The gonadotropin-releasing hormone (GnRH) system: a comparison between breeding and non-breeding naked mole rats *(Heterocephalus glaber)*

by

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DECLARATION

I, Caitlin Amy Smith, hereby declare that this thesis is based on my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University. I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

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Dedication:

This work is dedicated to my loving family for all their sacrifices and support.

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To my Lord and Saviour, I can do all things through You who gives me strength... (Philippians 4:13)

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LIST OF ABBREVIATIONS AND SYMBOLS

Tissue anatomy

3Vd	dorsal part of third ventricle
A	amygdala
AC	anterior commissure
bs	brain stem
c	cerebral hemispheres
CA3	hippocampal CA3 neurons (cornu ammonis, layer 3)
eh	cerebellum
cc	corpus callosum
CT	connective tissue
ec	external capsule
fi	hippocampal fimbriae
H	hypothalamus
is	internal capsule
LV	lateral ventricle
m	muscle
ME	median eminence
MEi	internal zone of ME
MEx	external zone of ME
MS	medial septum
0	ommatidia
oc	optic chiasm
of	region of the olfactory lobe
on	optic nerve
OVLT	organism vasculosum of the lamina terminalis
PC	piriform cortex
PO	preoptic area
PT	pituitary
PTa	anterior part of pituitary
PTp	posterior part of pituitary
PTs	pituitary stalk
SCN	suprachiasmatic nucleus
sg	sinus gland
td	tissue damage
VNO	vomeronasal organ

Amino acids

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic acid
Cys	Cysteine
Glu	Glutamic acid
Gln	Glutamine
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
pGlu	Pyroglutamate
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

General

	minus
	percent
a	**alpha**
	gamma
ACTH	**adrenocorticotropic hormone**
AKH	**adipokinetic hormone**
Attn	**attenuation**
AUFS	**absorbance units full scale**
bFGF	**basic fibroblast growth factor**
°C	**degrees celsius**
Cam	***Carausius morosus***
cGnRH	**chicken GnRH**
cHH	**crustacean hyperglycaemic hormone**
cm	**centimetres**
CRH	**corticotropin-releasing hormone**
DAB	**diaminobenzidine**
DAG	**diacyglycerol**
ELISA	**enzyme-linked immunosorbent assay**
Em	**emittance**
EtOH	**ethanol**
Ex	**excitation**
FSH	**follicle stimulating hormone**
g	**grams**
g	**gravitational constant**
GABA	**y-aminobutyric acid**
GAP	**GnRH associated peptide**
GPCR	**G-protein coupled receptor**
gpGnRH	**guinea pig GnRH**
GnIH	**gonadotropin-inhibitory hormone**
GnRH	**gonadotropin-releasing hormone**
GnRHR	**GnRH receptor**
GTP	**guano sine triphosphate**
h	**hour(s)**
H202	**hydrogen peroxide**
HPA	**hypothalamic-pituitary-adrenal axis**
hpg	**hypogonadal**
HrTH	**hypertrehalosaemic factor**
IGF	**insulin-like growth factor**
IgG	**immunoglobulin**
1P3	**inositol triphosphate**
-ir	**immunoreactive**
KC1	**potassium chloride**
kb	**kilo-base pair**
l	**litre**
LC-MS	**liquid chromatography mass spectrometry**
LH	**luteinizing hormone**
m	**milli**
M	**molar**

mGnRH	**mammalian GnRH**
MH^+	**monoisotopic mass**
min	**minute(s)**
m.i.u.	**milli international units**
n	**number**
NaC1	**sodium chloride**
NGtS	**normal goat serum**
nm	**nanometres**
OD	**optical density**
PBS	**phosphate buffered saline**
PCR	**polymerase chain reaction**
PFA	**paraformaldehyde**
pH	**hydrogen ion concentration**
PIP_2	**phosphatidylinositol biphosphate**
PKC	**protein kinase C**
PLC	**phospholipase C**
pmol	**picomol**
Prbo	***Procambarus bouvieri***
RPCH	**red pigment-concentrating hormone**
RP-HPLC	**reverse-phase liquid chromatography**
RT	**room temperature**
	seconds
TBS	**Tris-buffered saline**
TFA	**trifluoroacetic acid**
TGF	**transforming growth factor**
	micro
UV	**ultra violet**
vol	**volume**

ABSTRACT

Neuropeptides are well known to govern numerous biological functions and are found in all phyla studied to date. Probably the best known neuroendocrine system is the hypophyseal-portal system found in vertebrates, and one of the functions of this system is to mediate reproduction. Mammalian reproduction is controlled by a hormonal cascade which begins in distinct brain regions, namely the hypothalamus and the pituitary gland. Gonadotropin-releasing hormone (GnRH) is a neuropeptide typically produced in the hypothalamus. It is the key neuropeptide for initiating this cascade, and without it, reproduction cannot occur.

Naked mole rats *(Heterocephalus glaber)* have a rigid social hierarchy. The "queen" is the most dominant female and is the only female who breeds. All aspects of reproduction are suppressed in other females in the colony: these "subordinates" are in a prepuberty-like state as they do not ovulate or display breeding behaviours. They are, however, not infertile, and are capable of rising to the breeding position. Since GnRH is the "master hormone" of reproduction, this study investigates its role in the socially-induced suppression of reproduction in female *H. glaber*. Brains of breeding (n = 7) and non-breeding (n = 5) female naked mole rats were compared to determine any differences in brain size, particularly in regions related to GnRH production. Noteworthy morphological and physiological transformations accompany the change from subordinate to dominant social status, including a significant increase in body length (Mann Whitney U test; p = 0.005, U = 0.000), body mass (Mann Whitney U test; p = 0.009, 1.000) and pituitary width and length (Mann Whitney U test; p = 0.028, U = 0.500 and p = 0.018, U = 0.000, respectively).

Since little is known about the GnRH system in *H. glaber*, this study used immunocytochemistry to identify the distribution and abundance of GnRH neurons in the brains of both breeding and non-breeding females. GnRH neurons were found in the median eminence of the hypothalamus and in the anterior pituitary of both queens and subordinates, however in the brain of queen (n = 7) naked mole rats, there is a significantly larger area of immunoreactivity in comparison to the subordinate (n = 5)

brain tissue (Mann Whitney U = 4.000, p = 0.030). This suggests that, in subordinates, GnRH is inhibited at the level of production.

The amino acid structure of the form of GnRH found in the brain of the naked mole rat is currently unknown, therefore a pilot study was carried out, using synthetic mammalian GnRH (mGnRH), mouse brain tissue and naked mole rat pituitaries and hypothalami, to examine and modify (where necessary) the methodologies used for neuropeptide extraction, purification and identification. A limited number of naked mole rats were available as source tissue (n = 4), therefore this study also tested whether it is possible to extract and purify an unknown neuropeptide from only a few mammalian samples. Training for reverse-phase liquid chromatography (RP-HPLC) was achieved by practicing the necessary methods with crude extracts prepared from stick insect *(Carausius morosus)* corpora cardiaca, which also served to compare vertebrate and invertebrate neuroendocrine systems. Synthetic mGnRH was used to demonstrate repeatability of the protocol and to set up suitable conditions for elution of mGnRH: mGnRH elutes at — 12 min when a solvent gradient of 32 % - 47 % B is applied. Synthetic mGnRH was also used to establish the amount of peptide required for accurate identification of GnRH by antigenicity tests (ELISA) and mass spectrometry. However, when extracts of mouse or naked mole rat brain matter were applied to this system, purification of GnRH was unconvincing as either there was insufficient material, or, some endogenous factor was masking the GnRH. Future studies would benefit from using molecular techniques as they require smaller amounts of source tissue. Alternatively, a larger amount of source tissue would be necessary in order to proceed with biochemical studies.

While the impaired production of GnRH in subordinate naked mole rats seems to be linked to their prepuberty like state, it is unlikely that GnRH is the sole factor involved. Many other hormones (such as kisspeptin, gonadotropin-inhibiting hormone and neuropeptide Y) may influence GnRH and sexual maturity, and future studies would benefit from a multi-layered approach to investigate suppression of reproduction in naked mole rats.

CHAPTER ONE: INTRODUCTION

1.1. Neuropeptides

Co-ordinated communication between the different cells, tissues and organs of the body is vital for the survival of an organism (Bentley, 1998). Rapid responses are facilitated by the nervous system where nerve cells conduct electrical impulses (via the release of neurotransmitters) between the brain and the rest of the body. Slower, but longer lasting responses are modulated by glandular cells of the endocrine system, which release hormones (chemical messengers) into the blood stream that affect target organs (Hinson *et al,* 2007). Neurosecretory cells are specialised neurons which produce and secrete peptide hormones, also referred to as neuropeptide hormones. These neurosecretory cells do not make synaptic contact with effector organs or other nerves but rather project their axon terminals to blood vessels (or to storage glands, such as the corpora cardiaca in the case of insects, or sinus gland in crustaceans) into which the hormones are secreted (Hinson *et al,* 2007). Neurosecretory cells may, however, be innervated by other neurons which can activate or inhibit their secretion of neuropeptides. The functions of neuropeptide hormones are many, including modulating metabolism, colour change, reproduction and growth; possibly one of the best known neuroendocrine systems is that of the hypophyseal-portal system found in vertebrates (Bentley, 1998). Here, neurosecretory cells which reside in the brain, particularly in the median eminence (part of the ventral hypothalamus) release hormones into the hypophyseal portal vessels, which lie outside the blood brain barrier. Once released, the hormones act on target cells in the anterior pituitary gland to stimulate the release of other hormones necessary for reproduction, growth and metabolism (Hinson *et al,* 2007). The components of the hypophyseal-portal system, however, are not exclusive to vertebrates. Neurosecretory cells are found in all animal phyla studied to date and homologous systems in crustaceans and insects are well understood (Bentley, 1998). In crustaceans, neuropeptides, such as crustacean hyperglycaemic hormone (cHH, involved in glucose metabolism) are produced by neurosecretory cells in the X-organ, a neurohaemal organ found in crustacean eyestalk (Gide & Marco, 2006). These neuropeptides are then transported down the axons of the neurosecretory cells to a storage and release organ called the sinus gland (Marco

& Gale, 1999, Gäde & Marco, 2006). Similarly, in insects, neurosecretory cells in the retrocerebral glands, known as the corpora cardiaca and corpora allata, synthesise various hormones which are stored and released from the corpora cardiaca (Gäde & Marco, 2006). In all three systems, peptides are secreted by neuroendocrine cells in one part of the brain and stored and released by another part of the brain, the neurohaemal organ (Fig. 1).

1.2. Mammalian reproductive endocrinology

Mammalian reproduction is controlled primarily by hormones, secreted into the bloodstream from endocrine glands or neurosecretory cells, which bind to specific receptors at a target location (Hinson *et al,* 2007). Two such hormones are follicle stimulating hormone (FSH) and luteinizing hormone (LH), collectively known as gonadotropins. Both hormones are glycoproteins (i.e. a protein where short carbohydrate chains are attached to certain amino acids), synthesised by specialised cells (gonadotrophs) in the anterior pituitary, and act to regulate spermatogenesis and oogenesis, gonadal hormone secretion and the maintenance of gonad structure (Hinson *et al,* 2007). Gonadotropin secretion from the anterior pituitary is controlled by positive and negative feedback loops which are facilitated by a neuropeptide made in the hypothalamus, viz. gonadotropin-releasing hormone (GnRH): GnRH binds to specific receptors in the anterior pituitary to stimulate the secretion of gonadotropins (King & Millar, 1997). The importance of GnRH for reproduction was elegantly demonstrated in studies on the genetically mutated hypogonadal *(hpg)* mouse. The *hpg* mouse has a 33.5 kb deletion on chromosome 14 in the region coding for the GnRH precursor, hence the peptide is not produced; in humans, this deletion can occur on chromosome 8 (Williamson *et al,* 1991). The mutation causes depleted LH and FSH levels and the failure of gonad development after birth, the overall effect of which is the inhibition of reproductive activity (Charlton, 2004; Ebling, 2005). Tissue grafts of brain matter rich in GnRH neurons applied to the brains of *hpg* mice fully restored the ability to reproduce (Charlton, 2004). GnRH is regarded as the "master hormone" that governs reproductive function since, in the absence of this neuropeptide, reproduction cannot occur.

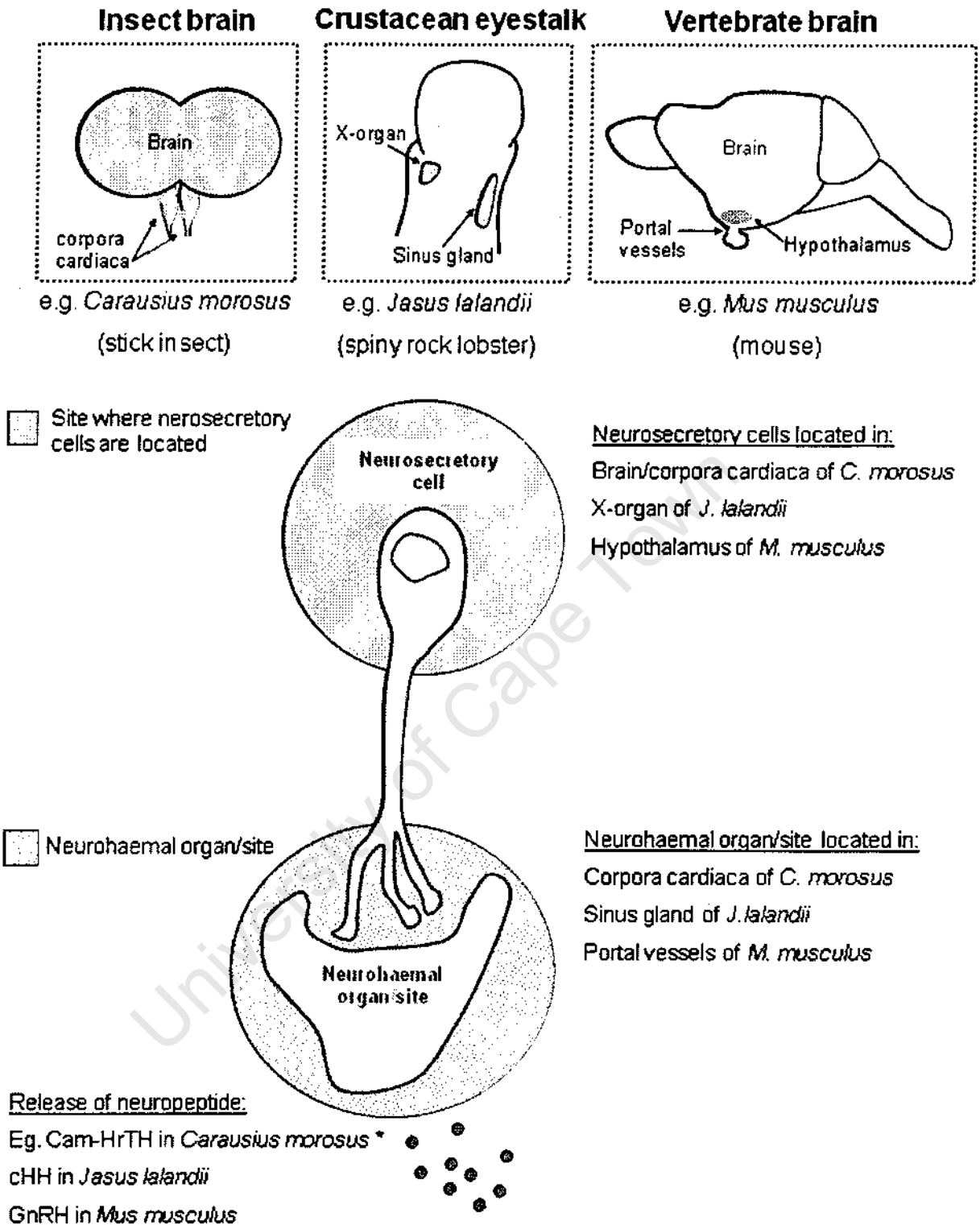


Figure 1: A schematic diagram of the generalised neuroendocrine systems in arthropods and vertebrates. Typically, neurosecretory cells reside in one region, such as the brain or X-organ of the crustacean eyestalk (shaded region) and secrete neuropeptides into a neurohaemal organ or site, such as the corpora cardiaca in insects, the sinus gland in crustaceans or the portal vasculature in vertebrates (stippled region). Adipokinetic hormones (and hence Cam-HrTH), which are involved in insect metabolism, are produced by neurosecretory cells within the corpora cardiaca.

1.3. Gonadotropin releasing hormone

1.3.1. GnRH structure and diversity

GnRH is a decapepeptide (i.e. 10 amino acid residues long) capped by an amidated (-NH2) C- terminus and an N- terminus which is blocked by a pyroglutamate (pGlu) (Millar, 2006). To date, 26 unique structural variants of this hormone have been isolated: 14 from vertebrate classes, 10 from protochordates (including one GnRH-like peptide) and two from the molluscs *Octopus vulgaris* and *Aplysia californica* (Fig. 2; Iwakoshi *et al,* 2002; Millar *et al,* 2008, Zhang *et al,* 2008). Each natural analog is named after the animal(s) or organ in which it was initially discovered. For example, the first form of GnRH to be characterised was isolated from the hypothalami of sheep and pigs, and the resulting elucidated sequence (Schally *et al,* 1971a; Schally *et al,* 1971b) was termed mammalian GnRH I (mGnRH, Fig. 2). However, it is now well known that mGnRH is found in a variety of animals, including non-mammalian species such as amphibians, jawed fish and the basal chordate amphioxus (King & Millar, 1997; Tello & Sherwood, 2009). An interesting variant of mGnRH has a hydroxyproline at position 9 ([Hyp ^{9}] GnRH) which differs from proline by the addition of a hydroxyl group (OH) to the amino acid (Gautron *et al,* 1991). To date, [Hyp^{9}] GnRH has been found in rats, hamsters and frogs (Somoza *et al,* 2002). The most widely distributed form of GnRH in vertebrates is called chicken GnRH II (cGnRH II). cGnRH II has been isolated from the brains of birds, amphibians, reptiles, bony, cartilaginous and jawed fishes, as well as from some mammals (King & Millar, 1997). It has also been established that multiple forms of GnRH can be found in a single animal (Somoza *et al,* 2002).

Most GnRH research has been conducted using vertebrate models, however, new data suggest that GnRH structures are also present in invertebrates. Perhaps the most conclusive evidence for the presence of GnRH in the invertebrate lineage comes from the octopus (Kah *et al,* 2007). Octopus GnRH, which was identified by chemical means and sequenced by mass spectrometry from the brain of *Octopus vulgaris,* consists of 12 amino acids and shares the pGlu and amidated terminal structures with that of other GnRHs (Fig. 2, Iwakoshi *et al,* 2002), while the organisation of octopus

precursor GnRH is also similar to the proGnRHs found in vertebrates (Kah *et al,* 2007). Less is known about GnRH in other invertebrates, although GnRH-like peptides have been identified in other molluscs (reviewed in Tsai, 2006), such as mussels, oysters, squid (Amano *et al,* 2008), the sea hare *Aplysia californica* (Zhang *et al,* 2000; Zhang *et al,* 2008) and cnidarians, but the exact function of the peptides remains unknown (Tsai, 2006). A GnRH-like peptide has also been cloned from the nematode *Caenorhabditis elegans* (Lindemans *et al,* 2009) and will be discussed in further detail in Section 1.3.6. A group of GnRH-like peptides that are steadily gaining interest from GnRH researchers is the adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family of peptides. These peptides are found in two different taxa with AKH found in insects and RPCH found primarily in crustaceans (Gäde & Marco, 2006). AKH is a hormone involved in the mobilisation of lipids in insect metabolism and was first isolated from the locust *Locusta migratoria.* This form is termed Locmi-AKH I, although multiple other forms have since been discovered and are thoroughly reviewed in Gäde (2009). The hormones may also be hypertrehalosaemic (HrTHs: increasing the level of the sugar trehalose in the haemolymph) or hyperprolinaemic (increasing the level of proline available for metabolism). AKH is particularly interesting as it is synthesised by neurosecretory cells of the corpora cardiaca/corpora allata in insects, a system which is functionally analogous to the hypophyseal-portal system in vertebrates. AKH also has a similar amino acid structure to GnRH (Fig.3). RPCH controls colour change in crustaceans by moving pigment granules within specialised colour cells (chromatophores), although it is has also been found in the green stink bug species *Nezara viridula* in which it has a lipid mobilising function (Gide *et al,* 2003). The complete primary structure of RPCH was first identified from the shrimp *Pandalus borealis* and is termed Panbo-RPCH. Another form of RPCH, identified in the water flea *Daphnia pulex* (termed Dappu-RPCH) is structurally different from Panbo-RPCH at three amino acid positions and has no pigment concentrating effect in the shrimp but does mobilise lipids in *N viridula* (Marco & Gäde, 2010). Although they occur primarily in different taxa, AKH and RPCH are grouped together because of their striking structural similarity (Fig. 3) as well as their ability to be cross active in the reciprocal systems (Gäde & Marco, 2006).

