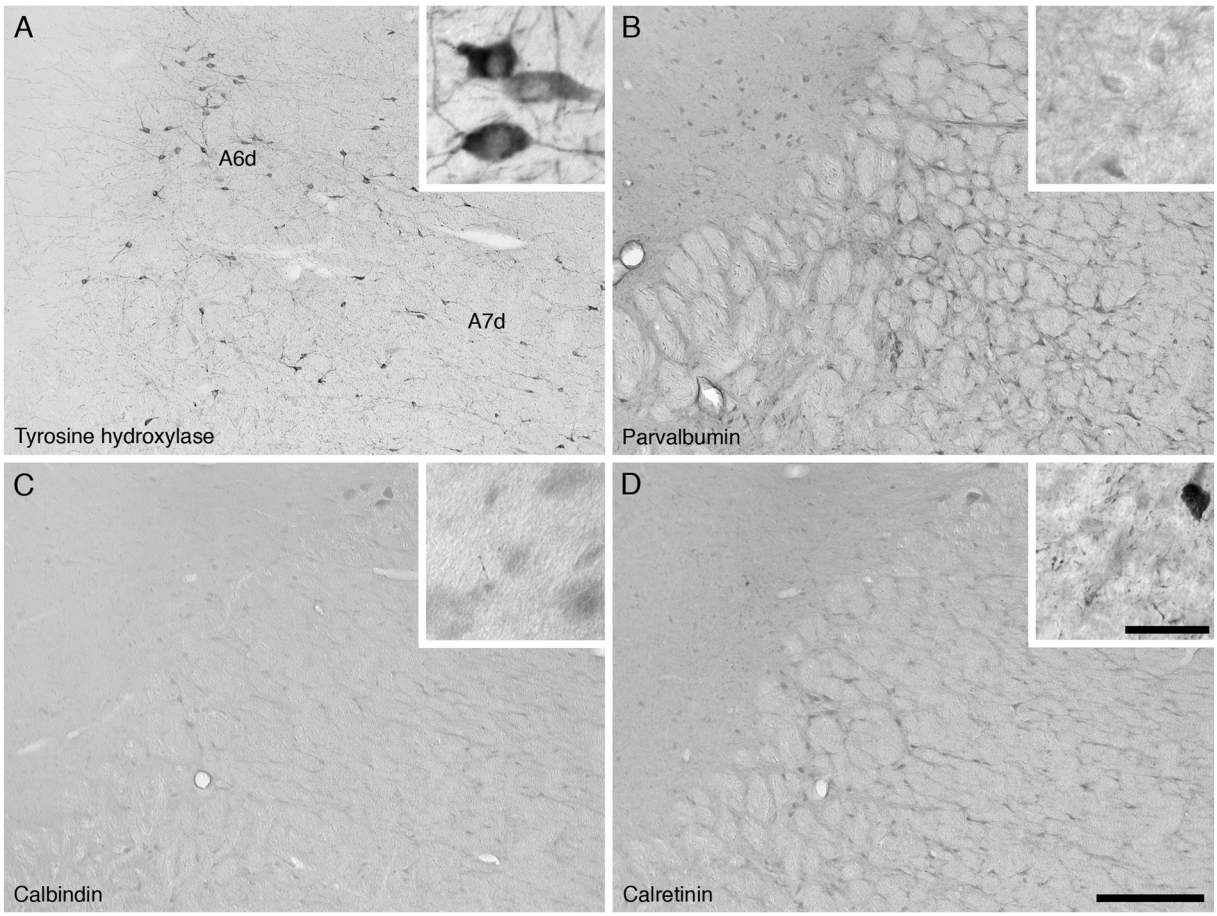

A
A6d
A7d
Tyrosine hydroxylase
B
Parvalbumin
C
Calbindin
D
Calretinin

Figure 3.7: Low (main image) and high (inset, dorsal raphe lateral division) magnification photomicrographs in the region of the dorsal raphe dorsal (**DRd**), ventral (**DRv**), peripheral (**DRp**) and lateral (**DRl**) divisions of the dorsal raphe nuclear complex in the blue wildebeest. **(A)** Neurons immunoreactive for serotonin showing the dorsal, ventral, peripheral and lateral divisions of the dorsal raphe complex in close proximity to the cerebral aqueduct (**ca**). **(B)** Parvalbumin immunoreactivity, note the absence of parvalbumin immunoreactive structures in these nuclei. **(C)** Calbindin immunoreactivity, note the absence of calbindin immunoreactive structures in these nuclei. (**D)** Calretinin immunoreactivity, note the low to moderate density of terminals and cells in these nuclei. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.

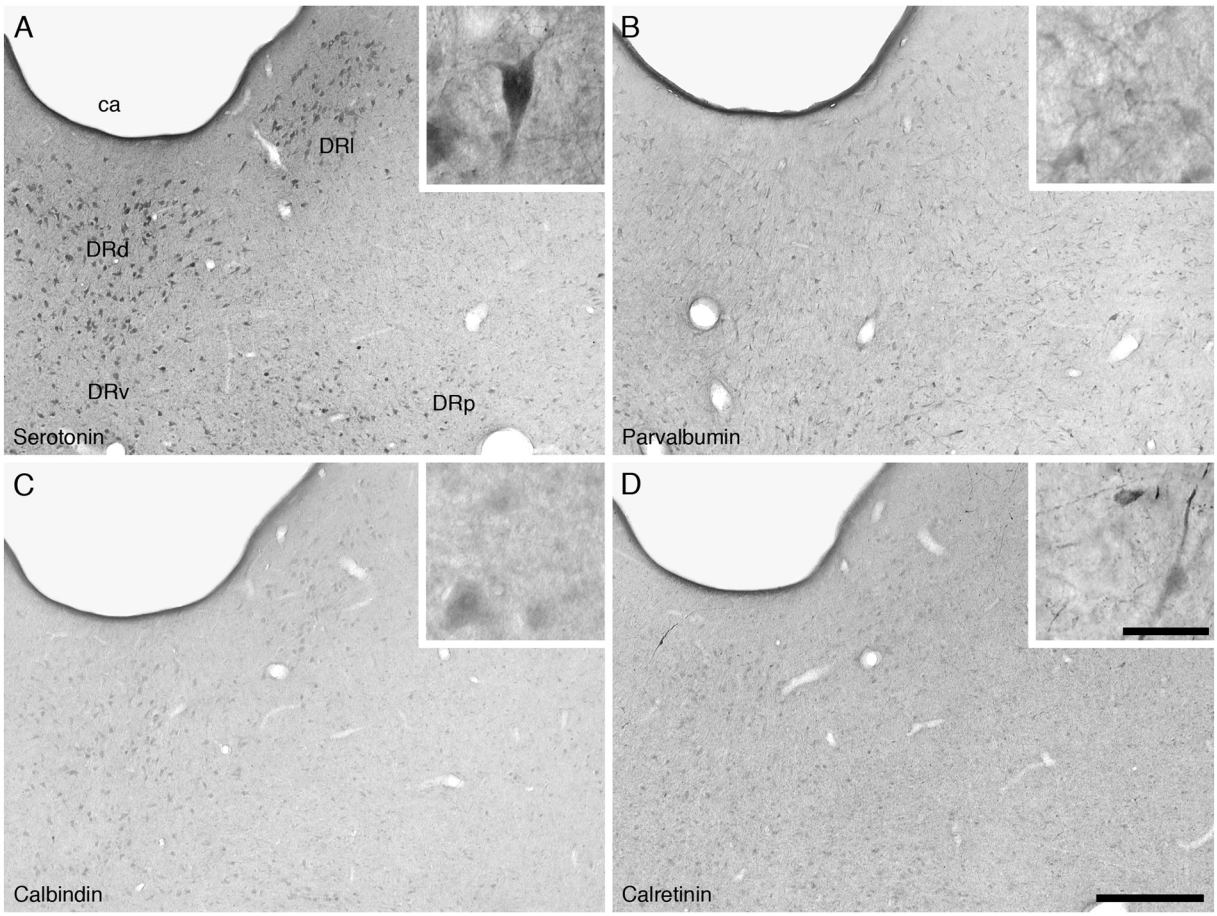

A
ca
DRl
DRd
DRv
DRp
Serotonin
B
Parvalbumin
C
Calbindin
D
Calretinin

Figure 3.8: Low (main image) and high (inset) magnification photomicrographs in the region of the border between the main magnocellular orexinergic cluster (**Mc**) and the parvocellular orexinergic cluster (**Pc**) in the hypothalamus of the blue wildebeest. **(A)** Neurons immunoreactive for orexin-A in the Mc and Pc clusters of the hypothalamus. Note the difference in size between the magnocellular and parvocellular neurons (**Inset A**). **(B)** Parvalbumin immunoreactivity, note the absence of parvalbumin immunoreactive structures in this hypothalamic region. **(C)** Calbindin immunoreactivity, note the occasional cell and terminals in this region of the hypothalamus. **(D)** Calretinin immunoreactivity, note the low to moderate density of cells and terminals in this region of the hypothalamus. Scale bar in **D** = 500 μm and applies to all, scale bar in **D** inset = 50 μm and applies to all insets. In all images medial is to the left and dorsal to the top.

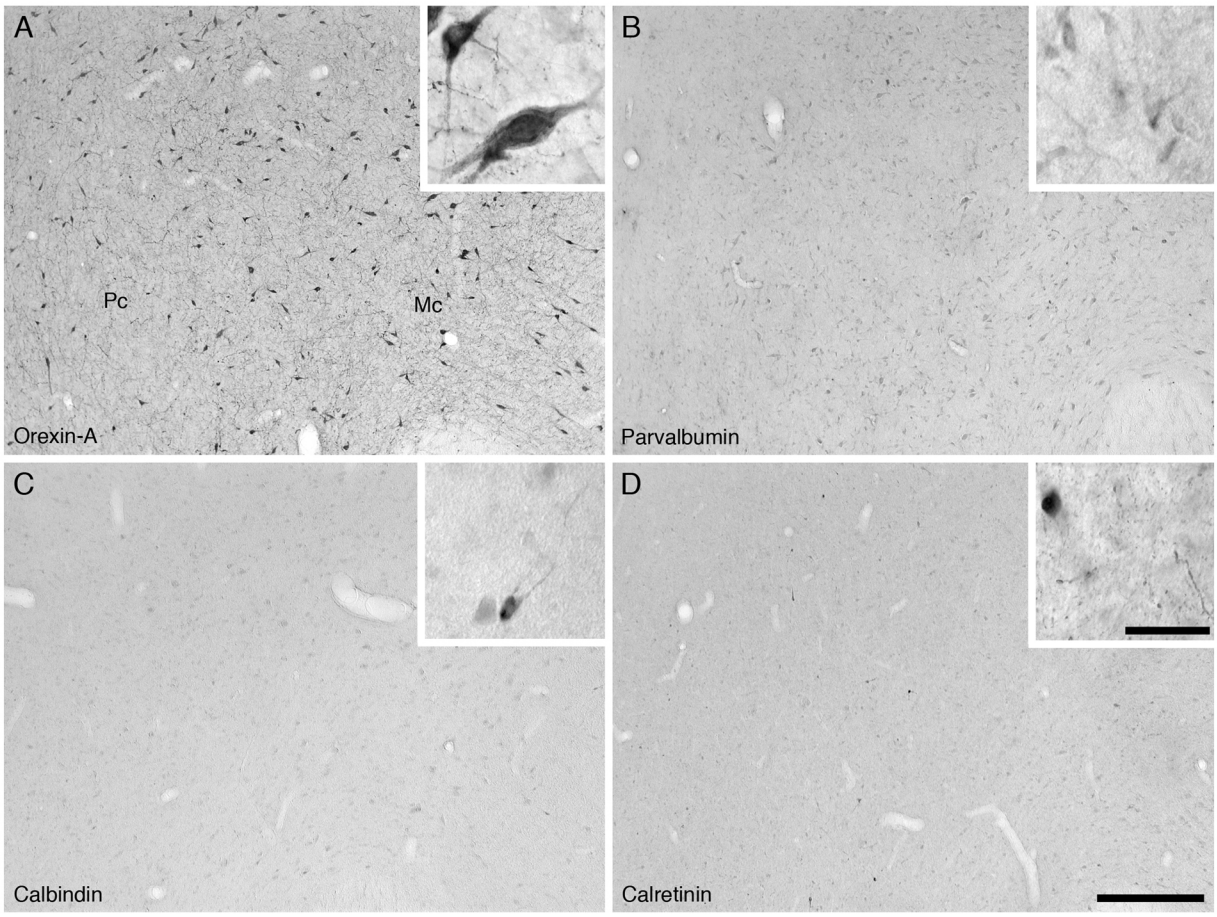
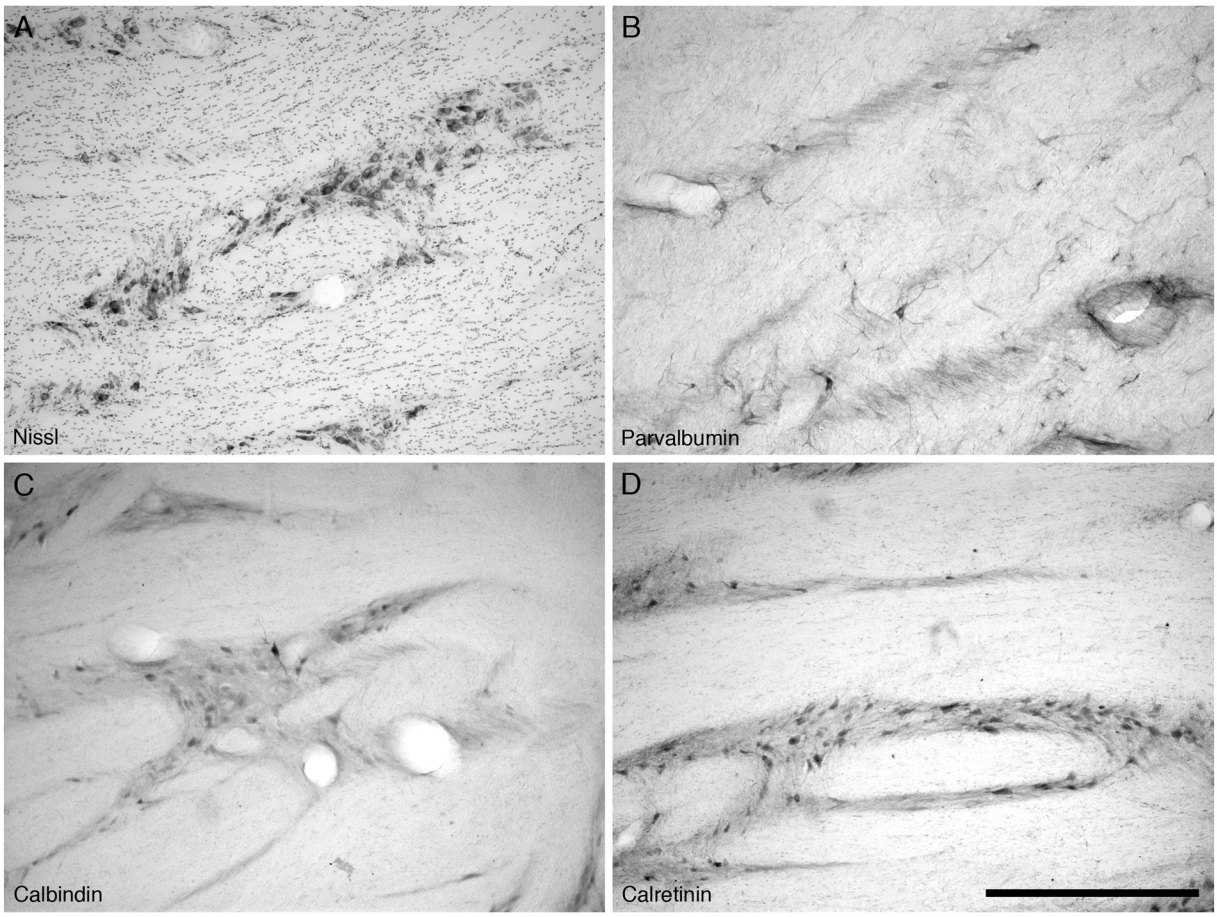

A
Pc
Mc
Orexin-A
B
Parvalbumin
C
Calbindin
D
Calretinin

Table 3.3. The density of neurons and terminal networks of the calcium binding proteins calbindin (CB), calretinin (CR) and parvalbumin (PV) in relation to various sleep-wake nuclei in the brain of the blue wildebeest. (-) none / very little. (+) low density. (++) moderate density. (+++) high density.

Sleep related nuclei	**Calbindin**		**Calretinin**		**Parvalbumin**	
	Neurons	**Terminal networks**	**Neurons**	**Terminal networks**	**Neurons**	**Terminal networks**
Cholinergic						
Islands of Calleja / Olfactory Tubercle	**+ / ++**	**+**	**++**	**++**	**++**	**++**
Medial septal nucleus	**- / +**	**- / +**	**+++**	**+++**	**++ / +++**	**++ / +++**
Diagonal band of Broca	**+ / ++**	**++**	**++ / +++**	**++ / +++**	**++ / +++**	**++ / +++**
Nucleus basalis	**+++**	**+++**	**++**	**++**	**++**	**+++**
Laterodorsal tegmental nucleus	**+**	**+**	**+ / ++**	**+ / ++**	**+ / ++**	**+ / ++**
Pedunculopontine tegmental nucleus	**+**	**+**	**+ / ++**	**++ / +++**	**+ / ++**	**+ / ++**
Catecholaminergic						
Compact subcoeruleus (A7sc)	**+ / ++**	**++**	**+ / ++**	**++ / +++**	**+ / ++**	**+ / ++**
Diffuse subcoeruleus (A7d)	**-**	**++**	**++**	**++ / +++**	**+ / ++**	**+ / ++**
Diffuse locus coeruleus (A6d)	**-**	**++**	**+ / ++**	**++**	**+ / ++**	**+ /++**
Serotonergic						
Dorsal raphe, interfascicular (DRif)	**++**	**++**	**++**	**++ / +++**	**+**	**+**
Dorsal raphe, ventral (DRv)	**++**	**++**	**+ / ++**	**++**	**-**	**+**
Dorsal raphe, dorsal (DRd)	**++**	**++**	**+ / ++**	**++**	**-**	**+**
Dorsal raphe, lateral(DRl)	**++**	**++**	**+**	**+ / ++**	**-**	**+**
Dorsal raphe, peripheral (DRp)	**++**	**++**	**+ / ++**	**++**	**-**	**+**
Dorsal raphe, caudal (DRc)	**- / +**	**+**	**+**	**+ / ++**	**-**	**+**
Orexinergic						
Main cluster	**+ / ++**	**+**	**++**	**++**	**- / +**	**- / +**
Optic tract cluster	**++ / +++**	**++ / +++**	**++ / +++**	**++ / +++**	**- /+**	**- /+**
Zona incerta cluster	**+ / ++**	**++**	**++**	**++**	**- / +**	**- / +**
Parvocellular cluster	**++**	**+ / ++**	**+**	**++**	**- /+**	**- /+**
Thalamic reticular nucleus	**+**	**-**	**+**	**+**	**+++**	**+++**

Figure 3.9: Photomicrographs of the thalamic reticular nucleus in the diencephalon of the blue wildebeest brain. **(A)** Nissl stain showing the low density of neurons within the thalamic reticular nucleus. **(B)**. Parvalbumin immunoreactivity, note the low density of parvalbumin immunoreactive cells. **(C)** Calbindin immunoreactivity, note the occasional calbindin immunoreactive cell. **(D)** Calretinin immunoreactivity, note the moderate density of calretinin immunoreactive cells and terminal networks. Scale bar in **D** = 500 μm and applies to all. In all images medial is to the left and dorsal to the top.


A
Nissl
B
Parvalbumin
C
Calbindin
D
Calretinin

3.4. Discussion

The current study, to the authors' knowledge, is the first to provide a detailed qualitative and partial quantitative description of the sleep-wake related nuclei in the brain of the blue wildebeest, *Connochaetes taurinus*. In the brain of the blue wildebeest, from the basal forebrain to the pons, the nuclear organization of the cholinergic, catecholaminergic, serotonergic and orexinergic systems for the most part, showed a corresponding nuclear organization to that reported in other mammals and more specifically the Cetartiodactyla (Manger et al., 2003; Bhagwandin et al., 2008; Gravett et al., 2009; Bux et al., 2010; Dell et al., 2016a,b,c; Mahady et al., 2017; Davimes et al., 2017). Furthermore, the description and distribution of the GABAergic system, which was examined through immunostaining for the calcium binding proteins calbindin, calretinin and parvalbumin, was also similar to that seen in other mammals (Bhagwandin et al., 2013; Dell et al., 2016a,b,c; Davimes et al., 2017). This indicates that sleep in the blue wildebeest is likely to show typically mammalian features in terms of the global brain activity of the generally recognized sleep states of mammals (Tobler, 1995).

3.4.1. Similarities and differences in the nuclear organization of the sleep-wake nuclei across mammals

The cholinergic nuclei located in the basal forebrain and pons of the blue wildebeest shared a similar location and nuclear organization as that found in all other mammals studied to date (e.g. Maseko et al., 2007; Gravett et al., 2009; Bux et al., 2010; Dell et al., 2010, 2016a,b,c). In the giraffe and river hippopotamus it was noted that the somata of the cholinergic LDT neurons were larger than those of the cholinergic PPT neurons (Bux et al., 2010; Dell et al., 2016c), in cetaceans no difference in somal size was noted (Dell et al., 2016a,b), but for the Goettingen mini-pig, Arabian oryx and blue wildebeest, the soma of the

LDT neurons were smaller than those of the PPT neurons (Mahady et al., 2017; Davimes et al., 2017). Thus, while the nuclear organization and topology of the cholinergic neurons involved in the sleep-wake cycle are consistent across Cetartiodactyla, and mammals more generally, there are variances that may influence the expression of sleep and global brain states across species. These observations need to be correlated with polysomnographic studies of sleep in the various species examined to determine what role this variance in pontine cholinergic relative cell size may play, especially in the expression of REM sleep across species (van Dort et al., 2015), as it has been noted that variances in the cholinergic neurons of the LDT and PPT in the rock hyrax (Gravett et al., 2009) does influence the expression of sleep states in this species (Gravett et al., 2012).

The locus coeruleus and dorsal raphe nuclear complexes of the blue wildebeest showed a similar nuclear organization as that of other Cetartiodactyla (Dell et al., 2016a,b,c; Bux et al., 2010; Davimes et al., 2017), and indeed several other mammalian species (e.g. Dell et al., 2010). This indicates that their role in the control of the sleep-wake cycle in the blue wildebeest is not likely to vary significantly from that seen in other mammals.

In contrast, the orexinergic system in the hypothalamus of the Cetartiodactyls, including the blue wildebeest, does show a significant difference to that observed in many other mammals, this being the presence of the parvocellular orexinergic neurons in the medial hypothalamus (Dell et al., 2012, 2016a,b,c; Davimes et al., 2017); however, the remainder of the orexinergic neurons, those classified as magnocellular, do show a similar organization to that seen in other mammals (Dell et al., 2013; Calvey et al., 2013, 2015). At present, this parvocellular medially located cluster of orexinergic neurons has been observed in all Cetartiodactyla studied, as well as the African elephant (Maseko et al., 2013). It has been noted that, in general, herbivores tend to sleep less (when body mass is taken into account) than either carnivores or omnivores (Siegel, 2005), and this is particularly apparent

in the African elephant (Gravett et al., 2017). The orexinergic system appears to be involved in both arousal and the stimulation of food intake (Sakurai et al., 1998; Wagner et al., 2000; McGranaghan and Piggins, 2001; Spinazzi et al., 2006; McGregor et al., 2011), indicating that changes to the orexinergic system are likely to affect both the sleep-wake cycle and food intake. Given that all the species in which the parvocellular orexinergic neurons have been found require large volumes of food (including both the herbivorous artiodactyls and elephants and the carnivorous cetaceans), and tend to sleep less than similar sized omnivorous or carnivorous mammals, it is likely that this parvocellular orexinergic cluster of neurons is involved in driving both increased food intake and increased time spent awake in order to allow time to ingest the increased amount of food required. While this functional relationship is speculative at present, investigating the presence or absence of the parvocellular orexinergic neurons in other large herbivores, such as perissodactyla and sirenia, along with measurements of the time spent sleeping in these species, may provide support to this concept.

Thus, despite the two differences discussed above, and differences in the total numbers of neurons belonging to different parts of the sleep neural network (Dell et al., 2016a,b,c; Davimes et al., 2017), the absence of other major differences observed in the neural systems that control and regulate the sleep-wake cycle in the blue wildebeest and other species indicates that these systems are strongly conserved across species (Tobler, 1995; Rattenborg et al., 2000; Lyamin et al., 2008; Dell et al., 2012, 2016a,b,c). This indicates the existence of a generalized underlying neural system controlling the sleep-wake cycle across mammalian species and the physiological and behavioural expression of mammalian sleep (Tobler, 1995).

3.4.2. Similarities and differences in the GABAergic systems of the sleep-wake nuclei across mammals

By using the calcium binding proteins parvalbumin, calretinin and calbindin, we were able to describe the density of three varieties of the GABAergic neurons and terminal networks in the different sleep-related nuclei (e.g. Bhagwandin et al., 2013; Dell et al., 2016a,b,c). The density of cells and terminals networks immunolabelled with antibodies to the calcium binding proteins in the sleep-related nuclei in the blue wildebeest are, in general, comparable to those distinguished in other Cetartiodactyla, but there are variances. Within the cholinergic nuclei of the blue wildebeest associated with sleep, and the locus coeruleus complex, an overall lower density of calbindin neurons and terminal networks was noted when compared to the harbour porpoise, minke whale, river hippopotamus and Arabian oryx (Dell et al., 2016a,b,c; Davimes et al., 2017). The overall density of the calretinin and parvalbumin neurons and terminal networks of the blue wildebeest within the cholinergic nuclei was similar to that seen in other Cetartiodactyla (Dell et al., 2016; Davimes et al., 2017), and even the giant Zambian mole rat (Bhagwandin et al., 2013). Thus, the GABAergic neurons containing calbindin appear to play a similar, although possible less strong, role in the cholinergic and noradrenergic nuclei involved with sleep in the blue wildbeest brain compared to other species where similar studies have been undertaken.

The overall density of calbindin and parvalbumin neurons and terminal networks in the serotonergic system of the blue wildebeest resembled those reported in other most Cetartiodactyls and the giant Zambian mole rat (Bhagwandin et al., 2013; Dell et al., 2016a,b,c); however, it should be noted that the density of calbindin neurons is lower in the serotonergic nuclei of the Arabian oryx compared to other species (Davimes et al., 2017). The density of the calretinin neurons and terminal networks within the serotonergic system is

lower in the blue wildebeest than that reported in other species (Bhagwandin et al., 2013; Dell et al., 2016; Davimes et al., 2017).

In the region of the hypothalamus where the orexinergic neurons were located, the densities of the calbindin and calretinin neurons in the blue wildebeest is similar to other Cetartiodactyls, but the density of calbindin terminal networks appears higher (Dell et al., 2016a,b,c; Davimes et al., 2017), while that of the calretinin terminal networks in the blue wildebeest corresponded with those reported in the Arabian oryx (Davimes et al., 2017). The densities of both the parvalbumin neurons and terminal networks in the blue wildebeest corresponded with those seen in the Arabian oryx, but was higher than that reported in other Cetartiodactyla (Dell et al., 2016a,b,c; Davimes et al., 2017).