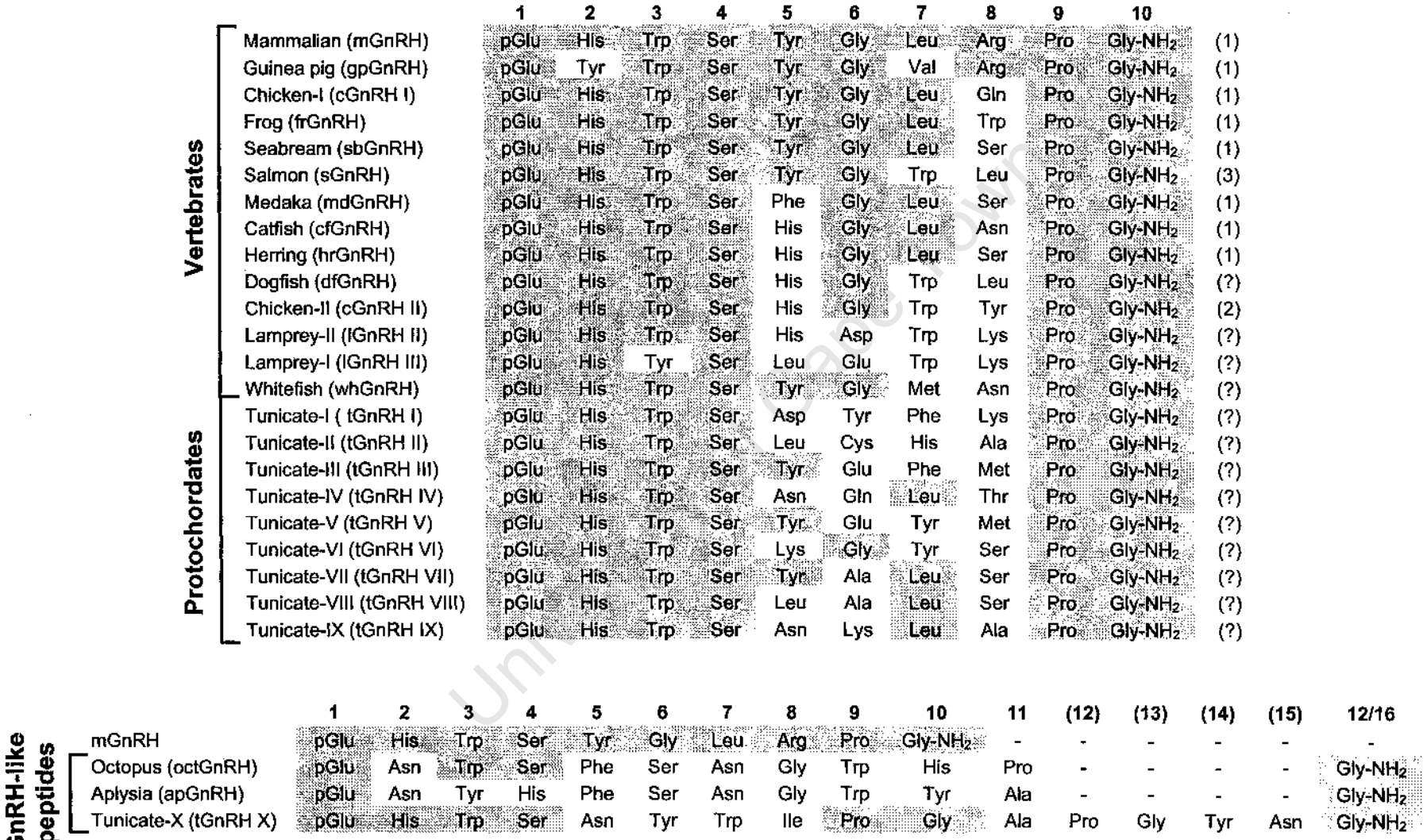


Figure 2: Amino acid sequences of the 23 naturally occurring structural variants of GnRH and 3 GnRH-like peptides which have been characterised to date. The grey boxed regions indicate residues which are highly conserved when compared to mGnRH. The numbers in brackets indicate the nomenclature according to functional and evolutionary grouping (i.e. GnRH 1, 2 or 3) while '?' indicates unknown evolutionary groupings. Modelled after Somoza *et al*, 2002; Kah *et al*, 2007, Millar *et al*, 2008 and Okubo & Nagahama, 2008.

	1	2	3	4	5	6	7	8	9	10	
mGnRH	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly	- NH_2
Panbo-RPCH	pGlu	Leu	Asn	Phe	Ser	Pro	Gly	Trp	-	-	- NH_2
Locmi-AKH I	pGlu	Leu	Asn	Phe	Thr	Pro	Asn	Trp	Gly	Thr	- NH_2
Cam-HrTH I	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp*	Gly	Thr	- NH_2
Cam-HrTH II	pGlu	Leu	Thr	Phe	Thr	Pro	Asn	Trp	Gly	Thr	- NH_2

Figure 3: The amino acid sequences of RPCH, an AKH and Cam-HrTH hormones in comparison to mammalian GnRH. Note the similarities in sequence length and terminal end structure. * indicates mannosylation of Trp^8 in Cam-HrTH I.

While structural variants do occur, if one considers the 600 million years that the GnRH peptide has been in existence, it has experienced few modifications (Millar *et al,* 2008). Chain length and N- (pGlu-His-Trp-Ser) and C- (Pro-Gly-NH2) terminal structures are conserved from protochordates to man (Millar, 2006), suggesting that these features may be important for the correct functioning of the peptide and, hence for the reproductive success and evolutionary survival of an organism and its species, considering the role of GnRH in reproduction exclusively (Sherwood *et al,* 1993). A comparison of the amino acid residues of GnRHs (Fig. 2) reveals that position 8 is the most variable, followed by positions 6, 5, 7 and 3 (King & Millar, 1997).

1.3.2. Nomenclature and evolution

At present, there is no official way in which to name GnRH analogs. Typically, the GnRH is named after the animal in which it was first discovered, however, complication arises when more than one form of GnRH is found in a single species. GnRH nomenclature can be viewed from two different perspectives, either in terms of gene evolution and function or in terms of amino acid structure. There are 26 different variants of GnRH (Millar *et al,* 2008), however, while structurally distinct, many of these variants perform the same function. For example, mammalian and guinea pig GnRH (mGnRH and gpGnRH) do not share the identical primary structure, but they perform the same function (Okubo & Nagahama, 2008); the genes that encode these hormones are considered to be orthologous i.e. genes from different species that evolve from a common ancestor and retain their function (Kah *et al,* 2007).

GnRHs can also be divided into three paralogous (i.e. genes that are related by duplication but can evolve new functions) groups, GnRH I, II and III. This division is based on gene evolution (Okubo & Nagahama, 2008) and, partially on the function, distribution in the brain and embryological origin of the GnRH neurons (Fernald & White, 1999; Dubois *et al,* 2002). GnRH II is conserved from bony fish to man, while GnRH I is variable, therefore the naming of different GnRH analogues applies only to GnRH I. GnRH I neurons arise in the olfactory placode: a thickening of embryonic epithelial tissue that later gives rise to the nasal epithelium and anterior pituitary (Whitlock, 2005). GnRH I is the primary form of the peptide found in the forebrain in the region of the preoptic area, hypothalamus and the pituitary, and its main role is in reproduction and in stimulation of the release of pituitary gonadotropins (Okubo & Nagahama, 2008). Of the structural forms known in vertebrates, eight of these are currently grouped together as GnRH I (Fig. 2). GnRH II neurons arise from the region of the mesencephalon, originating from the neural crest (Kah *et al,* 2007). The GnRH II gene is present in all teleost, amphibian, reptile and bird species studied to date but for unknown reasons has been inactivated (i.e. gene present but gene product absent) or deleted (i.e. total loss of the gene) in many mammalian species (Okubo Nagahama, 2008; Stewart *et al,* 2009). For example, humans posses a gene encoding GnRH II but lack a functional GnRHR II while in mice, the GnRH II gene is entirely absent (Millar, 2006; Okubo & Nagahama, 2008). GnRH II genes that produce functional gene products have only been identified in a few mammalian species, including humans, rhesus monkeys, various marsupials (metatherians, pouched mammals) such as the opossum *(Monodelphis domestica)* and the bandicoot *(Isoodon macrourus)* and only a few ancient placental (eutherian) species such as the musk shrew *(Suncus marinas)* (King & Millar, 1997; Dees *et al,* 1999). The neurons which produce GnRH II typically reside in nuclei of the mid brain and transport their products to various other parts of the brain (Millar, 2006; Fernald & White, 1999). The exact function of this paralog is unclear but it is thought to be involved in the coordination of reproduction and nutrient intake/energy balance (Schneider & Rissman, 2008). GnRH analogs classed as GnRH III have only been identified in some species of the teleost (Okubo & Nagahama, 2008). These neurons are thought to migrate from the olfactory placode (Whitlock *et al,* 2006; Kah *et al,* 2007) primarily to the terminal nerve/ventral telencephalon as well as other brain areas (Fernald & White, 1999). The function of GnRH III is largely unknown, although evidence

suggests that it is involved in reproductive behaviours such as nest-building and aggression (Okubo & Nagahama, 2008).

It is thought that, in evolutionary terms, the origin of the paralogous forms of GnRH arose by gene duplication (Dubois *et al,* 2002). The ancestral form of GnRH I in vertebrates is most certainly mGnRH (Okubo & Nagahama, 2008) as the amino acid sequence remains conserved in mammals, with only two amino acid substitutions to the ancestral type (Tyr replaces His at position 2 and Val replaces Leu at position 7) to form guinea pig GnRH (Okubo & Nagahama, 2008). The high degree of evolutionary stability of the peptide in mammals is thought to be as a consequence of the conserved evolution of its receptor (Kah, 2007, also Section 1.3.6). In the non-mammalian lineage, however, mGnRH underwent great structural diversification, while the GnRH II and GnRH III systems remain largely unchanged (Okubo & Nagahama, 2008). The current study is focussed on the hypothalamic form of GnRH (i.e. GnRH I), particularly mGnRH, therefore, other forms will not be discussed in detail.

1.3.3. GnRH I distribution

There are some interspecies differences in GnRH distribution, however, in vertebrates, GnRH I neurons typically migrate from the olfactory placode during embryological development to the forebrain, particularly in the regions of the preoptic area and the hypothalamus (Dubois *et al,* 2002). Immunocytochemical and radioimmunoassay studies show that in most animals, GnRH is typically found in greatest abundance in the median eminence, where the peptide is stored in nerve terminals prior to its release (Clarke & Pompolo, 2005). GnRH has also been found in the organism vasculosum of the lamina terminalis (OVLT, a small region in the preoptic area that is in association with the ventricles) of various mammals (Millar, 2006) but the function of the projections in this region remains unknown (Clarke & Pompolo, 2005). In some species, such as the sheep, a small population of GnRH neurons resides in the arcuate nucleus of the mediobasal hypothalamus (Clarke & Pompolo, 2005). GnRH I has also been identified by molecular methods, such as RT-PCR (reverse transcription polymerase chain reaction) in a host of different organs

and non-neural tissues outside of the brain of several mammalian and non-mammalian vertebrate species, although the function is unknown (Sherwood *et al,* 1993). GnRH and GnRHR I have been detected in tissues at various levels of the reproductive system, including the gonads (male and female), and mammalian breast tissue and placenta (Millar, 2006). GnRH I is also expressed in the human kidney and bone marrow (White *et al,* 1998) and the rat pancreas (Sherwood *et al,* 1993; Wang *et al,* 2001), and GnRHR I has been identified in the liver of goldfish (Habibi & Pati, 1993) and retina of voles (Wirsig-Wiechmann & Wiechmann, 2002). The distribution of GnRH (and its receptors) in such diverse parts of the body suggests that this hormone is not involved in regulating the synthesis of pituitary hormones exclusively, but has functions both within and outside the central nervous system.

1.34. Biosynthesis and storage

GnRH synthesis, like that of all other secreted peptides, begins in the cell nucleus. First, the GnRH gene is transcribed to RNA and once the RNA molecule has been processed, the mature mRNA migrates to the cell cytoplasm. The translation of mRNA into proGnRH (the precursor form of GnRH) then occurs in the Golgi apparatus and the endoplasmic reticulum (Yin & Gore, 2006). The GnRH precursor is comprised of three sections (Fig. 4): a signal sequence, the GnRH decapeptide and a 56 amino acid sequence known as gonadotropin-releasing hormone associated peptide (GAP, King & Millar, 1997). GAP is separated from GnRH by 3 amino acids (Gly-Lys-Arg) and during hormone processing, proGnRH and GAP are physically separated by proteolytic cleavage at the dibasic part of this site (Lys-Arg). Both GAP and the GnRH decapeptide are stored in the secretory vesicles prior to release. The role of GAP is currently unknown, but it is thought to aid in folding the precursor for accurate cleavage (King & Millar, 1997; Millar, 2006). After the GnRH peptide has been cleaved from GAP, the hormone undergoes further processing by cyclisation of Gln to pyro Glu at the N-terminus, and amidation of Gly at the C-terminus, where the Gly of the processing site becomes the NH2 donor (Millar, 2006).

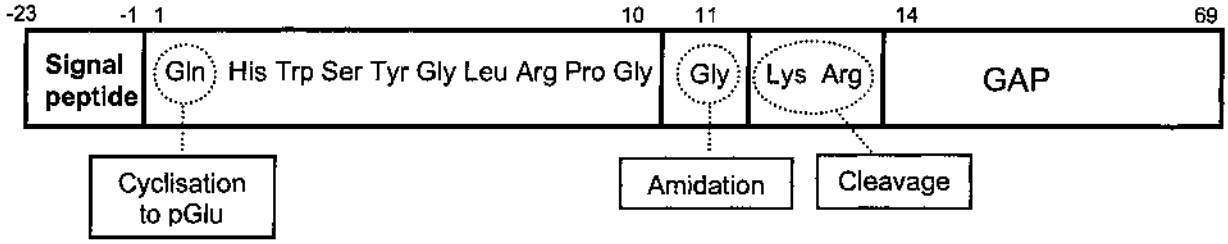


Figure 4: A schematic representation of the GnRH prohormone. In mGnRH, the precursor consists of a 23 amino acid precursor signal peptide followed by the GnRH decapeptide. Cleavage of the signal peptide reveals the Gln which cyclises, spontaneously or enzymatically, to pGlu. An additional Gly at position 11 amidates to form a NH_2 at the C-terminus of GnRH. Lys-Arg form the dibasic part of the cleavage site and are followed by the 56 amino acid sequence of GAP. Modelled after Millar, 2006.

1.3.5. GnRH genes

The mammalian GnRH gene was first described in humans by Seeburg and Adelman (1984). Since then, the DNA coding sequences for different GnRHs in numerous other species have been described (Sower *et* al, 2009). The GnRH gene of all animals studied to date shows a high degree of homology. The gene is approximately 4300 base pairs long and comprises of three large introns (non-coding part of the gene) and four relatively short exons (coding part of the gene). The size of the introns and exons may vary across species, but the overall modular organisation remains unchanged (reviewed by Fernald & White, 1999). The first exon encodes the 5'- untranslated region exclusively while the second and third exons encode proGnRH. This includes a signal peptide sequence at the N-terminus, the GnRH peptide, the proteolytic processing site and GAP. The fourth exon encodes the C-terminus of GAP and the 3'-untranslated region (Fernald & White, 1999).

1.3.6. Receptor and cell signalling

The GnRH receptor is a G-protein coupled receptor (GPCR) with seven transmembrane domains connected by three hydrophilic intracellular and extracellular loops (Fig. 5), (Schneider *et al*, 2006). All forms of the receptor, except mammalian GnRHR I, have a tail at the C-terminus which is responsible for rapid desensitization of the receptor (Millar, 2006; Tello & Sherwood, 2009). Desensitization is the process whereby prolonged exposure of the receptor to GnRH leads to the inhibition of gonadotropin release. When the GnRHR I is activated by GnRH, protein kinases are stimulated to phosphorylate (i.e. add a phosphorous group) intracellular domains (particularly the C-terminal tail) of the receptor. This results in uncoupling of the receptor from the G-protein, which inhibits all the intracellular pathways involved in second messenger production and LH release, and finally the receptor is internalised. Since the C-terminal tail is absent from the mammalian GnRHR I, the receptor does not desensitise very quickly, promoting a prolonged surge of LH required for ovulation (Millar, 2006).

When binding to the GnRH receptor, the terminal ends of the GnRH peptide are in close approximation to one another, forming a ß-II type fold, as they are directly involved in receptor activation (Millar, 2006) (Fig. 6). Binding of GnRH to the receptor triggers guanosine triphosphate (GTP) - binding proteins (specifically the subunit $G_{\alpha q/11}$) which activate the enzyme phospholipase C (PLC). PLC hydrolyses phosphatidylinositol 4, 5-biphosphate (PIP_2) to the second messengers inositol triphosphate (IP_3) and diacyglycerol (DAG) (Millar, 2006). Subsequently, $1P_3$ binds to receptors on the endoplasmic reticulum to release stored calcium ions (Ca^{2+}) which depolarise the cell and initiate gonadotropin release by exocytosis from vesicles within the gonadotroph. The Ca^{2+} ions from the endoplasmic reticulum are particularly important for initiating the rapid spike of LH required for ovulation, however, prolonged release of LH is still necessary for gonadal and gamete development. This sustained release of LH is mediated by protein kinase C (PKC), which is activated by DAG. PKC then activates L-type voltage operated Ca^{2+} channels, resulting in the influx of extracellular Ca^{2+} which replenishes the intracellular ion stores to prolong LH release (Fig. 5, Millar, 2006; Schneider *et al*, 2006). The release of FSH seems to be more closely linked to the amount of FSH in the gonadotroph available for secretion while LH secretion depends entirely on GnRH pulses. The stimulation of FSH and LH is also differentially influenced by GnRH pulse frequency. Low GnRH pulse frequencies preferentially stimulate FSH secretion while faster (e.g. hourly) GnRH pulse frequencies promote LH secretion (Millar, 2006)

The GnRH peptide interacts with specific receptors on the surface of pituitary gonadotrophs to stimulate the release of gonadotropins. Thus far, three forms of GnRH receptor (GnRHR) have been identified in vertebrates: type I, II and III, which parallel the evolution of GnRH I, II and III (Millar, 2006). All vertebrate GnRHs stimulate gonadotropin release in all vertebrates studied to date, however, the specificity of the different receptors and hence the magnitude of the gonadotropin response, vary. For example, GnRH I has a high affinity for the GnRHR I, whereas, other GnRHs bind with lower affinity to this receptor (King & Millar, 1997). The pre-vertebrate condition of GnRH and GnRH receptors has been studied in the invertebrate, amphioxus (a basal chordate). Using mass spectrometry, a peptide sequence identical to mGnRH, has been elucidated from the European amphioxus,

Branchiostoma lanceolatum (Chambery *et al,* 2009). Subsequently, Tello & Sherwood (2009) showed that four GnRH receptors are encoded in the genome of *Branchiostoma floridae,* the North American amphioxus species. These four GnRHR sequences were cloned, expressed in tissue culture and exposed to various forms of GnRH and GnRH related peptides, namely: GnRH I, GnRH II, octGnRH and silkworm AKH. Based on the affinity of the different ligands for the receptors, as well as the receptor sequence structure, the GnRHRs cluster into two distinct phylogenetic pairs: one pair shows high sequence similarity with vertebrate GnRHRs while the other pair shows a close phylogenetic relationship with octopus GnRH-like receptor and the AKH receptor. GnRHR orthologs have also been cloned from the mollusc *Crassostrea gigas* (Rodet *et al,* 2005), the fruit fly *Drosophila melanogaster* (Staubli *et al,* 2002) and the nematode *C. elegans* (Lindemans *et al,* 2009). Interestingly, the ligand of the GnRH-like receptor in *D. melanogaster* is not a vertebrate GnRH, but rather the GnRH-like peptide Drm-AKH *(Drosophila* AKH; Staubli *et al,* 2002). The work by Lindemans *et al* (2009) shows that the ligand for the GnRH receptor ortholog in *C. elegans* (which has similarities to both the GnRH and AKH receptors and is named Ce-GnRHR) is an AKH-GnRH-related peptide (i.e. shows homologies between the AKH and GnRH peptide), and that signalling through Ce-GnRHR modulates egg laying behaviour in *C. elegans.* This research suggests a common origin of GnRH and AKH and implies a crucial link between vertebrates and invertebrates in terms of the structure and functioning of this GnRH/AKH signalling system (Lindemans *et al,* 2009).

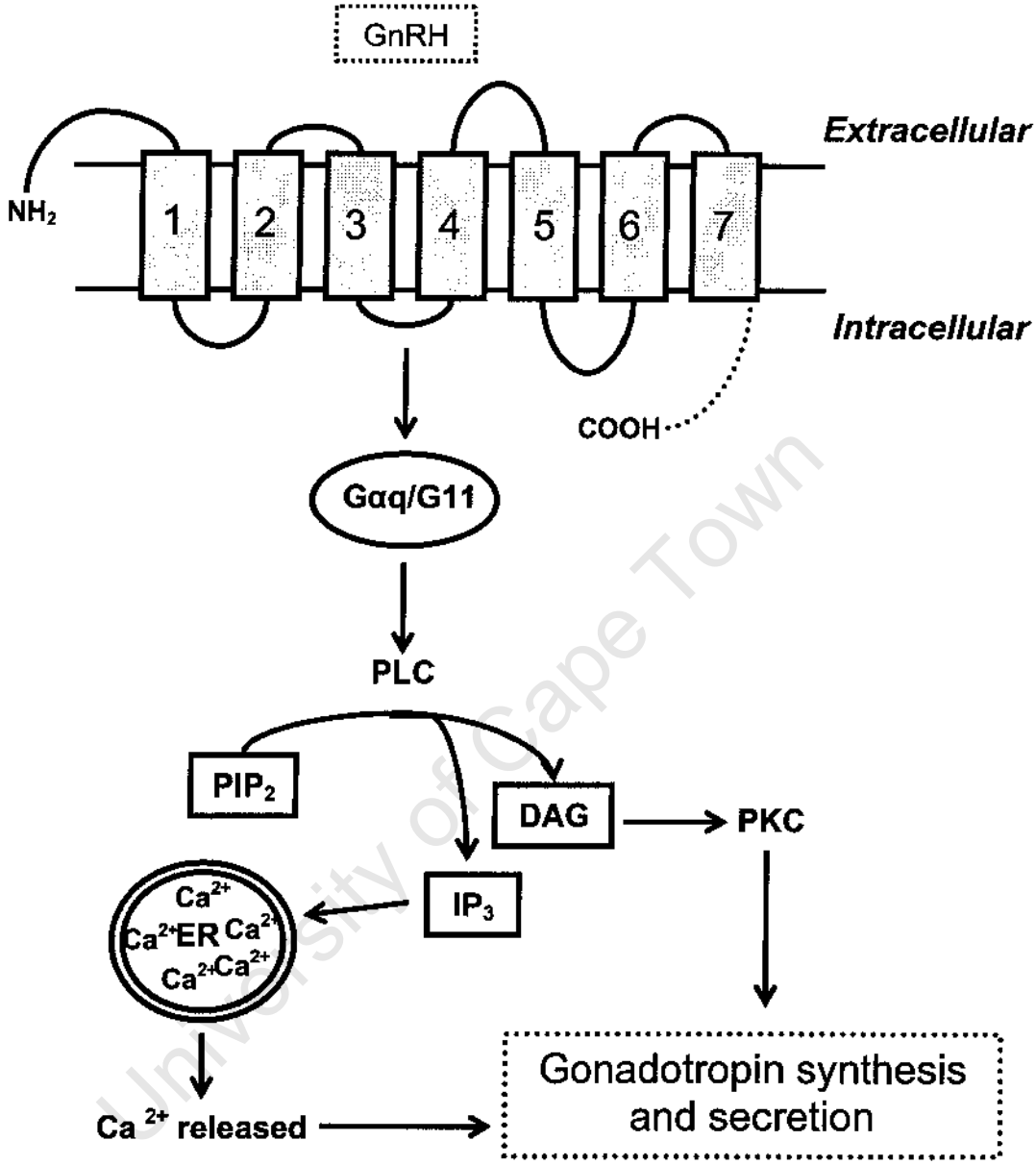


Figure 5: The basic signalling pathway of GnRH. Note the 7 transmembrane regions and the extra and intracellular loops of the receptor. The COOH tail (dotted line) is found in all receptor types except in the mammalian GnRHR. When GnRH binds to the receptor, the G-proteins G_q and G_{11} are triggered to activate phospholipase C (PLC). PLC converts phosphatidylinositol 4, 5-biphosphate (PIP_2) to the second messengers inositol triphosphate (IP_3) and diacyglycerol (DAG), which in turn stimulate calcium release from the endoplasmic reticulum or activate protein kinase C (PKC). PKC activates L-type voltage operated Ca^{2+} channels, resulting in the influx of extracellular Ca^{2+} which replenishes the intracellular ion stores to prolong LH release. Modelled after Millar, 2006

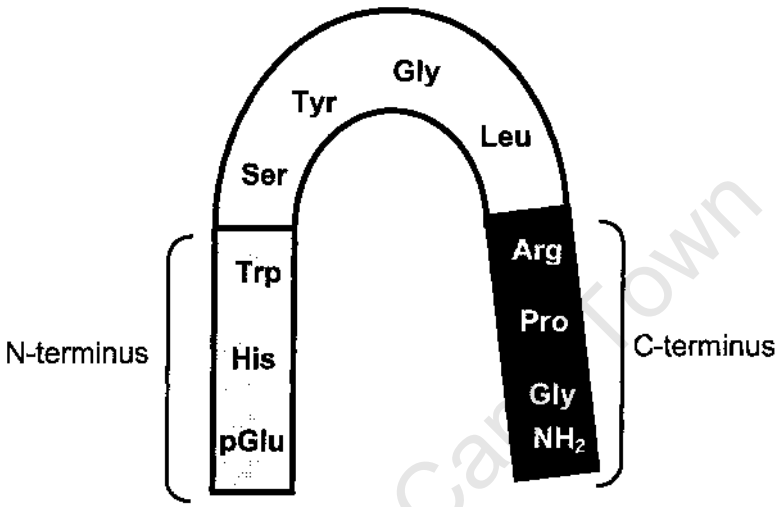


Figure 6: Representation of mGnRH in the folded form when bound to the GnRH receptor. Both N- (light grey) and C- (dark grey) termini are involved in receptor binding, while the N- terminal is involved in receptor activation exclusively. Adapted from Millar, 2005.

1.3.7. The role of GnRH in female reproductive physiology

The reproductive cycle of the mammalian female is divided into two phases, viz. the follicular phase and the luteal phase, the length of which is species dependent (Bentley, 1998). As illustrated in Fig. 7, during the follicular phase, GnRH is released from the hypothalamus and acts on the anterior pituitary to initiate FSH and LH secretion. It is during this phase that an oocyte (egg) contained within a primary follicle of the ovary begins to mature (primarily under the influence of FSH) and develop into a Graafian follicle i.e. a single oocyte surrounded by specialised granulosa cells which secrete estradiol (an estrogen). The elevated estradiol levels act in a positive feedback loop on the hypothalamus to increase the frequency of GnRH release. Typically, GnRH is released in regular, low amplitude pulses in order to maintain pituitary sensitivity to the hormone (Kalra *et al*, 1997; Millar, 2006). If the GnRH flow was continuous, the number of active GnRH receptors would decrease (i.e. down regulate) and the overall sensitivity of the gland to GnRH would be diminished (Fox, 2004). Late in the follicular phase, there is an increase in the frequency of GnRH release. This causes a surge of LH secretion from the anterior pituitary, which triggers ovulation: the rupturing of the Graafian follicle and the release of the oocyte into the uterine tube.

Ovulation is followed by the luteal phase. The high level of LH converts the ruptured Graafian follicle into an endocrine structure known as the corpus luteum, which secretes progesterone. Progesterone acts on the hypothalamus, forming a link in a negative feedback loop which results in decreased GnRH secretion and consequently a decrease in FSH and LH secretion. Towards the end of the luteal phase, if pregnancy has not occurred, the corpus luteum regresses, thus reducing plasma progesterone levels and GnRH secretion returns to its usual rate. At this point, a new cycle begins (Fox, 2004).

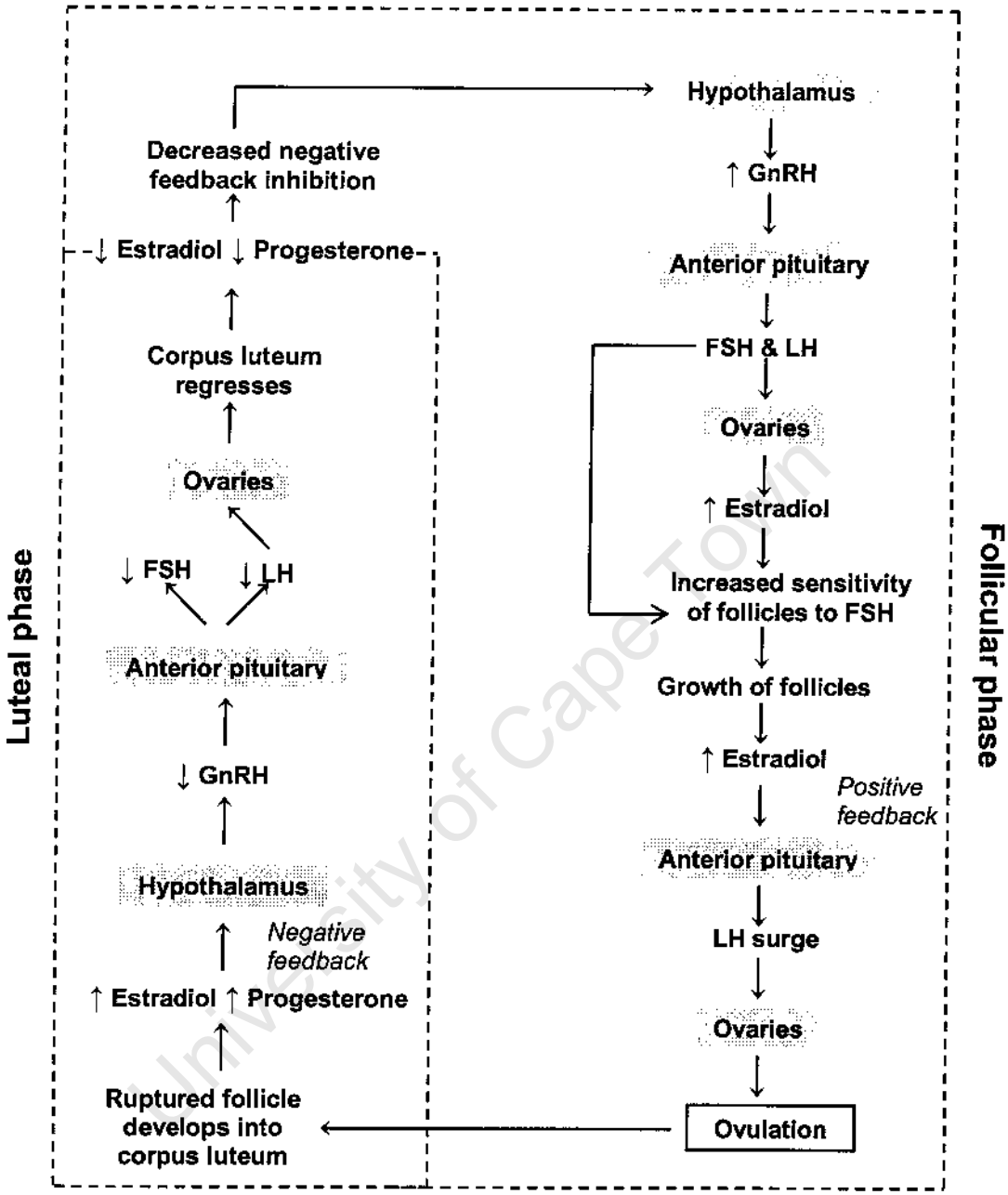


Figure 7: Generalised outline of the female reproductive cycle. Note the two phases, the luteal phase and the follicular phase. Organs/brain regions are indicated by shaded text. Adapted from Fox, 2004

1.3.8. The role of GnRH in males

Since the focus of this study is on the female reproductive system, the role of GnRH in male reproduction will only be dealt with briefly. As in the female reproductive system, GnRH is released in a pulsatile fashion which initiates the release of gonadotropins. This is important for stimulating the onset of puberty, and once sexually mature, the primary target organs for FSH and LH in the mammalian male are the testes. The testes are comprised of two main components: the seminiferous tubules and interstitial tissue. Spermatogenesis, which occurs in the seminiferous tubules, is facilitated by FSH. The interstitial tissue consists of Leydig cells that secrete testosterone, the hormone responsible for sexual maturation. LH targets the Leydig cells and stimulates the release of testosterone (Fox, 2004).

1.4. Reproductive suppression and cooperative breeding

Cooperative breeding is a reproductive system where individuals from a social group aid in the care of offspring, even if the young are not their own (Hatchwell & Komdeur, 2000; Holmes *et al,* 2009). In cooperatively breeding communities, reproduction is limited to a few breeding animals and in extreme cases to only one breeding pair. The reproductive capabilities of the remaining colony members are suppressed and they fulfil a 'helper' role (Faulkes & Bennett, 2001). Helpers are generally related to breeding individuals and they accrue "fitness" indirectly by ensuring that the genes of their relatives are successfully passed down to the young that they help (Hatchwell & Komdeur, 2000). Sociality and large group size confer certain benefits in terms of foraging and colony defense, but there is a direct negative impact on individuals due to limited breeding opportunities or because the reproductive capabilities of some are suppressed by the dominant individuals (Hatchwell & Komdeur, 2000).

Suppression of reproduction has been reported in a number of social mammalian species. Chemical signals, such as pheromones, and behavioural interactions are both employed by social species to suppress the reproduction of other individuals in the group (Faulkes & Abbott, 1993). Behavioural suppression occurs either by incest

avoidance (the innate response by most species to resist reproducing with close relatives), or by antagonistic behaviour from the dominant individuals. The latter form of behavioural suppression includes interference of the dominant animal with the courtship or mating behaviours of subordinates and infanticide (killing the offspring of subordinates) (Faulkes & Abbott, 1993). For example, subordinate male dwarf mongooses *(Helogale parvula)* are actively inhibited from breeding with the dominant female by aggressive behaviour from the dominant male (Keane *et al,* 1994) and subordinate grey wolves *(Canis lupis)* are inhibited from reproduction by interference from the breeding pair (Asa & Valdespino, 1998). Physiological suppression occurs either as a result of behavioural interactions which induce stress in the subordinates, or is mediated by chemical signals/urinary pheromones. Both mechanisms inhibit the hormonal pathways necessary for reproduction (Faulkes *et al,* 1990a). For example, female zebra fish *(Danio rerio)* use waterborne pheromones to suppress egg production of less dominant females (Gerlach, 2006), while chemical cues from dominant female marmoset monkeys *(Callithrix jacchus)* suppress LH secretion and ovulation in subordinate females (Barrett *et al,* 1990; Barrett *et al,* 1993).

1.4.1. Social suppression and the female reproductive system

In terms of physiology, suppression of female reproduction can occur at a number of different levels (Faulkes *et al,* 1990a). For example, sexual maturation is delayed in the prairie deer mouse *(Peromyscus maniculatus bairdii,* Wasser & Barash, 1983), yellow-bellied marmots *(Marmota flaviventris, Oli &* Armitage, 2003) and in Mongolian gerbils *(Meriones unguiculatus,* Clark & Galef, 2001). Reproduction may also be inhibited by a block or delay of sexual receptivity as seen in dwarf mongooses *(Helogale parvula)* (Keane *et al,* 1994). Ovulation can be delayed or blocked (as in gelada baboons, *Theropithecus gelada,* Wasser & Barash, 1983) and may be the result of decreases in LH pulse frequency or amplitude, or because of inhibition at the level of GnRH. Even if the female is reproductively receptive and copulation occurs successfully, suppression can still occur after fertilization, by failure of implantation, spontaneous abortion or early postpartum mortality.

A major factor which drives suppression of reproductive function is that of stress (Dobson & Smith, 2000). The female reproductive system is governed by the hypothalamic-pituitary-ovarian axis, however, this axis is closely linked with the hypothalamic-pituitary-adrenal axis (HPA) which mediates stress responses (Kalantaridou *et al,* 2004). When homeostatic balance is challenged (i.e. stressed) by factors such as starvation, fear or anxiety, the body releases a host of hormones to rectify the imbalance (Sapolsky, 2000; Creel, 2001) and attention is drawn away from sexual and feeding behaviours in order to deal with the stressor and ensure survival of the individual (Reeder & Kramer, 2005). The HPA axis can therefore exert an inhibitory force on female reproduction.

1.5. Mole rat reproduction: continuum of sociality in Bathyergidae

Members of the mole rat family Bathyergidae are found exclusively in Africa and within the 5 bathyergid genera, there is a continuum of sociality from a lifestyle that is solitary to one that is entirely colonial. Solitary mole rat species are of the genera *Bathyergus, Georychus* **or** *Heliophobius,* **while** *Cryptomys* **and** *Heterocephalus* **are genera containing social species (Honeycutt** *et al,* **1991a, Faulkes & Bennett, 2001). The Cape mole rat** *(Georychus capensis)* **and the Cape Dune mole rat** *(Bathyergus suillus)* **are solitary species which breed seasonally (Jarvis & Bennett, 1991). Mashona mole rats** *(Cryptomys darlingi)* **live in small colonies of approximately 7 individuals, including one breeding male and female. In this species, reproduction between non-breeders is innately suppressed by behavioural means, namely incest avoidance. Mashona mole rats are obligate outbreeders (i.e. will only breed with individuals that are from another, non-related colony) and this trait persists even if the breeding pair (and therefore the source of reproductive suppression) is removed from the colony (Bennett** *et al,* **1997). Damaraland mole rats** *(Cryptomys damarensis)* **live in slightly larger colonies than Mashona mole rats, averaging at 11 colony members, with the largest colony reported to have 41 individuals (Faulkes & Bennett, 2001). Here, too, there is an unequal distribution of reproduction within the colony, with only a single breeding female and 1 to 2 breeding males. In** *C. damarensis* **there are both behavioural and physiological mechanisms which bring about suppression of reproduction. Breeding behaviour of both sexes is suppressed by incest avoidance, but**

there is an additional inhibition of ovulation in Damaraland mole rat females to prevent pregnancy from possible sexual encounters with males that are from different colonies (Bennett *et al,* 1997). Naked mole rats *(Heterocephalus glaber)* are eusocial (a social structure that, until recently, has only been described in insects) which is defined as a group of organisms that meet the following criteria: a) a "reproductive division of labour" where different tasks, such as defense and gathering of food, are `assigned' to different members of the colony, depending on social and reproductive status, b) an overlap of generations and c) cooperative care of young (Jarvis, 1981; Lacey & Sherman, 1991; Faulkes & Bennett, 2001). All three criteria are met by the naked mole rats.

1.5.1. The naked mole rat (Heterocephalus glaber)

Naked mole rats are subterranean hystricomorphs of the mole rat family Bathyergidae (Fig. 8). They live in extensive burrow systems in east Africa, particularly Kenya, Ethiopia and Somalia (Honeycutt *et al,* 1991a) and feed on tubers and bulbs which are scarce in this arid region. As their name suggests (˙different headed, smooth'), naked mole rats are hairless except for vibrissae (long, stiff, sensory hairs) which are distributed sparsely on their otherwise naked bodies (Honeycutt *et al,* 1991a). Naked mole rats are well adapted to a burrowing lifestyle. They have reduced eyes and small ears which perceive sound in a range restricted to lower frequencies that are more suited to transmission through soil (Catania & Remple, 2002). Naked mole rats also have lips which are able to close behind their large incisors, thus preventing soil from entering their mouths while digging. These so-called extra-buccal incisors are also used in defense (Honeycutt *et al,* 1991a; Lacey *et al,* 1991). Because of their near blindness, naked mole rats have sensory and motor specialisations related to touch, such that an abnormally large proportion of the brain cortex is devoted to this sensation (Catania & Remple, 2002). Aside from their bizarre appearance and unusual habitat, naked mole rats are perhaps best known for their extreme form of sociality and reproduction. Naked mole rats display a high reproductive skew i.e. an unequal distribution of reproduction among the members of the social group (Faulkes & Bennett, 2001), where breeding is restricted to a single reproductive female, the queen, and one to three breeding males (Jarvis, 1991). The rest of the colony members

(subordinates) are not sterile, but rather reproductively quiescent. These subordinate individuals are responsible for colony maintenance, care of young and defense of the burrow system (Jarvis, 1991; Lacey & Sherman, 1991).

It is thought that cooperative breeding in this species evolved in response to the patterns of rainfall in the arid areas where mole rats typically live. Decreased rainfall affects the distribution of food and increases the energetic "costs" of foraging and dispersal (Faulkes & Bennett, 2001). If a naked mole rat finds a tuber, it is usually far too large for a single individual to consume. Ecologists suggest that communal living may encourage "sharing" behaviour, minimising energetic costs by increasing the chances of finding food, as well as providing some degree of protection. This extreme form of colonial living is also associated with complex social organisation: naked mole rat colonies are structured in a hierarchical manner, with the breeding female holding the most dominant position (Lacey & Sherman, 1991). The status of other colony members in the hierarchy can be determined by observing how individuals pass one another when they meet in a tunnel: higher ranking individuals pass over lower ranking individuals (Lacey *et al,* 1991). Clarke and Faulkes (1997) showed that dominance was not linked to age, but rather correlated to body weight, with heavier individuals being more dominant.

Figure 8: Naked mole rats in their sleeping chamber. Scale = 0.67cm

1.5.2. Naked mole rat reproduction

In the colonies of the eusocial naked mole rat, a single female (the queen) breeds with one to three males. There is a stark contrast of anatomy and physiology between breeding and non-breeding females, where queens are much larger and heavier than their non-breeding counterparts (Jarvis *et al,* 1991). An interesting feature of naked mole rat reproduction is the elongated spine of the breeding female during her first eight pregnancies. On average, the length of the queen's lumbar vertebrae can increase by a total of 32 %, making her the longest individual in the colony. This spine elongation is thought to facilitate the production of larger litters without affecting the ability of the queen to navigate through the narrow tunnels of her burrow (Dengler-Crish & Catania, 2009). Queens also display perforate vaginas (clearly visible as a dark pink line, the vaginal closure membrane is absent or thin) and prominent nipples (Faulkes *et al,* 1990a, Jarvis, 1991). Queens are spontaneous (or cyclic) ovulators (Margolis *et al,* 1995), where ovulation occurs at regular intervals and typically does not require the presence of a male to stimulate the LH surge (in contrast to 'induced ovulators', such as the musk shrew) (Holmes *et al,* 2009). The ovarian cycle length for breeding females is approximately 27 - 38 days with follicular and luteal phases approximately 6 and 28 days, respectively.