As is typical for the thalamic reticular nucleus across mammals (e.g. Bhagwandin et al., 2013), including all Cetartiodactyla studied, the neurons of this nucleus in the blue wildebeest were immunoreactive for parvalbumin (Dell et al., 2016a,b,c); however, neurons immunoreactive for calretinin, and the occassional neuron immunoreactive for calbindin, were found within the thalamic reticular nucleus of the blue wildebeest. The near-absence of calbindin neurons and terminal networks in the thalamic reticular nucleus of the blue wildebeest is similar to that reported for other Cetartiodactyla (Dell et al., 2016,a,b,c; Davimes et al., 2017). The presence of a low density of calretinin neurons and terminal networks in the blue wildebeest is similar to that reported for the Arabian oryx (Davimes et al., 2017) and river hippopotamus (Dell et al., 2016c), but less than that seen in the harbour porpoise and minke whale (Dell et al., 2016a,b). Variances in the neurochemistry of the thalamic reticular nucleus are likely to be important in regards to the physiological expression of sleep as the thalamic reticular nucleus plays a key role in sleep induction, maintenance, and the genesis of spindles and delta waves (Luppi et al., 2016).

Different populations of GABAergic neurons are known to be both promoters of sleep (Pace-Shott and Hobson, 2002; Jones, 2003; Gritti et al., 2003; Siegel, 2004) and promoters of wake (Luppi et al., 2016). The qualitative representation of the various populations of GABAergeic neurons in the sleep-wake related nuclei of the blue wildebeest is for the most part comparable to other mammals and other Cetartiodactyla. The comparable organization of the GABAergic neurons and terminal networks could be an indication of the conservation of the physiological features of sleep across mammalian species (Tobler, 1995).

3.4.3. What do these findings say about sleep in the blue wildebeest?

In this study we identified two characteristics of the sleep systems that appear to be primarily, but not strictly, characteristic of the Cetartiodactlya, that may have functional implications regarding sleep in the blue wildebeest, and more generally in the Cetartiodactyla. The first feature is the variability of the relative somal size of the cholinergic neurons in the LDT and PPT across Cetartiodactyla species, which has not been reported in other mammalian species. In the blue wildebeest, Arabian oryx and Gottingen mini-pig (Mahady et al., 2017; Davimes et al., 2017), the LDT neurons are smaller than the PPT neurons, they are similar in size in the cetaceans (Dell et al., 2016a,b), whereas the LDT neurons are larger than the PPT neurons in the giraffe and river hippopotamus (Bux et al., 2010; Dell et al., 2016c). The neurons of the LDT and PPT have been linked to the generation of REM sleep (Woolf, 1991; Pace-Shott and Hobson, 2002; Jones, 2007; Siegel, 2006), specifically REM sleep initiation, but not REM sleep duration (van Dort et al., 2015). It should be noted that REM sleep does not appear to occur in cetaceans (Lyamin et al., 2008). The variance in the relative somal size of the cholinergic LDT neurons compared to the PPT neurons within the Artiodactyls may indicate that the process of REM sleep initiation varies across the Artiodactyla, and the relative influence that LDT or PPT neurons

have on the process of REM initiation may vary across species. This may be related to the size of the forebrain in the different species, as the species with the relatively larger LDT neurons (giraffe and river hippopotamus) have larger brains than the species with relatively smaller LDT neurons (blue wildebeest and Gottingen mini-pig). This indicates that LDT neurons may play a stronger role in initiating forebrain/neural aspects of REM, while the PPT neurons may play a stronger role in initiating somatic aspects of REM. Detailed polysomnographic and single unit recording studies of the period of REM initiation in Artiodactyls with varied relative LDT:PPT somal sizes, as well as determining potential allometric aspects of the LDT:PPT somal sizes across further Artiodactyl species may reveal important clues regarding the functional implications of this variation regarding REM sleep initiation in Artiodactyls, and indeed across mammals more generally.

The second unusual characteristic is the medial parvocellular orexinergic cluster in the hypothalamus which, to date, has been observed only in Cetartiodacyla and the African elephant (Iqbal et al., 2001; Ettrup et al., 2010; Dell et al., 2012; Maseko et al., 2013; Dell et al., 2016a,b,c; Davimes et al., 2017). Orexinergic neurons play an important role in the maintenance of wakefulness, and play a role in feeding behavior and emotion and reward processes, with a deficiency of these neurons resulting in abnormalities in energy homeostasis, stress-related behaviour and narcolepsy (Schwartz and Roth, 2008; Lu and Zee, 2010; Tsujino and Sakurai, 2013; Sakurai, 2014). Given that all the species in which the parvocellular orexinergic neurons have been found feed more frequently for longer periods and tend to sleep less than similar sized omnivorous or carnivorous mammals, it is likely that this parvocellular orexinergic cluster of neurons is involved in driving both appetite and arousal to allow time to consume the greater amount of food required. Due to these animals needing to feed more frequently, the orexinergic neurons may prevent them from consolidating sleep into a single bout, which is observed in most large herbivores which

exhibit fragmented sleep-wake behaviour, or ‘polyphasic’ sleep (Tobler, 1995; Gravett et al., 2017). This would indicate that the blue wildebeest would exhibit polyphasic sleep and have shorter daily total sleep amounts than similar sized omnivorous or carnivorous mammals. Studies that measure total sleep time in blue wildebeest, and other species that exhibit the parvocellular orexinergic neuronal cluster, in their natural setting may be useful to determine whether this potential functional correlate is found. A recent study in the African elephant provides preliminary supportive evidence, as in its natural habitat the African elephants were found to only sleep for 2 hours per day (Gravett et al., 2017). Thus, the neuroanatomy of the sleep systems in the blue wildebeest do make specific, but testable, predictions regarding sleep in this species.

CHAPTER FOUR: **Sleep in two free-roaming blue wildebeest (*Connochaetes taurinus*), with observations on the concordance of polysomnographic and actigraphic techniques**

4.1. Introduction

Behaviourally, sleep is identified by sustained quiescence in species-specific postures accompanied by reduced responsiveness to external stimuli (Tobler, 1995; Lima et al., 2005; Zepelin et al., 2005). Electrophysiologically, mammalian sleep is usually composed of two states: rapid-eye-movement (REM) sleep and non-rapid eye movement sleep (non-REM) (Tobler, 1995; Lima et al., 2005; Lesku et al., 2006; Voirin et al., 2014; Berry et al., 2015). The two major states of sleep, non-REM and REM, alternate in a sleep cycle that is repeated one or more times during a sleep episode (Zepelin et al., 2005; Petersen, 2007; Capellini et al., 2008). Sleep architecture can be defined as the distribution of the sleep cycles, within a sleep episode, and also refers to the duration of these episodes and the phasing of sleep across the daily cycle (Lima et al., 2005). Sleep architecture varies extensively across mammalian species (Zepelin et al., 2005; Capellini et al., 2008; Voirin et al., 2014). There are studies that claim episodes of behavioural quiescence (rest) in invertebrates and non-mammalian vertebrates represent functional equivalents, and perhaps even homologues, of mammalian sleep (Rosenwasser, 2009). This in turn has blurred the traditional distinction between "sleep-wake" and "rest-activity" cycles (Rosenwasser, 2009). Comparative studies of mammalian sleep may provide insights into the function and evolution of sleep by identifying factors correlated with variations in sleep architecture (e.g. Siegel, 2005; Lesku et al., 2006, 2009).

Most studies examining sleep in mammals, specifically "wild" or non-traditional laboratory animals, have been performed under controlled conditions in a laboratory or

zoological facility with few studies being conducted on animals in their natural environment (e.g. Rattenborg et al., 2008; Lesku et al., 2011; Lyamin et al., 2012; Voirin et al., 2014; Rattenborg et al., 2016). The assumption made by the studies conducted in controlled conditions is that the interspecific differences in sleep observed in controlled environments somehow explains similar differences in the wild (Lesku et al., 2009).

It is not always possible to measure and record sleep polysomnographically (PSG) from animals in their natural environments, as PSG is invasive, requiring the surgical implantation of electrodes on the surface of the brain and other regions of the body. In contrast, actigraphy (ACT) has been shown to be a non-invasive method to objectively measure sleep and assess sleep disorders in humans (Sadeh and Acebo, 2002; Ancoli-Israel et al., 2003; Kanady et al., 2011; Shambroom et al., 2012). Furthermore, validation studies in humans, have shown that the concordance in sleep scoring between ACT and PSG ranges from 83 to more than 90% (Sadeh and Acebo, 2002; Ancoli-Israel et al., 2003; Kanady et al., 2011; Shambroom et al., 2012). While ACT is now commonly used in the study of sleep in humans, limited studies have made use of this methodology to study sleep in other mammals (e.g. Davimes et al., 2016; Gravett et al., 2017). The aim of this study is thus two-fold, first, to measure sleep polysomnographically in wild free-roaming blue wildebeest (*Connochaetes taurinus*) under the most natural conditions possible, and second, to establish the degree of concordance between ACT and PSG recordings taken simultaneously.

4.2. Materials and Methods

4.2.1. Experimental animals and site location

Two wild free-roaming adult male blue wildebeest were located and captured opportunistically in the Dinokeng Game Reserve with the assistance of Wildlife Assignments International (WAI). Each male had an estimated body mass of approximately 250 kg and

were estimated to be between 8 to 15 years in age by an experienced wildlife veterinarian. Permits from the Gauteng Provincial Government were obtained for the capture and transport of the animals from the wild. All animals were treated and used according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (AESC number: 2014/53D), which parallel those of the National Institutes of Health (NIH) for the care and use of animals in scientific experimentation.

The two adult male blue wildebeest were each housed in a 10 x 10 m^2 indoor and 10 x 10 m^2 outdoor enclosure on the premises of WAI and allowed to acclimatize for three weeks. The animals were kept in the indoor part of the enclosure at night and allowed out in the mornings and spent the day in the outdoor part of the enclosure. The WAI enclosures were cleaned every morning and the animals were fed lucerne and teff hay once a day and water was also made available *ad libitum*. Each animal was implanted with a Neurologger® (Vyssotski et al., 2006) that allowed the recording of EEG and EMG without cables or restraint and two actiwatches for activity recordings. After the surgical procedure (see below for details of the procedure), the animals were kept in the smaller enclosures described above for 5 days to allow post-surgical recovery and observation. Following this recovery period, the animals were moved to a larger 2-hectare enclosure (20 000 m^2, or 4.942 acres). The enclosure consisted of a fenced piece of land within the Dinokeng Reserve, which encompassed part of the natural habitat of the blue wildebeest. The animals had access to lucerne, teff hay and water; however, the animals preferred to graze on the available natural vegetation.

4.2.2. Recording period and environmental conditions

The period during which the recording took place was from February 26 to March 9, 2016. The average temperature for the recording period was 24°C, with a mean maximum

temperature of 37°C, a mean minimum temperature of 12°C, and an average daily rainfall of 0.21 mm. This weather data was recorded at the Pienaars River weather station, and provided by the Agriculture Research Council (ARC) of South Africa. Sunrise times for the recording period ranged from 05:59 (February 26) to 06:06 (March 9), and sunset times from 18:41 (February 26) to 18:28 (March 9). Sunrise and sunset times were obtained from freely accessible online databases (https://www.timeanddate.com).

4.2.3. Devices: Neurologger®

The Neurologger® (model: Neurologger 1, Evolocus LLC) used in the current study weighed 78 g, had approximate dimensions of 66 x 36 x 10 mm^3 (weight and dimensions include wax covering and batteries), and allowed simultaneous recording of six different channels (one EMG, two EEG and three for the accelerometer x, y and z planes). The Neurologger® had an onboard memory of 8 GB in the form of a removable microSD card and was powered by two Lithium ion batteries (Lithium Primary Battery, SW-AA11, 3.6 V, Tekcell, Vitzrocell Co. Ltd.). The sampling rate for each of the channels was set at 500 Hz (electrodes – LiFY Colorflex Li-HF 0.06mm2; Conductor configuration: Cu 30 x ø0.05mm; Insulation: PVC; impedance approximately 1.1Ω) and this configuration allowed for approximately 20 days of continuous recording. The Neurologger® unit was coated with two layers of biologically inert wax (SasolWax 1276, Sasol, Johannesburg, South Africa) and sterilized in a container containing formalin pellets for 48 h prior to implantation.

4.2.4. Devices: Actiwatches

Activity was logged in each animal using two subcutaneously implanted actiwatches, one on the side of the neck and one on the upper hindleg. The actiwatch is a wristwatch size, ambulatory device that is commonly used for measuring sleep in humans (e.g. Ancoli-Israel

et al., 2003; Shambroom et al., 2012). Within each actiwatch is a piezoaccelerometer device connected to a microchip that sums and records the number of acceleration events per minute. The Actiwatch Spectrum (Philips Respironics) was used in the current study. These devices have a mass of 25 g and approximate dimensions of 35 x 35 x 12 mm (weight and dimensions include wax covering). Each actiwatch was calibrated and programmed (data acquisition rate set at 1 min intervals) with the Philips Respironics Actiware 5 software, prior to implantation. The wristbands from the actiwatches were removed, and the watches were insulated with standard electrical insulation tape and covered with two coats of biologically inert wax (SasolWax 1276, Sasol, Johannesburg, South Africa) and sterilized in a container containing formalin pellets for 48 h prior to implantation.

4.2.5. Surgical Procedure

After the initial acclimatization to the WAI enclosure, surgical implantation of the Neurologger® and actiwatch devices were performed. With the help of an experienced wildlife veterinarian, the animals were anesthetized with weight-appropriate doses of thiofentanyl (4 mg of A30-80®, Wildlife Pharmaceuticals, South Africa) and azaperone (40 mg Stresnil®, Janssen Pharmaceutica, South Africa). Once safely immobilized, the animals were placed in sternal recumbency and then prepared for surgery. The head of the blue wildebeest was secured in an upright position through ropes tied to the horns and secured to a roof beam, to prevent movement and allow for the accurate placement of the EEG and EMG electrodes. The regions of the head and neck that would be manipulated during the surgery were shaved, and the skin cleaned with soap and water and disinfected with chlorhexidine (CHX, Kyron Laboratories, South Africa). During the surgical procedure the animal's heart rate and oxygen saturation were monitored. The animal received oxygen throughout the surgical procedure *via* a nasal catheter.

Under aseptic conditions, a mid-sagittal incision was made after which, the skin and underlying temporalis muscle were reflected to expose the region of the skull overlying the presumed visual cortex (based on comparative estimation of the location of visual cortex in domestic Artiodactyls). Using a cordless Dremel drill, four 2-mm-diameter holes were made (for placement of the EEG electrodes), with a fifth hole drilled posteriorly on the midline of the occipital crest for placement of the ground electrode. The four holes were drilled approximately 1 cm apart, and 2 cm lateral to the sagittal suture (two holes on each side of the midline) over the presumed visual cortex. The EEG electrodes were inserted in each of the holes in such a manner that the tips rested firmly on the surface of the dura mater. Once inserted, the electrodes were secured in place with dental cement. A 15 cm subdermal pocket was created on the left side of the neck for the placement of the Neurologger® unit, and the electrodes were fed through a subcutaneous tunnel from the neck to the skull. In addition to the EEG electrodes, two EMG electrodes were sutured into the nuchal musculature approximately 2 cm apart, near the location of the Neurologger® unit.

Subsequent to the Neurologger® implantation, each animal was implanted with two actiwatches. The two implantation sites (the right side of the neck and hind-leg) were shaved, washed and disinfected with chlorhexidine (CHX, Kyron Laboratories, South Africa). A small incision (approximately 5 cm in length) was made at each of the implantation sites and a subcutaneous pocket extending approximately 10 cm from the incision sites was created. One actiwatch was placed in each of these pockets.

All incision sites were sutured by means of intradermal and superficial stitches and sterilized with the topical antiseptic Necrospray® (Centaur Labs, Johannesburg, South Africa). Following the surgical procedure, each animal received weight appropriate doses of antibiotics and analgesics (8 ml of Draxxin®, Zoetis and 9 ml of Ketofen®, Zoetis) and was

returned to the WAI acclimatization enclosure. Once inside the enclosure the anesthesia was reversed by administering weight appropriate doses of naltrexone (50 mg of Trexonil®, Wildlife Pharmaceutical, South Africa), and the animals were closely monitored until they were able to stand and move around freely. The animals were examined each morning for 5 days by the attendant veterinarian, and were considered to have recovered well from the surgery.

4.2.6. Sleep Recording and Data Analysis

Following the five-day recovery period, the animals were moved to the 2 hectare, natural enclosure. The animals were allowed to acclimatize to the new environment for two days, following which 72 hours of continuous sleep recording commenced in both individuals simultaneously.

4.2.6.1. Polysomnography (PSG) analysis

The recorded data on the Neurologger® microSD was converted to float32 format and imported into Version 7.02 of the Spike 2 software (Cambridge Electronic Designs, UK) for visual scoring and analysis. Prior to scoring, DC remove was applied to all channels. The PSG data was visually scored in 1 minute epochs for 72 hours and designated as the states of Wake, non-rapid eye movement sleep (non-REM), or rapid eye movement sleep (REM), based on the characteristics of the EEG and EMG as described in detail below. An epoch was only assigned to one of these three states if the state occupied greater than 50% of the epoch. The following variables were calculated and averaged across the recording period for each blue wildebeest: the percent time spent in each state (Wake, non-REM sleep, and REM sleep) per 24 h, the percent of REM sleep in total sleep time (TST), the percent time spent in each state during the light period (sunrise to sunset) and during the dark period (sunset to sunrise). These variables were adapted from the analysis of Rattenborg et al., (2008) and Voirin et al.,

(2014) for ease of comparison. Additionally, the number of episodes and the mean duration of the episodes of each state for the 24 h, light and dark periods were calculated. An episode was defined as a succession of consecutive epochs of one state. From the 1-min scored data the modal state for 5-min was calculated and used to determine the onset and duration of the major sleep bouts for all animals. A sleep bout was defined as a period lasting at least 10 minutes (two consecutive 5-min sleep bouts without waking) and included both non-REM and REM epochs. The power spectrum for each of the defined states was calculated with the aid of the Spike 2 computer program (Hanning window, FFT number 512, sampling frequency 500 Hz, segment length 1.024 s). For the spectral power analysis, 10 episodes each of Wake, non-REM and REM from each animal (n = 20 for each state) were used.

4.2.6.2. Actigraphy (ACT) analysis

Phillips Respironics Actiware 5 was used to retrieve the recorded data from each of the implanted actiwatches. The data was exported to Microsoft Excel where it was scored and analysed. Data obtained from the neck and leg actigraphs were scored concurrently in 1-minute epochs as either Active or Inactive. For an epoch to be scored as Active either the neck or leg actigraphs had to have an activity score greater than zero. Inactive epochs were scored when both the neck and the leg actigraphs had an activity score equal to zero. The percent time spent in each state (Active and Inactive) for the 24 h, light (sunrise to sunset) and dark periods (sunset to sunrise) was calculated for all animals over the same recording days where PSG analysis was undertaken (see above). Total time spent Active/Inactive was determined for the 24 h, light and dark periods for all animals. From the 1-min scored data the modal state for 5-min was calculated and used to determine the onset and duration of the major Inactive bouts for both animals.

4.2.6.3. Concordance between polysomnography and actigraphy

In order to determine the degree of agreement between the two recording techniques (PSG and ACT, where Wake was considered equivalent to Active, and non-REM plus REM was considered equivalent to Inactive) we used Cohen's Kappa (κ), the variable of interest is categorical, which is a statistical method designed to take into account chance agreement (Watson and Petrie, 2010). When Cohen's kappa equals 1, there is complete agreement between the two techniques, while a kappa value equal to zero suggests that the agreement is no better than that which would be obtained by chance alone (Watson and Petrie, 2010). According to Watson and Petrie (2010) (adapted from Landis and Koch, 1977) there is no formal scale for Cohen's kappa, but suggest the following levels of agreement: Poor if $\kappa < 0.00$, Slight if $0.00 \leq \kappa \leq 0.20$, Fair if $0.21 \leq \kappa \leq 0.40$, Moderate if $0.41 \leq \kappa \leq 0.60$, Substantial if $0.61 \leq \kappa \leq 0.80$ and Almost perfect if $\kappa > 0.80$. All statistical analyses, where possible, were conducted using IBM SPSS Statistics software version 23 and PAST 3 (Hammer et al., 2001).

4.3. Results

4.3.1. State definitions

The recording of EEG and EMG allowed the definition of three distinct states in the blue wildebeest, wake, non-REM and REM sleep. Wake was characterized by low amplitude, high frequency EEG, with the EMG exhibiting a high and variable voltage and frequency (Fig. 4.1A). During Wake, the power spectra range was 0.1 – 12.5 Hz with a peak power at 8.7 Hz (Fig. 4.2). Non-REM sleep was identified by the low frequency and high-amplitude of the EEG, while the EMG voltage decreased, but was not atonic (Fig. 4.1B). Episodes of non-REM showed a power spectra range of 0.1 – 6.7 Hz with a peak at 2.9 Hz (Fig. 4.2). REM

sleep was associated with low amplitude mixed frequency EEG that resembled the Wake EEG, while the EMG activity exhibited consistent low amplitude nearing atonia (Fig. 4.1C). The power spectra during REM sleep showed a range of 0.1 – 12 Hz with a peak at 3.51 Hz, and also exhibited hippocampal theta activity with a range of 6.5 – 10.6 Hz and a peak at 8.6 Hz (Fig. 4.2).

4.3.2. Time spent in Wake, non-REM and REM states

Analysis of the polysomnographic recordings revealed that the average total daily sleep time (TST) for the blue wildebeest studied was 4.53 h (± 0.12 h). On average for each day, 19.47 h (± 0.12 h) was spent in a state of Wake, 4.26 h (± 0.11 h) spent in a state of non-REM sleep, and 0.28 h, approximately 17 min (± 0.01 h) spent in a state of REM sleep (Fig. 4.3A). TST during the light period was 1.41 h (± 0.06 h), with 11.59 h (± 0.06 h) spent in Wake, 1.32 h (± 0.05 h) in non-REM sleep and 0.09 h (± 0.01 h) in REM sleep (Fig. 4.3B). During the dark period, the TST observed was 3.12 h (± 0.10 h), with 7.88 h (± 0.09 h) spent in Wake, 2.94 h (± 0.08 h) spent in non-REM sleep and 0.18 h (SD ± 0.02 h) spent in REM sleep (Fig. 4.3C). REM sleep determined as a percentage of TST was 6.07 % over the full day, 3.24 % during the light period, and 5.88 % during the dark period.

4.3.3. Number and average duration of episodes of wake and sleep states

The average number of daily episodes for Wake was 44.50 (± 0.56), non-REM sleep was 34.17 (± 0.82) and REM sleep was 9.67 (± 0.41) (Fig. 4.4A). The average number of episodes during the light period was 20 (± 0.3) for Wake, 12 (± 0.48) for non-REM sleep and 3.33 (± 0.25) for REM sleep (Fig. 4.4B). During the dark period, the average number of episodes was 24.50 (± 0.57) for Wake, 22.17 (± 0.74) for non-REM sleep and 6.33 (± 0.5) for REM sleep (Fig. 4.4C). The average duration of the episodes for the 24 h period was 19.58

min (± 12.00 min) for the Wake state, 3.79 min (± 3.03 min) for the non-REM state, and 1.05 min (± 0.42 min) for the REM state (Fig. 4.5A). During the light period, the average duration of the episodes for Wake was 29.22 min (± 5.67 min), non-REM sleep was 3.7 min (± 2.05 min), and REM sleep was 1.08 min (± 0.26 min) (Fig. 4.5B). For the dark period, an average duration of 11.88 min (± 10.00 min) was found for the Wake state, 3.85 min (± 2.32 min) for the non-REM state, and 1.04 min (± 0.50 min) for REM sleep (Fig. 4.5C). Using 5-min modal times, the average daily onset of the main sleep bout (longest consecutive period of non-REM and REM sleep without any Wake) for both wildebeest occurred between 03:30 and 04:30 and lasted an average of 43.33 ± 9.83 min.

4.3.4. State transition probabilities

Transitions between the physiologically identified states were counted and summed for both animals for the entire recording period (Fig. 4.6). From the Wake state, the animals only transitioned to non-REM (100% of transitions), with no occurrences (0%) of transition from Wake to REM. From non-REM we observed that the state transitioned mostly to Wake (71.75%), with 28.25% of transitions from non-REM being to REM. From REM, 59.18% of state transitions were to non-REM, while 40.82% of state transitions were to Wake.

4.3.5. Actigraphy (ACT) recordings

Using actigraphy methods we could determine two states, Active and Inactive. The average daily time spent Inactive, as scored with actigraphy (see methods), was 4.77 h (± 0.18 h), while 19.23 h (± 0.18 h) was spent Active (Fig. 4.3A). During the light period 1.16 h (± 0.05 h) was spent Inactive, while 11.84 h (± 0.05 h) was spent Active (Fig. 4.3B). During the dark period, 3.6 h (± 0.18 h) was spent Inactive while 7.4 h (± 0.18 h) was spent in the Active state (Fig. 4.3C). The daily average number of episodes for Active was 131 (± 2.24)

and 114 (± 2.38) for Inactive (Fig. 4.4A). For the light period, 57 (± 1.5) episodes were scored as Active, while 47 (± 1.59) episodes were scored as Inactive (Fig. 4.4B). During the dark period, there was on average 74 (± 2.37) episodes spent Active and 68 (± 2.48) episodes spent Inactive (Fig. 4.4C). The average duration of these episodes on a daily basis was 3.53 min (± 13.05 min) for Active and 0.4 min (± 1.30 min) for Inactive (Fig. 4.5A). During the light period, the average episode duration was 5.72 min (± 10.40 min) for Active and 0.29 min (± 0.36 min) for Inactive (Fig. 4.5B). During the dark period, the average duration for Active episodes was 1.84 min (± 12.86 min) and Inactive episodes was 0.47 min (± 1.27 min) (Fig. 4.5C). Using the actigraphy scoring criteria, the average daily onset of the main sleep bout (longest uninterrupted period of Inactive) for both wildebeest occurred between 04:30 and 05:00 and averaged 49.16 min (± 10.68 min).

4.3.6. Concordance between polysomnography (PSG) and actigraphy (ACT)

In general, actigraphy methods tend to overestimate the total sleep time compared to PSG methods due to the inability of ACT to distinguish quiet wake from sleep states (Paquet et al., 2007). This was observed for the two blue wildebeest in the current study, where the total Inactivity measured by ACT for 24 hr was 4.77 h (±0.18 h), while TST (non-REM plus REM) using PSG was 4.53 h (± 0.12 h), ACT overestimating TST by a daily average of 14.2 min (± 12.98 min); however, when each day for each animal was analyzed individually, actigraphy did not always appear to overestimate the amount of time spent asleep. The ACT scoring for Animal A, on average for the 3 days analyzed, overestimated daily TST by 37.6 min (± 19.08 min), for the light period by 3 min (± 11.77 min), and the dark period by 34.6 min (± 21.17). In the case of Animal B, ACT scoring underestimated daily TST by 9.66 min (± 16.69 min), and by 33 min (± 9.83 min) in the light period. The ACT scoring overestimated the TST in the dark period by 23 min (± 18.22 min) in Animal B.

A Cohen's κ was run to determine if there was agreement between PSG and ACT, over the 3 days of recording for both blue wildebeest, for the measure of TST (Inactive equivalent compared to non-REM plus REM) and time spent awake (Wake compared to Active). There was fair agreement between the two techniques for sleep measurements, $\kappa = 0.306$ (95% CI, 0.281 to 0.330), $p < .0001$. The agreements were different for the individual animals, with animal A showing a fair agreement between the techniques for TST estimation, $\kappa = 0.388$ (95% CI, 0.355 to 0.421), $p < .0001$, whereas animal B showed a slight agreement between the techniques for TST estimation, $\kappa = 0.198$ (95% CI, 0.163 to 0.234), $p < .0001$ (Table 4.1).

Figure 4.1. Polygraphic traces illustrating examples of the electroencephalogram (**EEG**) and electromyogram (**EMG**) in **Wake**, **non-REM** sleep and **REM** sleep states in the blue wildebeest (*Connochaetes taurinus*). (**A**) Wake is characterized by a low-voltage, high-frequency EEG and high amplitude and variable EMG. (**B**) non-REM sleep is characterized by a low frequency, high amplitude EEG signal accompanied by a high frequency, low amplitude EMG signal. (**C**) REM sleep iss characterized by a high frequency, low amplitude EEG signal, resembling that of waking, accompanied by a low-amplitude EMG signal that contained large, high amplitude infrequent spikes indicative of muscle twitches.