Naked mole rats will readily inbreed and the queen needs to actively maintain the reproductive monopoly (Honeycutt *et al,* 1991b; Faulkes & Bennett, 2001). Hormone assays (of LH and progesterone) and histological studies show that both captive and wild subordinate females of this species do not have preovulatory follicles or corpora lutea present in their ovaries (Faulkes *et al,* 1990a) and hence, the levels of progesterone are undetectable. Faulkes *et al* (1990a) report that subordinates also do not ovulate due to lower levels of circulating LH relative to queens. The mechanisms underlying the reduced LH secretion and subsequent inhibition of ovulation in subordinates is poorly understood. Subordinates do possess a bioactive pool of LH which can be released if the GnRH stimulus is large enough (1 µg) (Faulkes *et al,* 1990b). However, low dose challenges (0.1 pig) of synthetic GnRH elicited little response in subordinates in comparison to the larger response in queens. Faulkes *et al* (1990b) suggest that the sensitivity of the subordinate pituitary to GnRH is reduced,

either by diminished numbers of GnRH receptors on the gonadotroph cell surface, or by some alteration of the intracellular pathways which occur after the ligand-receptor interaction. The study by Faulkes *et al* (1990b) also shows that priming of the subordinate pituitary is possible, as after four successive low dose challenges with GnRH, the LH response was equivocal to that in queens. However, the study by Faulkes *et al* (1990b) fails to disclose the form of GnRH used to conduct the experiment, and as discussed above, the gonadotropin response to GnRH stimulation depends greatly on the respective structures of GnRH and its receptor.

Because the subordinate females are not sterile, they are capable of becoming reproductively active at any time and therefore, the queen needs to actively maintain her dominant breeding position (Margulis *et al,* 1995). Initially, the physiological suppression in naked mole rats was thought to be mediated by olfactory cues. *H. glaber* is a subterranean species, and therefore their visual system is vestigial and they are heavily reliant on olfaction (Jarvis, 1991). Additionally, naked mole rats are known to make use of communal toilet chambers, the ideal site for spreading colony odour (the communal odour of colony members which helps to identify individuals from specific colonies, Lacey *et al,* 1991). The use of pheromones seemed a plausible explanation for the suppression of reproduction in naked mole rats. However, conclusive studies by Faulkes & Abbott (1993) and Smith *et al* (1997) have disputed this: in their experiments, subordinate females were prevented from directly interacting with the breeding individuals but they still had social contact with the rest of the colony members, as well as access to the communal toilet chambers, as did the queen. All of the experimental females showed signs of ovarian activation (monitored by increasing progesterone levels which indicate ovarian cyclicity) within three days of removal from contact with the breeding pair, while contact with the colony odour was still maintained. This suggested that the inhibition of ovulation in subordinate naked mole rats is maintained by physical contact with the queen rather than by pheromones.

Physical aggression is commonplace in *H. glaber* colonies and this aggression is identified by shoving behaviour, where two individuals push each other head to head resulting in one individual being pushed backwards along the tunnel. The queen is the most aggressive member of the colony and she initiates significantly more shoves per

unit time than any other individual (Clarke & Faulkes, 2001). She also preferentially directs aggressive behaviour towards females who threaten her dominant position by showing signs of reproductive activity (either at a behavioural or physiological level) (Margulis *et al,* 1995). How the queen distinguishes which subordinates are more likely to become reproductively active is currently unknown, however, olfaction may play a role as it does with other animals, although this still needs to be investigated. Interestingly, during pregnancy, when the queen is approaching parturition, subordinates may begin to develop nipples. This is possibly due to the queen being less agile in her highly gravid state, and thus her physical control over the colony weakens (Jarvis, 1991). If the queen dies, or is removed from the colony, the suppressive force of the queen on subordinate reproduction is lifted and a period of social instability ensues. Several non-reproductive females will begin to develop sexually and may show morphological and behavioural signs that their reproductive systems are no longer suppressed (e.g. development of a perforate vagina and nipples) after only 7 days, while elevated progesterone levels were measured after 19 days (Faulkes *et al,* 1990a, Margulis *et al,* 1995). These females will fight, often to the death, for the dominant position (Jarvis, 1991; Clarke & Faulkes, 1997; Lacey & Sherman, 1991). However, for 95 % of subordinates, this breeding opportunity never arises as queens have been shown to successfully defend their dominant position for as long as 13 years (Jarvis, 1991; Clarke & Faulkes, 1997; Smith *et al,* 1997).

1.5.3. Reproduction in male naked mole rats

Only one to three males mate with the queen and all other males are suppressed by the queen. Breeding males have higher levels of circulating LH, greater responses to exogenous GnRH challenges and higher concentrations of urinary testosterone (Faulkes & Abbott, 1991). Again, these results can only be interpreted qualitatively as the authors do not disclose whether the form of synthetic GnRH used in the study is the same as the form found in naked mole rats naturally. Interestingly, these endocrine differences do not suppress gamete development in subordinate males. In a study by Faulkes and Abbott (1991), subordinate males housed singly (i.e. removed from aggressive physical contact with the queen) attained testosterone and LH levels which mirrored that of breeding males. The hormone levels of the experimental subordinates

rose quickly (within approximately 5 days) and were sustained at high levels (testosterone: 8.2 ± 1.3 to 49.1 ± 5.5 ng/mg urinary creatinine; LH: 4.7 ± 1.0 to 19.8 ± 4.0 m.i.u/ml [milli International Units per milliliter]) for the duration of the experiment. Males paired with breeding females, however, displayed a more cyclical release of testosterone and LH, which synchronised with the ovarian cycle of the female (i.e. the levels of testosterone and LH were highest in the male when the female ovarian cycle was in the late luteal and early follicular phase). This indicated that the breeding female exerts some level of suppression, not only on the subordinate males and females, but also on breeding males. Since the varying levels of testosterone do not seem to affect gamete production, it is possible that the increased testosterone level in breeding males facilitates reproductive behaviours. By controlling the hormone levels of dominant males, the queen can select breeding partners from the colony and control when they are capable of breeding based on her reproductive cycle.

1.6. Neuropeptides and the control of reproduction

Since GnRH is the "master hormone" of reproduction, it, or other hormones further down the reproductive cascade are the likely targets of physiological suppression. GnRH secretion is controlled by the interplay between steroid hormones and feedback loops, however, there is little evidence to suggest that these steroids act directly on the GnRH neurons as few estrogen receptors have been found within GnRH cells (Fernald & White, 1999; Smith *et al,* 2006). Therefore other neuronal systems must be in place to act as conduits to relay information from steroid hormones to the GnRH neurons. Neurotransmitters elicit some effect, but the most significant group with the strongest effects seem to be that of neuropeptides and their receptors (Smith & Clarke, 2007).

1.6.1. Kisspeptin

In 1999, Dennis Lee and colleagues identified the cDNA of GPR54 (a novel G protein coupled receptor) in the brains of rats using polymerase chain reaction (PCR) studies. They also showed that this receptor is expressed in numerous brain areas as well as in

the liver and intestine. Subsequently it has been shown that mutations to GPR54 cause hypogonadotrophic hypogonadism (impaired gonadal hormone secretion from the gonads which leads to sexual immaturity) in mice (Smith & Clarke, 2007).

The natural ligand for this receptor was identified in 2001 and is called kisspeptin (Kotani *et al,* 2001; Muir *et al,* 2001; Ohtaki *et al,* 2001). Kisspeptin is the product of the KiSS-1 gene and is expressed in discrete regions in the forebrain, particularly in the hypothalamus (Messager *et al,* 2005; Smith *et al,* 2006) and is a major stimulator of GnRH release.This 54 amino acid peptide is part of the RF-amide neuropeptide family (i.e. terminating in —Arg-Phe-NH_2 at the C terminus, Seminara & Crowley, 2008). Immunocytochemical studies in mouse, rat and sheep tissue have shown GnRH and kisspeptin neurons in close proximity and that kisspeptin neurons project their axons to GnRH neurons (Smith & Clarke, 2007). GnRH neurons express GPR54 and therefore are a likely target for the direct action of kisspeptin (Messager *et al,* 2005; Smith *et al,* 2006). Kisspeptin has also been identified as a key trigger for the onset of puberty in mice and humans (Messager *et al,* 2005, Smith & Clarke, 2007). Puberty is the activation of the reproductive system and in terms of neuroendocrinology, it is defined as the reactivation of GnRH secretion and maturation of the reproductive system (Ebling, 2005). Leading up to this change in reproductive status, there is increased innervation of GnRH cells by kisspeptin neurons (demonstrated by dual-immunofluorescence studies), as well as an increase in the expression of KiSS-1, particularly in the region of the hypothalamus (Clarkson & Herbison, 2006), suggesting that kisspeptin is involved in the reactivation of GnRH neurons at puberty (Smith & Clarke, 2007).

1.6.2. Gonadotropin-inhibitory hormone

Gonadotropin-inhibitory hormone (GnIH) is a 12 amino acid peptide from the RF-amide family of neuropeptides (Ubuka *et al,* 2006). It was first and recently identified in the brain of the quail, where studies showed that it directly inhibited the release of gonadotropins from cultured anterior pituitary cells (Ubuka *et al,* 2006). Administration of exogenous GnIH also rapidly reduced the levels of plasma LH in Siberian hamsters (Kriegsfeld *et al,* 2006) and male quail (Ubuka *et al,* 2006). GnIH

has since been identified in the hypothalamus of a number of bird, fish, amphibian and mammalian species where its role is that of a GnRH antagonist (Bentley *et al,* 2006; Tsutsui Osugi, 2009). In the mammals studied to date (mice, rats and Siberian hamsters) GnIH cell bodies are found in the hypothalamus and their nerve fibres project to a number of different brain regions, including the anterior hypothalamus, the arcuate nucleus and the internal zone of the median eminence (Kriegsfeld *et al,* 2006). More than 40 % of GnRH neurons receive GnIH neuron projections and a putative GnIH receptor is expressed on GnRH cells. This suggests that GnIH neurons directly inhibit GnRH neurons, which in turn inhibit the synthesis and secretion of gonadotropins. GnIH neurons also extend their axons to multiple areas in the brain, such as the brainstem and limbic system, which do not contain GnRH neurons and are not directly involved in reproductive processes. This implies the potential of multiple regulatory roles for GnIH as well as the monitoring of various other factors which may influence reproduction, such as food intake and stress (Bentley *et al,* 2006; Kriegsfeld *et al,* 2006). One of the proposed mechanisms employed by GnIH to inhibit GnRH is to suppress the rate of firing of the GnRH neuron (Ducret *et al,* 2009). GnIH G-protein coupled receptors have also been identified in the pituitary, suggesting that GnIH inhibits gonadotropin secretion directly, or via the inhibition of GnRH secretion (Ubuka *et al,* 2006; Tsutsui *et al,* 2007). In female hamsters, double-labelled immunofluorescence for GnIH and estrogen receptor-a showed a large group of GnIH neurons expressed estrogen receptors, suggesting that GnIH neurons may be activated or inactivated by sex steroids (Kriegsfeld *et al,* 2006), hence providing the link between steroid and GnRH secretion.

In the brains of seasonally breeding birds, a strong association between GnIH and melatonin has been identified. Breeding seasonality and the day to day establishment of breeding cycles is controlled by an internal 'biological clock', or circadian rhythm. Circadian rhythms are driven largely by light/dark cycles which stimulate the release of hormones and train the animal to anticipate the daily changes in the environment (Goldman, 1999). In endocrinological terms, circadian rhythms are maintained by the secretion of melatonin from the pineal gland (Fox, 2004). In the avian brain, melatonin is key to the production of GnIH by inducing the expression of GnIH mRNA and maturation of GnIH peptide (Ubuka *et al,* 2006). Present findings suggest

a putative role of GnIH as a GnRH antagonist which, with the aid of melatonin, regulates the reproductive axis (Kriegsfeld, 2006). However, all studies to date have focussed on non-fossorial mammals whose circadian rhythms are well understood.

1.6.3. Other factors influencing GnRH production and secretion

1.6.3.1. Glial cells

Glia are specialised, non-neural cells that reside throughout the body in close approximation to neurons (Fox, 2004). They have a variety of functions which include providing mechanical and physiological support, electrical insulation between neurons and aiding in the maintenance of homeostasis (Garcia-Sergura & McCarthy, 2004). Like nerve cells, glia are involved in intercellular communication, however, the mechanisms employed for this are somewhat different. Glial cells do not use synaptic contacts, but rather link with other cells via membrane bound receptors or intercellular channels (called gap junctions) which facilitate the transfer of small signal molecules from one cell to another (Ojeda & Ma, 1999). This communication aids in the integration of neural and chemical signals which ultimately brings about some cellular response, such as increasing the expression of receptors or stimulating axonal growth (Garcia-Sergura *et al,* 2008). Specialised glial cells (called tanycytes) line the dorsal part of the median eminence and send projections to the external zone. Here, the end feet of the projections abut the endothelial cells of the portal vessels. These end feet block synaptic contact of GnRH neurons with the portal vasculature, thus inhibiting the release of GnRH into the bloodstream (Garcia-Segura *et al,* 2008). When there is an increased demand for GnRH, the end feet of the tanycytes retract, allowing the GnRH neurons to re-establish contact with the portal vessels (Ojeda & Ma, 1999). Pituicytes (glial cells of the pituitary gland) also behave in this manner. This glial cell plasticity seems to be linked with steroid hormones, where an increase in testosterone or estrogen promotes the secretion of GnRH (Garcia-Segura & Melcangi, 2006). For example, exposure of the foetal brain (of guinea pigs, mice, sheep, pigs and rhesus monkeys) to testosterone results in alterations to the function, number of synaptic inputs to the GnRH neurons and area of each glial cell. In rats, the surface area and the number of processes per glial cell decreases, in response to high estradiol levels,

to facilitate the GnRH-induced surge of LH (Garcia-Segura *et al,* 2008). In adult primates, ovariectomy, and therefore a decrease in sex steroid levels, causes an increase in the number of glial cell projections (which blocks GnRH release into the portal vessels) and decreases the number of synaptic inputs innervating GnRH neurons. The tanycyte processes have also been observed to extend and retract following the natural hormonal fluctuations of the oestrus cycle (Garcia-Segura *et al,* 2008).

1.6.3.2. Growth factors

GnRH neurons can also be regulated by growth factors, which act via cytoplasmic or transmembrane receptor-linked tyrosine kinases (an enzyme that phosphorylates tyrosine residues) (Ojeda & Ma, 1999). These growth factors can bind directly to receptors on the GnRH neuron, or via receptors on glial cells. Growth factors acting on GnRH directly include transforming growth factor-ß1 (TGF- ß1), basic fibroblast growth factor (bFGF) and insulin-like growth factor 1 (IGF-1). TGF- 131 is released by glial cells and acts to stimulate GnRH release, as well as to modulate GnRH gene expression (Garcia-Segura & McCarthy, 2004). IGF-1 in rats is released by glial cells and has been shown to facilitate GnRH secretion (Garcia-Segura & McCarthy, 2004). IGF-1 is also produced by the GnRH neurons themselves and may act as auto- or paracrine factors to enhance GnRH release at puberty (Garcia-Segura *et al,* 2008). bFGF induces neuronal differentiation and enhances processing of the prohormone (Ojeda & Ma, 1999). Transforming growth factor-a (TGFa) and neuregulins (NRGs) stimulate GnRH secretion (in rats and mice) via receptors on glial cells and are particularly active around the time of puberty (Ojeda & Ma, 1999).

1.6.3.3. Neuropeptide

Energy balance is tightly linked to reproductive function and the onset of puberty (Gamba & Pralong, 2006). Little is known about the exact mechanisms involved, but animals need to have an adequate energy store in order to progress through pubertal development to attain sexual maturity. Neuropeptide Y (NPY) is a 36 amino acid neurotransmitter/neuropeptide found in numerous parts of the central and peripheral

nervous system, and the primary site of NPY production is in the arcuate nucleus (Meczekalski & Warenik-Szymankiewicz, 1999). NPY is involved in stimulatory regulation of appetite (Gamba & Pralong, 2006) where fasting or starvation is associated with a decrease in the neuropeptide, while exogenous administration of NPY strongly induces an increase in appetite (Meczekalski & Warenik-Szymankiewicz, 1999; Kalra & Kalra, 2004). NPY also has an inhibitory effect on LH release and suppresses sexual behaviour and motivation (Genazzani *et al,* 2000; Kalra & Kalra, 2004). Receptors for NPY have been found on GnRH neurons which is evidence to suggest the direct action of NPY on GnRH neurons (Gamba & Pralong, 2006). NPY is released in a cyclical fashion which is closely linked to the pulsatile release of GnRH (Kalra & Kalra, 2004). When unfavourable metabolic conditions arise, GnRH neurons are inhibited by an increase in NPY secretion. In juveniles (mice), this inhibits the onset of puberty and in adults it inhibits reproductive activity while promoting appetite (Genazzani *et al,* 2000).

1.7. Aims of this study

The naked mole rat displays many biological anomalies. One of which is the socially-induced reproductive suppression of subordinates. Most studies to date have tackled this enigma by focusing on the behavioural aspects of naked mole rat reproduction, revealing little about the underlying mechanisms at play in this unusual reproductive regime. This study aims to use a physiology and biochemistry based approach to investigate how reproductive suppression may be maintained in this species.

Naked mole rats display a large degree of sexual dimorphism, not between different sexes, but rather between females of different breeding status. A recent paper by Holmes *et al* (2007) suggests that a change in social status can result in some form of brain remodelling. Therefore, this study aims to ascertain whether the somatic size differences observed in naked mole rats persists in brain morphology.

Physiologically, subordinates are also different to queens in that they have very low levels of circulating LH and no detectable levels of progesterone (Faulkes *et al,* 1990a). There is clearly some disruption of the hormonal cascade responsible for

reproductive processes, but where exactly this disruption occurs is not yet known. Since GnRH is the hormone which controls the production and release of reproductive hormones, it is very likely that in *H. glaber* GnRH is somehow suppressed, resulting in inhibition of reproduction in subordinates. Little is known about the GnRH system in naked mole rats and therefore another aim of this project is to use immunocytochemistry to identify where in the naked mole rat brain these GnRH neurons reside and then to use this information to deduce whether there is a significant difference between the amount and distribution of GnRH in breeding and non-breeding naked mole rat females.

The form of GnRH found in naked mole rats is currently unknown. Therefore, a pilot study was developed, with the aim of using synthetic mGnRH, insect tissue and brain tissue from mice to critically examine the steps involved in extraction and purification of neuropeptides with the future aim of purifying the form of GnRH found in naked mole rats and elucidating its amino acid structure. In this laboratory, there is little naked mole rat tissue available for experimental purposes, therefore another aim of the pilot study was to establish the methods required for optimal extraction and purification of neuropeptides from a limited amount of source material.

Finally, secretion of chemical messengers which mediate various biological functions is an ancient form of cellular communication. The similarities between the AKH/RPCH and GnRH signalling systems (as outlined in Section 1.3.6. and Lindemans *et al,* 2009) suggest that they may have diverged from an ancient lineage, therefore, this study also served as a comparison with invertebrate neuropeptide systems.

CHAPTER TWO: MATERIALS AND METHODS

2. Materials and methods

All of the experiments carried out during this study were in compliance with the University of Cape Town (UCT) animal ethics board (Faculty of Health Sciences Ethics approval number: REC.REF 008/041; Zoology Department Ethics approval number: 2008N12/CS). Most of the experimental work was carried out in the Zoology Department, UCT, South Africa. Dissections of mouse brain and all tissue processing for immunocytochemistry (including embedding and sectioning procedures) were performed at the Faculty of Health Sciences in the Animal Unit and the Histology laboratory, respectively.

2.1. Study animal: naked mole rat *(Heterocephalus glaber)*

2.1.1. Animal husbandry

A total of 16 adult naked mole rat females (breeding and non-breeding) from different colonies, between 5 and 20 years old, were used in this study. 5 subordinates, 5 intact queens and 2 ovariectomised queens (which had been ovariectomised for approximately 5 years) were used in the immunocytochemical part of this study, while the brains of 4 subordinates were used in the peptide extraction and isolation study. These live specimens were supplied by Dr Justin O'Riain from colonies bred in captivity in the Zoology Department at UCT. The naked mole rats live in a system of interconnected plastic burrows maintained at 28 °C ± 2 °C and fitted with small incandescent lamps to provide a heat source for basking 24 hours (h) a day. Animals have free access to the numerous tunnels which connect to toilet chambers, feeding and sleeping areas. Weaned animals do not actively drink but are sustained on a daily diet of chopped fruit and vegetables including apple, butternut, potato, sweet potato, carrot, green pepper and spinach.

2.1.2. Euthanasia and tissue collection

Naked mole rat specimens were euthanized by halothane inhalation (administered by Dr Justin O'Riain) and then rapidly decapitated in preparation for dissection. Brain tissue used for extraction of neuropeptides was carefully dissected, placed in liquid nitrogen within 10 minutes (min) of euthanasia and stored at - 80 °C until required. For morphological and histological studies, brain tissue was exposed by removing the scalp and underlying bony skull. The entire head was then fixed overnight at 4 °C in a 4 % paraformaldehyde solution (PFA) in phosphate buffered saline (PBS: 0.14 M NaCl, 3 mM KC1, 0.01 M Na2HPO4 and 2 mM KH2PO4 at pH 7.2); the brain was removed from the skull the following day. The fixed tissue samples were then taken to the histology laboratory for further processing (Section 2.3).