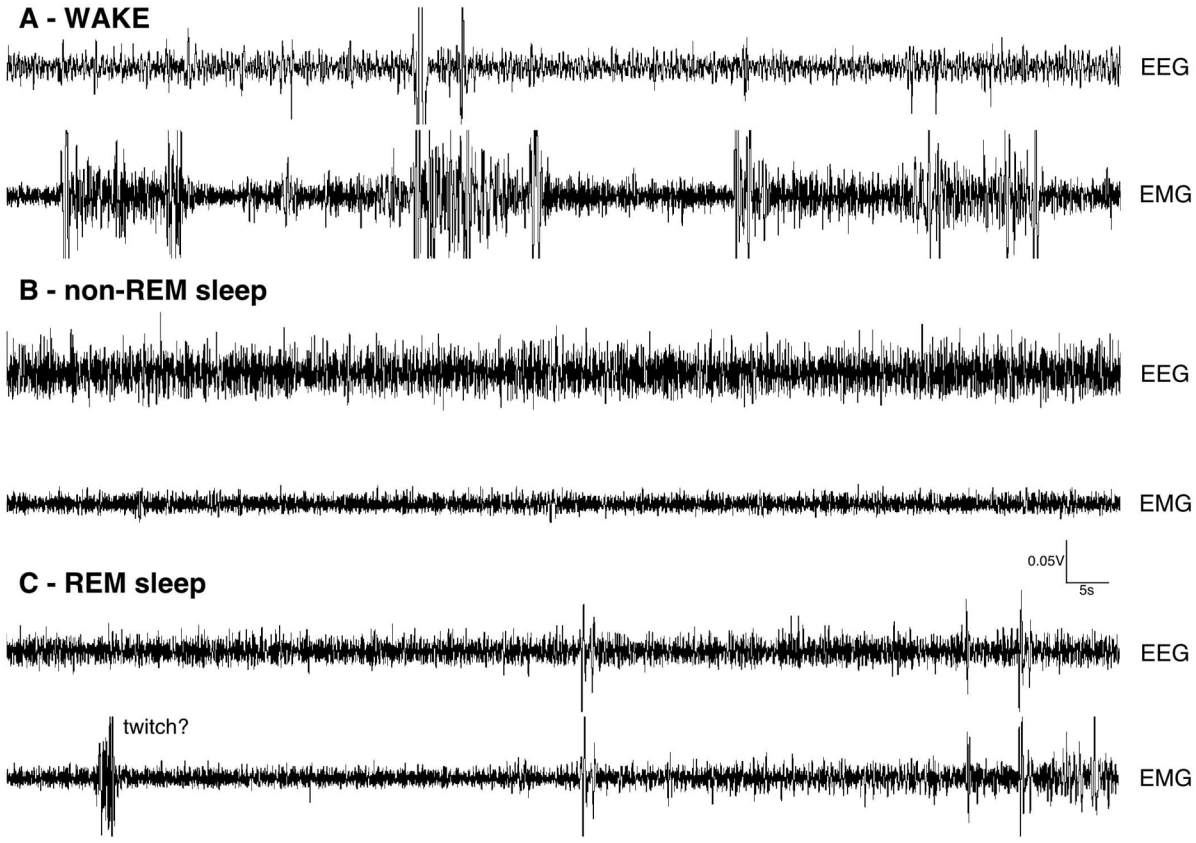

A - WAKE
EEG
EMG
B - non-REM sleep
EEG
EMG
0.05V
5s
C - REM sleep
EEG
twitch?
EMG

Figure 4.2. Graph indicating the spectral power range characteristic of **Wake**, **non-REM** and **REM** sleep in the blue wildebeest.

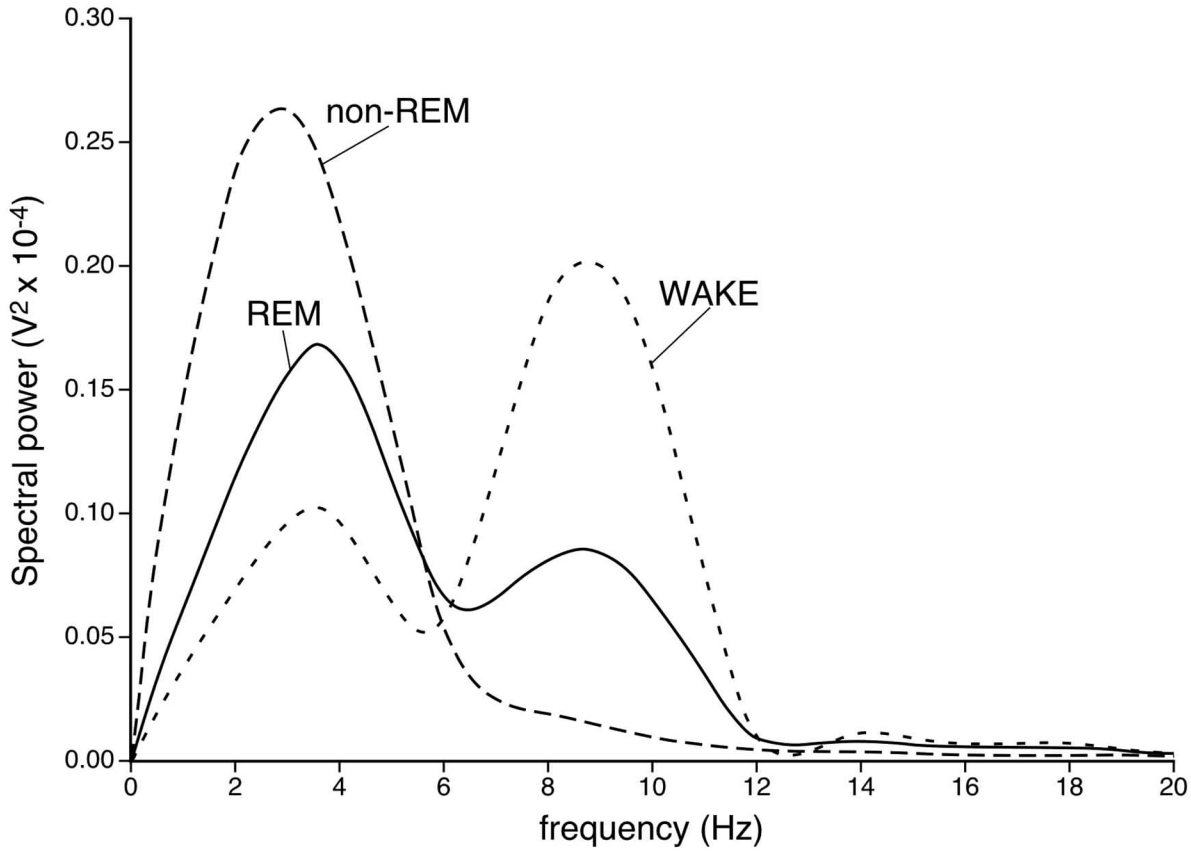

0.30
0.25
0.20
0.15
0.10
0.05
0.00
Spectral power (V2 x 10-4)
non-REM
REM
WAKE
0
2
4
6
8
10
12
14
16
18
20
frequency (Hz)

Figure 4.3. Histograms depicting the total amount of time spent in each of the polysomnographically (**PSG**, **Wake**, **non-REM** and **REM**) and actigraphically (**ACT**, **Active**, **Inactive**) defined states for (**A**) the 24 hour period, (**B**) the light period, and (**C**) the dark period, for both analytical methods. **BW A** – blue widlebeest animal A, **BW B** – blue wildebeest animal B.

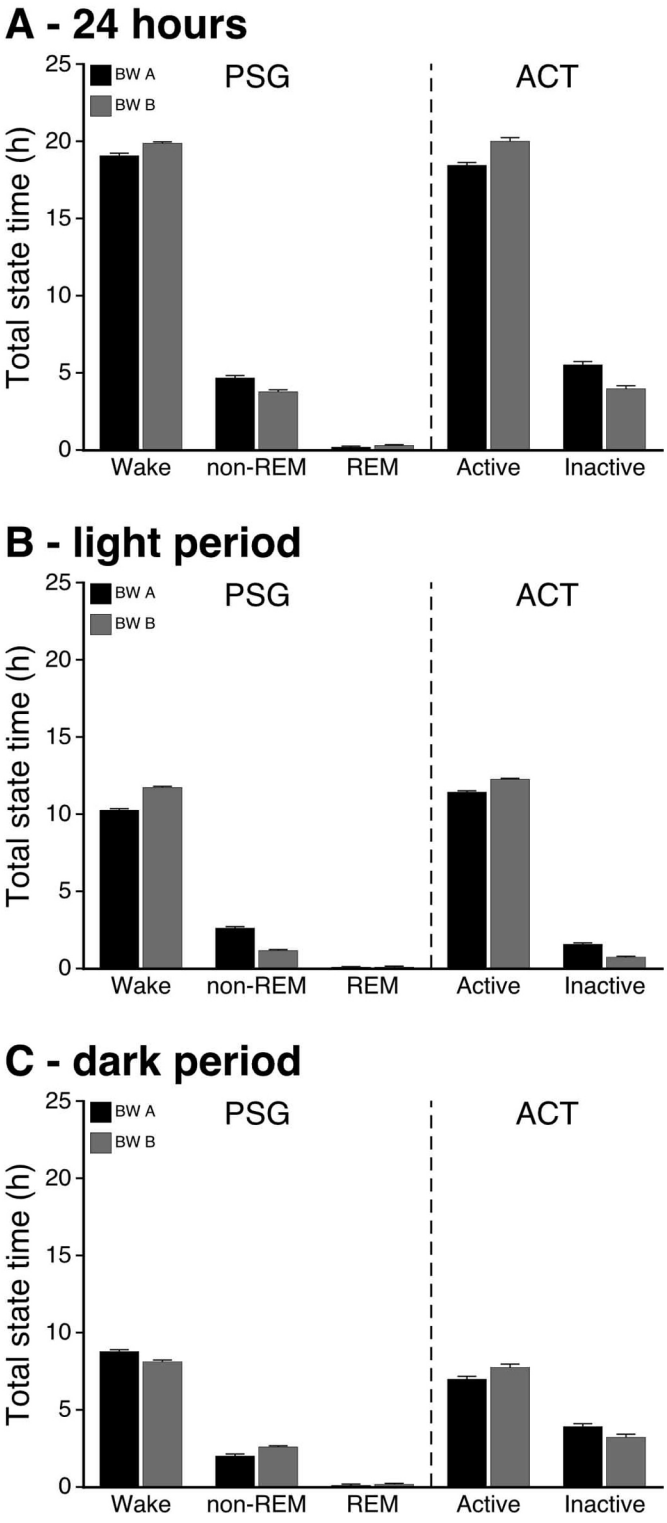

A - 24 hours
BW A
BW B
PSG
ACT
Total state time (h)
25
20
15
10
5
0
Wake
non-REM
REM
Active
Inactive
B - light period
BW A
BW B
PSG
ACT
Total state time (h)
25
20
15
10
5
0
Wake
non-REM
REM
Active
Inactive
C - dark period
BW A
BW B
PSG
ACT
Total state time (h)
25
20
15
10
5
0
Wake
non-REM
REM
Active
Inactive

Figure 4.4. Histograms depicting the average number of episodes for each of the polysomnographically (**PSG**, **Wake**, **non-REM** and **REM**) and actigraphically (**ACT**, **Active**, **Inactive**) defined states for (**A**) the 24 hour period, (**B**) the light period, and (**C**) the dark period, for both analytical methods. **BW A** – blue widlebeest animal A, **BW B** – blue wildebeest animal B.

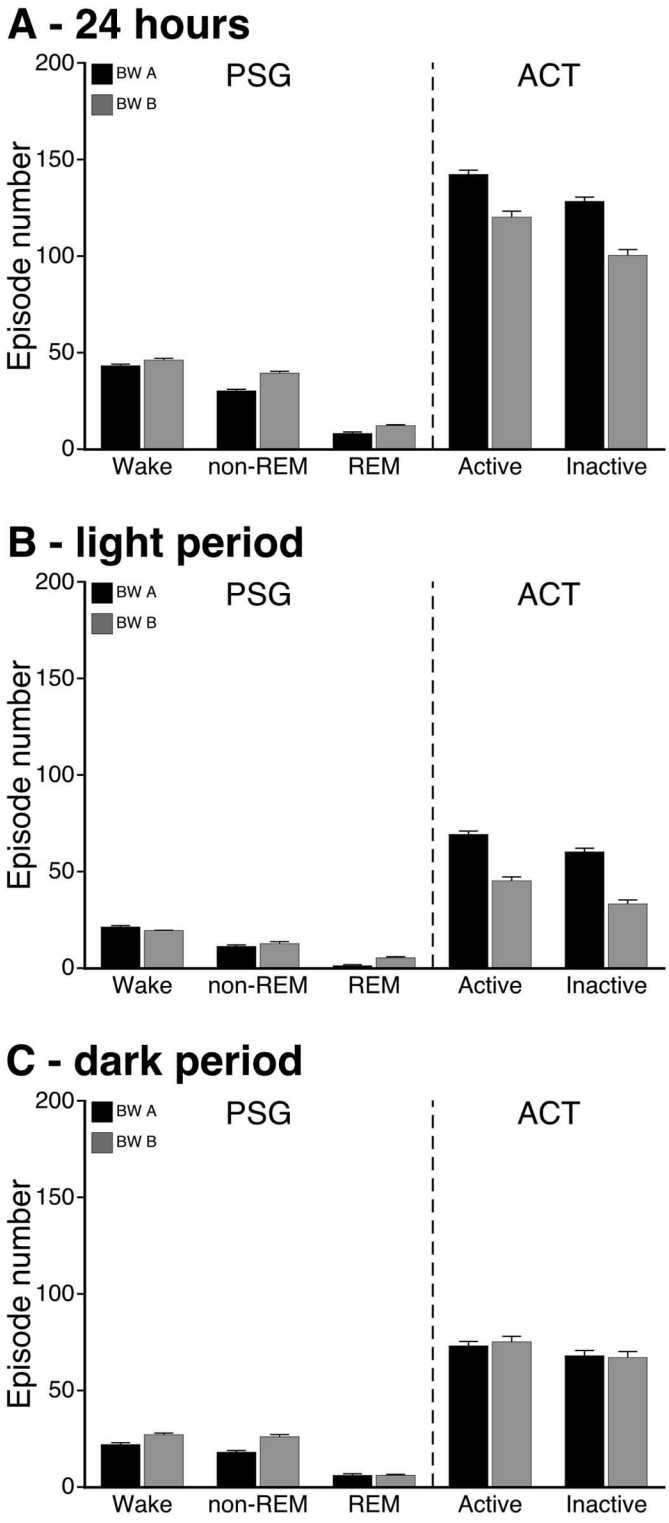

A - 24 hours
BW A
BW B
PSG
ACT
Episode number
200
150
100
50
0
Wake
non-REM
REM
Active
Inactive
B - light period
BW A
BW B
PSG
ACT
Episode number
200
150
100
50
0
Wake
non-REM
REM
Active
Inactive
C - dark period
BW A
BW B
PSG
ACT
Episode number
200
150
100
50
0
Wake
non-REM
REM
Active
Inactive

Figure 4.5. Histograms depicting the average episode duration for each of the polysomnographically (**PSG**, **Wake**, **non-REM** and **REM**) and actigraphically (**ACT**, **Active**, **Inactive**) defined states for (**A**) the 24 hour period, (**B**) the light period, and (**C**) the dark period, for both analytical methods. **BW A** – blue widlebeest animal A, **BW B** – blue wildebeest animal B.

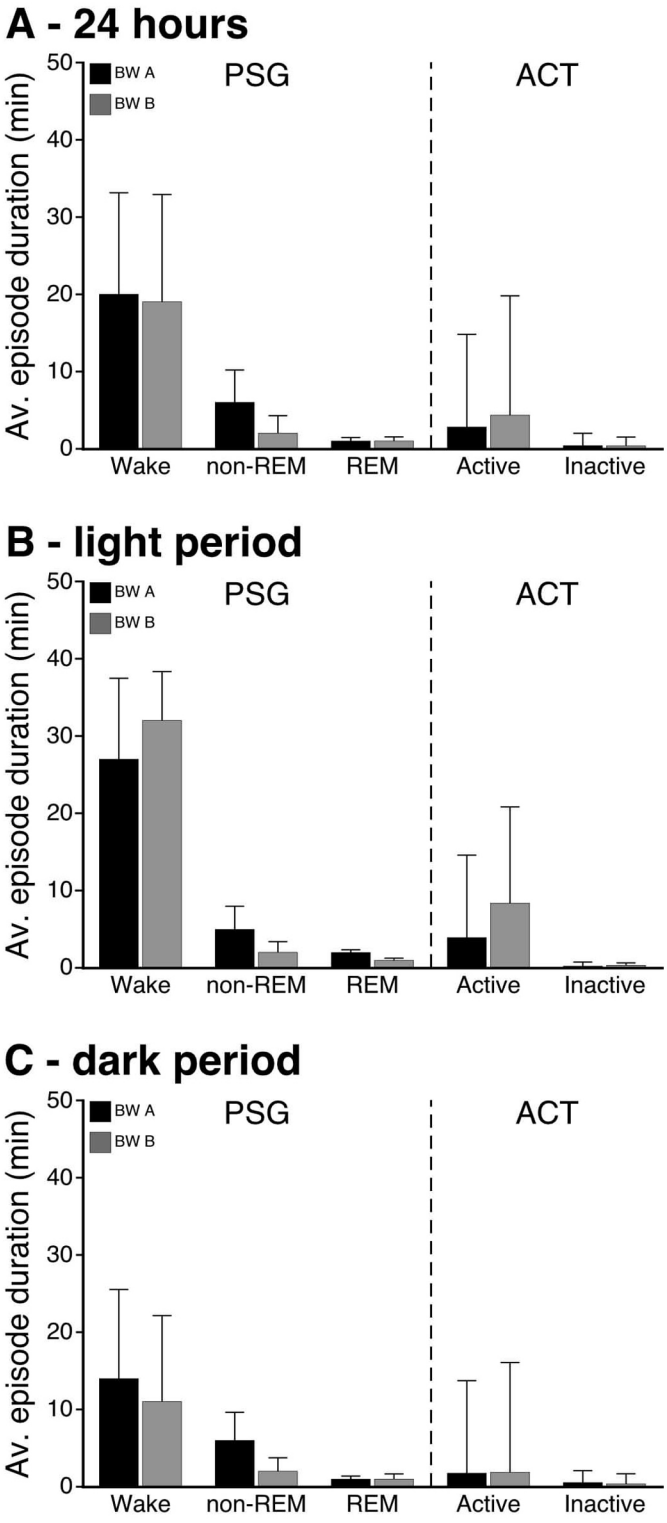

A - 24 hours
BW A
BW B
PSG
ACT
Av. episode duration (min)
50
40
30
20
10
0
Wake
non-REM
REM
Active
Inactive
B - light period
BW A
BW B
PSG
ACT
Av. episode duration (min)
50
40
30
20
10
0
Wake
non-REM
REM
Active
Inactive
C - dark period
BW A
BW B
PSG
ACT
Av. episode duration (min)
50
40
30
20
10
0
Wake
non-REM
REM
Active
Inactive

Figure 4.6. Flow diagram illustrating the occurrence of state transitions (represented as a percentage) between **Wake**, **non-REM** sleep and **REM** sleep states with the pooled data from both animals studied over the 72 h recording period analyzed.

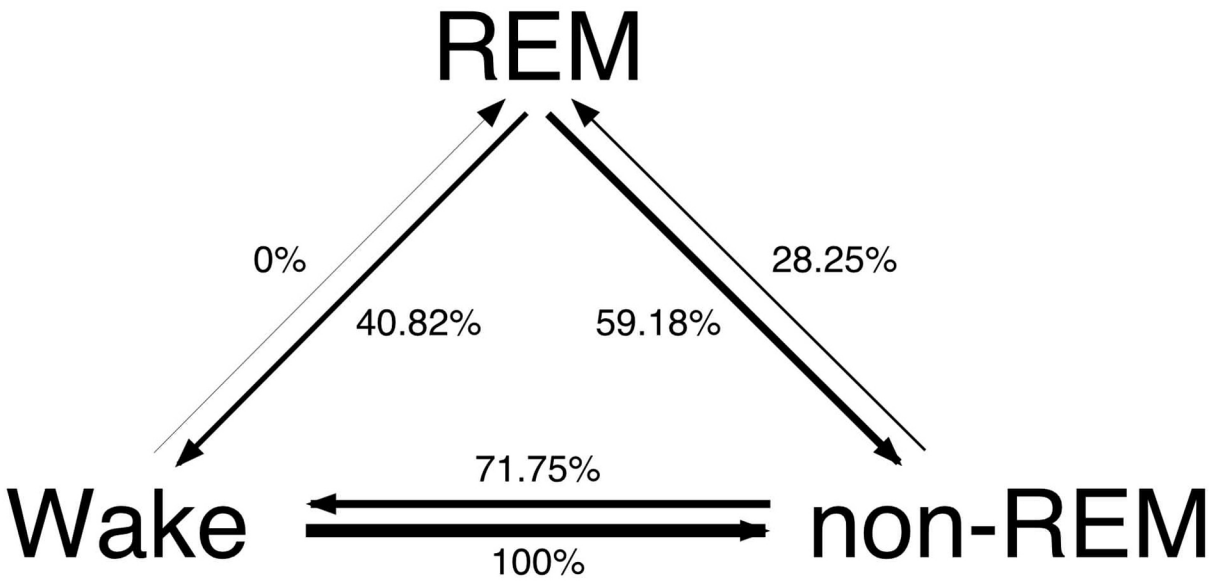

REM
0%
40.82%
59.18%
28.25%
71.75%
Wake
non-REM
100%

Figure 4.7. Graph plotting the relationship between body mass (***Mb***, in kilograms, **kg**) and total sleep time (***TST***, in hours, **h**) for herbivores, including the blue wildebeest. While the regression indicates a lower TST with increased body mass, this regression only approaches significance for the herbivores included for analysis. Note that in relative terms the total sleep time for the blue wildebeest recorded in the current study appears to correlate with the data obtained from other herbivores. The data used in this plot is derived mainly from Siegel (2005), with data for the African elephant from Gravett et al. (2017) and the Arabian oryx from Davimes et al. (2018).

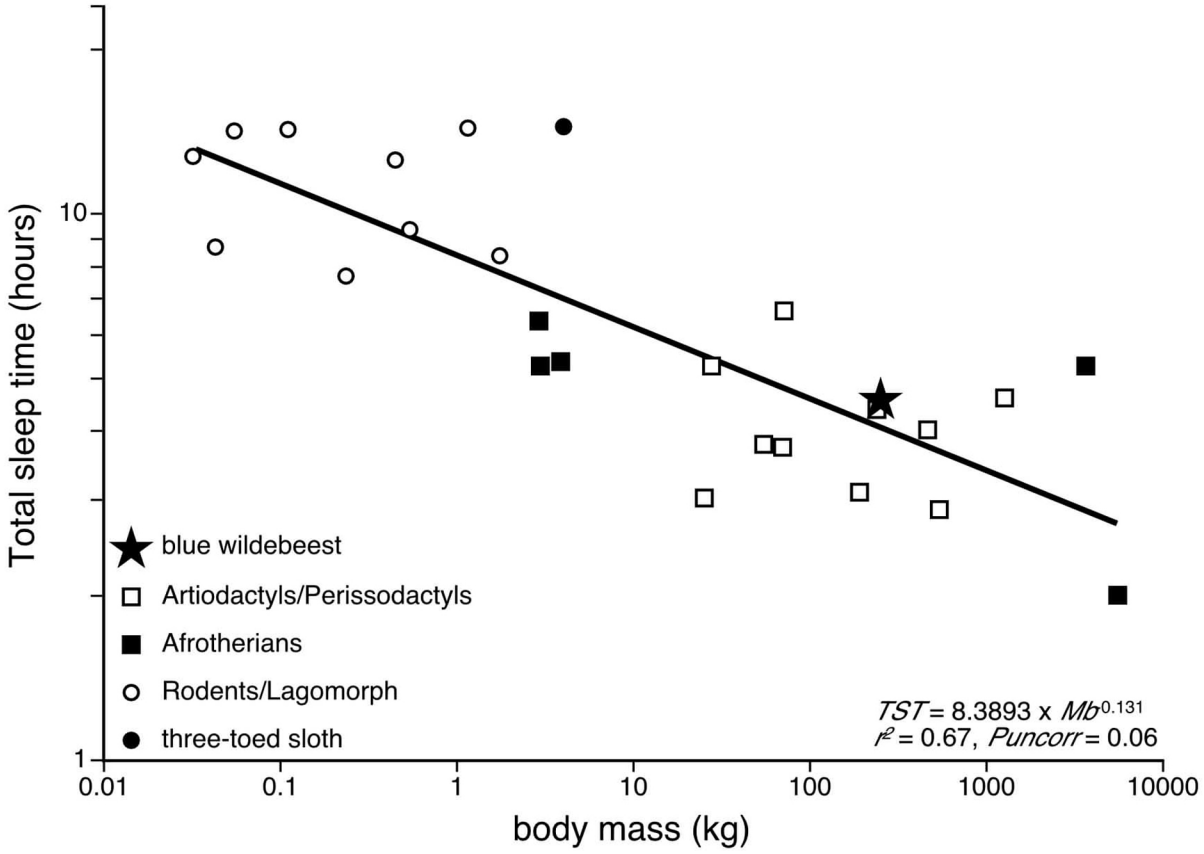

Total sleep time (hours)
10
1
0.01
0.1
1
10
100
1000
10000
body mass (kg)
blue wildebeest
Artiodactyls/Perissodactyls
Afrotherians
Rodents/Lagomorph
three-toed sloth
TST = 8.3893 x Mb^0.131
r² = 0.67, Puncorr = 0.06

Table 4.1: Cohen's κ showing the agreement between polysomnography (**PSG**) and actigraphy (**ACT**) recordings, for the individual days of recording for each blue wildebeest, for the measure of total sleep time (combined **non-REM** and **REM** compared to **Inactive**) and wake (**Wake** compared to **Active**). The statistically significant results are indicated by an asterix.

						95% CI	
Animal	Day	Cohen's κ	Level of agreement	p value	Std. Error	Lower Limit	Upper Limit
A	1	0.596	moderate	0.000 *	0.025	0.546	0.645
	2	0.241	fair	0.000 *	0.034	0.175	0.307
	3	0.301	fair	0.000 *	0.035	0.232	0.370
B	1	0.273	fair	0.000 *	0.044	0.188	0.359
	2	0.313	fair	0.000 *	0.036	0.242	0.383
	3	0.034	poor	0.188	0.038	0	0.109

4.4. Discussion

The present study examined sleep in the blue wildebeest, in a naturalistic setting, by means of two different measures of sleep, polysomnography (PSG) and actigraphy (ACT). Our recordings indicate that the blue wildebeest shows the standard non-REM and REM states of sleep observed in most mammals studied, without any additional unusual sleep states being observed. Moreover, they slept mostly at night, in the early hours of the morning, and exhibited polyphasic sleep, all of which could be considered typical for an Artiodactyl based on studies of domestic species. PSG showed that daily TST in the blue wildebeest for a 24-hour period averaged approximately 4.5 h, with approximately 4.3 h spent in non-REM sleep and 0.3 h (18 min) spent in REM sleep, with the remainder of the day, approximately 19.5 h spent in Wake. This amount of sleep appears to be what one would expect based on the body mass of the blue wildebeest in comparison to measures of total sleep time in other Artiodactyls (Siegel, 2005, see below). ACT showed that the blue wildebeest spent on average approximately 19.2 h Active and 4.8 h Inactive each day. Thus, the total daily sleep amount was similar between the two methods, but ACT led to a slightly higher amount of time spent in sleep/Inactive, by around 15 minutes each day, indicating that during the analysis of the ACT data a portion of quiet waking is likely the cause of the slightly inflated total daily sleep time.