2.1.3. Animal morphometrics

Live animals were weighed (Lasec top loading balance model MP1100B) just prior to euthanasia and their body mass was measured in grams (g) and recorded to the nearest decimal place. Age was also noted (information supplied by Dr Justin O'Riain) and body length was measured in centimeters (cm) from the rostral tip of the snout to the base of the tail (excluding the tail) for each individual. Other life history information, such as social rank and sexual status (i.e. breeding females with perforate vaginas or non-breeding females with non-perforate vaginas) was also recorded. Immediately after dissection the width and the length of the naked mole rat brain was measured in millimeters (mm) to the nearest two decimal points using vernier calipers. Brain length was measured from the rostral tip of the cerebral hemispheres (excluding the olfactory lobes) to the most caudal point of the cerebellum. Brain width was measured between the two widest points on the lateral edges of the cerebral hemispheres. Dimensions of the hypothalamus and pituitary were obtained *in situ,* as well as via the eye-piece graticule of a light microscope after the tissue had been sectioned, stained and fixed onto slides. Hypothalamus and pituitary length were measured in the rostrocaudal plane between the widest two points along the midline, while hypothalamus and pituitary width were measured in the same manner but in the mediolateral plane.

2.2. Control animals: Husbandry, euthanasia and tissue collection

2.2.1. Mouse (Mus musculus)

Laboratory mice *(Mus musculus)* were obtained from the Animal Unit, Faculty of Health Sciences. All of the mice used in this study were wild-type males (i.e. not knock-out strains) of two different strains; C57/B16 with characteristic black fur and the albino strain Balb/C. C57/B16 mice were used in the immunocytochemical part of the study, while both C57/B16 and Balb/C mice were used for peptide extraction. The mice were euthanized by carbon dioxide inhalation (administered by Mr Noel Markgraaf), decapitated and brain tissue was immediately dissected after euthanasia. For histological studies, the tissue was fixed in 4 % PFA overnight at 4 °C and processed and embedded into wax blocks the following day (Section 2.3). For peptide extraction brain tissue was carefully dissected, placed in liquid nitrogen within 8 min of euthanasia and stored at - 80 °C until required.

2.2.2. Indian stick insect (Carausius morosus)

Adult Indian stick insects *(Carausius morosus)* were obtained from the insectaria of Prof. Gerd Gäde in the Zoology Department, UCT. The insects are kept at 25 °C ± 2 °C and are subjected to a light/dark cycle of 14 h light and 10 h dark. They are fed *ad libitum* with ivy *(Hedera helix)* or the leaves from *Rhamnus prinoides* (commonly known as `blinkblaar'). Stick insects were anaethetised on ice and the dorsal surface of the head was carefully shaved off with a scalpel blade to reveal the brain and corpora cardiaca. The corpora cardiaca were dissected with the aid of iridectomy scissors and fine forceps under a dissecting microscope and placed in 80 % chilled methanol for immediate extraction of hypertrehalosaemic neuropeptides (Section 2.5).

2.2.3. Spiny lobster (Jasus lalandii)

Tissue sections from the eyestalks of the rock lobster species *Jasus lalandii* were obtained from Dr. Heather G. Marco (Zoology Department, UCT). The tissue had already been dissected, sectioned and fixed onto slides in preparation for histological study as per the methods outlined by Marco and Gäde (1999).

2.3. Histology and immunocytochemistry

2.3.1. Tissue embedding, sectioning and histostaining

PFA-fixed brain tissue was rinsed in PBS for 5 min and then placed into an automatic tissue processor (Shandon Elliot SE 400) for dehydration through a series of increasing ethanol (EtOH) concentrations (at room temperature, RT) and infiltration in hot paraffin wax (2 x 1 h 70 % EtOH, 2 x 2 h 90 % EtOH, 3 x 2 h 100 % EtOH, 2 x 2 h xylene, 2 x 2 h wax). The entire brain was then sectioned at 5µm in the coronal plane using a Leica rotary microtome (model 2125RT). Most of the sections were stored in boxes at RT until use in immunocytochemistry, while some tissue sections were selected at regular intervals from the ribbon of sections and put onto glass slides. The sections were heat fixed at 37 °C, and stained with Mayers haematoxylin and eosin (prepared by the Histology laboratory) for anatomical orientation of the remaining brain sections. Briefly, the sections were dewaxed in xylene (10 min), rehydrated in a series of decreasing ethanol concentrations (2 x 1 min in 100 % EtOH, 1 x 1 min in each 90 %, 70 %, 50 % and 35 % EtOH) and finally rinsed under running tap water (2 min). The slides were then immersed in Mayers haematoxylin (5 min), excess dye was rinsed off before briefly differentiating the stain (3 seconds) in a 1 % acid alcohol solution (70 % EtOH (99): HCl (1) vol/vol) and finally rinsing in tap water (3 min) to produce the blue colour of the stain. The same sections were then stained with eosin (2 min), rinsed with tap water, dehydrated in a series of increasing EtOH concentrations (35 — 100 % EtOH) and finally glass coverslips were mounted with Entellan (a permanent mounting medium; supplied by Merck).

2.3.2. Comparative immunocytochemical studies

2.3.2.1. Primary and secondary antisera

The primary antiserum utilised for detecting GnRH in this study (antibody-678, King *et al,* 1994), was kindly donated by Prof. Arieh Katz (Institute of Infectious Disease and Molecular Medicine, UCT). This polyclonal antibody was raised in rabbits and binds specifically to the N- and C- termini of all GnRH peptides studied to date. Antibody-678 was used in both the immunocytochemical and enzyme-linked immunosorbent assay (ELISA, Section 2.7) studies at dilutions of 1:250 and 1:100, espectively. These optimal concentrations of primary antiserum were determined in a pilot study (in the laboratory of Dr Marco) by testing a series of dilutions (1:100 to 1:1000) in immunocytochemistry and ELISA. For the immunocytochemical studies, goat anti-rabbit immunoglobulin (IgG) conjugated to horse-radish peroxidase (supplied by Sigma) was used as a secondary antibody at a working dilution of 1:100. For the ELISA studies (Section 2.7), goat anti-rabbit antibody conjugated to alkaline phosphatase (supplied by Sigma) was used as a secondary antibody at a dilution of 1:2000.

A polyclonal antibody raised in rabbits against crustacean hyperglycaemic hormone (cHH) of the crayfish *Procambarus bouvieri* was supplied by Dr Heather Marco. This primary antiserum (code-named anti Prbo-cHH) was used at a working dilution of 1:1000 in conjunction with the secondary antibody goat anti-rabbit IgG conjugated to horse-radish peroxidase (1:100).

2.3.2.2. The indirect peroxidase procedure

Wax sections were mounted onto glass slides and heat-fixed overnight at 37 °C. The following day, the tissue sections were prepared for immunocytochemistry by dewaxing them in xylene (10 min), followed by rehydration in a series of decreasing ethanol concentrations (3 x 1 min in 100 % EtOH, 1 x 1 min in each 90 %, 70 % and 50 %), and finally into distilled water. Endogenous peroxidases were blocked by continually flooding the slides with a 1 % hydrogen peroxide solution in methanol

(H202-Me0H), for 30 min and then rinsed under running tap water (5 min). Non-specific binding of the antibody was blocked by applying 10 % normal goat serum (NGtS, supplied by Sigma), diluted in distilled water, to the sections, for 1 h at RT. After incubation, the 10 % NGtS was removed and replaced with 50 ,1l of either primary antibody diluted in PBS and 2 % NGtS, or only in PBS with 2 % NGtS. The sections were incubated with the appropriate primary antibody (Section 2.3.2.1) in a sealed damp chamber overnight at 4 °C. The following day the sections were rinsed in PBS (3 x 5 min) before applying 50 pi of the secondary antibody (Section 2.3.2.1) diluted in PBS and 2 % NGtS, to each tissue section and left to incubate in a sealed container, for 1 h at RT. Thereafter, the sections were rinsed in a series of buffers (3 x 5 min in PBS and 1 x 5 min in tris-buffered saline [TBS: 0.01 M NaC1 in 0.05 M Tris-HC1]). The tissue was then treated with 2 ml of a 0.05 M Tris-HC1 solution (pH 7.6) containing 0.01 % H202 and 1 mg of the substrate, diaminobenzidine (DAB, supplied by Sigma), for 5 min at RT, to visualise the immunoreactive (-ir) structures in either brain or eyestalk tissue. The reaction product can be identified as a rust brown colour. The sections were rinsed under running tap water (5 min), dehydrated in a series of increasing ethanol concentrations, 35 — 100 % EtOH, cleared in xylene, mounted with Entellan and coverslipped. If a section became damaged during the staining procedure, such that the pituitary gland or hypothalamus was obscured, the immunocytochemical procedure was repeated on a section adjacent to the one which was damaged.

2.3.2.3. Procedural controls

The binding specificity of the antibodies was tested in two ways: 1) by omission of the primary antibody (or replacement of the primary antibody with either PBS, NGtS or another antibody which was not specific to the antigen) to test specificity of secondary antibody, and 2) by the application of primary antibody that had been preabsorbed with the antigen to form an antibody-antigen complex. The antibody-antigen complexes were prepared by adding 12 ill of primary antibody (diluted 1:60 in PBS) to 100 pmol of dried synthetic mGnRH (supplied by Sigma). This solution was left at RT for approximately 4 h, vortexing occasionally to ensure that as much of the antibody could bind to the antigen as possible and finally storing overnight at 4

°C. The following day the complexes were used as primary antibody stock for the immunocytochemical procedure. Unblocked antibody solutions (using the same primary antiserum) were subjected to the same treatment as the preabsorbed antigen-antibody solutions, except that synthetic peptide was omitted; these served as controls.

2.3.3. Microscopy, photomicrography and GnRH-ir staining measurement

Slides were viewed using a Nikon Stereoscopic Zoom SMZ1500 dissecting microscope with C-W1OX eyepieces and a HP PLan Apo 1 x objective, and a Nikon Compound Eclipse 50i compound microscope. Photomicrographs were taken with a Nikon DS DS-U2 digital camera and Nikon NIS-Elements documentation digital imaging software, at set magnification (x 10 or x 40), exposure (30 ms) and gain (1.4) settings. The imaging software provides a conversion factor from pixels to gm; this was recorded for every picture. Each photograph that contained GnRH-ir structures was exported to Adobe photoshop (version 7.01) where contrast and brightness were corrected (where necessary) and the colour balance of each picture was altered such that the area of GnRH staining was enhanced (see appendix I for details). The stained area was selected using Photoshop's 'magic wand' tool which selects pixels based on their colour composition. The area of staining was calculated for each section by measuring the number of pixels that were stained and converting them to µm2 by means of the software conversion factor. The area values for each section were then added to obtain a single figure representing the staining observed in the brain of each animal. These values were then compared statistically.

2.4. Statistics

Brain morphometric data as well as the areas of GnRH-ir for intact and ovariectomised queens were compared using Lord's range test for small samples (Lord, 1947). All other data comparisons between queens and subordinates were tested using Mann Whitney U tests (Statistica version 8).

2.5. Peptide isolation

This study uses a series of standard techniques to investigate neuropeptide hormones. Neuropeptides were extracted from tissues and purified using reverse-phase high-performance liquid chromatography (RP-HPLC). Fractions collected from RP-HPLC were then tested for the presence of GnRH by using enzyme-linked immunosorbent assays (ELISA).

2.5.1. Crude extract preparation

2.5.1.1. Naked mole rat and mouse brain tissue

In preparation for RP-HPLC, crude extracts were prepared by homogenising brain tissue according to methods outlined by Myers and Patonay (2006). Whole brains were homogenised on ice in glass test tubes with 1.5 ml acetonitrile (supplied by Microsep) per brain using an Ultra-Turrax (model TP18/10, Janke and Kunkel) tissue homogeniser. In some cases, the pituitary and/or hypothalamus was homogenised separately from the rest of the brain tissue and these smaller tissue samples were disrupted by ultrasonic action (Branson Sonifier cell disrupter B-30) on ice in 1 ml acetonitrile. The homogenate was divided into 2 ml aliquots (where necessary) and centrifuged for 30 min at 4 °C, 2500 *g* in an Eppendorf centrifuge (model 5415R). The resulting supernatant was collected and dried using a Bachofer speed vacuum concentrator. Pellets were suspended in 1 ml acetonitrile for re-extraction and centrifuged, as described above. The resulting supernatant was pooled with the first extract, vacuum-dried and stored at - 20 °C.

2.5.1.2. Stick insect corpora cardiaca

The cells of the corpora cardiaca glands were disrupted by ultrasonication (Section 2.5.1.1) in 80 % chilled methanol. The homogenate was centrifuged (Heraeus Sepatech Biofuge model 15) for 3 min at 11000 *g*, the supernatant collected, vacuum-dried and stored at - 20 °C.

2.6. Peptide purification

2.6.1. Prepurification of tissue extracts

Crude extracts of mouse and naked mole rat brain tissue were prepurified by solid phase extraction according to Myers and Patonay (2006). Briefly, a SEP-PAK $_{C18}$ cartridge (supplied by Waters Corporation) was conditioned with methanol and then rinsed with loading solution (HPLC grade water (96): glacial acetic acid (3): TFA (1), vol/vol). The dried crude extracts were reconstituted in loading solution and applied to the cartridge. Sequentially, solution 2 (loading solution (70): methanol (30) vol/vol) and solution 3 (3 % acetic acid (30): methanol (70) vol/vol) were applied to the cartridge to elute peptide material; which was followed by a final rinse of 100 % methanol. In each of the steps, a volume of 3 ml was applied to the cartridge; the resulting eluant was collected, vacuum dried and stored at - 20 °C. Synthetic mGnRH (60 pmol) was subjected to the same procedure of solid phase extraction and at each step (including all washing steps) fractions were collected and dried.

2.6.2. Purification by reverse-phase high performance liquid chromatography

RP-HPLC was used to separate peptides in prepurified brain extracts of naked mole rats. Synthetic peptide, extracts of corpora cardiaca and prepurified extracts of mouse brain were used to optimise the methodology for neuropeptide isolation and purification.

2.6.2.1. Equipment and solvents

The components of the RP-HPLC system used in this study are as follows: pump A (Gilson model 305), pump B (Gilson model 302), $_{C18}$ column (Nucleosil, 250 mm x 4.6 mm, solid phase particle size 5 pm) fitted with a $_{C18}$ guard column (lcm), column heater set to 30 °C (Jones Chromatography), 500 sample loop, solvent degasser (Jasco model DG-1580-54), UV detector (Gilson model 112), intelligent fluorescence detector (Jasco model FP-920), a dynamic mixer (Gilson model 881), a manometric

module (Gilson 802) and Kipp and Zonen BD 40 and 41 pen recorders. A flow rate of 1 ml/min was maintained. Solvent A was 0.11 % trifluoroacetic acid (TFA, supplied by Sigma) in HPLC grade water (obtained from Prof. Wolf Brandt, Department of Molecular and Cell Biology, UCT). Solvent B was 0.1 % TFA in 60 % acetonitrile (Romil, HPLC grade, supplied by Microsep). The eluant was monitored by both UV (214 nm, sensitivity 0.02 Absorbance Units Full Scale; AUFS) and fluorescence (excitation 276 nm, emittance 350 nm, gain 100, attenuation 8) detectors. The system was flushed with 99 % solvent B daily to elute very hydrophobic biological material from the column. Additionally, 25 % solvent B was injected onto the column and subjected to the solvent gradient routinely to assess carry-over of brain material from previous runs. During such 'clean up' runs, one minute fractions were collected, vacuum-dried and tested for presence of GnRH using ELISA (Section 2.7).

2.6.2.2. Method development and procedural controls

2.6.2.2.1. Purification of hypertrehalosaemic factors

Training for the RP-HPLC method began by extracting and purifying hypertrehalosaemic hormones (Cam-HrTH I and II) from the corpora cardiaca of Indian stick insects as the chromatographic profile of these neuropeptides is well known (Gade, 1984; Gäde, 1990). Dried corpora cardiaca extract was reconstituted in 25 % solvent B, passed over the RP-HPLC column and subjected to a solvent gradient of 43 — 53 B in 20 min. Peak material was hand-collected, vacuum-dried and stored at - 20 °C.

2.6.2.2.2. Synthetic gonadotropin releasing hormone

Further optimisation of RP-HPLC methodology was attempted with synthetic mGnRH using a solvent gradient of 32 - 47 % solvent B in 15 min. Known amounts of synthetic mGnRH were injected onto the RP-HPLC column and the chromatographic profile (e.g. retention time, peak height) was established under these conditions for comparative purposes.

2.6.2.2.3. Purification of neuropeptides from the mouse and naked mole rat brain

Prepurified extracts of mouse brain were re-dissolved in 25 % B and applied to the RP-HPLC column (using the established solvent gradient, Section 2.6.2.2.2) in amounts equivalent to half, one or two brains per run. Fractions were manually collected either per minute of the run, or by targeting specific peaks. These samples were then vacuum dried and either tested in ELISA using the GnRH antibody or sent for mass spectrometric analysis. This procedure was repeated with prepurified extracts of 4 naked mole rat brains and the eluant was collected at every minute of the run. The purified extract of 1 naked mole rat brain was retained for ELISA (Section 2.7), while the remaining material was tested by mass spectrometry (Section 2.8).

2.7. Peptide identification by enzyme linked immunosorbent assays (ELISA)

ELISA was used to locate the GnRH antigen in fractions collected either during crude extraction (Section 2.5.1), prepurification (Section 2.6.1) or purification by RP-HPLC (Section 2.6.2). This method was also used in conjunction with synthetic peptide to determine the optimal concentrations of primary antisera for use in immunocytochemistry.

Antigen (5 — 10 Ill of crude extract, synthetic peptide or HPLC fractions, plated singly) was pipetted onto a 96 well microtitre plate (F96 maxisorp immuno plate, Nunc) and dried at 37 °C. 100 of coating buffer (0.1 M sodium carbonate, pH 9.6) was then applied and left overnight in a damp chamber at 4 °C to block non-specific binding of the primary antibody to the plate. The following day, the coating buffer was discarded and the wells were washed, 3 x 5 min, with 200 of 0.02 M PBS (pH 7.4, containing 0.1 % Tween-20 and 0.02 % sodium azide). Following the washes, 100 ill of the primary antibody or PBS was applied to the experimental or negative control wells, respectively. The plate was then covered and incubated at 37 °C for 1.5 h after which the wells were washed as before. 100 of secondary antibody was then applied, and the microtitre plate was sealed and incubated for a further 1.5 h at 37 °C.

After incubation, the wells were washed with PBS containing only Tween (3 x 5 min). 100 of the substrate chromagen (5 mg para-nitrophenyl phosphate disodium, supplied by Sigma, in 20 ml of 0.1 M carbonate buffer, pH 9.6) was applied to each well. The plate was sealed and placed in a dark chamber and left on a shaker at RT for 20 min for colour development. The optical density (OD) of each well was determined at 405 nm using a Biorad microplate reader (model 550).

The sensitivity of this assay to the GnRH peptide was determined by constructing a standard curve in which the amount of peptide was plotted against optical density. Known amounts of synthetic hormone, ranging from 0.1 pmol to 400 pmol mGnRH were tested with three different primary antibody concentrations (1:250, 1:500 and 1:1000).

2.8. Peptide identification by mass spectrometry

Purified HPLC fractions were analysed by Dr Stefan Walter (University of Osnabrµck, Germany) by LC-MS (liquid chromatography — electrospray ionization mass spectrometry). A series of tubes containing synthetic mGnRH that ranged from 20 pmol to 140 pmol were analysed to establish the optimal quantity of GnRH required for unequivocal identification by mass spectrometry.

CHAPTER THREE: RESULTS

3.1. Morphometrics of the naked mole rat

3.1.1. Body morphometrics

Body mass and length (from the rostral tip of the snout to the base of the tail, excluding the tail) of 12 female naked mole rats (n = 5 and 7 for subordinates and queens, respectively) were measured; statistical analyses showed that mass and length differed significantly between the two groups of animals (Mann Whitney U test; U = 1.000, p = 0.009; U = 0.000, p = 0.005, respectively, Fig. 9). Queen naked mole rats are significantly heavier and longer than their non-breeding counterparts.