4.4.1. Comparisons to sleep in other Artiodactyla

The vast majority of systematic studies of mammalian sleep have been conducted in only a few domesticated species including rats, mice, cats, dogs, monkeys and humans within well controlled settings (Lesku et al., 2006; Siegel, 2008), with very few studies of mammalian sleep being undertaken in their natural habitat (Rattenborg et al., 2008; Voirin et al., 2014; Yetish et al., 2015; Davimes et al., 2018). Along with the cetaceans, the blue

wildebeest is a species assigned to the superorder Cetartiodactyla; however, comparing sleep between blue wildebeest (or indeed other terrestrial Artiodactyls) and cetaceans is not tenable due to the specialized nature of cetacean sleep phenomenology (Lyamin et al., 2008), thus we, for the most part, restrict our comparisons to other members of the terrestrial Artiodactyls.

Within the Artiodactyla, the majority of polysomnographically conducted sleep studies have been undertaken on the domesticated species belonging to this order. The TST per day reported for the cow (*Bos taurus*) was 3.97 h (Ruckebusch, 1972), sheep (*Ovis aries*) 3.85 h (Ruckebusch, 1972), goat (*Capra aegagrus*) 5.4 h (Bell and Itabisashi, 1973), pig (*Sus scrofa*) 7.82 h (Ruckebusch, 1972; Robert and Dellaire, 1986), and in the non-domesticated Arabian oryx (*Oryx leucoryx*) 6.60 h in winter and 3.77 h in summer (Davimes et al., 2018) and in the current study blue wildebeest 4.5 h. Behaviourally, the TST of the giraffe (*Giraffa camelopardalis*) was reported to be 4.6 h (Tobler and Schwierin, 1996). Thus, the result of an average daily total sleep time of 4.5 h for the two blue wildebeest investigated in the current study appears to be well within the range of phylogenetically related terrestrial species, even though the current study was undertaken in as close to natural conditions as possible, which has been shown to lower total sleep time in mammals (Rattenborg et al., 2008; Yetish et al., 2015).

If we broaden our comparison to encompass all mammalian herbivores in which sleep has been recorded, as suggested by Siegel (2005, the data provided includes other species of Artiodactyls, Perissodactyls, Rodents, domestic rabbit, three-toed sloth and Afrotherians), we find that the total sleep time for the blue wildebeest is very similar to what would be predicted based on their body mass in comparison to other herbivores (Fig. 4.7). As the majority of the data used to calculate the regression presented in Figure 4.7 is taken from studies of species in controlled environments (be they wild or domesticated species), it would

appear that for the blue wildebeest investigated in the current study, that either the time spent acclimatizing to the controlled environment has increased their amount of daily sleep (Rattenborg et al., 2008), or that irrespective of the setting in which sleep recording occurs, this is the normal amount of daily sleep for the blue wildebeest. Further studies of more individual wildebeest in different settings are needed to determine whether either of these potential explanations of the total sleep time in the blue wildebeest is correct, or whether additional as yet unidentified factors may be playing a role.

Concerning the percentage of TST occupied by REM sleep, the blue wildebeest exhibited 6.18 % of TST being REM sleep, which is comparable with the 4.7% observed behaviourally in the giraffe (Tobler and Schwierin, 1996) and 4.18% observed physiologically during winter sleep in the Arabian oryx (Davimes et al., 2018, but see Davimes et al., 2018 concerning suppression of REM sleep in the summer in Arabian oryx). In contrast, the domestic Artiodactyls (cow – 18.91%, sheep – 14.72%, pig – 29.13%, goat – 13.15%) all had substantially higher percentages of TST occupied by REM sleep. Thus, there appears to be a domestic *vs* wild dichotomy in the Artiodactyls, where while total sleep times appear to be consistent across both domestic and wild Artiodactyl species, the proportion of sleep occupied by REM is substantially higher in the domestic species compared to the wild species. Indeed, the three wild Artiodactyl species (blue wildebeest, giraffe and Arabian oryx) appear to have approximately 15 min of REM sleep per day, while the domesticated cow, sheep and goat have approximately 45 min of REM sleep per day, with the domestic pig having about 2 h 15 min of REM sleep per day

The average daily amount of REM sleep for a given mammalian species appears to be strongly related to maturity at birth, with precocial mammals having less REM as adults, while altricial mammals have more REM as adults (Siegel, 2005); however, here we have noted that domestication appears to be a factor in increasing the amount of daily time spent in

REM sleep, at least in the Artiodactyls. This may be a result of the artificial selection processes leading to the domestic varieties of mammals, where neotenous, or juvenile, features are selected for as they increase the tameness and manageability of the animals for human purposes – "the domesticated phenotype" (e.g. Diamond et al., 2002; Agnvall et al., 2017). Perhaps the increased amount of REM sleep, both in actual terms and as a percentile of total sleep time, in domestic Artiodactyls is part of the domesticated phenotype. An analogy may be drawn between humans and chimpanzees, where humans sleep on average for 8 h per day, with 23.75% of total sleep time being REM (Meddis, 1983), while chimpanzees sleep for an average of 10.8 h per day, with 15% of this sleep being REM (McNew et al., 1971; Balzamo et al., 1972). Thus, even though the chimpanzee sleeps for 2.8 h more per day than the human, the chimpanzee experiences only around 97 min per day of REM, while the human experiences 114 min of REM. Do humans living in the industrialized world show aspects of "the domesticated phenotype" regarding REM sleep? Further studies on REM sleep in humans leading a non-industrialized lifestyle (e.g. Yetish et al., 2015) may provide an answer to this intriguing possibility.

The lowered amount of REM in the wild Artiodactyls compared to the domestic Artiodactyls, may also be a reflection of the difference in the extent of exposure to the natural environment, where in the Arabian oryx, extremely hot weather has been observed to lower the amount of time spent in REM sleep each day (Davimes et al., 2018). In addition, the lower amounts of REM sleep in wild Artiodactyls are difficult to reconcile with the REM sleep – memory consolidation hypothesis (Vertes and Eastman, 2000; Siegel, 2001), as one would imagine that when experiencing the various environmental pressures that are alleviated in domesticated species (such as the uncertainty of food, environmental conditions and the risk of predation), that consolidations of memories would be important for survival of these animals living in the natural habitat when contrasted with the domesticated environment.

Important insights into the function and evolution of sleep could be attained by expanding the comparative sleep databases and by examining how sleep varies across species with differing physiological and ecological specializations under the most naturalistic conditions (Tobler, 2005; Siegel, 2008; Aulsebrook et al., 2016).

4.4.2. Comparison of Actigraphy and Polysomnography as measures of total sleep time and patterns in the blue wildebeest

Polysomnography (PSG) is considered the gold-standard of sleep measurement as it provides the ability to distinguish the various global neuronal states of a mammal, namely Wake, non-REM and REM sleep, but PSG is usually performed in a controlled laboratory setting. As mentioned above, there are discrepancies regarding the total sleep time measured in the laboratory compared to a more natural setting (Rattenborg et al., 2008). Obtaining high-quality EEG recordings from free-roaming animals under naturalistic settings can be challenging. PSG was recorded in dairy cows using non-invasive surface-attached electrodes to measure different vigilance states (Ternman et al., 2012). Unfortunately, this method is not suitable for most animals, especially those in naturalistic settings. One difficulty with this method is that the animals may simply remove the electrodes, as was seen in the case of the dairy cows (Ternman et al., 2012), and this is likely to be more exaggerated in non-domestic species. Another drawback of the surface-attached electrodes is that they are susceptible to artefacts from muscle activity and movements being recorded (Rattenborg et al., 2017). Stable EEG recordings can be obtained from electrodes placed on the dura mater overlying the brain, as done in the current study; however, this then becomes an invasive procedure. Although the EEG provides a direct measure of sleep-related brain activity, by itself it provides an incomplete view of sleep (Rattenborg et al., 2017). It gives us insight to the

vigilance states of the animal, but not necessarily how the animal structures it sleep under different ecological factors especially for extended periods of time.

Accelerometers have been used to study a wide range of behavioural and physiological aspects of animals in their natural habitat, including foraging, reproduction, activity, energy budgets, and locomotion (Brown et al., 2013; Wang et al, 2015). In a study on free-ranging pumas (*Puma concolor*), accelerometer measurements were validated successfully against behavioural observations (Wang et al., 2015), being able to accurately predict periods of no movement, low acceleration (i.e., stalking, walking) and high acceleration movements (i.e., trotting and running) in unobserved wild animals. Actigraphy (ACT) has also been shown to be a minimally-invasive method to objectively measure sleep/inactivity during prolonged naturalistic investigations in mammals (Davimes et al., 2016; Gravett et al., 2017). The accuracy of sleep measurement using accelerometers in free-roaming large mammals still needs to be validated against polysomnographical measures of sleep. In humans, there are a number of studies that have evaluated the ability of actigraphy to discriminate between sleep and wake as defined by PSG criteria (de Souza et al., 2003; Paquet et al., 2007; Sadeh 2011; Shambroom et al., 2012). To the author's knowledge the current study is the first attempt at comparing the accuracy of actigraphic vs polysomnographic measures of sleep in a large non-human non-domestic mammal recorded from in a naturalistic setting.

While validation studies of ACT with PSG in humans, have shown that the concordance in sleep scoring between the two methods ranges from 83 to more than 90%, in the current study of the blue wildebeest, it was found that the concordance between ACT and PSG revealed only a fair agreement of 30% and differences were noted in the different animals and on different days. In addition, on average the ACT method led to an approximately 15 min/day overscore of total sleep time, which would likely be considered

quiet wake from PSG recordings. One crucial difference between the current study and those performed in humans, is the manner in which inactivity, or sleep, is assigned on the basis of the ACT recordings. In the current study each animal was implanted with two actiwatches, one in the neck and one in the leg. Each minute that showed no activity in both actiwatches in one animal was scored as "Inactive" (any activity was scored as "Active"), and from the one minute scored data the modal state for five minute was calculated and assigned as either "Inactive" or "Active". This was then compared to the PSG five mintue modal data to determine the extent of correlation. In contrast, studies in humans have developed complex algorithms to improve the concordance between PSG and ACT in human studies (de Souza et al., 2003; Paquet et al., 2007; Sadeh, 2011). Despite these methodological differences, it appears that, as a first attempt to determine whether ACT can be used as a successful replacement for PSG for the study of sleep of large mammals in their natural habitat, ACT can prove to be a useful method in this endeavour. However, at this stage further studies across more individuals of the current species and a broader range of species is required in order to determine to what extent ACT recordings can be regarded as true sleep as opposed to the less proscriptive terms used here such as "Inactive" and "Active". It appears likely that with further studies and increased stringency on the analysis of the ACT recordings, that ACT could be used successfully to determine what happens to total sleep times and sleep architecture in large, free-roaming mammals for extended periods of time (although these recordings could not determine what happens to specific sleep states, such as non-REM and REM). It should also be noted that the development of potential algorithms to increase the accuracy of ACT when recorded in parallel with PSG, are likely to be species or phylogenetic group specific. Such validation of ACT methodology for the accurate assessment of sleep would expand the opportunities for studying sleep in various large mammalian species in their natural habitat, providing accurate quantifiable information that can be related to a

variety of environmental conditions, including, for example, changes in weather patterns (seasonally, daily, or with specific weather phenomenon), levels of predation risk, mating and birthing schedules, and long-distance migrations. Thus, properly validated ACT may provide a method to broaden our understanding of mammalian sleep and how this relates to the natural habitat and life histories of mammals.

CHAPTER FIVE: **Male blue wildebeest significantly increase activity levels during the breeding season (rut), but not at the expense of the time spent at rest**

5.1. Introduction

For a few weeks each year in the southern African sub-region, sometime in the period of April to June depending on environmental conditions coinciding with the end of the rainy season, the blue wildebeest enter their breeding season, also known as the rut (Estes, 1969). Sexually active male blue wildebeest (bulls) are typically territorial, and defend their territories with ritualized threat displays that, when not sufficient to deter an intruder, may develop into horn-sparring or head-butting contests (Estes, 1969). Territorial defence becomes more intense during the rut, with the wildebeest bulls forming an exploded lek system, where they vigorously defend their harems and territories (the size of which is dependent on population density, Talbot and Talbot, 1963). It has been reported that when a wildebeest bull is protecting a harem in his territory during the rut, he neither eats nor rests (Estes, 1997). This indicates that the most successful sexually active territorial bulls would need to go without rest (including sleep) for extended periods.

Sleep is a physiological and behavioural imperative that normally occurs on a daily basis, but no function that can explain the huge variation in total sleep times observed across species has been identified (Lesku et al., 2012; Siegel, 2012). Sleep may be viewed as a state of adaptive inactivity that increases the efficiency of activity by regulating its timing and reducing energy use when activity is not beneficial (Lesku et al., 2012; Siegel, 2012). This means that animals can go without sleep when specific demands, such as mating, favour being awake (Lesku et al., 2012; Siegel, 2012). Lesku et al. (2012) showed that male pectoral sandpipers (*Calidris melanotos*) are able to maintain high neurobehavioural functioning even

though the time spent sleeping during a 3-week period of intense male-male competition for access to fertile females is greatly reduced; however, sleep loss appeared to be compensated for by a greater sleep intensity (Lesku et al., 2012). In a different context, great frigatebirds are able to minimize the amount of sleep during long flights, reducing total sleep time from 12.8 hours per day down to 0.7 hours per day (Rattenborg et al., 2016). Such observations are yet to be made in mammals, but the sexually active territorial wildebeest bulls during the rut appear to be potential candidates that may substantially lower, or even eliminate, the time spent at rest (presumably some portion of which would be sleep) during the mating season or rut (Estes, 1997).

To test this possibility we examined daily activity and inactivity patterns in a total of four dominant male blue wildebeest by means of actigraphy (e.g. Davimes et al., 2016) for a period of three months, which included the time of the mating season or rut. In addition, we measured faecal androgen metabolite levels (Ganswindt et al., 2002; Hodges et al., 2010) and subcutaneous temperature (Kortner and Geiser, 1994; Grigg et al., 2009), both of which have variances known to relate to the mating season or rut in other mammalian species.

5.2. Material and Methods

5.2.1. Experimental animals and site location

All animals were treated according to the guidelines of the University of the Witwatersrand Animal Ethics Committee (approval AESC number 2015/09/39/C), which parallel those of the National Institutes of Health (NIH) for the care and use of animals in scientific experimentation. Permission and permits to undertake the research were granted from the Gauteng Department of Agriculture and Rural Development Division of Nature Conservation and the Dinokeng Game Reserve. Four free-roaming adult male blue wildebeest, which were the dominant bull in their respective herds, were located

opportunistically in the Dinokeng Game Reserve. Each male had an estimated body mass of approximately 250 kg and were estimated to be between 8 to 15 years in age. All animals were judged to be in good condition by an experienced wildlife veterinarian. Dinokeng Game Reserve is located in the north-eastern part of the Gauteng Province of South Africa (latitude° S: 25.403236; longitude° E: 28.296906) encompassing nearly 18 500 ha. The grassland and bushveld bioregions support an abundance of wildlife, with Dinokeng Game Reserve housing many large herbivorous and carnivorous mammal species. The climate in this region can be described as warm and temperate, and the area experiences summer rainfall patterns, between November and February, in the form of thunderstorms that can be variable and erratic (Low and Rebelo, 1996; Rutherford et al., 2006).

5.2.2. Recording period and environmental conditions

The period during which the recording of activity, subcutaneous temperature and the measurement of faecal androgen metabolite levels (see below) took place was from February 15 to May 11, 2016. The average temperature for the recording period was 21°C, with a mean maximum temperature of 39°C, a mean minimum temperature of 1°C, and an average daily rainfall of 0.07 mm. This weather data was recorded at the Pienaars River weather station, and provided by the Agriculture Research Council (ARC) of South Africa. Sunrise times for the recording period ranged from 05:52 (February 15) to 06:36 (May 11), and sunset times from 18:50 (February 11) to 17:31 (May 11). Sunrise and sunset times were obtained from freely accessible online databases.

5.2.3. Activity and temperature data loggers

Activity was logged in each animal using two subcutaneously implanted actiwatches, one the side of the neck and one on the upper hindleg. The actiwatch is a wristwatch size, ambulatory device that is commonly used for measuring sleep in humans (e.g. Ancoli-Isreal et al., 2003; Shambroom et al., 2012). Within each actiwatch is a piezoaccelerometer device connected to a microchip that sums and records the number of acceleration events per minute. The Actiwatch Spectrum (Philips Respironics) was used in the current study. These devices have a mass of 25 g and approximate dimensions of 35 x 35 x 12 mm. Each actiwatch was calibrated and programmed (data acquisition rate set at 1 min intervals) with the Philips Respironics Actiware 5 software, prior to implantation. Subcutaneous temperature was logged using an iButton subcutaneously implanted with the neck actiwatch in each animal (see below). The iButton is a microchip enclosed in a round stainless steel case and has a diameter of approximately 16 mm and a depth of 5 mm. The iButton thermochron hi-resolution (DS 1922L, Fairbridge Technologies) is a multi-use temperature data logger with a range of -40 to 80°C and measuring accuracy of ± 0.5°C. The sampling frequency for each iButton was set to 30 minutes with the Cold Chain ThermoDynamics software (version 4.9). Prior to implantation, the wristbands were removed from the actiwatches and all actiwatches and iButtons were wrapped with standard electrical insulation tape and then covered in 2 coats of biologically inert wax (SasolWax 1276, Sasol, Johannesburg, South Africa). The devices were then placed in a container with formalin pellets for sterilization for a minimum of 24 hours prior to the implantation procedure.

5.2.4. Surgical implantation and removal of actiwatches and iButtons

The four blue wildebeests were darted and immobilized with weight appropriate doses of thiofentanyl (4 mg of A30-80®, Wildlife Pharmaceuticals, South Africa) and azaperone

(40 mg Stresnil®, Janssen Pharmaceutica, South Africa). Following immobilization, the animals were placed in sternal recumbency, respiration and body temperature were monitored, and the animals received oxygen *via* a nasal tube throughout the procedure. The two implantation sites (the side of the neck and hind-leg) were closely shaved, cleaned and disinfected with chlorhexidine (Kyron, South Africa). Sterile drapes were clamped in place over the implantation sites to isolate the cleaned areas. A small incision (approximately 5 cm in length) was made at each of the implantation sites and a subcutaneous pocket extending approximately 10 cm from the incision sites was created. The sterilized actiwatches were rinsed in sterile saline and inserted into each pocket. A sterilized iButton was rinsed in sterile saline and placed into the pocket created on the side of the neck in addition to the actiwatch. The incision sites were sutured closed by means of intradermal and superficial stitches and sterilized with the topical antiseptic spray Necrospray® (Centaur Labs, Johannesburg, South Africa). Following the surgical procedure each animal was fitted with a radio transmitter collar (African Wildlife Tracking, Pretoria, South Africa) and plastic ear tags for individual identification (yellow, green, blue and white) and then received weight appropriate doses of antibiotics and analgesics (8 ml of Draxxin®, Zoetis and 9 ml of Ketofen®, Zoetis). The radio transmitter collar was placed on each of the animals to monitor their recovery post-surgery and to relocate them on a weekly basis (to collect faecal samples) and after the 3-month recording period (to retrieve the implants). To reverse the anaesthesia, each animal was injected with weight appropriate doses of naltrexone (50 mg of Trexonil®, Wildlife Pharmaceutical, South Africa) and was monitored until they were able to stand unassisted and move off freely. The time period for the entire surgical procedure was less than 1 hour, from the time the animal was darted to the time the reversal drugs were administered.

Following the recording period, the implanted animals were located and immobilized in the same manner as described for the implantation. Once the animal was immobilized the

radio collar was removed and the implant sites located. These areas were once again closely shaved and disinfected and the devices retrieved. The incisions were sutured closed and sterilized by spraying liberally with a topical antiseptic spray (Necrospray®, Centaur Labs, South Africa). The animals received weight appropriate doses of antibiotics (20 ml Lentrax®, Merial, South Africa) and the reversal drug, naltrexone (40 mg Trexonil®, Wildlife Pharmaceuticals, South Africa). No animals exhibited any ill effects of the implantation procedure, and all were in good health following the recording period.

5.2.5. Organization of data for analysis

The data presented herein is the grouped data for all four individual wildebeest, with results provided throughout as the mean ± the standard deviation (SD). The data was combined into group data as all individual wildebeest showed similar patterns of activity, inactivity, subcutaneous temperature and faecal androgen metabolite (fAM) levels, with individual data not contradicting the group patterns described; thus, for brevity only the group data is presented. The data was analysed for the three-month recording period and also for the average 24-hour period. The data was then divided into periods for analyses and was described as pre-rut, rut and post-rut. The rutting period was determined by the highest peak in the average fAM level and other factors described in the results section (see also Figs. 5.1, 5.2). Pre-rut is the time period before the rut and post-rut is the time period after the rut. The data recorded on the first four days following implantation was excluded from any analysis, allowing time for the animals to recover from any physical and chemical complications of the surgical procedure.

5.2.6. Activity analyses

Phillips Respironics Actiware 5 was used to retrieve the recorded data from each of the implanted actiwatches. The raw data was exported to Microsoft Excel for analysis and the raw counts of each of the individual actiwatches were summed for each day. For each individual actiwatch, the highest summed activity count was recorded as maximum, or 100%, activity. The remaining counts for each actiwatch for each day were then converted to a percentage of the maximum activity. This process allowed for inter-device variability and sensitivity of the raw activity counts, allowing for unbiased comparisons between the individual actiwatches and between animals. For statistical comparisons 21 days pre-rut, rut and post-rut, were compared to determine whether differences in activity levels between the three distinct periods occurred. Thus, data for percentage maximum activity of each neck actiwatch and each leg actiwatch on each day was pooled for the four animals (giving 84 data points for neck activity for each period, pre-rut, rut and post-rut). This pooled data was averaged and used to determine the levels of daily activity for the group between the recording periods as well as being compared statistically between periods.

5.2.7. Inactivity analyses

Data obtained from the neck and leg actigraphs were scored concurrently in 1-minute epochs as either active or inactive. For an epoch to be scored as active either the neck or leg actigraphs had to have an activity score greater than zero. Inactive epochs were scored when both the neck and the leg actigraphs had an activity score equal to zero. From the 1 minute scored data the modal state for 5 minutes was calculated and used to determine, for each animal, total inactive time each day, number of inactive episodes each day and the average duration of inactive episodes. This data was then averaged across the individuals on a daily basis to determine group level changes across the entire recording session, and for statistical

comparisons 21 days pre-rut, rut and post-rut, were compared to determine whether differences between the three distinct periods occurred. In addition, this data was used to determine the daily patterns of inactivity during the three distinct periods.

5.2.8. Subcutaneous temperature

The iButton recordings were downloaded and analysed using ColdChain ThermoDynamics version 4.9 and exported to Microsoft Excel for further analysis. To determine average daily subcutaneous temperature, the 48 daily recordings from each of the four animals on the same day were summed and divided by 192 (48 recordings, 4 animals). For statistical comparisons, averages from 21 days pre-rut, rut and post-rut, were compared to determine whether differences between the three distinct periods occurred. To obtain the daily pattern of variation in subcutaneous temperature, the time matched recordings from each animal across the 24 hour period were summed and averaged across the pre-rut, rut and post-rut periods (21 days per period, 4 animals). In order to determine the daily range of subcutaneous temperature, the lowest recorded temperature was subtracted from the highest recorded temperature for each animal for each day. These were then averaged for the group on a daily basis and plotted for the entire recording session to examine differences, or for statistical comparisons, averages from 21 days pre-rut, rut and post-rut, were compared.

5.2.9. Measurement of faecal androgens

Faecal samples were collected from each of the four male blue wildebeest at least once a week for the duration of the experimental period, with the animals being located using radio telemetry. In addition to the four males, faecal samples were also collected from randomly selected juveniles (n =20) of unknown sex for assay validation purposes. A total of n = 51 samples were collected. For each sample, approximately 40 g of fresh faecal matter

was collected in a plastic container as soon after defecation as possible and placed immediately on ice in the field and subsequently stored at the end of the day at -20 °C until analysis. Faecal samples were taken from the centre of the stool to avoid any cross contamination with urine, soil or water.

Frozen faecal samples were subsequently lyophilised and pulverised using a metal strainer allowing the removal of coarse material. Following this, 100 - 110 mg of faecal powder was mixed with 3 ml of 80 % ethanol and the suspension vortexed for 15 minutes. Subsequently the samples were centrifuged for 10 minutes at 1500 x g. The supernatant was transferred into micro-centrifuge-tubes and stored at -20°C until used for the enzyme immuno assay (EIA) analysis (Scheun et al. 2017).

All steroid extracts were measured for immunoreactive faecal androgen metabolite (fAM) concentrations using an EIA first described by Palme and Möstl (1993) and previously been used to reliably monitor fAM alterations in other mammals (Ganswindt et al., 2002). The EIA used and antibody against testosterone-3-CMO-BSA. Further assay characteristics, including a full description of the assay components and cross-reactivities, is provided by Palme and Möstl (1993). The EIA was biologically validated for blue wildebeest by comparing fAM concentrations from individuals at different maturation stages, with significantly higher fAM concentrations found in adult males 0.71 ± 0.33 µg/ g DW (mean ± SD)) compared to juveniles (0.46 ± 0.33 µg/ g DW) ($t_{31,20}$=2.67; P=0.01).
The assays were performed on microtiter plates according to the protocol described by Ganswindt et al. (2002). Intra- and Interassay coefficients of variation (CV), determined by repeated measurements of high and low value quality controls ranged between 5.0% - 5.1% (Intra-assay CV) and 11.1% - 15.6% (Inter-assay CV) respectively. The sensitivity of the EIA used was 18 ng/g. All faecal samples were prepared and analysed in the Endocrine Research Laboratory, Faculty of Veterinary Science, University of Pretoria. The individual fAM for

each focal animal for the 3-month recording period was pooled to obtain the overall average for the group.

5.2.10. Statistical Analysis

To compare the activity levels prior to, during, and after the rut, a Mann-Whitney test was conducted with a Bonferroni correction to determine the statistical significant differences at the $p < 0.05$ level. To compare the effects of the different factors during the periods, pre-rut, rut and post-rut, a one-way ANOVA (type III) was conducted and post-hoc (Tukey HSD) pairwise comparisons was used to determine the statistical significant differences at the $p < 0.05$ level, with animals as a random independent variable, thereby accounting for the repeated measures obtained from each animal. To determine the relationships between the ambient air temperature, inactivity and subcutaneous temperature, a multiple regressions and correlations test was conducted. The statistical analyses were conducted using IBM SPSS statistics software version 23.0 and PAST version 3.14 (Hammer et al., 2001).

5.3. Results

The current study examined how the rut affects both activity and inactivity levels and timing in the blue wildebeest. Based on three observations: (1) an increase in the amount of daily activity recorded in the neck and leg actiwatches; (2) an increase in the levels of fAM; and (3) a major increase in the daily range of subcutaneous body temperatures recorded; the blue wildebeest rut was determined to occur between the 27th of March and the 16th of April, 2016 (Figs. 5.1, 5.2). In contrast to these changes, the timing of daily activity and inactivity patterns did not vary substantially with the rut compared to the pre-rut and post-rut periods, although the amount of time spent inactive did increase substantially and significantly after the rut (Figs. 5.1, 5.2). Throughout the recording period, the average daily ambient

temperatures decreased steadily, along with the change of season, and the subcutaneous body temperature followed this pattern, although it was not as marked (Figs. 5.1, 5.2). Thus, in general we observed that during the rut, the male wildebeest experienced higher levels of activity, fAM, and a greater range of body temperatures, but this did not preclude the wildebeest from having sufficient time each day to rest during the rut, although a major increase in the time spent at rest after the rut was observed.

5.3.1. Activity levels prior to, during, and after the rut

In the pre-rut period, the average daily percentage of maximum neck activity was 54 ± 16%, while that for the leg was 45 ± 12% (Fig. 5.2). During the rut these counts increased significantly, with the daily percentage of maximum neck activity being 67 ± 16% and leg activity being 62 ± 18% during the rut period (Mann-Whitney, Bonferroni corrected, $p < 0.001$). Following the rut, in the post-rut period, the percentage of maximum neck and leg activity were both statistically significantly lower than both the rut and pre-rut (Mann-Whitney, Bonferroni corrected, $p < 0.001$), with the average daily percentage of neck activity being 45 ± 17%, while that of the leg was 37 ± 21 %. Thus, the rut was the period with the greatest overall level of activity, while the post-rut was the period with the lowest overall level of activity (Figs. 5.1A-C; 5.2A, B).

5.3.2. Timing of activity prior to, during, and after the rut

Throughout the recording period, the four male blue wildebeest showed a similar architecture regarding the timing of activity (Figs. 5.3, 5.4). From midnight to the pre-dawn period there was a steady decrease in the levels of activity, with the pre-dawn period being the time of least activity. Around sunrise a substantive matutinal peak, lasting approximately 3 hours, in activity was observed prior to a decline in activity levels throughout the day. Prior

to, and around the time of sunset, a substantive vespertine peak, lasting approximately 3 hours, in activity was observed, which was followed by a significant drop in activity levels prior to a steadily increasing level of activity until midnight. When compared across the three periods, pre-rut, rut and post-rut, specific differences in the activity patterns were noted. While the midnight to pre-dawn decline in activity was present during all three periods, the intensity of this decline was more prevalent during the rut and even more so during the post-rut period, leading to a more noticeable pre-dawn nadir. The matutinal and vespertine activity peaks did not change between the three periods, but the level of activity between these two major activity peaks was lower during the rut compared to the pre-rut period, and lower in the post-rut period compared to the rut. The post-vespertine dip in activity levels was less apparent during the rut than the pre- and post-rut periods, as was the steady increase in activity levels until midnight. Thus, while the general pattern of activity remains similar across the three periods, the relative amounts of activity at different periods during the day did change in subtle ways.

5.3.3. Inactivity prior to, during, and after the rut

The average daily time spent inactive was 1.23 ± 1.24 h for the 3-month recording period. An ANOVA on the effects of the periods on the total hours spent inactive yielded a significant difference between the periods ($F(2;6) = 42.47$, $p < 0.001$). During the pre-rut period, an average of 0.64 ± 0.68 h per day was spent inactive. The average daily time spent inactive increased slightly during the rut to 0.88 ± 0.78 h, which was a significant increase compared to pre-rut inactivity levels (Tukey's HSD, $p = 0.044$). During the post-rut period, the average daily time spent inactive increased to 2.57 ± 1.29 h, which was significantly higher than both the pre-rut period (Tukey's HSD, $p < 0.001$) and the rut (Tukey's HSD, $p < 0.001$) (Figs. 5.1C; 5.2C). Thus, the amount of time spent inactive, with much of this time

presumably being spent in sleep, did not change by much between the pre-rut and rut, but increased significantly, by 2.92 (rut) to 3.95 (pre-rut) times, in the post-rut period.

For each day, the main rest episode was identified as the longest consolidated period of inactivity recorded in a 24 h period. During the pre-rut, rut and post-rut periods the onset of the main rest episode occurred on average at 03h17 ± 1.88 h, 04h21 ± 1.42, and 04h05 ± 2.02 h, respectively, and had an average duration of 32.16 ± 14.16 min, 34.09 ± 17.21 min and 50.06 ± 32.80 min, respectively. The duration of the main rest episode was significantly different between the three periods (ANOVA, $F(2;6) = 34.53$, $p = 0.001$). Thus, in addition to resting more during the post-rut period, the average length of the main rest episode was significantly longer the post-rut period compared to the pre-rut period (Tukey's HSD, $p < 0.001$) and the rut (Tukey's HSD, $p < 0.001$) (Fig. 5.2); however, no difference in the length of the main rest episode was observed between the pre-rut period and the rut (Tukey's HSD, $p = 0.062$).

On each of the recording days multiple episodes of inactivity were observed, indicating that blue wildebeest, like most mammals, are likely to be polyphasic sleepers. Throughout the three-month recording period the average number of daily inactive episodes was 14.77 ± 14.83 During the pre-rut, rut and post-rut periods, the average number of daily inactive episodes was 7.70 ± 8.22, 10.50 ± 9.39, and 30.89 ± 15.51 respectively. An ANOVA on the effect of the periods on the number of daily inactive episodes yielded significant variation between the periods ($F(2;6) = 42.47$, $p < 0.001$). The number of daily inactive episodes was significantly different between the pre-rut period and the rut (Tukey's HSD, $p = 0.044$) as well as between the post-rut period and both the pre-rut period and the rut (Tukey's HSD, $p < 0.001$ for both instances). Thus, the amount of time spent inactive indicates the possibility of an inactivity rebound period following the sustained increased levels of activity

during the rut (see above). This increase in daily amounts of inactivity specifically marked the end of the rut (Fig. 5.1).

5.3.4. Timing of inactivity prior to, during, and after the rut

Throughout the three-month recording period, the majority of time spent inactive occurred during the night, with an average percentage of time spent inactive during the day being 16.69 ± 22.65% and 76.69 ± 30.17% during the night. During the pre-rut, rut and post-rut periods the average percentage of time spent inactive during the day was 15.59 ± 25.49%, 18.93 ± 22.83% and 16.26 ± 16.50%, respectively, and during the night it was 72.49 ± 36.49%, 77.5 ± 27.07% and 83.74 ± 16.50%, respectively. Thus, during the post-rut period, more time was spent inactive at night than in the pre-rut and rut periods.

A clear pattern in the timing of inactive episodes was observed throughout the recording period (Fig. 5.5). Between midnight and sunrise, the majority of inactive episodes were observed, but during the rut there was a distinct spike of inactivity between 04h00 and 06h00, while during the post-rut period this spike of inactivity was larger, and occurred over a longer time period, between 02h00 and 06h00. Between sunrise and sunset, the amount of inactivity was low, but there was a noticeable increase in inactivity at this time during the post-rut period. Between sunset and midnight, a number of inactive episodes occurred, but the number was far greater during the post-rut period compared to the pre-rut period and the rut (Fig. 5.5). These observations are consistent with the activity patterns described above, and the general increase in inactivity levels in the post-rut period compared to the pre-rut and rut periods. The very distinct pre-dawn spike in inactivity during the rut indicates that it is likely that all the wildebeest recorded during this period were likely to be sleeping at this time of the day. Despite these variances, the general temporal pattern of inactivity did not differ between the three periods.

5.3.5. Levels of faecal androgen metabolites prior to, during, and after the rut

The mean fAM level was 0.76 ± 0.25 µg/g DW during the pre-rut period, 1.04 ± 0.23 µg/g DW during the rut, and 0.36 ± 0.11 µg/g DW during the post-rut period (Fig 5.2F). There was a significant difference in fAM levels between the three periods (ANOVA, $F(2; 9) = 14.03$, $p = 0.002$), with significantly lower fAM levels during the post-rut period compared to the pre-rut (Tukey's HSD, $p = 0.008$) and the rut period (Tukey's HSD, $p = 0.002$) (Fig. 5.2F). Levels of fAM did not differ significantly between the pre-rut and rut period (Tukey's HSD, $p = 0.325$).

5.3.6. Average daily subcutaneous temperature prior to, during, and after the rut

The average daily subcutaneous temperature showed a steady decline over the three-month recording period, with temperatures being approximately 38.5°C at the start of the recording period and 36.5°C towards the end of the recording period, and the overall average being 37.43 ± 0.78°C (Fig. 5.1E). An ANOVA showed a significant difference between the periods for average daily subcutaneous temperature ($F(2;4) = 16.49$, $p = 0.012$). During the pre-rut, rut and post-rut periods the average daily subcutaneous temperature was 38.04 ± 0.36°C, 37.12 ± 0.52°C, and 36.64 ± 0.63°C, respectively. This drop in subcutaneous temperature was significantly different between the pre-rut and rut periods as well as between the post rut and both the pre-rut period and the rut (Tukey's HSD, $p < 0.001$ for all instances) (Fig. 5.2G).

5.3.7. Average daily variation in subcutaneous temperature prior to, during, and after the rut

Unlike the steady decline in average daily subcutaneous temperature, the average daily variation in subcutaneous temperature (the daily maximum subcutaneous temperature recorded minus the daily minimum subcutaneous temperature recorded), exhibited an increased variance during the rut and post-rut periods compared to the pre-rut period (Fig. 5.1F). There was a significant difference between the periods for the daily variation in the subcutaneous temperature (ANOVA, $F(2;4) = 16.49$, $p = 0.029$). During the pre-rut period, the average daily subcutaneous temperature range was 1.91 ± 0.82°C, and increased significantly to an average of 4.01 ± 1.35°C (Tukey's HSD, $p < 0.001$) (Fig. 5.2). During the pot-rut period the average daily subcutaneous temperature range increased to 4.49 ± 1.49°C which was significantly higher compared to both the pre-rut period (Tukey's HSD, $p < 0.001$) and the rut (Tukey's HSD, $p = 0.011$) (Fig. 5.2H). This increased variance in the subcutaneous temperature is reflected across the day, with substantial dips in subcutaneous temperature observed prior to sunrise and following sunset, as well as a greater increase in subcutaneous temperature during the afternoon (Fig. 5.6). This increased range of daily subcutaneous temperatures specifically marked the beginning of the rut (Fig. 5.1F).

5.3.8. Average daily ambient air temperature prior to, during, and after the rut

The recording period spanned the days from February 15 through to May 11, meaning that the recording period spanned the last two weeks of summer, and all but the last two weeks of autumn. As would be expected, the average daily ambient air temperature showed a steady decline during this period, although there was substantial variation (Fig. 5.1G). The average daily ambient air temperature over the three-month recording period was 21.04 ± 3.72°C. An ANOVA on the daily ambient temperature yielded significant variation among

the periods ($F(2;84) = 57.19$, $p < 0.001$). During the pre-rut period, the average daily ambient air temperature was 23.80 ± 2.45°C, which was significantly higher than the average recorded during the rut period, which was 19.77 ± 2.28°C (Tukey's HSD, $p < 0.001$). During the post-rut period, the average daily ambient air temperature dropped to 17.32 ± 2.58°C, and this average was statistically significantly lower than that recorded during both the pre-rut (Tukey's HSD, $p < 0.001$) and rut periods (Tukey's HSD, $p = 0.004$) (Fig. 5.2I).

5.3.9. Average daily ambient temperature and average daily time inactive

It is well known that seasons affect the amount of rest/sleep experienced, generally with more rest/sleep during the cooler seasons (e.g. Tobler et al., 1992; Yetish et al., 2015), and as the recordings undertaken herein covered the dates from 15 February (late summer) to 11 May (late autumn), the effect of season on daily time spent inactive needs to be addressed. Over the recording period average daily temperatures dropped from close to 24°C to around 17°C. During the post-rut period, the period with the coolest average temperatures, the amount of inactivity was the highest, but when we compare across the entire recording period (Fig. 5.7), while we do see a trend towards increasing time spent inactive on a daily basis with cooler temperatures, this is a trend that does not have a strong predictive value. Rather, it would appear that the major increase in daily inactivity during the post-rut period is driving this trend. While we cannot rule out completely that cooler temperatures play a role in the increased amount of time spent inactive over the recording session, any role would be minor in comparison to that driven by other factors such as the rut, or recovery from the extensive activity during the rut.

Figure 5.1: The average daily percentage maximum of neck (**A**) and leg (**B**) activity, the average daily time inactive (**C**), the average faecal testosterone levels (**D**), the average daily subcutaneous temperature (**E**), the average daily variation in subcutaneous temperature (**F**) of the blue wildebeest and the average daily ambient air temperature in Dinokeng Game Reserve (**G**). The graphs represent these different factors across the 3 month recording period, with each bar/point representing each day (dates provided in graph **G** and apply to all), including its standard deviation for the entire recording session. The three distinct periods within the recording session, Pre-rut, Rut and Post-rut are indicated at the top of graph **A**, with the shaded area in each graph showing the rut period specifically. Graphs **A** and **B** show the average daily percentage maximum for the neck and leg activity, respectively. Note the increase in the average neck and leg activity during the rut. Graph **C** shows the average daily time spent inactive measured in hours. Note the increase in the time spent inactive during the post-rut period. Graph **D** shows the average faecal androgen metabolite concentrations for the group of 4 blue wildebeest. Note the peak in testosterone during the rut period and the decline in the post-rut period. Graph **E** shows the average daily subcutaneous temperature. Note the steady decline of the daily subcutaneous temperature over the 3-month recoding period. Graph **F** shows the average daily variation in the subcutaneous temperature (the daily maximum subcutaneous temperature minus the daily minimum subcutaneous temperature) for the recording period. Note the increase in the variation during the rut and post-rut period. Graph **G** shows the average daily ambient air temperature of the 3-month recording period in Dinokeng Game Reserve. Note the steady decrease in temperature associated with the changing seasons.

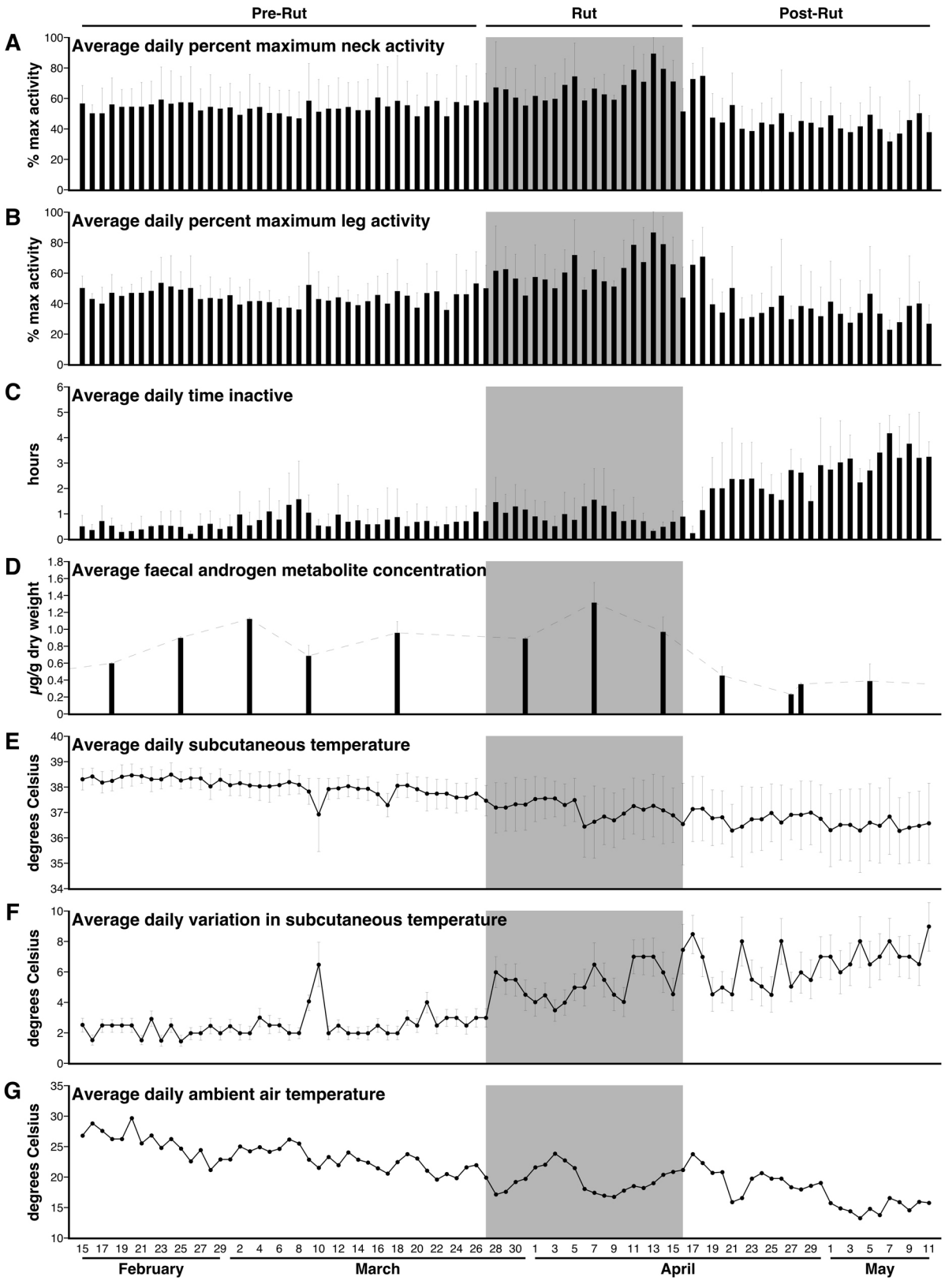

Pre-Rut
Rut
Post-Rut
A
Average daily percent maximum neck activity
% max activity
B
Average daily percent maximum leg activity
% max activity
C
Average daily time inactive
hours
D
Average faecal androgen metabolite concentration
µg/g dry weight
E
Average daily subcutaneous temperature
degrees Celsius
F
Average daily variation in subcutaneous temperature
degrees Celsius
G
Average daily ambient air temperature
degrees Celsius
15 17 19 21 23 25 27 29 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 1 3 5 7 9 11
February
March
April
May

Figure 5.2: Box and whisker plots showing the distributions of group averages of daily counts of percentage maximum neck (**A**) and leg (**B**) activity, the daily time inactive (**C**), the duration of main rest episode (**D**), the number of daily rest episodes (**E**), the faecal androgen metabolite concentration (**fAM**) (**F**), the daily subcutaneous temperature (**G**), the daily variation in subcutaneous temperature (**H**) of the blue wildebeest, and the daily ambient air temperature in Dinokeng Game Reserve (**I**) during the three periods, Pre-rut, Rut and Post-rut. * indicates which are statistically significantly different, ° indicates outliers, • indicates extreme values. Note the statistically significant increases in neck and leg activity during the rut period (**A**, **B**), the increase in inactivity in the post-rut period (**C**), the increased length of the main rest episode in the post-rut period (**D**), and the increase in the number of rest episodes in the post-rut period (**E**). Moreover, the rut is marked by an increase in the levels of faecal testosterone (**F**) and a greater range in daily subcutaneous temperature (**H**). **Av** – average, **Subcut** – subcutaneous, **temp** – temperature.

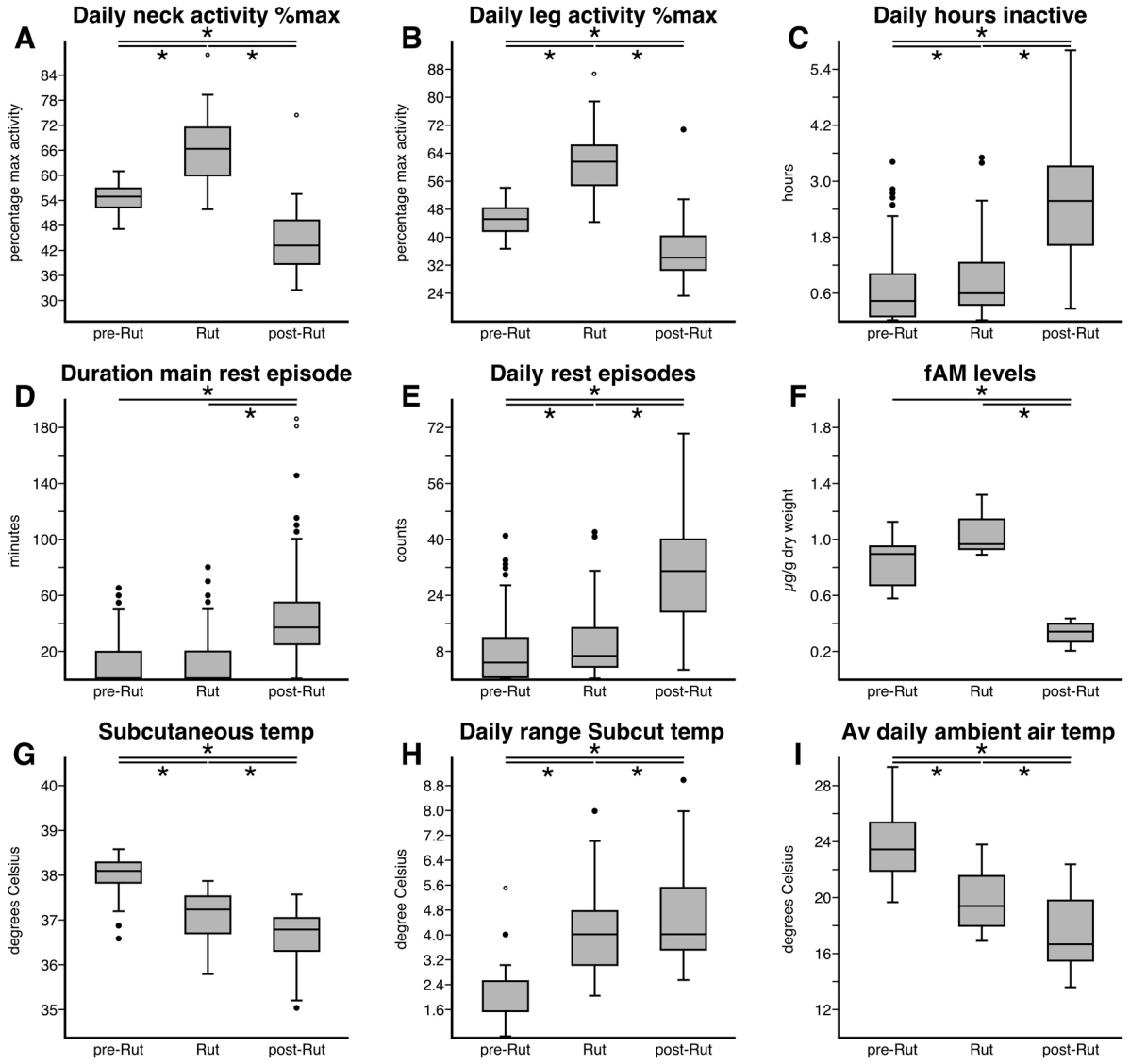

A
Daily neck activity %max
percentage max activity
84
78
72
66
60
54
48
42
36
30
pre-Rut
Rut
post-Rut
B
Daily leg activity %max
percentage max activity
88
80
72
64
56
48
40
32
24
pre-Rut
Rut
post-Rut
C
Daily hours inactive
hours
5.4
4.2
3.0
1.8
0.6
pre-Rut
Rut
post-Rut
D
Duration main rest episode
minutes
180
140
100
60
20
pre-Rut
Rut
post-Rut
E
Daily rest episodes
counts
72
56
40
24
8
pre-Rut
Rut
post-Rut
F
fAM levels
µg/g dry weight
1.8
1.4
1.0
0.6
0.2
pre-Rut
Rut
post-Rut
G
Subcutaneous temp
degrees Celsius
40
39
38
37
36
35
pre-Rut
Rut
post-Rut
H
Daily range Subcut temp
degree Celsius
8.8
8.0
7.2
6.4
5.6
4.8
4.0
3.2
2.4
1.6
pre-Rut
Rut
post-Rut
I
Av daily ambient air temp
degrees Celsius
28
24
20
16
12
pre-Rut
Rut
post-Rut

Figure 5.3: The average percentage maximum of the daily timing of neck activity for each 5 minutes over a 24 hour period. Graphs **A**, **B** and **C** show the neck average percentage maximum for the pre-rut, rut and post-rut periods, respectively. The shaded area indicates the period between sunset and sunrise. Each bar represents the 5 minute average of the full group of animals over a 21 day period (i.e. each five minutes averaged for 21 days pre-rut, 21 day during the rut, and 21 days post-rut) over the 24 hour period. Note the steady decrease in the levels of activity from midnight to pre-dawn, and the substantive matutinal and vespertine peaks. Although the general pattern of activity remains similar across the periods, the intensity of the pre-dawn decline in activity is more evident in the rut (**B**) and post-rut (**C**) periods.

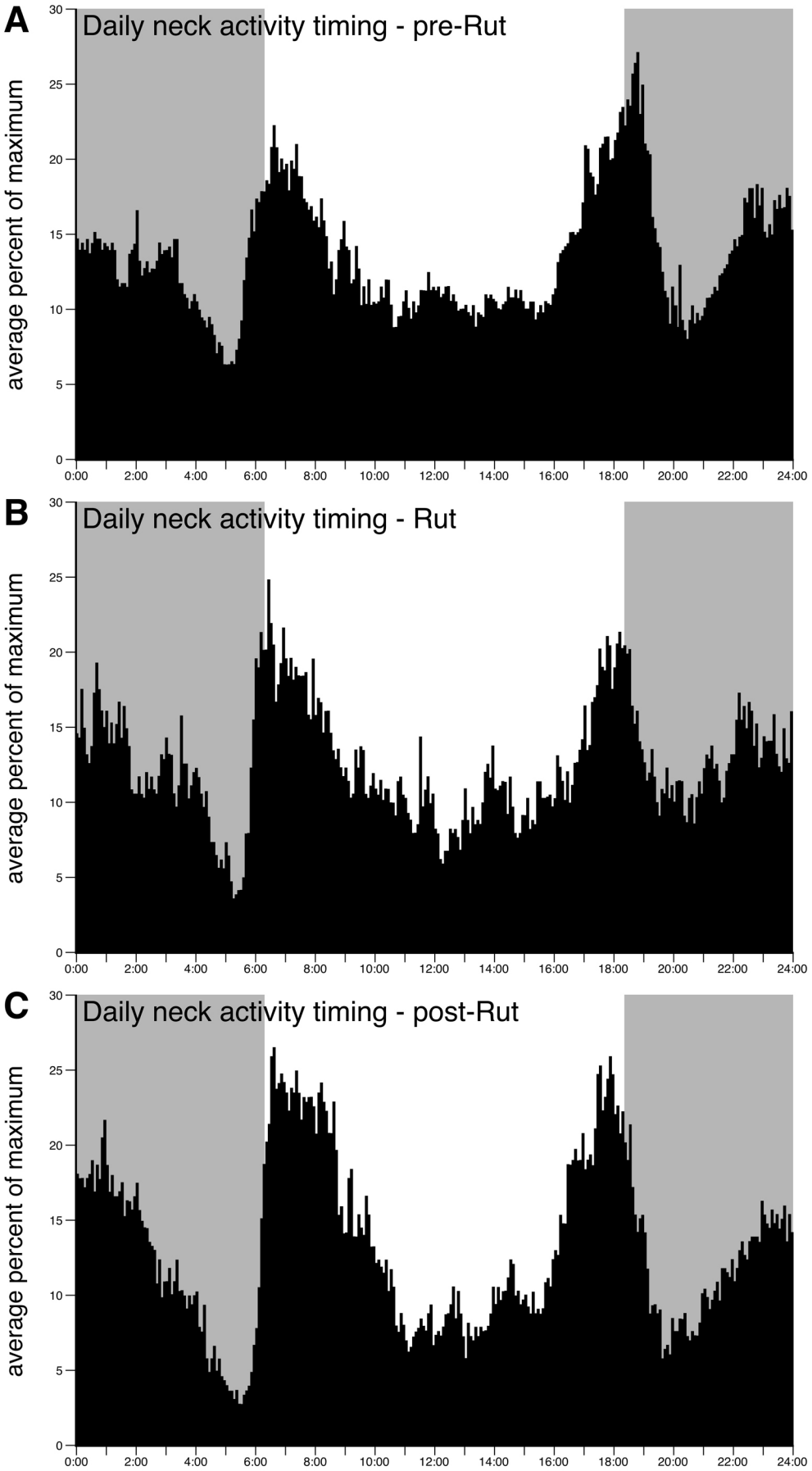

A
Daily neck activity timing - pre-Rut
average percent of maximum
B
Daily neck activity timing - Rut
average percent of maximum
C
Daily neck activity timing - post-Rut
average percent of maximum
0
5
10
15
20
25
30
0:00
2:00
4:00
6:00
8:00
10:00
12:00
14:00
16:00
18:00
20:00
22:00
24:00

Figure 5.4: The average percentage maximum of the daily timing of leg activity for each 5 minutes over a 24 hour period. Graphs **A**, **B** and **C** show the leg average percentage maximum for the pre-rut, rut and post-rut periods, respectively. The pattern of activity is essentially similar to that seen for the neck (see Fig. 5.3). Same conventions as in Figure 5.3.