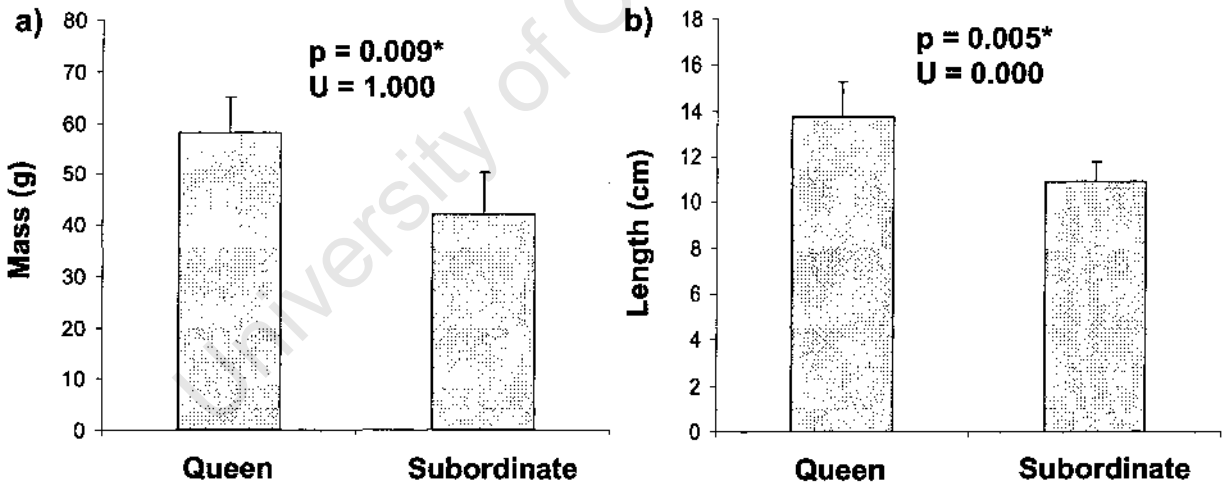
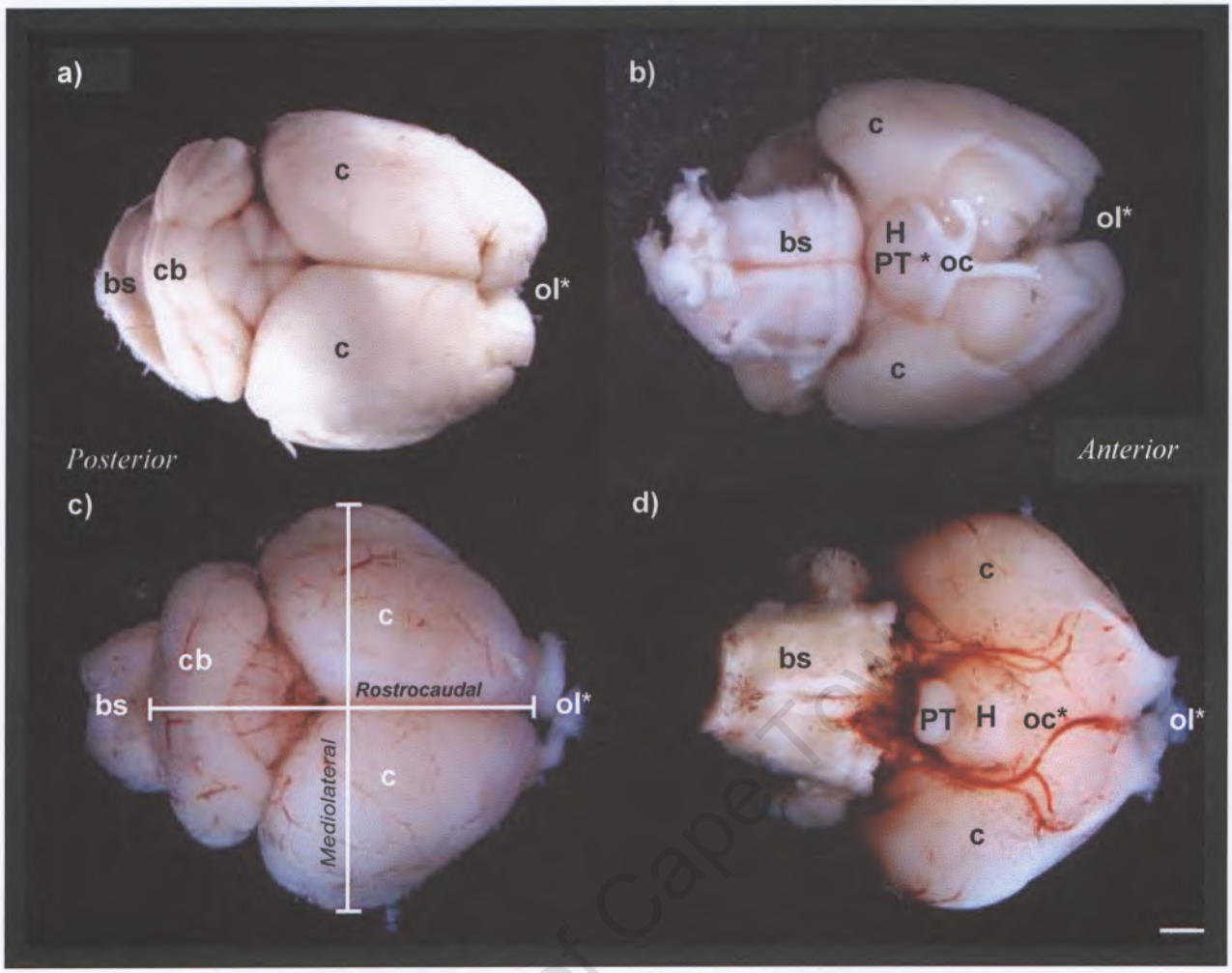


Figure 9: Body mass (a) and length (b) of queen (n = 7) and subordinate (n = 5) naked mole rats represented as mean ± SD. Significant differences (*) were calculated using Mann-Whitney U tests ($p \leq 0.05$)

3.1.2. General organisation of the naked mole rat brain

Brain regions were identified using a stereotaxic atlas of the naked mole rat brain (Xiao *et al,* 2006). In comparison to the uniformly oblong shape of the mouse brain, the naked mole rat brain is oval in shape with distinct widening of the cerebral hemispheres at the midpoints on the rostral-caudal axis (Fig.10). Other external characteristics of the mouse and naked mole rat brain, such as the position of the cerebellum and the cerebral hemispheres, are homologous. The hypothalamus, one of the sites typically associated with GnRH production, was identified as a bulbous region that lies approximately midway along the rostral-caudal axis of the ventral part of the brain (Fig. 10 b, d). In the rostro-caudal plane, the hypothalamus lies posterior to the optic chiasm (where the optic nerves cross') and anterior to the brain stem (Fig. 10 b, d). Internal diagnostic features in the region of the hypothalamus include the presence of the hippocampal fimbriae and prominent third ventricles (Fig. 11 b). The median eminence was identified in the ventral hypothalamus as a thin band of tissue that lies ventral to the third ventricle (Fig.11 b). It is divided into two main regions, the internal and external zone of the median eminence (enlarged region of Fig. 11 b). The medial septum and the preoptic area are two other areas of the brain associated with GnRH neurons (Dubois *et al,* 2002); they lie anterior to the hypothalamus in the region of the optic chiasm (Fig. 10 b; 11 a). Internal diagnostic features in this area of the brain include the lateral ventricles and the anterior commissure (Fig. 11 b).

[1] **Note that the optic chiasm is present in the naked mole rat brain but is not visible in Figure 10 d as it was removed during dissection.**



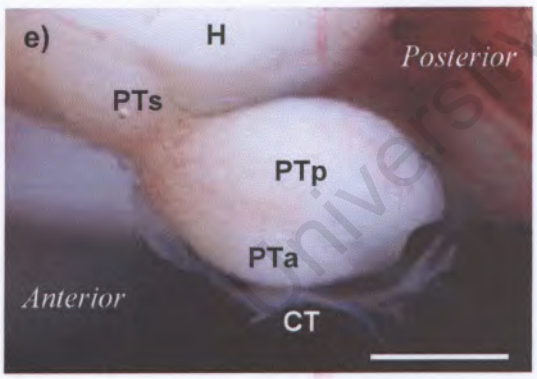


Figure 10: Gross morphology of the mouse (a, b) and naked mole rat (c, d) brain shown dorsally (a, c) and ventrally (b, d). Rostrocaudal and mediolateral measurements were taken of the naked mole rat brain from the points indicated in (c). (e) Detail of the naked mole rat pituitary gland, viewed laterally.

Scale bar = 1mm

Abbreviations

bs	brain stem	ol	region of the olfactory lobe
c	cerebral hemispheres	PT	pituitary
cb	cerebellum	PTa	anterior pituitary
CT	connective tissue	PTp	posterior pituitary
H	hypothalamus	PTs	pituitary stalk
oc	optic chiasm	*	denotes brain regions not visible in this picture

3.1.3. Brain morphometrics

The brains of naked mole rats were measured to compare brain morphology between queen (breeding; n = 7) and subordinate (non-breeding; n = 5) females. The breeding female group was comprised of ovariectomised queens (n = 2) and intact (i.e. possessing functional reproductive organs) queens (n = 5); statistical analysis of morphometric data in the queen group (performed via the Lord's Range test for small samples) showed that there were no significant differences in brain, hypothalamus or pituitary widths and lengths within this group (all $p > 0.05$; results were only deemed significant if the p value was lower than 0.05). Hence, the data from intact and ovariectomised queens were pooled and the combined group is referred to as 'queens' for the rest of this study. The brains, hypothalami and pituitary glands of the naked mole rats were measured in the rostrocaudal (i.e. length, see Fig. 10 c) and mediolateral (i.e. width, see Fig. 10 c) planes to gauge brain size. These measurements were then compared between the queen and subordinate groups of naked mole rats. Width and length of the brain and the hypothalamus did not differ significantly between the two groups (Mann Whitney U test, all $p > 0.5$, Fig. 12 a, b), whereas, width and length of the entire pituitary differ significantly (Fig. 12 c) with that of queen naked mole rats being wider and longer than those of subordinates. Qualitatively, most of the size difference in the pituitary gland seems to be attributed to the posterior part of the pituitary, with smaller posterior pituitaries consistently observed in the subordinates (Fig 13). This size disparity of the different pituitary regions (i.e. anterior and posterior) could not be accurately quantified as in some dissections (n = 1 subordinate pituitary, n = 3 queen pituitaries) the posterior pituitary detached from the rest of the brain and was processed and sectioned separately. In such cases, it was not easy to retain the *in situ* orientation of the posterior pituitary and the plane in which the tissue was sectioned was not known, hence width/length dimensions of the posterior pituitary could not be accurately measured from the resulting sections for light microscopy. Histologically, the pituitary glands of queens and subordinates also differ in structure. The most rostral end of the anterior pituitary was thin, loop shaped and sparsely surrounded by connective tissue in the majority of subordinate ***H. glaber*** (Fig. 13 a, c). The equivalent region in the queen naked mole rats was generally round, large and densely surrounded with connective tissue (Fig. 13

b, d). One of the subordinates, however, had a more 'queen-like' pituitary, which was larger and surrounded with connective tissue (results not shown). This individual was also the heaviest and longest of the subordinate group. Interestingly, this individual, unlike the other subordinates, was semi-perforate (i.e. showed signs of partial reproductive activation; not shown) and if excluded from the statistical analysis, the p -value representing the degree of difference between queen and subordinate pituitary widths decreased from 0.028 to 0.011.

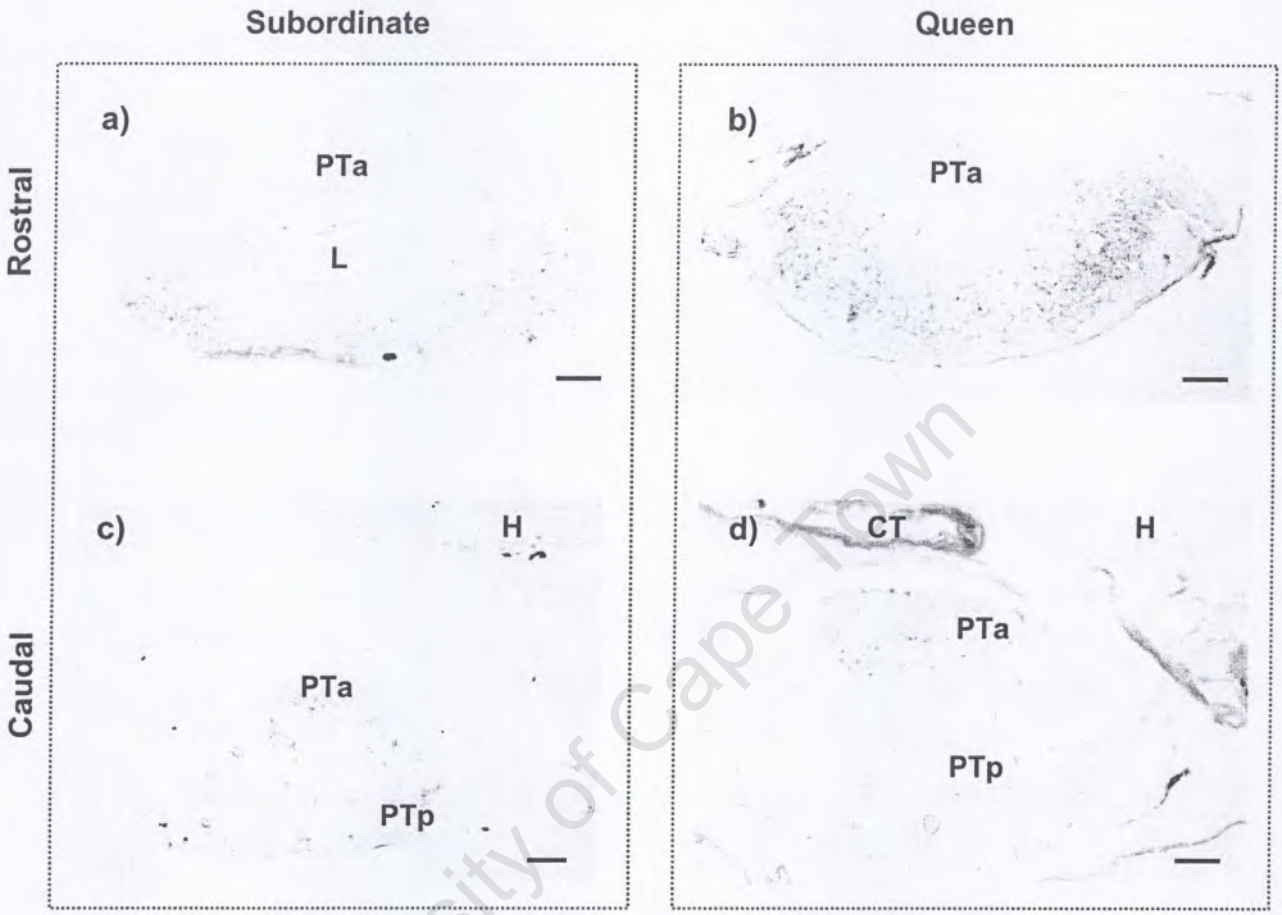


Figure 13: Morphology of the pituitary of subordinate (a, c) and queen (b, d) naked mole rats. These photomicrographs are from equivalent regions in the queen and subordinate brain. Note the loop shape of the most rostral part of the anterior pituitary of subordinate (a) compared to the oval shape of the queen (b) pituitary gland at approximately the same brain region. At the most caudal part of the subordinate (c) and queen (d) anterior pituitary, one can see the appearance of the posterior pituitary. Note the size difference between the posterior pituitary of the queen and subordinate, as well as the abundance of connective tissue surrounding the gland in the queen brain. Scale = 50μm

Abbreviations:

PTa	anterior pituitary	H	hypothalamus	L	loop shape
PTp	posterior pituitary	CT	connective tissue		

3.2. Comparative immunocytochemical studies

3.2.1. Procedural controls

3.2.1.1. Immunocytochemistry on crustacean tissue

Methodological training began by performing immunocytochemistry on eyestalk tissue of the spiny (rock) lobster *Jasus lalandii*. An immunostaining protocol, characterised antibodies, as well as appropriately prepared tissue sections were readily available in the laboratory (see Marco & Gäde, 1999). Rust-brown staining was clearly observed in the region of the sinus gland after treating the tissue sections with an antibody directed towards crustacean hyperglycaemic hormone (cHH, specifically Prbo-cHH) (Fig. 14 a). No staining was observed in adjacent sections where anti Prbo-cHH was omitted (Fig. 14 b) or replaced with another primary antibody which was not specific for the antigen (such as antibody-678 which binds specifically to GnRH rather than cHH, results not shown). Endogenous peroxidase activity (i.e. non-specific background staining caused by naturally occurring peroxidases reacting with DAB) in the spiny lobster tissue was eliminated successfully by applying a 1 % H202-Me0H solution to the tissue sections before applying the antisera.

3.2.1.2. Immunocytochemistry on mammalian tissue

Once the immunocytochemical procedure was learnt with crustacean tissue, the protocol was applied to mammalian tissue sections using antibody-678 (1:250), which binds to the N- and C- terminals of GnRH peptides. The laboratory mouse was used as a control for GnRH neuron distribution and antibody specificity in mammalian tissue. The mouse is an ideal control animal as its size approximates that of a naked mole rat and it has long been a model for mammalian endocrine studies, including the distribution of GnRH in the brain (Douglas, 1976; Gill *et al*, 2008). Immunocytochemistry on the brain tissue of mice revealed a typical pattern of mGnRH staining, according to Douglas (1976) and Gill *et al* (2008) with most of the GnRH neurons occurring in the median eminence of the hypothalamus (Fig. 15); a few neurons were located in the region of, and just posterior to, the optic chiasm and in the mediobasal hypothalamus (results not shown). No staining was observed in

adjacent sections where the antibody-678 was omitted (Fig. 16 a) or replaced with another primary antibody which was not specific for the antigen (such as anti Prbo-cHH, results not shown). Endogenous peroxidase activity was also successfully eliminated by applying a 1 % H202-Me0H solution. Detection of GnRH in the brain of the mouse indicates that this immunostaining method is suitable to apply to mammalian tissue.

3.2.1.3. Antibody specificity

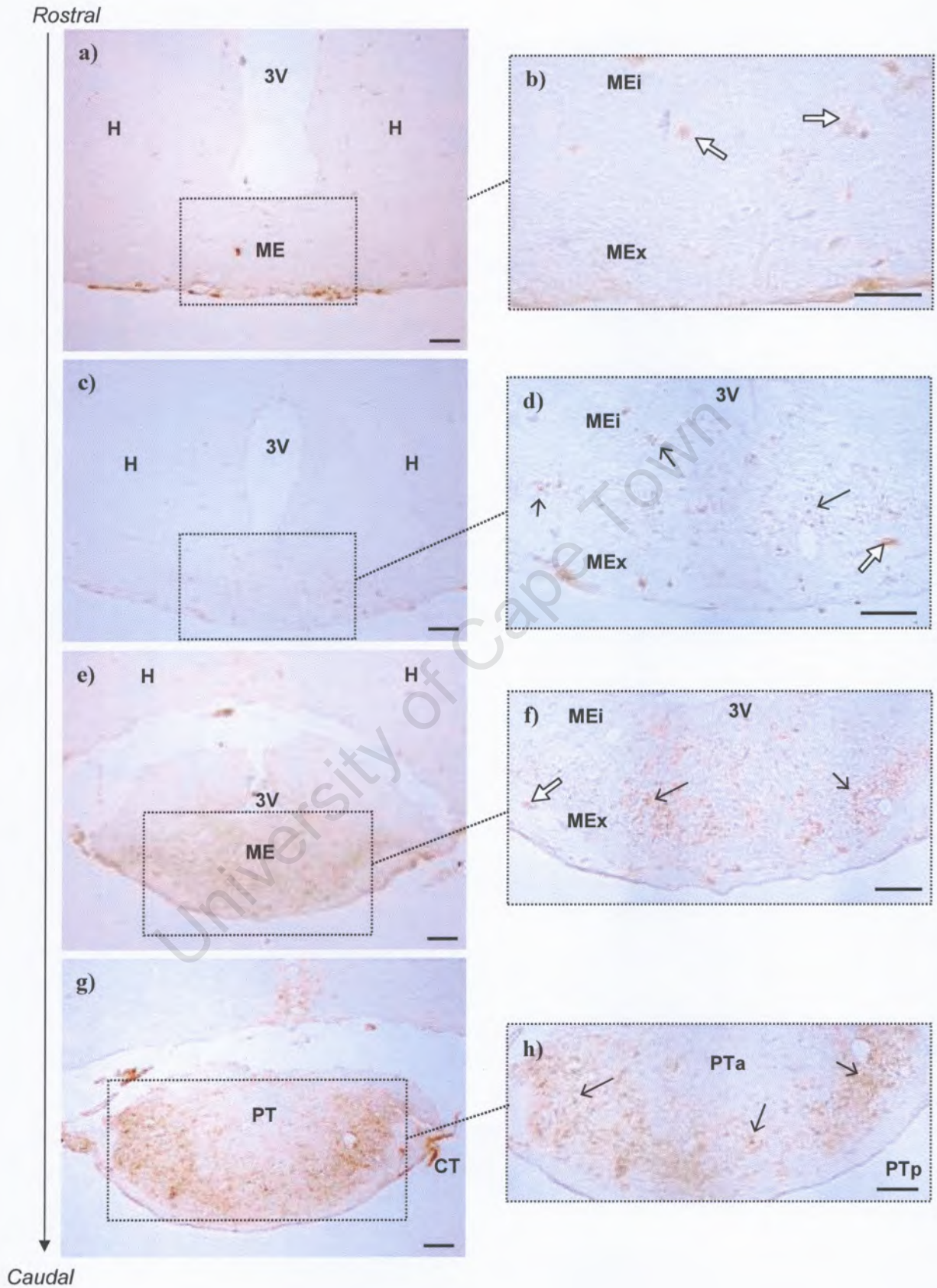
In all of the immunocytochemical studies, no immunoreactivity was observed when the primary antibody was omitted (results not shown). This indicated specificity of the secondary antibody for the primary antibody. The specificity of the primary antibody for the GnRH peptide was tested by preabsorption studies on both mouse and naked mole rat tissue. When preabsorbed antibody-antigen complexes (prepared with antibody- 678 and synthetic mGnRH) were applied to tissue sections, staining was abolished as the primary antibody had bound completely to the synthetic peptide (Fig. 16 a, b). Tissue sections adjacent to those treated with the complexes were incubated with antibody-678 that had not been preabsorbed with synthetic peptide. These control sections showed immunoreactive staining, indicating that the antibodies retained their binding ability after they had undergone same preparation method as the antibody-antigen complexes (Fig. 16 a, b).