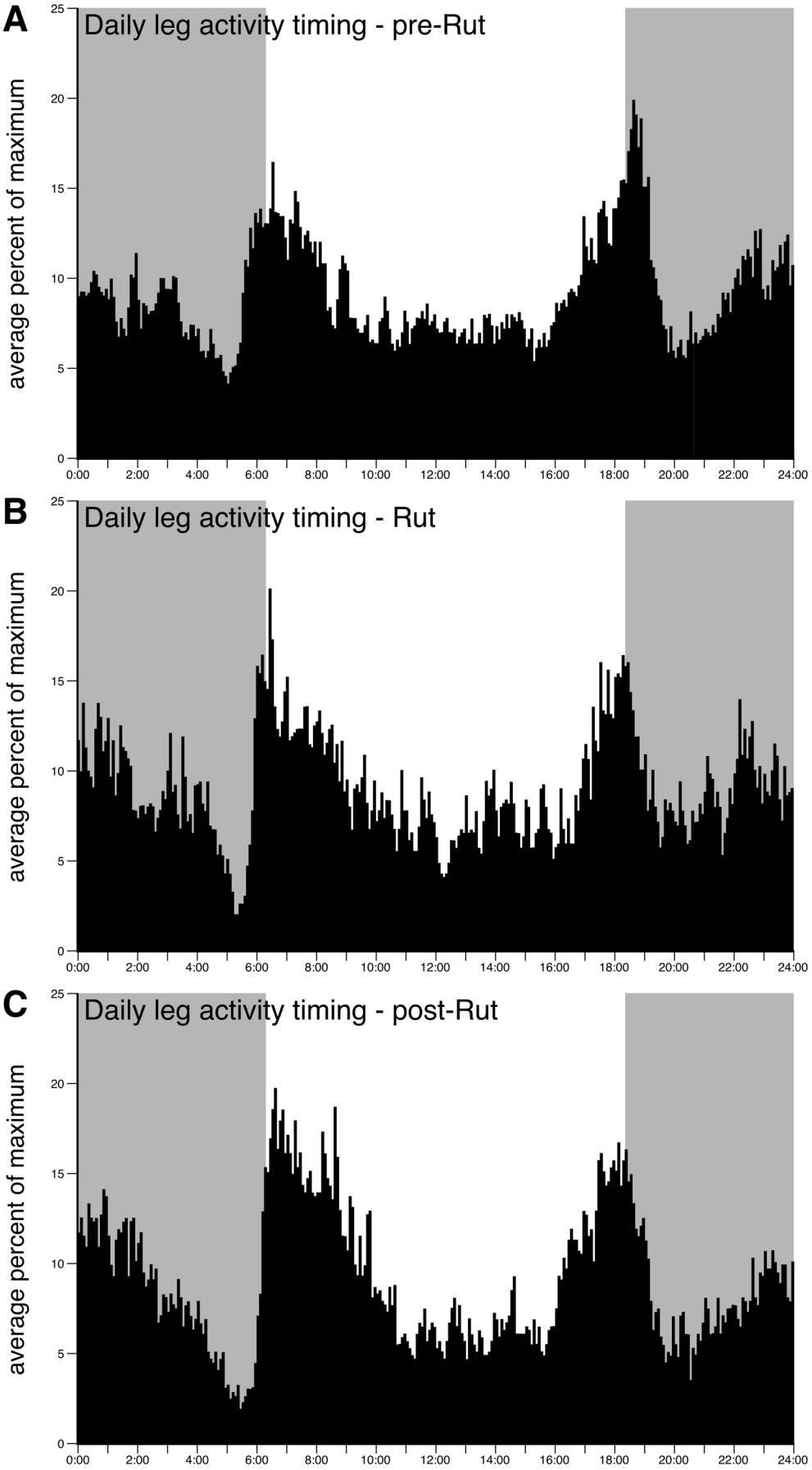

A
Daily leg activity timing - pre-Rut
average percent of maximum
B
Daily leg activity timing - Rut
average percent of maximum
C
Daily leg activity timing - post-Rut
average percent of maximum
0
5
10
15
20
25
0:00
2:00
4:00
6:00
8:00
10:00
12:00
14:00
16:00
18:00
20:00
22:00
24:00

Figure 5.5: Graphs illustrating the total counts of inactivity for any given 5 min period scored over 21 days in the pre-rut (**A**), rut (**B**) and post-rut (**C**) for all animals. The grey regions represent the period between sunset and sunrise. Note that there was a clear pattern of the timing of inactive episodes observed throughout the recording period. The majority of inactive episodes were observed between midnight and sunrise, but during the rut (**B**) there was a distinct spike of inactivity between 04:00 and 06:00, while during the post-rut (**C**) this spike of inactivity was larger, but occurred over a longer time period, between 02:00 and 06:00.

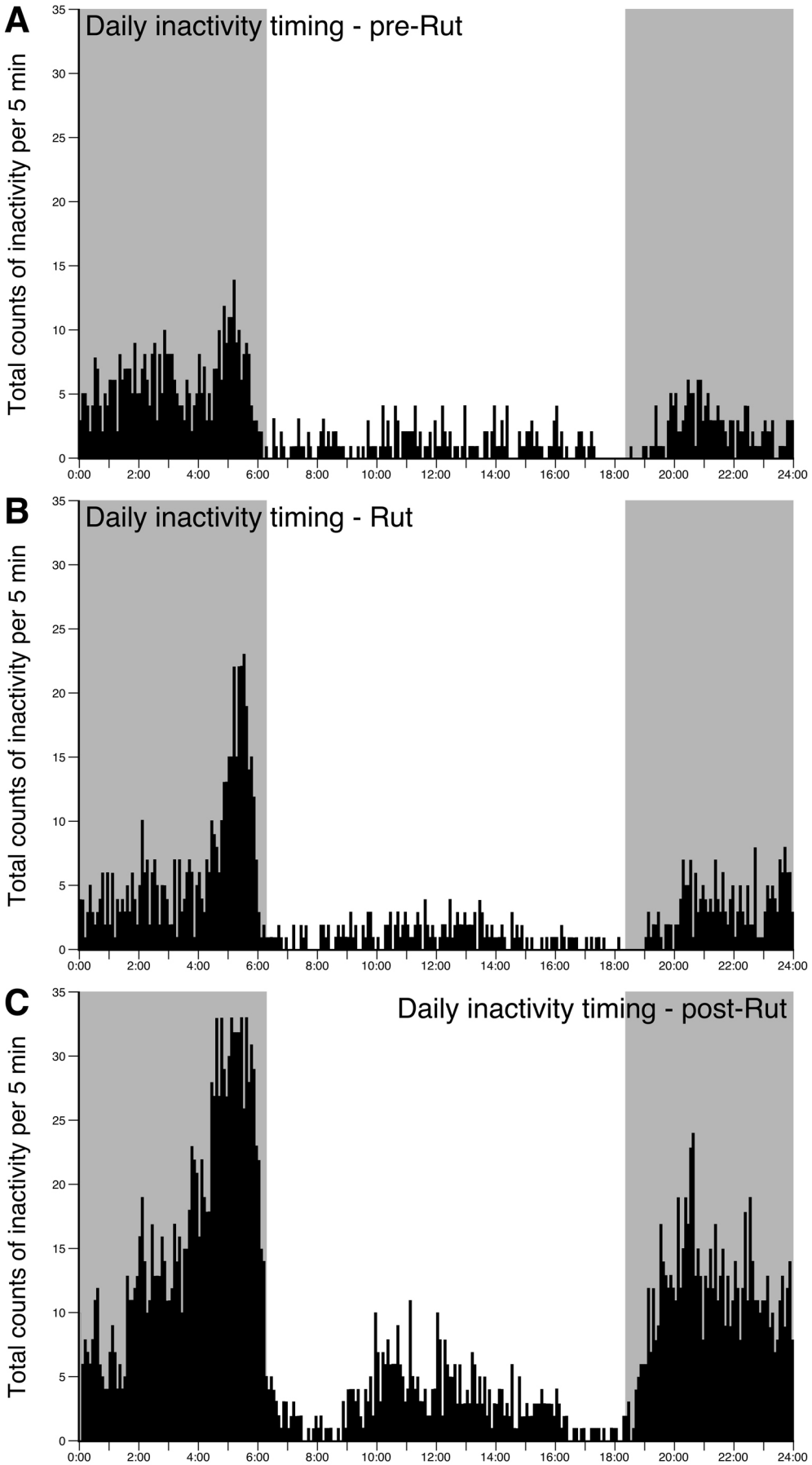

A
Daily inactivity timing - pre-Rut
Total counts of inactivity per 5 min
B
Daily inactivity timing - Rut
Total counts of inactivity per 5 min
C
Daily inactivity timing - post-Rut
Total counts of inactivity per 5 min
0
5
10
15
20
25
30
35
0:00
2:00
4:00
6:00
8:00
10:00
12:00
14:00
16:00
18:00
20:00
22:00
24:00

Figure 5.6: The daily pattern of subcutaneous temperature over a 24 hour period, each 30 minute recording averaged over all animals for 21 days in the pre-rut (**A**), rut (**B**) and post-rut (**C**). The grey regions represent the period between sunset and sunrise. Note the increased variance in the subcutaneous temperature reflected across the day during the rut (**B**) and post-rut (**C**) periods, with substantial dips in subcutaneous temperature observed around sunrise and sunset.

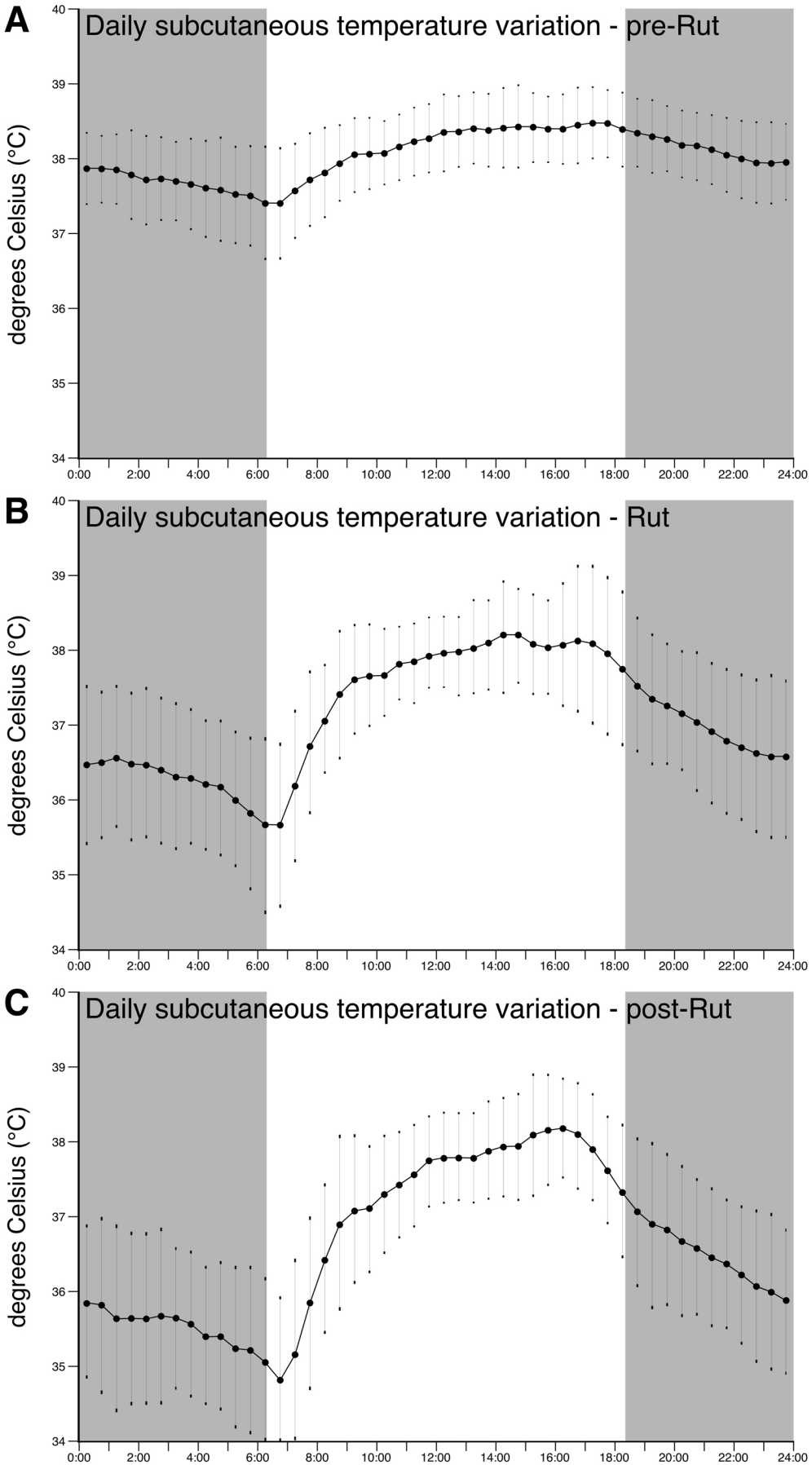
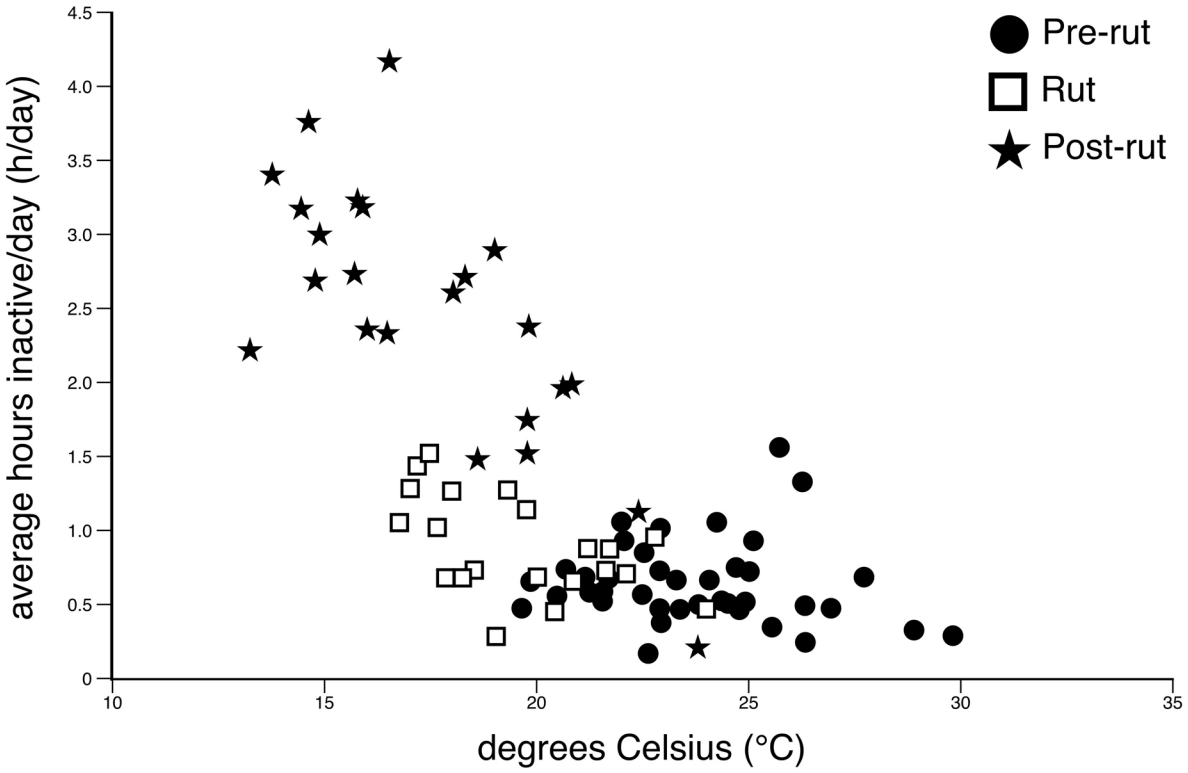

A
Daily subcutaneous temperature variation - pre-Rut
B
Daily subcutaneous temperature variation - Rut
C
Daily subcutaneous temperature variation - post-Rut
degrees Celsius (°C)
40
39
38
37
36
35
34
0:00
2:00
4:00
6:00
8:00
10:00
12:00
14:00
16:00
18:00
20:00
22:00
24:00

Figure 5.7: Graph showing the lack of a clear relationship between average ambient air temperature (°C) and the average hours of inactivity per day for the four male wildebeest recorded during the pre-rut (filled circles), rut (open squares) and post-rut (filled stars). The recording period covered the dates from 15 February (late summer) to 11 May (late autumn), with average daily temperatures dropping from close to 30°C to around 13°C. While there is clearly a trend for increased time spent inactive with the cooler weather, this trend is not strongly predictive.


Pre-rut
Rut
Post-rut
average hours inactive/day (h/day)
degrees Celsius (°C)
0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
10
15
20
25
30
35

5.4. Discussion

In the current study we used actigraphy to explore the assertion that during the rut, or breeding season, when a blue wildebeest bull is maintaining a harem in his territory, he neither eats nor rests (Estes, 1997). The concept that the male blue wildebeest will not rest during the rut indicates that it is unlikely to spend any time asleep, despite sleep being one of the biological imperatives of mammalian biology (Mignot, 2008). Our findings indicate that during the rut, the male blue wildebeest, while having significantly higher activity levels than either before or after the rut, do not increase the daily amount of time spent active at the expense of time spent resting (potentially asleep). Following the rut there appears to be a significant increase in the amount of time spent resting, which may be seen as a period where the male wildebeest recuperate following the high activity levels during the rut.

5.4.1. Defining the rut

The wildebeest rut is a notoriously difficult period to *a priori* define accurately, with a variable beginning and ending that is related to changes in environmental conditions (Skinner and Chimimba, 2005). In the present study, instead of attempting to define the period of the rut prior to undertaking the study, we decided to record various parameters over an extended period, allowing us to *a posteriori* define the rut period with greater precision. As with other mammalian species that exhibit a rut period (Geist, 1965; Geist and Walther, 1974; Poole 1987; Balmford et al., 1993; Carranza et al., 1996; Whittle et al., 2000; Mysterud et al., 2002), related androgen levels of the male wildebeest under study increased (by approximately 1.4 times) during the rut period compared to the pre-rut period, and decreased substantially after the rut (to almost a third of the level observed during the rut). The beginning of the rut was marked by three additional specific measures taken in the current study, these being significant increases in the average daily levels of activity recorded by

both the neck and leg actiwatches, and a significant increase in the daily variation of subcutaneous temperature. The end of the rut was also defined by three additional specific measures, these being significant decreases in the average daily levels of activity recorded by both the neck and leg actiwatches, and by a significant increase in the amount of inactivity recorded. By combining the results for the faecal androgen metabolite levels, daily activity levels, daily inactivity levels, and daily subcutaneous temperature variations, we were able to reliably define a period of three weeks, from the 27th of March to the 16th of April, as the 2016 rut period for the four male blue wildebeest studied. During this period, the male blue wildebeest under study also displayed typical rut behaviour such as increased calling, foaming at the mouth and multiple attempts to mount receptive cows (Estes, 2014). Thus, our *a posteriori* definition of the rut appears to be supported by several independent measurements, and provides confidence in the comparisons made between the pre-rut, rut and post-rut periods made herein.

5.4.2. Activity levels before, during and after the rut

Our observations indicate that the rut period was clearly the most active time throughout the recording period. Overall activity levels during the rut were 1.2 – 1.4 times higher than during the pre-rut period, and were 1.5 – 1.7 times higher than the post-rut period. Thus, in agreement with qualitative observations made regarding activity levels of the male wildebeest during the rut (Estes, 1997), we find that quantitatively the 21-day rut period is the most active time for the male wildebeest. This appears to support the concept that that male wildebeest are protecting a harem within a territory (Estes, 1997), and that due to the possibility of intruders, the activity levels are consistently high during this time in order to prevent loss of the harem and breeding rights. Interestingly, during the 21 days following the rut, which we term the post-rut period, the activity levels were substantially lower than both

the rut and pre-rut periods. This indicates that post-rut, the male wildebeest likely need substantially more rest for recovery after the period of intense activity during the rut.

5.4.3. Inactivity levels before, during and after the rut

One of the central aims of the current study was to test whether, during the rut, the male wildebeest did not rest at all, as indicated by Estes (1997). In the current study we used a very strict definition of rest, this being that both the neck and leg activity meters, combined, had to show modal count of no activity (i.e. an activity score of zero) for a period of five minutes. This strict definition of rest, indicating no major or consistent movement of the animals for a five-minute period, likely means we are underscoring rest as a whole, but does allow us to be confident in our quantitative conclusions regarding variations in the amount of rest. Contrary to what is qualitatively reported by Estes (1997), we find that during the pre-rut, rut and post-rut, rest occurred on a daily basis. The amount of daily rest obtained by the male wildebeest in the pre-rut and rut was similar, and with our strict scoring criteria for inactivity, amounted to 40 – 50 min of daily inactivity. Thus, even during the rut, the blue wildebeest appeared to rest for the same amount of time each day as during the pre-rut period. The conclusion derived by Estes (1997) appears to be understandable from the perspective that this daily rest during the rut occurred mostly between 4 to 6 am, a time when observations of male wildebeest activity or inactivity are unlikely to have been made with any consistency.

Interestingly, in the post-rut period, the amount of daily rest increased substantially compared to the pre-rut and rut periods. We observed that the amount of rest in the post-rut period increased to approximately 2.5 h per day, which is around 3 –4 times greater than that observed daily in the pre-rut and rut periods. This finding mirrors the lowered amount of activity observed in the post-rut period compared to the rut, and indicates that the post-rut

period appears to represent a time of the year when the male blue wildebeest are undergoing a sustained period of increased rest in response to the sustained period of increased activity leading up to and during the rut. The emphasis placed on this substantial increase in rest in the post-rut period is possibly tempered by the reduced photoperiod as well as cooler weather conditions observed during this time. These changes in the weather conditions may play a role in the increased amount of rest observed in the post-rut period; however, it is unlikely that weather conditions alone account for the major increase in daily rest time observed in the post-rut period and that indeed much of this increase in daily rest can be attributed to a period of prolonged recovery after a period of intense activity.

5.4.5. Sleep and the rut

One of the central biological imperatives of mammalian existence is the need to undergo the physiological and behavioural process of sleep on a daily basis (Campbell and Tobler, 1984; Tobler, 1995; Siegel, 2005). Lack of sleep can lead to a range of neural and behavioural complications (see review by Boonstra et al., 2007) that can compromise survival. Thus, the indication by Estes (1997) that the male wildebeest do not rest during the rut also indicates that they do not enter the physiological and behavioural process of sleep during the rut. As outlined above, it is clear that the male wildebeest do rest, for around 50 min per day, during the rut, and it is likely that much of this time, if not more given the strict scoring criteria applied to rest in the current study, will be spent in sleep. Thus, despite the overwhelming need to protect a territory, harem and breeding rights during the rut, the male wildebeest appear to obtain sleep on a daily basis. This finding complements previous findings in birds, where during breeding season total sleep time is reduced significantly for male pectoral sandpipers (Lesku et al., 2012), with a similar observation being made in great frigatebirds during long flights (Rattenborg et al., 2016), but both species do sleep, despite

being under intense pressure not to sleep. Interestingly, in the male pectoral sandpipers, the reduced amount of sleep may be compensated for by a greater sleep intensity, allowing for the shortened daily sleep times (Lesku et al., 2012), and this may also apply to the male blue wildebeest. However, in order to determine whether this is the case in the wildebeest, polysomnographic recordings would be required during the pre-rut, rut and post-rut, and this is presently beyond our technical capacities. In addition, to make categorical statements regarding sleep in the blue wildebeest using actigraphy, the scoring of the actigraphy data needs to be correlated with polysomnographic measures of sleep in free-roaming wildebeest, which may then allow long-term investigation of sleep over different periods of the year in the blue wildebeest.

5.4.6. Summary

The present study demonstrates that a variety of measures indicate that the male blue wildebeest do enter a specific and consolidated rut period that lasts for approximately 21 days. Despite previous qualitative reports (Estes, 1997), the male blue wildebeest do rest on a daily basis during the rut, and while the amount of rest may be low, it does not appear to be substantially lower than in the period prior to the rut. It appears that in the post-rut period a substantive increase in rest occurs, potentially allowing the male wildebeest time to recover following a period of intense activity. The blue wildebeest appears to form an exploded lek system for mating, and thus may represent only a portion of the spectrum of variation in rest and sleep across Artiodactyls that show this system of mating. Other Artiodactyls, such as the Uganda kob (*Adenota kob thomasi*), which form an intense and localized classical lek system (Buechner and Roth, 1974), may show reduced or absent rest during the breeding season. It would be of interest to use actigraphy to test these potential variations in the amount of daily rest obtained in lek systems of varying intensity and size to determine whether a true

spectrum exists, or whether at a certain level of intensity related to different lek systems the biological imperative of sleep is delayed for a specific period of time and the effect this may have on survival.

CHAPTER SIX: **Summary and Final Discussion**

In the present thesis, the global aim was to investigate sleep in blue wildebeest from a neuroanatomical, physiological and behavioural point of view. Furthermore, it also set out to understand how this species sleeps in its natural environment and the effects that natural behaviours can have on sleep, specifically investigating the influence of the rut on sleep/rest phasing and architecture in dominant breeding males of this species. Neuroanatomically, the somnogenic systems of the blue wildebeest showed a corresponding nuclear organization to that reported in other mammals and more specifically the Cetartiodactyla. The blue wildebeest were found to be primarily diurnal, polyphasic sleepers that have an average of 4.53 hours of sleep per night. It was also observed that there were no changes to the sleep phasing and architecture of the blue wildebeest during the rut, there were however changes to the total time spent in a state of inactivity. The studies presented in this thesis form the baseline for further studies of the influence of the environment and natural behaviours on sleep in the blue wildebeest. More broadly, these studies establish a framework from which studies of sleep not only the blue wildebeest, but in many other large mammals in their natural environment, can be established and compared in a full quantitative manner.

6.1. Neurobiology of sleep

The sleep-wake related neuronal systems have demonstrated some evolutionary trends, and while these systems are quite similar across species for the most part, differences do occur (Manger, 2005). These differences, for the most part, appear to be order-specific variations (Manger, 2005; Maseko et al., 2007; Dell et al., 2010; Calvey et al., 2013; Kruger et al., 2015), although as more species are examined, certain species-specific variations are beginning to be noted. To the author's knowledge, the study presented in this thesis (Chapter 3) is the first study of the sleep-wake related nuclei in the brain of the blue wildebeest,

broadening the basis for comparison across the Cetartidactyls (Dell et al., 2016a,b,c). One of the findings of interest in Cetartidoactyls species that have been studied relates to the relative somal size of the cholinergic neurons in the LDT and PPT. The blue wildebeest, together with the Goettingen mini pig and Arabian Oryx, exhibited larger cholinergic neurons in the PPT than in the LDT (Ettrup et al., 2010; Mahady et al., 2017; Davimes et al., 2017). This differed from the cetaceans where there was no distinguishable difference in somal size between the cholinergic neurons of the LDT and PPT (Dell et al., 2016 a,b). The cholinergic neurons in the LDT of the giraffe (Bux et al., 2010) and the river hippopotamus (Dell et al., 2016c) were found to be larger than the cholinergic neurons in the PPT. The cholinergic neurons in the LDT and PPT are reported to predominantly begin firing just before REM sleep or wake, suggesting that they may help promote transitions from NREM to REM sleep (van Dort et al., 2015; Scammell et al., 2017). Could the somal size of the cholinergic neurons play a role in how rapidly a species transitions from one state to the next? Cetaceans do not present with REM sleep (Lyamin et al., 2008), and they exhibit no distinguishable difference in the cholinergic neurons in the LDT and PPT, which may support this idea. Alternatively, the variation in relative size between the LDT and PPT neurons may relate to size of the Artiodactyl forebrain (cetaceans being an exception here), as those species with larger brains (giraffe and river hippopotamus) have larger LDT neurons than those with smaller brains (pig, blue wildebeest and Arabian oryx). With further investigations into Artiodactyl species of differing brain sizes, an allometric relationship between brain size and/or body size and the relative size of these cholinergic neurons may be revealed.