3.2.2. GnRH detection in H. glaber by immunocytochemistry

The brains of 7 queens and 5 subordinates were examined for immunoreactivity of GnRH neurons, using antibody-678 (1:250). Prior to the current work, a pilot study was conducted in the laboratory Dr Marco where immunocytochemistry was performed using sections from the entire brain of a naked mole rat. In the pilot study, immunoreactivity was only detected in the hypothalamic and pituitary regions and therefore, only these regions were targeted in the current study. Mean + SD of 19.70 ± 2.21 and 19.40 ± 2.20 sections were selected in the queen and subordinate brains, respectively, each spaced approximately 1.8 mm apart in the rostrocaudal plane, which spanned the full length of the hypothalamus. Immunoreactive staining with the GnRH antibody-678 was found in both subordinate and queen naked mole rat brain tissue (Fig. 17; 18). Of the total number of sections that were examined, 8.14 ± 2.03 sections (mean ± SD) contained immunoreactivity in the queen brain, while 8.60 ± 1.01 sections contained immunoreactivity in the subordinate brain. The number of hypothalamic sections that contained staining is not significantly different between queens and subordinates (Mann Whitney U test, U = 13.000, p = 0.94).

Immunoreactive cells were found neither in any region above the basal part of the third ventricle nor in the preoptic area. All staining of cell bodies was restricted to the region of the median eminence in the basal part of the hypothalamus (queen: Fig. 17 a — f, m; subordinate: Fig. 18 a — d) and the anterior part of the pituitary (queen: Fig 17 g — 1; subordinate: Fig. 18 g — i). Compared with the GnRH-ir cell distribution in the mouse brain, the immunoreactive GnRH cells in naked mole rat (both the queen and the subordinate) brain tissue lie more towards the caudal end of the hypothalamus with the largest proportion of staining concentrated in the region of the anterior pituitary.

The distribution of the staining differed somewhat between queens and subordinates. From rostral to caudal, staining in queen naked mole rats was typically found in the external zone of the median eminence with a few cells scattered dorsally in the internal zone (Fig. 17 d — f, m). Caudal to this staining, GnRH-ir neurons were found concentrated in the lateral edges of the anterior pituitary, with only a few cells found



3.3. Pilot studies for isolation, purification and identification of neuropeptides

3.3.1. Method training: synthetic mGnRH and insect neuropeptides

Training for RP-HPLC (including handling samples, injecting samples onto a column, collection of fractions, use of detectors and their sensitivity settings, correct use of columns and pre-columns, preparation of solvents, designing gradients and overall maintenance of RP-HPLC equipment) was achieved by practicing the necessary methods with crude extract prepared from stick insect *(C. morosus)* corpora cardiaca. The corpora cardiaca were identified as pearly-white spindle shaped structures which lie ventro-caudally to the stick insect brain (Fig. 19). Figure 20 shows a typical separation pattern when a crude methanolic extract of 2 /2 corpora cardiaca are subjected to a gradient of 43 % - 53 % B in 20 minutes. Fluorescence detection could be used as both forms of Cam-HrTH have a tryptophan at position 8 (Fig. 3), which has an absorbance of 276 nm and a detectable emittance at 350 nm (Gäde, 2009). The resulting UV and fluorescence chromatograms show two well resolved peaks: the major peak represents the hypertrehalosaemic hormone Cam-HrTH II (retention time = 13 minutes, — 49.5 % B; Fig. 20), while the minor peak represents Cam-HrTH I (retention time = 8min, — 47 % B: Fig. 20), both of which are known to have a trehalose mobilising effect in stick insects (Gade, 1985). Based on peak height, Cam-HrTH I is less abundant in the stick insect corpora cardiaca than Cam-HrTH II.

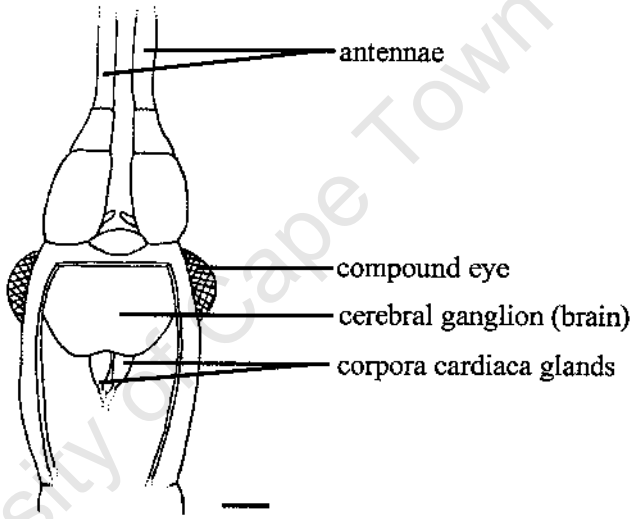


Figure 19: The location of the corpora cardiaca glands in the head of the stick insect, *C. morosus*: the head capsule has been opened dorsally to reveal the underlying neural tissue. Scale bar = 0.1 cm

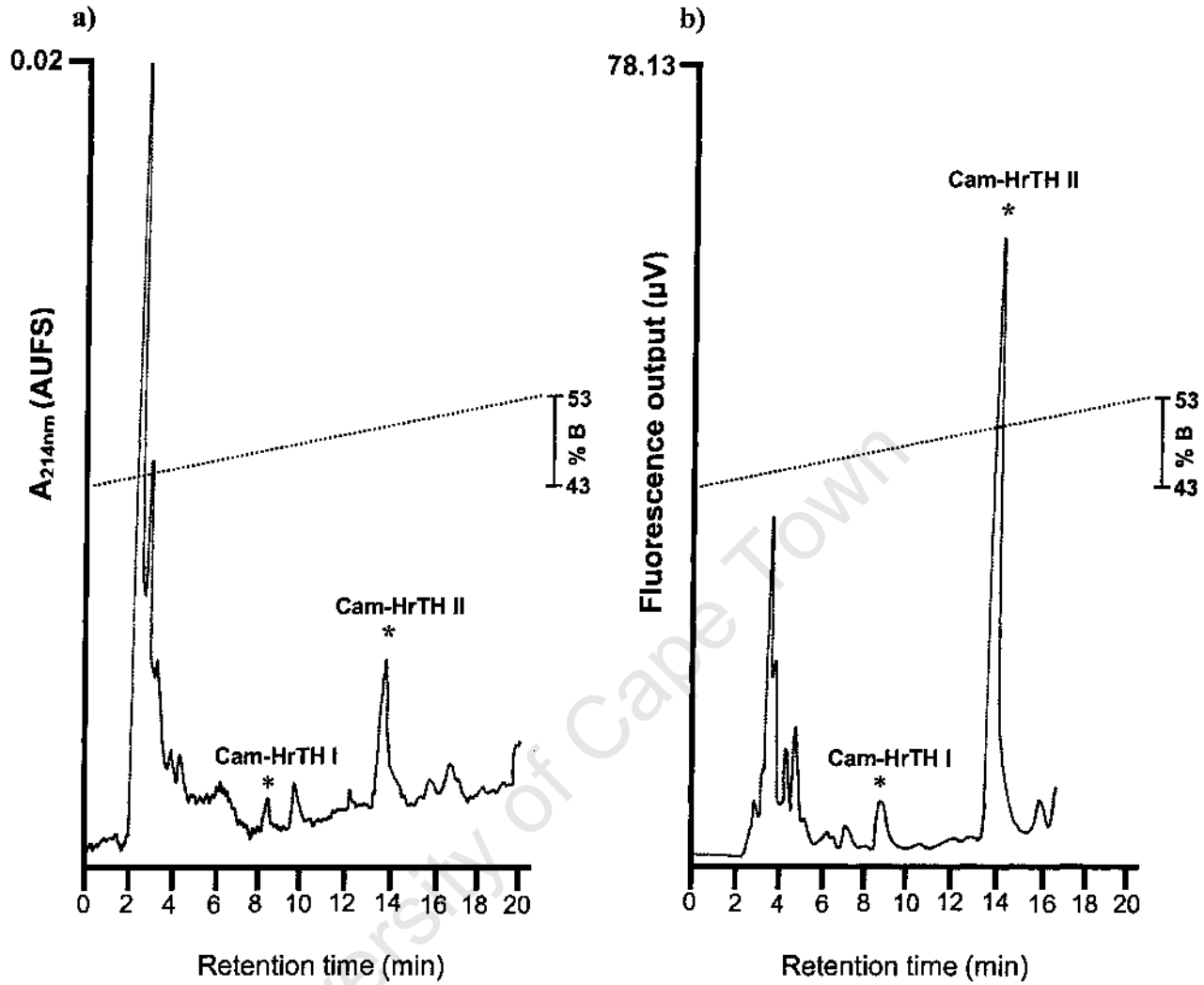


Figure 20: UV absorbance (a), measured at 214 nm in AUFS (absorption units full scale) and fluorescence (b) measured at excitation 276 nm/emission 350 nm, profiles of methanolic extract from corpora cardiaca of *Carausius morosus*. Separation of Cam-HrTH I and II was attained with a linear gradient of 43 % - 53 % solvent B in 20 minutes at a flow rate of 1 ml/min (section 2.6.2.1).

In order to isolate, purify and identify GnRH from mammalian tissues, synthetic mGnRH was used as a proxy for the behaviour of naturally occurring GnRH during the different purification steps of this study. Synthetic mGnRH was used to establish a suitable gradient for elution with RP-HPLC under specific conditions. The same HPLC system, column and solvents were used for elution of mGnRH as for the hypertrehalosaemic hormones of stick insects, but the solvent gradient was modified to 32 % - 47 % B in 15 minutes. mGnRH has a Trp at position 3 and therefore fluorescence detection could be used. Figures 21 and 22 show a major, sharp peak at retention time —12 min (or — 42 % **B)** monitored with fluorescence detection, after different amounts of synthetic mGnRH (200 pmol, 60 pmol) were subjected to the above mentioned solvent gradient. This pattern and peak height/area were consistent over several applications of the same amount of mGnRH, concluding that: i) the gradient of 32 % - 47 % B in 15 minutes is suitable for isolation/purification of mGnRH, and ii) mGnRH elutes with approximately 25 % acetonitrile and not very hydrophobic.

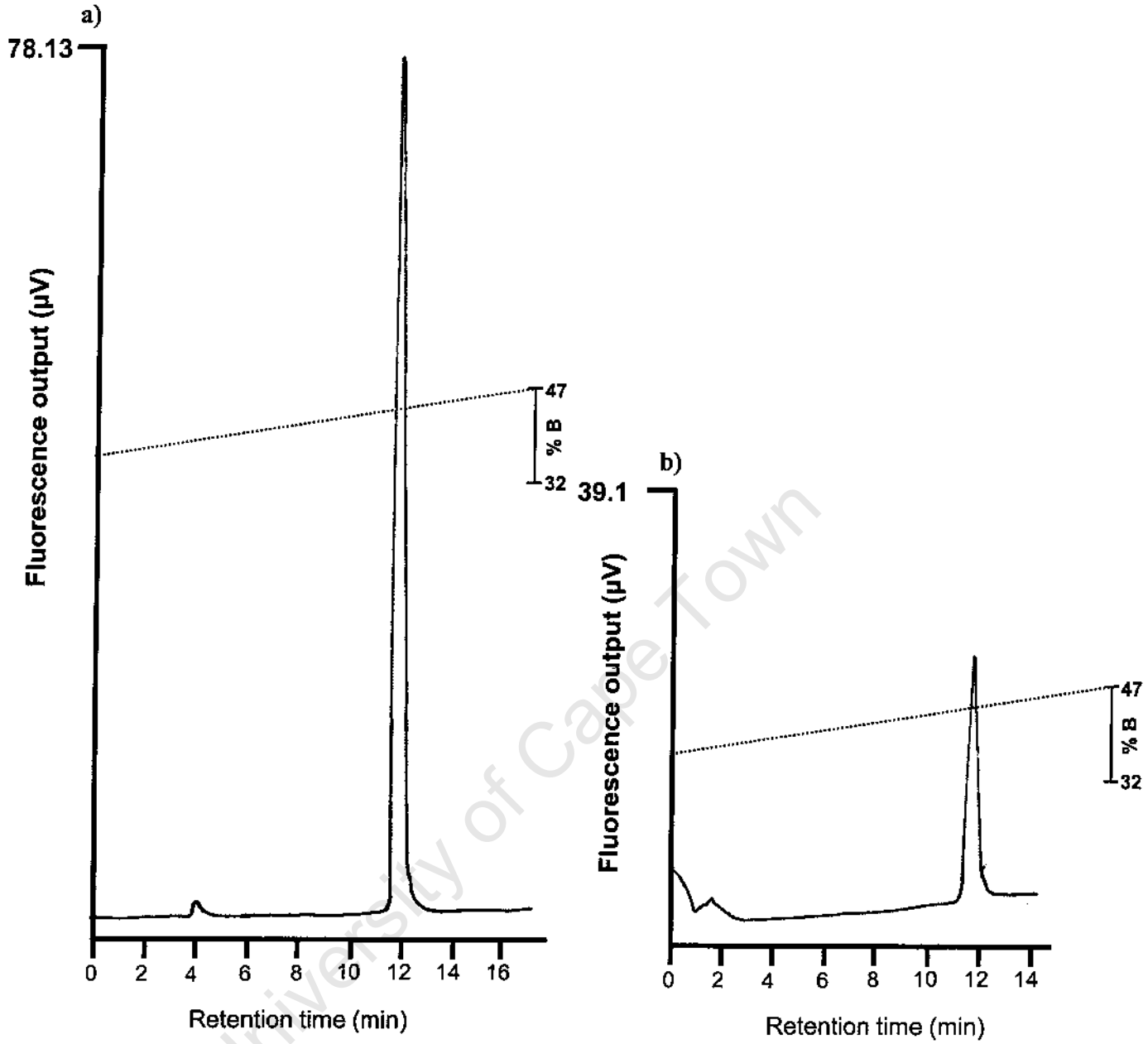


Figure 21: Fluorescence chromatograms, measured at excitation 276 nm/emission 350 nm, of 200 pmol (a) and 60 pmol (b) of synthetic mGnRH passed directly over RP-HPLC with a linear gradient of 32 % - 47 % solvent B in 15 minutes at a flow rate of 1 ml/min. Note the clear peaks at ~ 12 minutes.

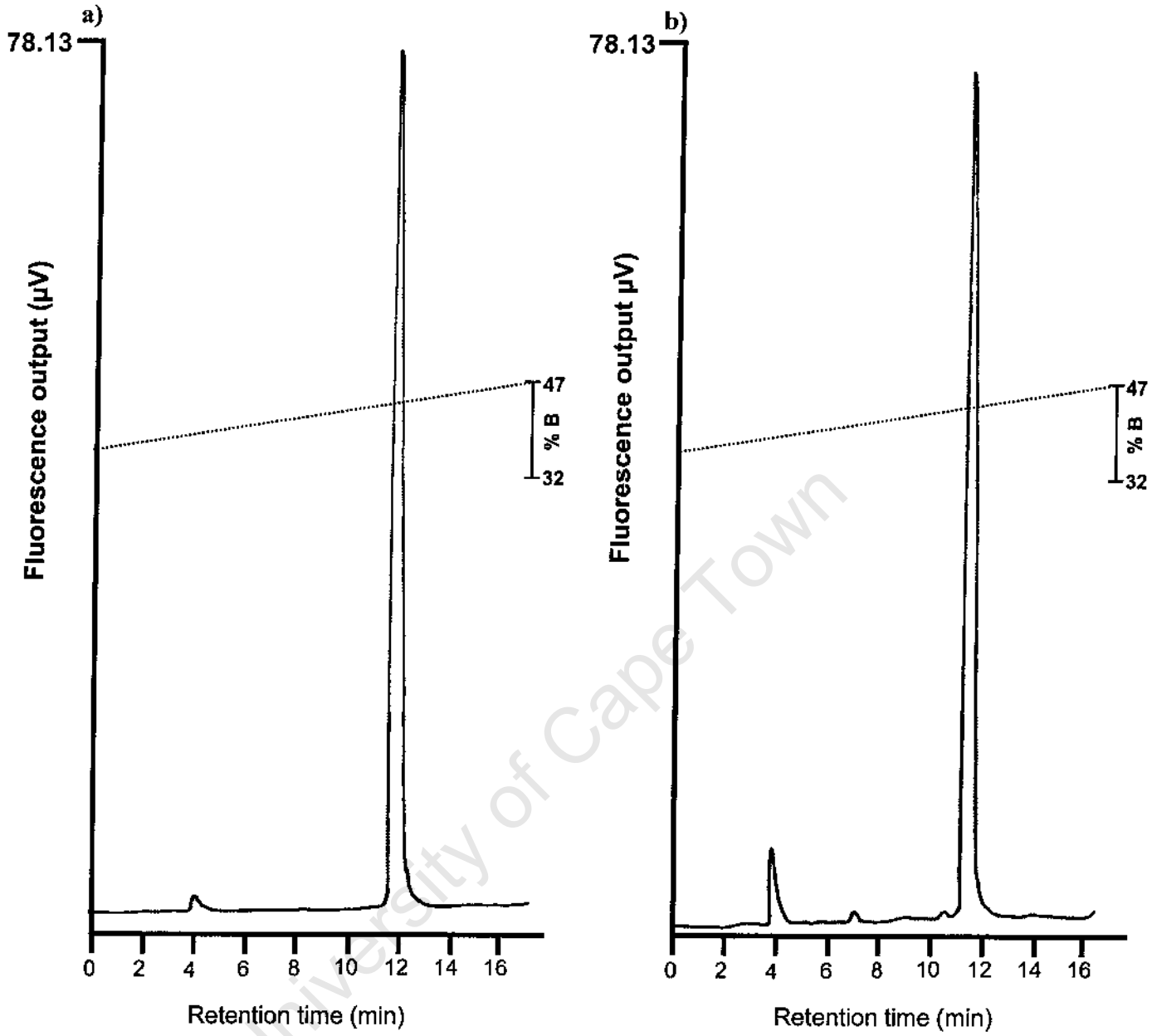


Figure 22: Fluorescence chromatograms, measured at excitation 276 nm/emission 350 nm, of 200 pmol synthetic mGnRH injected directly onto the RP-HPLC column a), and b) 200 pmol of synthetic mGnRH that had been subjected to a mock homogenisation, SEP-PAK prepurification (eluted with 70 % MeOH) and subsequently vacuum dried. Notes the peaks at ~12 minutes. Gradients and solvents as in Figure 21. The hump around minute 4 in both figures is an injection peak and the small humps around minute 7 and 10 in (b) are very likely a small amount of breakdown product.

3.3.2. Biochemical characterisation of mGnRH: pilot study for other GnRHs

Identification of a single synthetic peptide is relatively easy when only that pure substance was injected onto the column. However, identification of specific peptides from a complex mixture (e.g. a crude extract of biological tissue) is not as straight forward as many peaks are detected (for example, Fig. 20). Hence, other means of detection and identification are required. In this part of the pilot study, identification by means of antigenicity (using antibody-678 and direct ELISA) and mass spectrometry were established for synthetic mGnRH.

3.3.2.1. ELISA

Whilst learning the direct ELISA method, it was established that several of the reagents used in the study produce significant levels of non-specific background staining (Table 1). Typically, a well on the microtitre plate containing only substrate material (i.e. para-nitrophenyl phosphate disodium) is used as a reference, or 'blank', to determine the baseline OD value, above which results are considered as positive immunoreactivity. In this study, PBS had relatively high levels of non-specific immunoreactivity (Table 1). Therefore PBS was used as a reference point for all ELISA studies. If an OD reading is negative, it indicates that the level of immunoreactivity of the sample is lower than the level of background stain and therefore is not significant.

Table 1: ELISAs (using antibody-678; 1:250) were used to check for the effect of non-specific immunoreactivity of the different reagents used in this study. OD readings are represented as the mean value for a number of different tests (n) ± standard deviation.

Reagent	Mean OD ± SD	Number of repeats (n)
PBS	0.068 ± 0.023	7
25 % solvent B (15 % acetonitrile)	0.062 ± 0.021	5
Distilled water	0.049 ± 0.023	3

Note: optical density was measured at 405 nm 20 min after the substrate had been applied. A well containing only substrate was used as a reference or "blank".

A standard curve was constructed to establish the sensitivity of the assay to detecting GnRH (Fig. 23). Primary antibody dilutions of 1:250, 1:500 and 1:1000 were compared (Fig. 23 a) and a concentration of 1:250 was selected for further ELISAs as it gave the highest optical density values above non-specific background staining. The OD values for the immunoreactivity of GnRH were linear between 0.1 pmol and 0.5 pmol, above which the curve began to plateau, therefore the standard curve (Fig. 23 b) was constructed using OD values obtained from a range of 0.1 pmol to 0.5 pmol (which can be used for quantitative purposes). The plateau indicates the point of saturation of the binding sites on the microtitre plate and hence, the values which lie below the point of saturation represent the optimal amount of GnRH that this assay is capable of detecting, whilst retaining its accuracy (Kemeny & Challacombe, 1989). Since the R^2 value is high (0.9886, i.e close to 1) one can more accurately infer that the relationship between the two variables (i.e. optical density and the amount of GnRH) is linear. Therefore, given one of the variables the other can be predicted with reasonable accuracy. Throughout the ELISA studies, no immunoreactivity (above background levels) was observed if the primary or secondary antibodies were omitted.

3.3.2.2. Mass spectrometry

While known amounts of synthetic peptide are visible as clear peaks during RP-HPLC (when using the parameters described in Section 2.6), it was important to establish the optimal amount of peptide required for identification of GnRH by mass spectrometry. A series of different amounts of synthetic GnRH, ranging from 20 pmol to 140 pmol, were sent to Osnabrück to determine the optimal amount of peptide required for detection by mass spectrometry. The monoisotopic mass [MH^+] of mGnRH was predicted to be 1182.8 using Protein Prospector (version 5.0.0) and mass analysis of synthetic peptide showed the same value (1182.8) (Fig. 24). The doubly charged ion [M+2H] of molecular weight 592.0 was also visible. The minimum amount of GnRH required for easy and reliable mass spectrometric detection using the facilities in Osnabrück was 40 pmol.