Recent studies have also examined the non-cholinergic neurons of the PPT and LDT (Boucetta et al., 2014; Cox et al., 2016; Scammell et al., 2017). Most glutaminergic neurons in this region begin firing just before and continue during REM sleep and many also fire during wake (Boucetta et al., 2014; Cox et al., 2016; Scammell et al., 2017). The GABA-

ergic neurons in the LDT and PPT seem mainly Wake active, but some may be more active during REM sleep than in non-REM sleep (Boucetta et al., 2014; Cox et al., 2016; Scammell et al., 2017). Thus, distinguishing how these different neuronal populations work together to regulate REM sleep could prove to be an important goal for future research. These observations need to be correlated with polysomnographic studies of sleep in the various species examined to determine what role this variance in pontine cholinergic relative cell size may play, especially in the expression of REM sleep and the transitioning from one state to the next across species (van Dort et al., 2015; Scammell et al., 2017).

The other feature of the neural systems involved in sleep regulation that was observed to be unusual in the blue wildebeest is the presence of the parvocellular orexinergic neurons in the medial hypothalamus, a feature observed in other Cetartiodactyls (Dell et al., 2012, 2016a,b,c; Davimes et al., 2017), the African elephant (Maseko et al., 2013), and in unpublished studies from the laboratory of the Equid brain (plains zebra, mountain zebra, domestic horse and domestic donkey, Manger, pers. comm.). In contrast, the more laterally placed orexinergic neurons, those classified as magnocellular, show a similar organization to that seen in other mammals (Dell et al., 2013; Calvey et al., 2013, 2016). Thus, this novel group of medially located orexinergic neurons appears to be found in herbivores that require a large daily food intake of a low calorie feed, and that also appear to sleep substantially less than species with high calorie feed (Siegel, 2005). The cetaceans are unusual in this respect, as they have the parvocellular orexinergic neurons (Dell et al., 2012, 2016b) but have a high calorie diet. Is the presence of parvocellular orexinergic neurons in the cetaceans due their phylogenetic relationship to Artiodactyls, inheriting this trait from the ancestral Artiodactyls from which they evolved?

To date no studies of the function of these parvocellular orexinergic neurons have been undertaken, but for the magnocellular orexinergic neurons it has been shown that they

are important for maintaining long periods of wake (Shiraishi et al., 2000; Kanda et al., 2016; Scammell et al., 2017; Yamashita et al., 2017). Animals with disrupted orexin signalling have a very similar phenotype – chronic often severe sleepiness and dysregulated REM sleep (Shiraishi et al., 2000; Kanda et al., 2016; Scammell et al., 2017; Yamashita et al., 2017). These animals are also shown to have much shorter wake bouts than usual and sudden transitions from wake into REM sleep-like states (Shiraishi et al., 2000; Kanda et al., 2016; Scammell et al., 2017; Yamashita et al., 2017). The orexin neurons are scattered across the lateral hypothalamic area (LHA), and these cells project to all the wake-promoting brain regions of the brain (Scammell et al., 2017; Yamashita et al., 2017). The LHA and the ventromedial hypothalamic nucleus (VMH) have been implicated in ingestive behaviour, energy balance and body mass regulation (Shiraishi et al., 2000; Stuber and Wise, 2016; Scammell et al., 2017; Yamashita et al., 2017). The LHA is more closely associated with the initiation of eating, whereas the VMH mediates the cessation of eating (Shiraishi et al., 2000; Stuber and Wise, 2016; Scammell et al., 2017; Yamashita et al., 2017). Electrolytic lesions of the LHA supresses feeding and drinking while lesioning the VMH promotes feeding and body weight gain (Shiraishi et al., 2000; Stuber and Wise, 2016; Scammell et al., 2017; Yamashita et al., 2017). Since the orexin system is selectively activated by signals indicating severe nutritional depletion, it would be highly adaptive for a hungry animal not only to seek sustenance, but also to remain fully alert (Rodgers et al., 2002).

Given that all the species in which the parvocellular orexinergic neurons have been found require large volumes of food (including both the herbivorous artiodactyls and elephants and the carnivorous cetaceans), and tend to sleep less than similar sized omnivorous or carnivorous mammals (Siegel, 2005), it is possible that this parvocellular orexinergic cluster of neurons is involved in driving both increased food intake and increased time spent awake in order to allow time to ingest the increased amount of food required. This

concept could be tested with the use of single unit neural recordings of the parvocellular orexinergic neurons (for example in domesticated species such as sheep or goats) while these animals undergo controlled fasting, potentially giving us insight to the role of these parvocellular orexinergic neurons. The neuroanatomy of the sleep systems provides insights into the function of sleep, by relating differences and similarities in sleep to the anatomical organization of the sleep systems.

6.2. Sleep in the wild

A recent trend in the study of sleep and sleep evolution is to study sleep behaviour and neurophysiology in more naturalistic environments compared to the traditional controlled laboratory settings. In Chapter Four of this thesis sleep in the blue wildebeest was examined in a naturalistic setting by means of two different measures of sleep, polysomnography (PSG) and actigraphy (ACT). The results showed that the standard Wake, non-REM and REM sleep states observed in most animals studied (Tobler, 1995), were also observed in the blue wildebeest. PSG showed daily total sleep time (TST, non-REM plus REM sleep time) for the blue wildebeest for a 24-hour period averaged approximately 4.5 hours, whereas ACT showed that the blue wildebeest spent on average approximately 4.8 hours Inactive each day. While there are numerous studies that have validated the ability of ACT to discriminate between sleep and wake as defined by PSG criteria in humans, to the author's knowledge this is the first attempt at validation of ACT to measure sleep in a large free-ranging mammal. The concordance between ACT and PSG revealed a fair agreement of 30%, but differences were note in the different animals and on different days. In addition, on average the ACT method overscored TST by approximately 15 min/day. In general, ACT tends to overestimate TST because ACT is prone to misinterpreting quiet wakefulness as sleep (Paquet et al., 2007; Shambroom et al., 2010). In humans, it appears that ACT also tends to be less accurate in

populations showing fragmented sleep compared to healthy subjects (Paquet et al., 2007). Studies on insomniac populations have shown ACT to overestimate TST (from 14 to 60 mins) compared to PSG (Paquet et al., 2007), which is comparable to what was observed in the polyphasic blue wildebeest. It should be noted that there are various devices used for actigraphy, most modelled for the use in humans. Accelerometers, specifically tri-axial accelerometers, have been used to study a wide range of behavioural and physiological aspects of animals in their natural habitat, including foraging, reproduction, activity, energy budgets, and locomotion (Brown et al., 2013; Wang et al, 2015). The advantage of using tri-axial accelerometers are that one is able to identify the animals given movements over space and time and the ability to measure movement behaviour of free-roaming animals over biologically and ecologically significant evens and periods (Brown et al., 2013; Wang et al., 2015). It appears likely that with further studies and increased stringency on the analysis of the ACT recordings, that ACT could be used successfully to determine what happens to total sleep times and sleep architecture in large, free-roaming mammals for extended periods of time (although these recordings could not determine what happens to specific sleep states, such as non-REM and REM). Ideally these ACT recordings will be deployed with other sensors such as location recordings (GPS) and physiological recordings (e.g. heart rate, body temperature). Despite the potential concerns with using ACT to model sleep in large free-roaming mammals, the current study is encouraging, in that it indicates that with further studies this minimally invasive method of sleep recording could be employed to provide very useful and accurate information regarding the total sleep time and sleep phasing in the natural environment. One such example, elaborated on below, was the use of ACT to examine sleep in male wildebeest during the mating season (or rut).

6.3. Factors influencing sleep

Animals in the wild are faced with numerous biological and ecological demands for survival, such as foraging for food, mating strategies and defending themselves against competitors and predators. Do these selective pressures influence the amount of time that individuals can devote to sleep? In Chapter Five of the thesis, it was examined whether the rut of the blue wildebeest had an impact on the total sleep time and sleep architecture, as it had been previously reported that the male blue wildebeest do not rest at all during the rut (Estes, 1969). The results indicated that during the rut, the male blue wildebeest, while having significantly higher activity levels than either before or after the rut, did not increase the daily amount of time spent active at the expense of time spent resting (potentially asleep). Following the rut there appeared to be a significant increase in the amount of time spent resting, which may be seen as a period where the male wildebeest recuperate following the high activity levels during the rut. In addition, the timing of daily activity and inactivity patterns did not vary substantially with the rut compared to the pre-rut and post-rut periods. The blue wildebeest appears to form an exploded lek system for mating, and thus may represent only a portion of the spectrum of variation in rest and sleep across Artiodactyls that show this system of mating. Other Artiodactyls, such as the Ugandan kob (*Kobus kob thomasi*), which form an intense and localized classical lek system (Buechner and Roth, 1974), may show reduced or absent rest during the breeding season. It would be of interest to use combine the use of polysomnography to validate actigraphy in a semi-controlled naturalistic setting, and then to test potential variations in the amount of daily rest obtained in lek systems of varying intensity and size to determine whether a true spectrum in the amount of sleep experienced by males during the rut exists, or whether at a certain level of intensity related to different lek systems the biological imperative of sleep is delayed for a specific period of time and the effect this may have on survival. This type of study would form the

mammalian equivalent to studies of this sort previously undertaken in avian species, where male sandpipers were observed to substantially reduce the amount of daily sleep during the mating season (Lesku et al., 2012).

As mentioned, there are multiple potential factors that could directly effect total sleep time and sleep phasing in the natural environment of mammals. The use of validated ACT methodology to study sleep times and phasing in large mammals could lead to significant advances in our understanding of sleep in free-roaming large mammals (e.g. Gravett et al., 2017), and even in humans leading a pre-industrial lifestyle (e.g. Yetish et al., 2015). The effect of various features of the environment on sleep, such as risk of predation, conspecific and interspecific densities (for competition to resources), migrations, and seasonal and short term meteorological variations, could all be accurately assessed with validated ACT. The data obtained could dramatically improve our understanding of what could be considered natural sleep, contributing to the field of "sleep ecophysiology" (Aulsebrook et al., 2016).

6.4. Future directions

The studies presented in this thesis have shown that the neural systems involved in the production and regulation of sleep, as well as the physiological expression of sleep in terms of states expressed, total sleep times and sleep phasing, in the blue wildebeest are what might have been predicted for an Artiodactyl of its body mass. In addition, the concordance between ACT and PSG methods have opened up the possibility of studying the effect of variations in environmental conditions on sleep in the blue wildebeest, with the study of the rut being an example of research areas of interest that could be explored. These studies have established a methdological and ideological baseline from which future studies of sleep in the wild could be developed.

In terms of the blue wildebeest in particular, while the studies presented here have contributed to our understanding of sleep in this species, there are many more questions regarding sleep in the blue wildebeest that could be explored. The concordance of ACT with PSG needs to be explored further to develop truly representative algorithms of the relationship between ACT and PSG, with the aim to obtain concordance values between the two measures that allow for accurate estimations of sleep times and phases when only using ACT. However, once this is complete, then sleep in a range of envrionmental conditions in which the wild blue wildebeest occur could be investigated. Are there differences between the adult males and females of the species in sleep? Could sleep during ontogeny in the blue wildebeest be accurately measured? How does sleep in blue wildebeest vary with predation risk? How does female blue wildebeest sleep vary during the breeding season, gestation, birth (peri-natal period) and in the early post-natal period? What effects do seasonal variations and environmental variations in temperature, rain, humidity and solar radiation have on sleep? Blue wildebeest form the numerical majority of animals that undertake the great migration in the Serengeti, thus ACT may be able to inform us regarding sleep during migration. Thus, the baseline formed in the current study has opened up many potential avenues for the further study of sleep in the blue wildebeest.

In addition to the blue wildebeest, the current series of studies does pave the way for examining sleep across a range of mammalian species, especially the more readily accessible herbivores. In all species the data generated in the current studies and the questions outlined above for further study in the blue wildebeest could be applied to all large ungulates. It would appear that the concordance of ACT with PSG is likely to be a species specific concordance, indicating that in each species targetted for study, the baseline studies presented herein for the blue wildebeest would need to be undertaken, prior to the ecologically relevant questions regarding sleep being investigated in detail. These types of studies would assist in developing

our understanding of mammalian sleep in the natural habitat, as well as provide data relevant to understanding the evolution of sleep in mammals. Thus, as with any series of scientific studies, while some answers to specific questions have been obtained, further questions arise.

As a final consideration, the study of sleep in the blue wildebeest raised the interesting question of the effect of domestication on REM sleep in mammals. It appears that in the domesticated animals, potentially including humans, that REM sleep occupies a far greater proportion of the total sleep time than that observed in phylogenetically related non-domesticated species. It would be of specific interest to expand this question to various wild and domesticated animals, for example the domestic dog compared to the wolf, the zebra compared to the domestic horse, domestic cats compared to wild cats, and humans living an industrialized lifestyle compared to those living a traditional non-industrialized lifestyle. Studying sleep in feral mammals may also be of interest in this regard. The various questions raised by the studies presented herein, underscore the importance of comparative sleep research and what we can learn about sleep, its evolution and eventually its function from an approach that examines sleep in its natural setting.

REFERENCES

Acerbi A, and Nunn CL. Predation and the phasing of sleep: an evolutionary individual-based model. Anim Behav 2011; 81: 801-811.

Agnarsson I, and May-Collado LJ. The phylogeny of Cetartiodactyla: The importance of dense taxon sampling, missing data, and the remarkable promise of cytochrome b to provide reliable species-level phylogenies. Mol Phylogenet Evol 2008; 48: 964–985.

Agnvall B, Belteky J, Katajamaa R, Jensen P. Is evolution of domestication driven by tameness? A selective review with focus on chickens. App Anim Behav Sci 2017: in press. doi: 10.1016/j.applanim.2017.09.006.

Allison T, and Cicchetti PV. Sleep in mammals: Ecological and constitutional correlates. Science 1976; 194: 732-734.

Allison T, and Van Twyver H. The evolution of sleep. Nat Hist 1972; 79: 56–65.

Ancoli-Isreal S, Cole R, Alessi C, Chambers M, Moorcroft W, Pollak CP. The role of actigraphy in the study of sleep and circadian rhythms. Sleep 2003; 26: 342-392.

Andersson MB. Sexual selection. Princeton University Press, Princeton, New Jersey, 1994; pages 143 – 182.

Aulsebrook AE, Jones TM, Rattenborg NC, Roth TC, Lesku JA. Sleep ecophysiology: intergrating neuroscience and ecology. Trends Ecol Evol 2016; 31: 590-599.

Ball NJ. The phasing of sleep in animals. In: Stampi C, ed. Why We Nap. Evolution, Chronobiology and the Function of Polyphasic and Ultrashort Sleep. Boston: Birkhauser, 1992; pages: 31-49.

Balmford A, Bartoš L, Brotherton P, Herrmann H, Lancingerova J, Mika J, Zeeb U. When to stop lekking: density-related variation in the rutting behaviour of sika deer. J Zool 1993; 231: 652-656.

Balzamo E, Bradley RJ, Rhodes JM. Sleep ontogeny in the chimpanzee: from two months to forty-one months. Electroenceph Clin Neurophysiol 1972; 33: 47-60.

Barton RA, and Capellini I. Sleep, evolution and brains. Brain Behav Evol 2016; 87: 65-68.

Beletsky LD, and Orians GH. Site fidelity and territorial movements of males in a rapidly declining population of yellow-headed blackbirds. Behav Ecol Sociobiol 1994; 34: 257-265.

Bell FR, and Itabisashi T. The electroencephalogram of sheep and goats with special reference to rumination. Physiol Behav 1973; 11: 503-514.

Berry HH. Aspects of wildebeest *Connochaetes taurinus* ecology in the Etosha National Park - a synthesis for future management. Madoqua 1997; 20: 137-148.

Berry RB, Brooks R, Gamaldo CE, Harding SM, Lloyd RM, Marcus CL and Vaughn BV for the American Academy of Sleep Medicine. The AASM Manual for the Scoring of Sleep and Associated Events: Rules, Terminology and Technical Specifications, Version 2.2. www.aasmnet.org. Darien, Illinois: American Academy of Sleep Medicine, 2015.

Bhagwandin A, Fuxe K, Bennett NC, Manger PR. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brains of two species of African mole-rat. J Chem Neuroanat 2008; 35: 371–387.

Bhagwandin A, Gravett N, Bennett NC, Manger PR. Distribution of parvalbumin, calbindin and calretinin containing neurons and terminal networks in relation to sleep associated nuclei in the brain of the giant Zambian mole-rat (*Fukomys mechowii*). J Chem Neuroanat 2013; 52: 69-79.

Blumberg MS, and Rattenborg NC. Decomposing the evolution of sleep: Comparative and developmental approaches. In: Kaas J, ed., Evolution of Nervous systems. Oxford: Elsevier 2017; 2e, pages: 523-545.

Boonstra TW, Stins JF, Daffertshofer A, Beek PJ. Effects of sleep deprivation on neural functioning: an integrative review. Cell Mol Life Sci 2007; 64: 934–946.

Boucetta S, Cissé Y, Mainville L, Morales M, Jones BE. Discharge profiles across the sleep–waking cycle of identified cholinergic, GABAergic, and glutamatergic neurons in the pontomesencephalic tegmentum of the rat. Int J Neurosci 2014; 34: 4708-4727.

Brown D, Kays R, Wikelski M, Wilson R, Klimley AP. Observing the unwatchable through acceleration logging of animal behavior. Animal Biotelemetry 2013, 1:20 https://doi.org/10.1186/2050-3385-1-20.

Brown RE, Basheer R, McKenna JT, Strecker RE, McCarley RW. Control of sleep and wakefulness. Physiol Rev 2012; 92: 1087–1187.

Bubenik A. Wild u. Hund 1960; 63: I960.

Buechner HK, and Roth DH. The lek system in Uganda kob antelope. American Zoologist 1974; 14: 145-162.

Bux F, Bhagwandin A, Fuxe K, Manger PR. Organization of cholinergic, putative catecholaminergic and serotonergic nuclei in the diencephalon, midbrain and pons of sub-adult male giraffes. J Chem Neuroanat 2010; 39: 189-203.

Byers J, Hebets E, Podos J. Female mate choice based upon male motor performance. Anim Behav 2010; 79: 771-778.

Byers JA, Moodie JD, Hall N. Pronghorn females choose vigorous mates. Anim Behav 1994; 47: 33-43.

Calisi RM, and Bentley GE. Lab and field experiments: Are they the same animal? Horm Behav 2009; 56:1-10.

Calvey T, Patzke N, Kaswera C, Gilissen E, Bennett NC, Manger PR. Nuclear organization of some immunohistochemically identifiable neural systems in three Afrotherian species – *Potomogale velox*, *Amblysomus hottentotus* and *Petrodromus tetradactylus*. J Chem Neuroanat 2013; 50-51: 48-65.

Calvey T, Alagaili AN, Bertelsen MF, Bhagwandin A, Pettigrew JD, Manger PR. Nuclear organization of some immunohistochemically identifiable neural systems in two species of the Euarchontoglires: a lagomorph, *Lepus capensis*, and a Scadentia, *Tupaia belangeri*. J Chem Neuroanat 2015; 70: 1-19.

Calvey T, Patzke N, Bennett NC, Consolate KK, Gilissen E, Alagaili AN, Mohammed OB, Pettigrew JD, Manger PR. Nuclear organisation of some immunohistochemically identifiable neural systems in five species of insectivore—Crocidura cyanea, Crocidura olivieri, Sylvisorex ollula, Paraechinus aethiopicus and Atelerix frontalis. J Chem Neuroanat 2016; 72: 34-52.

Campbell SS, and Tobler I. Animal sleep: a review of sleep duration across phylogeny. Neurosci Biobehav Rev 1984; 8: 269-300.

Capellini I, Barton RA, Mcnamara P, Preston BT, Nunn CL. Phylogenetic analysis of the ecology and evolution of mammalian sleep. Evolution 2008a; 62: 1764-1776.

Capellini I, Nunn CL, McNamara P, Preston BT, Barton RA. Energetic constraints, not predation, influence the evolution of sleep patterns among mammals. Funct Ecol. 2008; 22: 847-853.

Capellini I, Preston BT, McNamara P, Barton RA, Nunn CL. Ecological constraints on mammalian sleep architecture. In: McNamara P, Barton RA, Nunn CL, eds. Evolution of Sleep: Phylogenetic and functional perspectives. Cambridge University Press, 2010 pages: 12-33.

Carranza J, Fernandez-Llario P, Gomendio M. Correlates of territoriality in rutting red deer. Ethology 1996; 102: 793-805.

Carskadon MA, and Dement WC. Monitoring and staging human sleep. In: Kryger MH, Roth T, and Dement WC, eds. Principles and practice of sleep medicine. St. Louis: Elsevier Saunders 2011; 5e pages: 16-26.

Chae KY, Kripke DF, Poceta JS, Shadan F, Jamil SM, Cronin JW, Kline LE. Evaluation of immobility time for sleep latency in actigraphy. Sleep Med 2009; 10: 621-625.

Cox J, Pinto L, Dan Y. Calcium imaging of sleep-wake related neuronal activity in the dorsal pons. Nat Commun 2016; 7: 10763.

Daan, S. How and Why? The lab versus the field. Sleep and Biol Rhythms 2011; 9: 1-2.

Davimes JG, Alagaili AN, Gravett N, Bertelsen MF, Mohammed OB, Ismail K, Bennett NC, Manger PR. Arabian oryx (*Oryx leucoryx*) respond to increased ambient temperatures with a seasonal shift in the timing of their daily inactivity patterns. J Biol Rhythms 2016; 31: 365-374.

Davimes JG, Alagaili AN, Bennet NC, Mohammed OB, Bhagwandin A, Manger PR, Gravett N. Nuclear organization and morphology of the sleep related nuclei in the brain of the Arabian oryx, *Oryx leucoryx*. J Chem Neuroanat 2017; 81: 53-70.

Davimes JG, Alagaili AN, Bhagwandin A, Bertelsen MF, Mohammed OB, Bennett NC, Manger PR, Gravett N. Seasonal variations in sleep of free-ranging Arabian oryx (*Oryx leucoryx*) under natural hyper-arid conditions. Sleep 2018; in press. doi:10.1093/sleep/zsy038.

De Souza L, Benedito-Silva A, Pires M, Poyares D, Tufik S, Calil HM. Further validation of actigraphy for sleep studies. Sleep 2003;1: 81-85.

Dell LA, Kruger JL, Bhagwandin A, Jillani NE, Pettigrew JD, Manger PR. Nuclear organization of cholinergic, putative catecholaminergic and serotonergic systems in the brains of two megachiropteran species. J Chem Neuroanat 2010; 40: 177-195.

Dell LA, Patzke N, Bhagwandin A, Bux F, Fuxe K, Barber G, Siegel JM, Manger PR. Organization and number of orexinergic neurons in the hypothalamus of two species of Cetartiodactyla: A comparison of giraffe (*Giraffa camelopardalis*) and harbour porpoise (*Phocoena phocoena)*. J Chem Neuroanat 2012; 44: 98-109.

Dell LA, Kruger JL, Pettigrew JD, Manger PR. Cellular location and major terminal networks of the orexinergic system in the brain of two megachiropterans. J Chem Neuroanat 2013; 53: 64–71.

Dell LA, Patzke N, Spocter MA, Siegel JM, Manger PR. Organization of the sleep-related neural systems in the brain of the harbour porpoise (*Phocoena phocoena*). J Comp Neurol 2016a; 524: 1999-2017.

Dell LA, Karlsson KÆ, Patzke N, Spocter MA, Siegel JM, Manger PR. Organization of the sleep-related neural systems in the brain of the minke whale (*Balaenoptera acutorostrata*). J Comp Neurol 2016b; 524: 2018-2035.

Dell LA, Patzke N, Spocter MA, Bertelsen MF, Siegel JM, Manger PR. Organization of the sleep related neural systems in the brain of the river hippopotamus (*Hippopotamus amphibius*): a most unusual Cetartiodactyl species. J Comp Neurol 2016c; 524: 2036-2058.

Diamond J. Evolution, consequences and future of plant and animal domestication. Nature, 2002; 418: 700-707.

Dorst J and Dandelot P. Larger mammals of Africa. Houghton Mifflin; 1970.

Estes R. Territorial behavior of the wildebeest (*Connochaetes taurinus* Burchell, 1823). Z. Tierpsychol. 1969; 26: 284-370.

Estes R. The Behaviour Guide to African Mammals. Russel Friedman, Halfway House, South Africa 1997; pages: 150-156.

Estes R. The Gnu's World: Serengeti Wildebeest Ecology and Life History. Univ. of California Press 2014; pages: 218-219.

Ettrup KS, Sorensen JS, Bjarkam CR. The anatomy of the Göttingen minipig hypothalamus. J Chem Neuroanat 2010; 39: 151-165.

Ferguson AV, and Samson WK. The orexin/hypocretin system: critical regulator of neuroendocrine and autonomic function. Front Neuroendocrinol 2003; 24: 141-150.

Ferreira G, Meurisse M, Tillet Y, Levy F. Distribution and co-localization of choline acetyltransferase and P75 neurotrophin receptors in the sheep basal forebrain: implications for the use of a specific cholinergic immunotoxin. Neurosci 2001; 104: 419-439.

Fryxell JM, Mosser A, Sinclair ARE, Packer C. Group formation stabilizes predator–prey dynamics. Nature 2007; 449: 1041-1044.

Fuller RW. Mechanisms and functions of serotonin neuronal systems. Ann N Y Acad Sci 1996; 780:176-184.

Funston PJ, Mills MG, Biggs HC, Richardson PRK. Hunting by male lions: ecological influences and socioecological implications. Anim Behav 1998; 56: 1333-1345.

Gallyas F. Silver staining of myelin by means of physical development. Neurolog Res 1979; 1: 203-209.

Ganswindt A, Heistermann M, Borragan S, Hodges JK. Assessment of testicular endocrine function in captive African elephants by measurement of urinary and fecal androgens. Zoo Biol 2002; 21: 27–36.

Geist V, and Walther F. The behaviour of ungulates and its relation to management. IUCN Publications, Morges, Switzerland 1974.

Geist V. On the rutting behavior of the mountain goat. J Mammal 1965; 45: 551-568.

Gonzalez AA. 2016. Sleep stage scoring. Accessed 15 October 2017, httpd://emedicine.medscape.com/article/1188142-overview.

Gravett N, Bhagwandin A, Fuxe K, Manger PR. Nuclear organization and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the rock hyrax, *Procavia capensis*. J Chem Neuroanat 2009; 38: 57-74.

Gravett N, Bhagwandin A, Lyamin OI, Siegel JM, Manger PR. Sleep in the rock hyrax, *Procavia capensis*. Brain Behav Evol 2012; 79: 155-169.

Gravett N, Bhagwandin A, Sutcliffe R, Landen K, Chase MJ, Lyamin OI, Siegel JM, Manger PR. Inactivity/sleep in two wild free-roaming African elephant matriarchs - Does large body size make elephants the shortest mammalian sleepers? PLoS ONE 2017; 12: e0171903.

Grigg G, Beard L, Dörges B, Heucke J, Coventry J, Coppock A, Blomberg S. Strategic (adaptive) hypothermia in bull dromedary camels during rut; could it increase reproductive success? Biol Lett 2009; 5: 853–856.

Gritti I, Manns ID, Mainville L, Jones BE. Parvalbumin, calbindin, or calretinin in cortically projecting and GABAergic, cholinergic, or glutaminergic basal forebrain neurons of the rat. J Comp Neurol 2003; 458: 11-31.

Gunderson HJG, and Jensen EBV. The efficiency of systematic sampling in stereology and its prediction. J Microsc 1987; 147: 229-263.

Gunderson HJG, Jensen EBV, Kiêu K, Neilsen J. The efficiency of systematic sampling in stereology – reconsidered. J Microsc 1999; 193: 199-211.

Hammer, Harper DAT, Ryan PD. PAST: Paleontological statistics software package for education and data analysis. Paleontol Electron. 2001; 4: 9.

Hänninen L, Makela J, Rushen J, de Passille A, Saloniemi H. Assessing sleep state in calves through electrophysiological and behavioural recordings: a preliminary study. Appl Anim Behav Sci 2008; 111: 235–250.

Hayward MW, Hayward GJ, Tambling CJ, Kerley GIH. Do lions *Panthera leo* actively select prey or do prey preferences simply reflect chance responses via evolutionary adaptations to optimal foraging? PLoS ONE 2011; 6: e23607.

Hodges K, Brown J, Heistermann M. Endocrine monitoring of reproduction and stress. In: Kleiman DG, Thompson KV, Kirk Baer C, eds. Wild Mammals in Captivity: Principles and Techniques for Zoo Management. Chicago: The University of Chicago Press. 2010; pages: 447-468.

Hurelbrink CB, Lewis SJ, Barker RA. The use of the Actiwatch-Neurologica system to objectively assess the involuntary movements and sleep-wake activity in patients with mild-moderate Huntington's disease. J Neurol 2005; 252: 642-647.

Immelmann K, Gebbing H. Schlaf bei Giraffiden. Ethology 1962; 19: 84-92.

Iqbal J, Pompolo S, Sakurai T, Clarke IJ. Evidence that orexin-containing neurons provide direct input to gonadotrophin-releasing hormone neurones in the bovine hypothalamus. Neuroendocrinol 2001; 13: 1033-1041.

Joiner WJ. Unraveling the evolutionary determinants of sleep. Curr Biol 2016; 26: R1073-R1087.

Jones BE. Arousal systems. Front Biosci 2003; 8: s438-s451.

Jones EG. The Thalamus. Cambridge University Press, Cambridge UK. 2007.

Kanady JC, Drummond SPA, Mednick SC. Actigraphic assessment of a polysomnographic-recorded nap: a validation study. J Sleep Res 2011; 20: 214–222.

Kanda T, Tsujino N, Kuramoto E, Koyama Y, Susaki EA, Chikahisa S, Funato H. Sleep as a biological problem: an overview of frontiers in sleep research. The Journal of Physiological Sciences 2016; 66: 1-3.

Kirsch D, Harding SM, Eichler AF. Stages and architecture of normal sleep. *UpToDate*. Retrieved November 1, 2017 from https://www.uptodate.com/contents/stages-and-architecture-of-normal-sleep.

Kitahama K, Nagatsu I, Pearson J, Catecholamine systems in mammalian midbrain and hindbrain: theme and variations. In: Smeets WJAJ, Reiner A, eds. Phylogeny and development of catecholamine systems in the CNS of vertebrates. Cambridge University Press, Cambridge, 1994; pages: 183-205.

Klemm WR. Sleep and paradoxical sleep in ruminants. Exp Biol Med 1966; 121: 635-638.

Körtner G, and Geiser F. Body temperature rhythms and activity in reproductive *Antechinus* (Marsupialia). Physiol Behav 1994; 58: 31-36.

Krasnec MO, Cook CN, Breed MD. Mating systems in sexual animals. Nature Education Knowledge. 2012; 3: 72.

Kruger JL, Patzke N, Fuxe K, Bennett NC, Manger PR. Nuclear organization of cholinergic, putative catecholaminergic, serotonergic and orexinergic systems in the brain of the African pygmy mouse (*Mus minutoides*): Organizational complexity is preserved in small brains. J Chem Neuroanat 2012; 44: 45-56.

Landis JR, and Koch GG. The measurement of observer agreement for categorical data. Biometrics 1977; 33: 159–74.

Leshin LS, Kraeling RR, Kiser TE. Immunocytochemical localization of the catecholamine-synthesizing enzymes, tyrosine hydroxylase and dopamine- b-hydroxylase, in the hypothalamus of cattle. J Chem Neuroanat 1995a; 9: 175-194.

Leshin LS, Kraeling RR, Kiser TE, Barb CR, Rampacek GB. Catecholaminergic region A15 in the bovine and porcine hypothalamus. Brain Res Bull 1995b; 37: 351-358.

Leshin LS, Kraeling RR, Kineman RD, Barb CR, Rampacek GB. Immunocytochemical distribution of catecholamine-synthesizing neurons in the hypothalamus and pituitary gland of pigs: tyrosine hydroxylase and dopamine- b-hydroxylase. J Comp Neurol 1996; 364: 151-168.

Lesku JA, Roth TC, Amlaner CJ, Lima SL. A phylogenetic analysis of sleep architecture in mammals: the integration of anatomy, physiology, and ecology. Am Nat. 2006; 168: 441–453. pmid:17004217

Lesku JA, Roth II TC, Rattenborg NC, Amlaner CJ, Lima SL. History and future of comparative analyses in sleep research. Neurosci Biobehav Rev 2009; 33: 1024–1036.

Lesku JA, Meyer LC, Fuller A, Maloney SK, Dell'omo G, Vyssotski AL, Rattenborg NC. Ostriches sleep like platypuses. PLoS One 2011; 6: e23203

Lesku JA, Rattenborg NC, Valcu M, Vyssotski AL, Kuhn S, Kuemmeth F, Heidrich W, Kempenaers B. Adaptive sleep loss in polygynous pectoral sandpipers. Science 2012; 337: 1654-1658.

Lima SL, Rattenborg NC, Lesku JA, Amlaner CJ. Sleeping under the risk of predation. Anim Behav 2005; 70: 723-736.

Low AB, and Rebelo AG. Vegetation of South Africa, Lesotho and Swaziland. DEAT (Department of Environment and Tourism), Pretoria 1996; 85 pages.

Lu BS, and Zee PC. Neurobiology of sleep. Clin Chest Med 2010; 31: 309–318.

Luppi P-H, Peyron C, Fort P. Not a single but multiple populations of GABAergic neurons control sleep. Sleep Med Rev 2016; 32: 85-94.

Lyamin OI, Manger PR, Ridgway SH, Mukhametov LM, Siegel JM. Cetacean sleep: An unusual form of mammalian sleep. Neurosci Biobehav Rev 2008; 32: 1451-1484.

Lyamin OI, Kosenko PO, Vyssotski AL, Lapierre JL, Siegel JM, and Mukhametov LM. Study of sleep in a walrus. Dokl Biol Sci 2012; 444: 188-91.

Mahady LJ, Perez SE, Emerich DF, Wahlberg LU, and Mufson EJ. Cholinergic profiles in the Goettingen miniature pig (*Sus scrofa domesticus*) brain. J Comp Neurol 2017; 525: 553–573. doi:10.1002/cne.24087

Manger PR, Ridgway SH, Siegel JM. The locus coeruleus complex of the bottlenose dolphin (*Tursiops truncatus*) as revealed by tyrosine hydroxylase immunohistochemistry. J Sleep Res 2003; 12: 149-155.

Manger PR. Establishing order at the systems level in mammalian brain evolution. Brain Res Bull 2005; 66: 282–289.

Manger PR, Pillay P, Maseko BC, Bhagwandin A, Gravett N, Moon DJ, Jillani N, Hemingway J. Acquisition of brains from the African elephant (*Loxodonta africana*): perfusion-fixation and dissection. J Neurosci Methods 2009; 179: 16-21.

Manger PR, Hemingway J, Haagensen M, Gilissen E. Cross-sectional area of the elephant corpus callosum: comparison to other Eutherian mammals. Neuroscience 2010; 167: 815-824.

Manger PR, Prowse M, Haagensen M, Hemingway J. Quantitative analysis of neocortical gyrencephaly in African elephants (*Loxodonta africana*) and six species of cetaceans: comparison with other mammals. J Comp Neurol 2012; 520: 2430-2439.

Maseko BC, Bourne JA, Manger PR. Distribution and morphology of cholinergic, putative catecholaminergic and serotonergic neurons in the brain of the Egyptian Rousette flying fox (*Rousettus aegyptiacus*). J Chem Neuroanat 2007; 34: 108-127.

Maseko BC, Spocter MA, Haagensen M, Manger PR. Volumetric analysis of the African elephant ventricular system. Anat Rec 2011; 294: 1412-1417.

Maseko BC, Spocter MA, Haagensen M, Manger PR. Elephants have relatively the largest cerebellum size of mammals. Anat Rec 2012; 295: 661-672.

Maseko BC, Patzke N, Fuxe K, Manger PR. Architectural organization of the African elephant diencephalon and brainstem. Brain Behav Evol 2013; 82: 83-128.

McGranaghan PA, and Piggins HD. Orexin-A-like immunoreactivity in the hypothalamus and thalamus of the Syrian hamster (*Mesocricetus auratus*) and Siberian hamster (*Phodopus sungorus*), with special reference to circadian structures. Brain Res 2001; 904: 234-244.

McGregor R, Wu MF, Barber G, Ramanthan L, Siegel JM. Highly specific role of hypocretin (orexin) neurons: differential activation as a function of diurnal phase, operant reinforcement vs. operant avoidance and light level. J Neurosci 2011; 31: 15455–15467.

McNamara P, Capellini I, Harris E, Nunn L, Barton RA, Preston B. The phylogeny of sleep database: a new resource for sleep scientists. Open Sleep J 2008; 1: 11-14.

McNew JJ, Howe RC, Adey WR. The sleep cycle and subcortical-cortical EEG relations in the unrestrained chimpanzee. Electoenceph Clin Neurophysiol. 1971; 30: 489-503.

Meddis R. On the function of sleep. Anim Behav 1975; 23: 676–691.

Meddis R. The evolution of sleep. In: Mayes A, ed. Sleep Mechanisms and Functions in Humans and Animals. Van Nostrand Reinhold, Wokingham. 1983; Pages 57-95.

Mignot E. Why we sleep: The temporal organization of recovery. PLoS Biol 2008; 6: e106.

Moruzzi G, and Magoun H. Brainstem reticular formation and activation of the EEG. Electroenceph Clin Neurophyiol 1949; 1: 455-473.

Muller MN, and Wrangham RW. Dominance, aggression and testosterone in wild chimpanzees: a test of the 'challenge hypothesis'. Anim Behav 2004; 67: 113-123.

Mysterud A, Coulson T, Stenseth NC. The role of males in the dynamics of ungulate populations. J Animl Ecol 2002; 71: 907-915.

O'Leary MA. Parsimony analysis of total evidence from extinct and extant taxa and the Cetacean-Artiodactyl question (Mammalia, Ungulata). Cladistics 1999; 15: 315–330.

Ohno K, and Sakurai T. Orexin neuronal circuitry: Role in the regulation of sleep and wakefulness. Front Neuroendocrinol 2008; 29: 70–87.

Østergaard K, Holm IE, Zimmer J. Tyrosine hydroxylase and acetylcholinesterase in the domestic pig mesencephalon: an immunocytochemical and histochemical study. J Comp Neurol 1992; 320: 149–166.

Owen-Smith N. Changing vulnerability to predation related to season and sex in an African

ungulate assemblage. Oikos 2008; 117: 602–610.

Pace-Shott EF, and Hobson JA. Basic mechanisms of sleep: new evidence on the neuroanatomy and neuromodulation of the NREM-REM cycle. In: Davis KL, Charney D, Coyle JT, Nemeroff C, eds. Neuropsychopharmacology: The Fifth Generation of Progress. Lippincott, Williams, & Wilkins, Philadelphia, Pennsylvania, 2002; pages: 1859-1877.

Palme R, and Möstl E. Biotin-streptavidin enzyme immunoassay for the determination of oestrogens and androgens in boar faeces. In: Proceedings of the 5th Symposium on the Analysis of Steroids, Budapest, Hungary 1993; pages: 111–117.

Paquet J, Kawinska A, Carrier J. Wake detection capacity of actigraphy during sleep. Sleep 2007; 30:1362–1369.

Petersen CCH. Higher nervous function In: Petersen OH, ed. Lecture notes Human physiology. 5th Edition. Blackwell Publishing Ltd, 2007 Pages 213-226.

Poole JH. Rutting behavior in African elephants: the phenomenon of musth. Behaviour 1987; 102: 283-316.

Price SA, Bininda-Emonds OR, Gittleman JL. A complete phylogeny of the whales, dolphins and even-toed hoofed mammals (Cetartiodactyla). Biol Rev 2005; 80: 445-473.

Rattenborg NC, Amlaner CJ, Lima SL. Behavioral, neurophysiological and evolutionary perspectives on unihemispheric sleep. Neurosci Biobehav Rev 2000; 24: 817–842.

Rattenborg NC, Mandt BH, Obermeyer WH, Winsauer PJ, Huber R, Wikelski M, Benca RM. Migratory sleeplessness in the white-crowned sparrow (*Zonotrichia leucophrys gambelii*). PLoS Biol 2004; 2: e212.

Rattenborg NC, Voirin B, Vyssotski AL, Kays RW, Spoelstra K, Kuemmeth F, Heidrich W, Wikelski M. Sleeping outside the box: electroencephalographic measures of sleep in sloths inhabiting a rainforest. Biol Lett 2008; 4:402-5.

Rattenborg NC, Voirin B, Cruz SM, Tisdale R, Dell'Omo G, Lipp HP, Wikelski M, Vyssotski AL. Evidence that birds sleep in mid-flight. Nat Comm. 2016; 7: 12468.

Rattenborg NC, de la Iglesia HO, Kempenaers B, Lesku JA, Meerlo P, Scriba MF. Sleep research goes wild: new methods and approaches to investigate the ecology, evolution and functions of sleep. Phil Trans R Soc B 2017; 372: 20160251.

Robert S, and Dallaire A. Polygraphic analysis of the sleep-wake states and the REM sleep periodicity in domesticated pigs (*Sus scrofa*). Physiol Behav 1986; 37: 289-293.

Rodgers RJ, Ishii Y, Halford JC, Blundell JE. Orexins and appetite regulation. Neuropeptides 2002; 36: 303-325.

Rosenwasser A. Functional neuroanatomy of sleep and circadian rhythms. Brain Res Rev 2009; 61: 281-306.

Ruckebusch Y, Bell FR, Barbey P, Guillemot P, Serthelon JP. Etude polygraphique et comportementale des états de veille et de sommeil chez la vache (*Bos taurus*). In Annales de Recherches Vétérinaires 1970; 1: 41-62.

Ruckebusch Y. The relevance of drowsiness in the circadian cycle of farm animals. Anim Behav 1972; 20: 637-643.

Rutherford MC, Mucina L, Powrie LW. Biomes and Bioregions of Southern Africa. In: Mucina L, and Rutherford MC eds. The vegetation of South Africa, Lesotho and Swaziland. South African National Biodiversity Institute, Pretoria. Strelitzia 2006; 19: 30-51.

Rye DB, Wainer BH, Mesulam MM, Mufso EJ, Saper CB. Cortical projections arising from the basal forebrain: A study of cholinergic and noncholinergic components employing combined retrograde tracing and immunohistochemical localisation of choline acetyltransferase. Neuroscience 1984; 13: 627-643.

Sadeh A, and Acebo C. The role of actigraphy in sleep medicine. Sleep Med Rev 2002; 6: 113–124.

Sadeh A. The role and validity of actigraphy in sleep medicine: An update. Sleep Med Rev 2011; 15: 259-267.

Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, Williams SC, Richardson JA, Kozlowski GP, Wilson S, Arch JR, Buckingham RE, Haynes AC, Carr SA, Annan RS, McNulty DE, Liu WS, Terret JA, Elshourbagy NA, Bergsma DJ, Yanagisawa M.

Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 1998; 92: 573–585.

Sakurai T. The neural circuit of orexin (hypocretin): Maintaining sleep and wakefulness. Nat Rev Neurosci 2007; 8: 171-181.

Sakurai T. The role of orexin in motivated behaviours. Nat Rev Neurosci 2014; 15: 719-731.

Saper CB, Chou TC, Scammell TE. The sleep switch: hypothalamic control of sleep and wakefulness. Trends Neurosci 2001; 24: 726-731.

Scammell TE, Arrigoni E, Lipton JO. Neural circuitry of wakefulness and sleep. Neuron. 2017; 93: 747-765.

Schmidt MH. The energy allocation function of sleep: A unifying theory of sleep, torpor and continuous wakefulness. Neurosci Biobehav Rev 2014; 47: 122-153.

Schwartz JRL, and Roth T. Neurophysiology of sleep and wakefulness: Basic science and clinical implications. Curr Neuropharmacol 2008; 6: 367-378.

Schwartz MD, and Kilduff TS. The neurobiology of sleep and wakefulness. Psychiatr Clin North Am 2015; 38: 615-644.

Selebatso M, Fynn R, Maude G. Adaptive activity patterns of a blue wildebeest population to environmental variability in fragmented, semi-arid Kalahari, Botswana. J Arid Environ 2017; 136:15-18.

Shambroom JR, Fabregas S, Johnstone J. Validation of an automated wireless system to monitor sleep in healthy adults. J Sleep Res 2012; 21: 221–230.

Shiraishi T, Oomura Y, Sasaki K, Wayner MJ. Effects of leptin and orexin-A on food intake and feeding related hypothalamic neurons. Physiol Behav 2000; 71: 2512-61.

Scheun J, Bennett NC, Nowack J, Ganswindt A. Reproductive behaviour, testis size and faecal androgen metabolite concentrations in the African lesser bushbaby. J Zool 2017; 301: 263-270.

Siegel JM. The REM sleep-memory consolidation hypothesis. Science 2001; 294: 1058-1063.

Siegel JM. The neurotransmitters of sleep. J Clin Psychiat 2004; 65: 4-7.

Siegel JM. Clues to the function of mammalian sleep. Nature 2005; 437: 1264-1271.

Siegel JM. The stuff dreams are made of: anatomical substrates of REM sleep. Nat Neurosci 2006; 9: 721-722.

Siegel JM. Do all animals sleep? Trends Neurosci 2008; 31: 208-213.

Siegel JM. Sleep viewed as a state of adaptive inactivity. Nat Rev Neurosci 2009; 10: 747-753.

Siegel JM. Suppression of sleep for mating. Science 2012; 337: 1610.

Siegel JM. Evolution of sleep (Sleep phylogeny). Encyclopaedia of Sleep 2013; 1: 38-42.

Skinner JD, Chimimba CT. The Mammals of the Southern African Subregion. Cambridge University Press, Cape Town 2005.

Smeets WJAJ, and González A. Catecholamine systems in the brain of vertebrates: new perspectives through a comparative approach. Brain Res Rev 2000; 33: 308–379.

Smithers, RHN. The mammals of Rhodesia, Zambia and Malawi. London: Collins. 1966

Spinazzi R, Andries PG, Rossi GP, Nussdorfer GG. Orexins in the regulation of the hypothalamic–pituitary–adrenal axis. Pharmacol Rev 2006; 58: 46–57.

Stuber GD, and Wise RA. Lateral hypothalamic circuits for feeding and reward. Nat Neurosci 2016; 19: 198.

Talbot LM, and Talbot MH. The wildebeest in western Masailand, East Africa. Wildlife Monogr 1963; 12: 88.

Ternman E, Hänninen L, Pastell M, Agenäs S, Nielsen PP. Sleep in dairy cows recorded with a non-invasive EEG technique. Appl Anim Behav Sci 2012; 140: 25-32.

Thaker M, Vanak AT, Owen CR, Ogden MB, Slotow R. Group dynamics of zebra and wildebeest in a woodland savanna: effects of predation risk and habitat density. PLoS ONE 2010; 5: e12758.

Tillet Y, and Kitahama K. Distribution of central catecholaminergic neurons: a comparison between ungulates, humans and other species. Histol Histopathol 1998; 13: 1163-1177.

Tillet Y, and Thibault J. Catecholamine-containing neurons in the sheep brainstem and diencephalon: immunohistochemical study with tyrosine hydroxylase (TH) and dopamine-b-hydroxylase (DBH) antibodies. J Comp Neurol 1989; 290: 69-106.

Tillet Y. Catecholamine neuronal systems in the diencephalon of mammals. In: Smeets WJAJ, and Reiner A, eds. Phylogeny and Development of Catecholamine Systems in the CNS of vertebrates. Cambridge University Press, Cambridge, 1994; pages: 207-246.

Tobler I, and Schwierin B. Behavioural sleep in the giraffe (*Giraffa camelopardalis*) in a zoological garden. J Sleep Res 1996; 5: 21–32. pmid:8795798.

Tobler I, Franken P, Trachsel L, Borbély AA. Models of sleep regulation in mammals. J Sleep Res 1992; 1: 125–127. doi:10.1111/j.1365-2869.1992.tb00024.x.

Tobler I. Napping and polyphasic sleep in mammals. In: Dinges DF, and Broughton RJ, eds. Sleep and Alertness: Chronobiological, Behavioural Medical Aspects of Napping New York: Raven Press, 1989; pages: 9-30.

Tobler I. Is sleep fundamentally different between mammalian species? Behav Brain Res 1995; 69: 35-41.

Tobler I. Phylogeny of sleep regulation. In: Kryger MH, Roth T, Dement WC eds. Principles Practice of Sleep Medicine. Elsevier/Saunders, Philadelphia, 2005; Pages. 112-125.

Törk I. Anatomy of the serotonergic system. Ann N Y Acad Sci 1990; 600: 9–35.

Tsujino N, and Sakurai T. Role of orexin in modulating arousal, feeding, and motivation. Front Behav Neurosci 2013; 7: 1-14.

und Halbach OV, Dermietzel R. Neurotransmitters. In: Neurotransmitters and neuromodulators: Handbook of receptors and biological effects. John Wiley & Sons, 2006 2e; pages 46-139.

Ursin R. Serotonin and sleep. Sleep Med Rev 2002; 6: 57-69.

Van Dort CJ, Zachs DP, Kenny JD, Zheng S, Goldblum RR, Gelwan NA, Ramos DM, Nolan MA, Wang K, Weng FJ, Lin Y, Wilson MA, Brown EN. Optogenetic activation of cholinergic neurons in the PPT or LDT induces REM sleep. Proc Natl Acad Sci U.S.A 2015; 112: 584-589.

Vertes RP, and Eastman KE. The case against memory consolidation in REM sleep. Behav Brain Sci. 2000; 23: 867-876.

Voirin B, Scriba MF, Martinez-Gonzalez D, Vyssotski AL, Wikelski M, Rattenborg NC. Ecology and neurophysiology of sleep in two wild sloth species. Sleep. 2014; 37(4): 753.
Von Richter W. Territorial behaviour of the black wildebeest Connochaetes gnou. Afr Zool 1972; 7: 207-231.

Vrahimis S, and Kok OB. Daily activity of black wildebeest in a semi-arid environment. Afr J Ecol 1993; 31: 328–336.

Vyssotski AL, Serkov AN, Itskov PM, Dell'Omo G, Latanov AV, Wolfer DP, Lipp HP. Miniature neurologgers for flying pigeons: multichannel EEG and action and field potentials in combination with GPS recording. J Neurophysiol. 2006; 95: 1263-1273.

Wagner D, Salin-Pascual R, Greco MA, Shiromani PJ. Distribution of hypocretin-containing neurons in the lateral hypothalamus and c-fos-immunoreactive neurons in the VLPO. Sleep Res Online 2000; 3: 35–42.

Wang Y, Nickel B, Rutishauser M, Bryce CM, Williams TM, Elkaim G, Wilmers CC. Movement, resting, and attack behaviors of wild pumas are revealed by tri-axial accelerometer measurements. Movement Ecology 2015; 3: 2.

Watson PF, and Petrie A. Method agreement analysis: A review of correct methodology. Theriogenology 2010; 73: 1167–1179.

West MJ, Slomianka L, Gundersen HJG. Unbiased stereological estimation of the total number of neurons in the subdivision of the rat hippocampus using the optical fractionator. Anat Rec 1991; 231: 482 – 497.

West MJ. Seterological methods for estimating the total number of neurons and synapses: issues of precision and bias. Trends Neurosci 1999; 22: 51- 61.

Whittle CL, Bowyer RT, Clausen TP, Duffy LK. Putative pheromones in urine of rutting male moose (*Alces alces*): evolution of honest advertisement? J Chem Ecol 2000; 26: 2747-2762.

Woolf NJ. Cholinergic systems in mammalian brain and spinal cord. Prog Neurobiol 1991; 37: 475-524.

Yetish G, Kaplan H, Gurven M, Wood B, Pontzer H, Manger PR, Wilson C, McGregor R, Siegel JM. Natural sleep and its seasonal variations in three pre-industrial societies. Curr Biol 2015; 25: 2862-2868.

Yamashita T, and Yamanaka A. Lateral hypothalamic circuits for sleep–wake control. Curr Opin Neurobiol 2017; 44: 94-100.

Zepelin H, Siegel JM, Tobler I. Mammalian sleep. In: Kryger MH, Roth T, Dement WC eds. Principles Practice of Sleep Medicine. Elsevier/Saunders, Philadelphia, 2005; pp. 91–100.

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2012/16/2B

APPLICANT: **Ms N Gravett**

DEPARTMENT: **Anatomical Sciences**

PROJECT TITLE: **Nuclear organization and morphology of the systems involved in the sleep-wake cycle in the brain of the blue wildebeest, Connocheates taurines**

Number and Species

3 Blue Wildebeest

Approval was given for the use of animals for the project described above at an AESC meeting held on **29 May 2012**. This approval remains valid until **31 May 2014**.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and to the following additional conditions:

Conditions:

- Include details of transportation of animals (tissue samples) if applicable and ensure permits are in place.
- Category should be 2B.

Signed: _____ Date: 15 June 12
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: _____
(Registered Veterinarian)

cc: Supervisor:
Director: CAS

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/11/44/C

APPLICANT: Dr N Gravett

SCHOOL: School of Anatomical Sciences
DEPARTMENT:
LOCATION:

PROJECT TITLE: Neuronal organisation and distribution of the systems that control the sleep-wake cycle in different species of ungulates

Number and Species

3 Wild Blue Wildebeest, 3 Wild Plain Zebra, 3 Wild Arabian Oryx

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/11/24. This approval remains valid until 2017/12/01.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____ Date: 1st Dec 2015
(Chairperson, AESC)

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ Date: 1 December 2015
(Registered Veterinarian)

cc: Supervisor: N/A
Director: CAS

Works 2000/Iain0015/AESCCert.wps

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2014/53/D

APPLICANT: Dr G Gravett

SCHOOL: Anatomical Sciences

LOCATION: Faculty of Health Sciences

PROJECT TITLE: *Actigraphy: A novel approach to study sleep in free-roaming ungulates in their natural habitat*

Number and Species

3 Blue Wildbeest
3 Zebras
3 Gemsbok
3 Arabian Oryx

Approval was given for to the use of animals for the project described above at an AESC meeting held on 23 September 2014. This approval remains valid until 22 September 2016.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None.

Signed: _____ (Chairperson, AESC) Date: 19/01/2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 19/01/2015

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2015/09/39/C

APPLICANT: Dr N Gravett

SCHOOL: School of Anatomical Sciences
DEPARTMENT:
LOCATION:

PROJECT TITLE: The effect of the rut (mating season) on sleep architecture and duration in free-roaming male blue wildebeest (Connochaetes taurinus)

Number and Species

4 Blue Male Adult Wildebeest

Approval was given for the use of animals for the project described above at an AESC meeting held on 2015/09/29. This approval remains valid until 2017/10/12.

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

None

Signed: _____ (Chairperson, AESC) Date: 2nd November 2015

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 3rd November 2015

cc: Supervisor: N/A
Director: CAS

Works 2000/lain0015/AESCCert.wps

0503525n:Philander_Illke_PhD_thesis_Turnitin.pdf

ORIGINALITY REPORT

27%	17%	14%	18%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

MATCH ALL SOURCES (ONLY SELECTED SOURCE PRINTED)

15%

★ Submitted to University of Witwatersrand

Student Paper

Exclude quotes	On	Exclude matches	Off
Exclude bibliography	On		