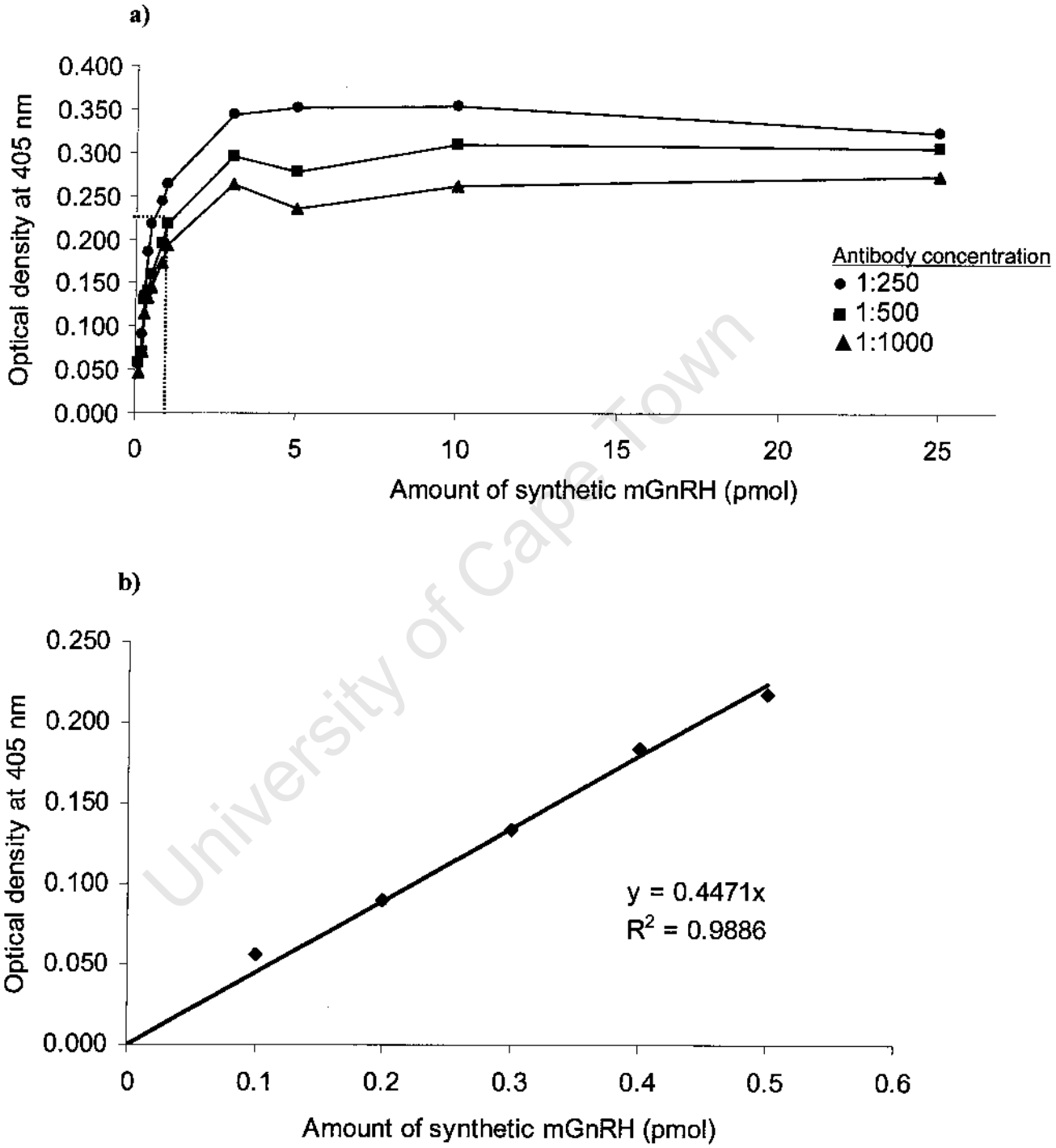


Figure 23: Standard curves constructed using synthetic mGnRH. a) 0.1 to 25 pmol of synthetic mGnRH were prepared in PBS and used in ELISA with antibody-678 at concentrations of 1:250, 1:500 and 1:1000. OD was measured at 405 nm 20 minutes after the substrate chromagen was applied, using PBS as a reference point. b) A standard curve was constructed in the linear part of the curve in a) (indicated by a dotted line, representing 0.1 pmol – 0.5 pmol) when using antibody-678 at a concentration of 1:250. Both the equation of the straight line and the R^2 value are given.

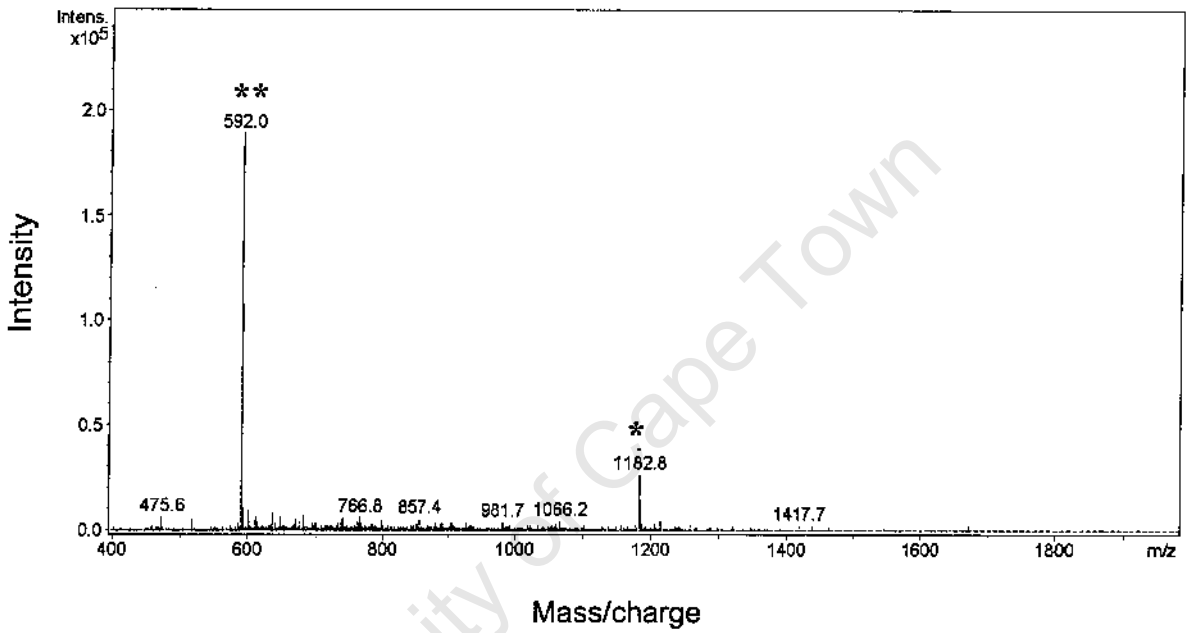


Figure 24: Electrospray ionization mass spectrum of 40 pmol of synthetic mGnRH. The MH^+ of mGnRH is 1182.8 (*), while the doubly charged peptide is 592 (**).
Figure provided by Dr. Stefan Walter

3.3.3. Isolation procedures for GnRHs: pilot study with synthetic mGnRH and mammalian tissue

The procedures followed to extract and isolate putative GnRHs are shown in Figure 25. The repeatability of the entire procedure was first demonstrated several times with 200 pmol and 60 pmol synthetic mGnRH. This also served to establish the extent of peptide lost during the extraction procedures. Known amounts of synthetic GnRH (200 pmol or 60 pmol) were mock treated to the same conditions as crude extracts that had undergone the homogenisation and prepurification process (Fig. 25). Fractions were collected at each washing step, dried, run over HPLC and tested for the presence of immunoreactive material using ELISAs. All washing steps from prepurification were analysed for GnRH immunoreactivity: GnRH was only detected by ELISA in the fraction where the SEP-PAK column had been washed with 70 % methanol; this was substantiated by RP-HPLC (Fig. 22 b). No trace of GnRH was detected in any of the SEP-PAK washing steps, including the final rinsing step. The chromatograms (Fig 22) and ELISA reading (Table 2) of GnRH that had been `prepurified' and GnRH that had been directly injected on the HLPC column were comparable, indicating that little or no peptide was lost during the prepurification process when mGnRH was applied on its own to these procedures (or without mouse or naked mole rat extract).

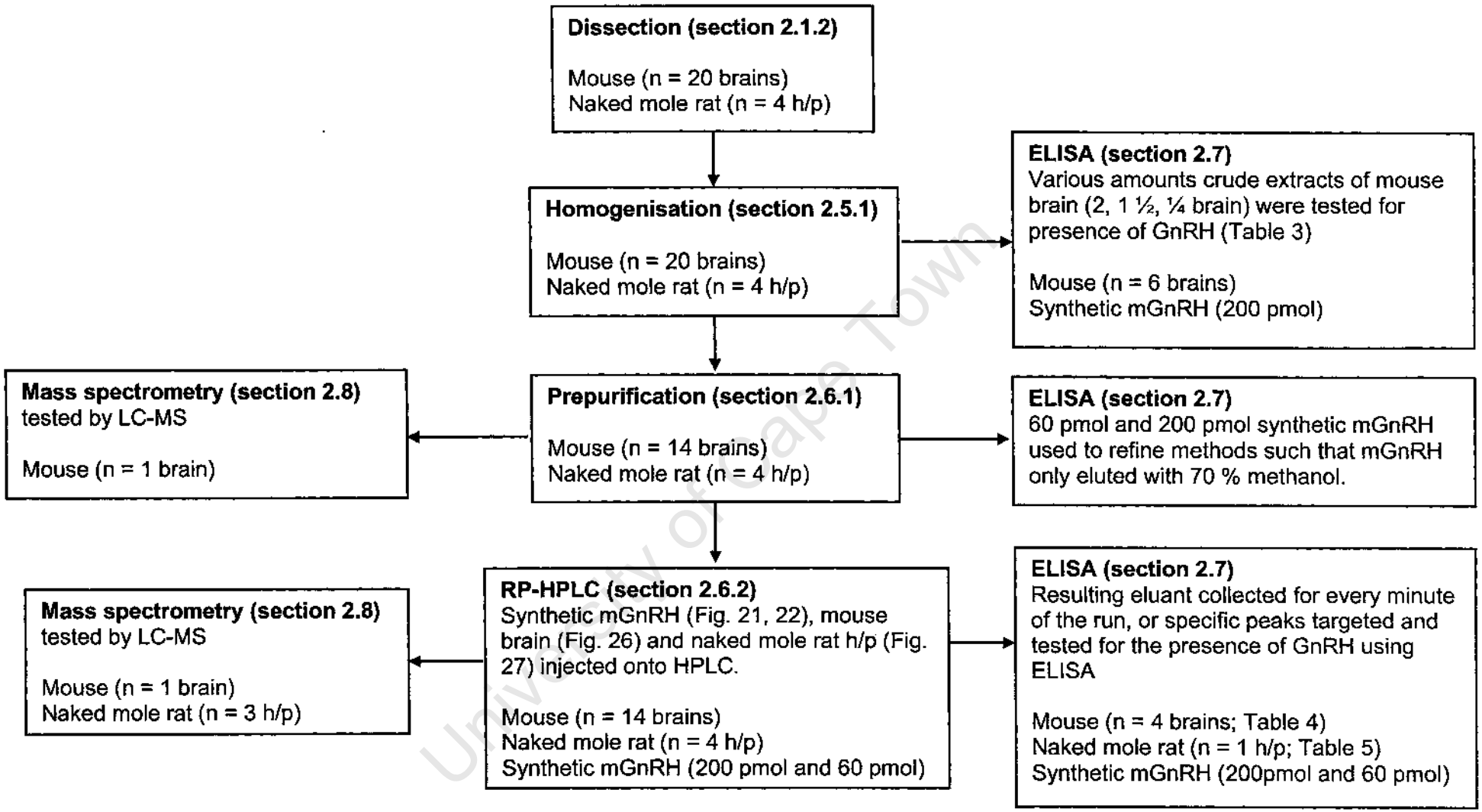


Figure 25: An outline of the methodologies used to attempt extraction, purification and identification of GnRH from mouse brain (n = 20) and naked mole rat hypothalami and pituitaries (h/p; n = 4). Synthetic mGnRH was first used to establish suitable conditions for extraction, purification and identification of this neuropeptide.

3.3.3.1. Trials with mammalian tissue: mouse

Dissections of brain tissue were only carried out on fresh tissue as old or frozen tissue became viscous and difficult to work with as it had lost its structural integrity, risking loss or damage to the neuropeptides. Therefore, to ensure that as little peptide degraded as possible, only freshly killed animals were used for dissection and brain matter was stored at — 80 °C immediately. The outline of the methods used are summarised in Figure 25: briefly, homogenisation proceeded as described in sections 2.5.1 and a proportion (equivalent to 6 brains) of crude extract of mouse brain was plated in ELISA, in different amounts (either equivalent to 2, 1, ½ or ¼ mouse brain), to test for the presence of GnRH prior to the prepurification and RP-HPLC procedures. The remaining crude extract was prepurified as described in Section 2.6.1 and the resulting fractions were vacuum-dried, resuspended in 10 ill — 50 ill of 25 % B, injected onto the RP-HPLC column and subjected to the gradient described in Section 2.6.2. The eluate was hand collected (either for each minute of the program, or targeting specific peaks), vacuum dried and tested for the presence of GnRH using ELISA.

When different amounts of crude extract of mouse brain, equivalent to either 2, 1, ½ or ¼ mouse brains, were tested for the presence of GnRH using ELISA, the immunoreactivity above background staining of PBS was very weak (OD < 0.04) (Table 3). When 60 pmol of synthetic mGnRH (that had not been passed over RP-HPLC) was plated on its own as a control, the OD reading was — 0.280, however, when 1 or ¼ extract of mouse brain was plated together (in the same well) with 60 pmol of synthetic mGnRH the OD values remained low and nowhere near the level of immunoreactivity observed for the control of 60 pmol of synthetic mGnRH (Table 2, 3).

Table 2: Optical density values of 60 pmol of synthetic mGnRH that was plated directly onto an ELISA plate vs. 60 pmol of synthetic mGnRH that was passed through mock homogenisation, prepurification and RP-HPLC procedures prior to ELISA.

Optical density values of 60 pmol synthetic mGnRH	
Plated directly onto ELISA (n = 4)	**Passed through the whole procedure (n = 3)**
0.288	0.304
0.337	0.325
0.279	0.231
0.210	
Mean ± SD: 0.279 ± 0.052	**Mean ± SD: 0.287 ± 0.049**

Table 3: The optical density values for ELISAs of different amounts (represented in Note: Primary antibody 678 (1:250) was used and optical density was measured at 405 nm 20 minutes after the substrate had been applied, using the immunoreactivity of PBS as a reference.

Well contents (number of brains)	**Optical Density**
Mouse brain (2)	-0.042
Mouse brain (1)	-0.026
Mouse brain (1)	-0.015
Mouse brain (1) + 60 pmol mGnRH	0.033
Mouse brain (½)	0.033
Mouse brain (¼)	0.014
Mouse brain (¼) + 60 pmol mGnRH	0.085
60 pmol mGnRH (passed through HPLC)	0.287

Note: Primary antibody 678 (1:250) was used and optical density was measured at 405 nm 20 minutes after the substrate had been applied, using the immunoreactivity of PBS as a reference.

Figure 26 shows the RP-HPLC chromatographic separation at a gradient of 32 % - 47 % B in 15 minutes of one mouse brain that had been prepurified; the RP-HPLC eluant was monitored by both UV and fluorescence detection. The pattern and peak heights were consistent over several applications of mouse brain to RP-HPLC. A number of peaks are visible on both chromatograms but the resolution of the peaks (i.e. the clear separation of peaks) is poor and the peaks are small. Eluant was collected from each minute that elapsed for the duration of the run and the resulting 15 fractions were tested singly for the presence of GnRH using ELISAs. Out of all 15 fractions, only minute 10 (t_{10}) had very faint cross-reactivity with antibody-678 above background staining, indicating the potential presence of GnRH (Table 4).

In the approach described above, each minute was collected and peak material was not targeted specifically. As a result, it is likely that there is some dilution effect due to the fact that a single peak can be spread over more than one minute. Considering that the retention time of synthetic mGnRH (when subjected to the same gradient under the same conditions) was 12 minutes, the peak(s) most likely to represent mGnRH in the mouse should occur between 10 and 13 minutes.

Referring to Fig. 26, there are 3 small peaks in the region between 10 and 13 minutes (named peak 1, 2 and 3). Peak 1 eluted at 10.5 minutes, peak 2 eluted at 11 minutes and peak 3 eluted at 13 minutes. A repeat experiment was conducted where a single, prepurified mouse brain was passed over the RP-HPLC column using the prescribed gradient. Instead of collecting fractions for every minute that elapsed, the eluant from the three peaks between 10 and 13 minutes was collected and the material from each peak was tested for the presence of GnRH using ELISA and antibody-678. The OD values corresponding with the three peaks were very low, and remained so even when the amount of source tissue was doubled (i.e. 2 prepurified mouse brains applied to RP-HPLC) (Table 4).

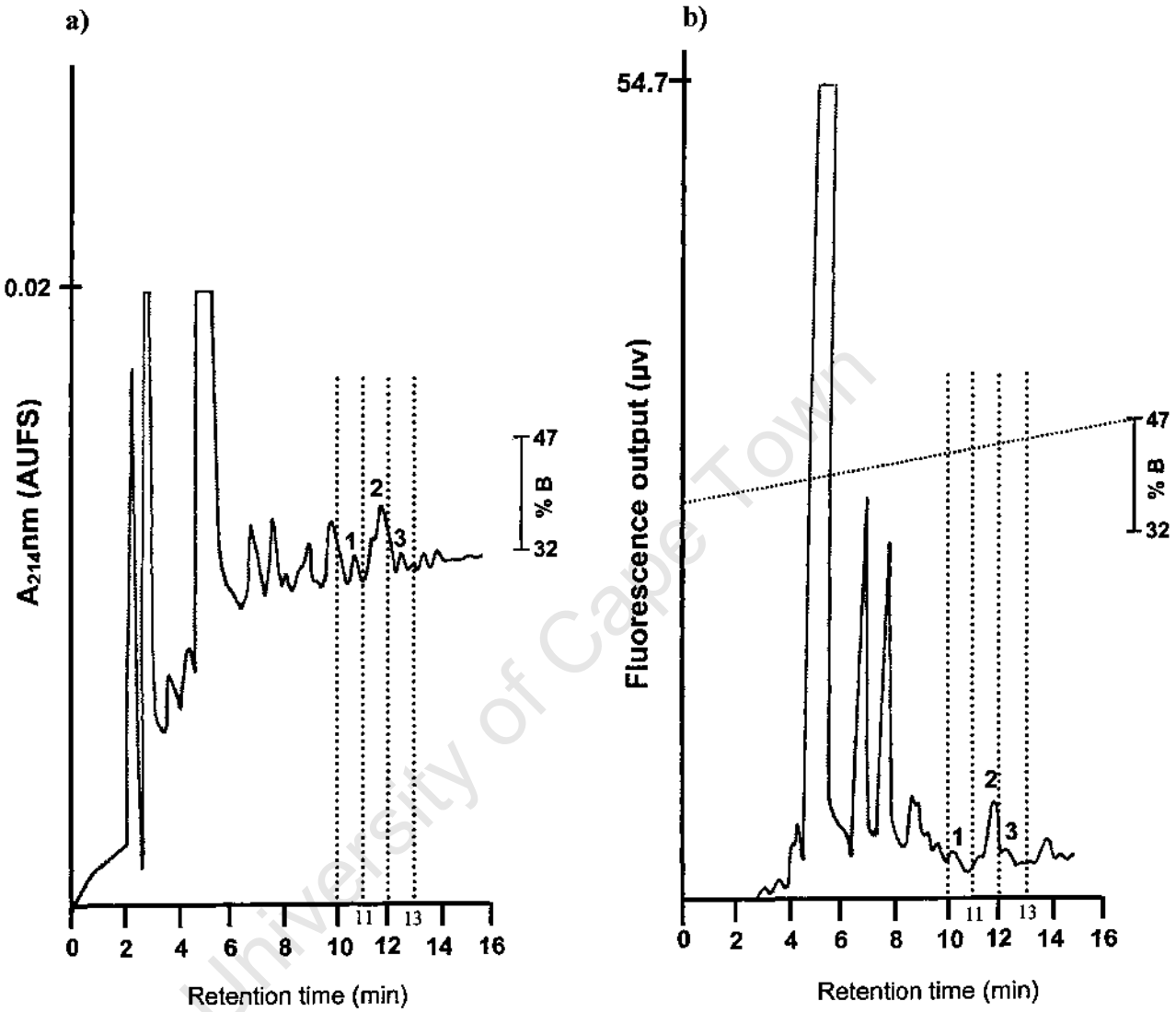


Figure 26: UV absorbance (a), measured at 214 nm in AUFS (absorption units full scale) and fluorescence (b) measured at excitation 276 nm/emission 350 nm, profiles of prepurified extract from the brain of 1 mouse. Note peaks 1, 2 and 3. Gradients and solvents as in Figure 21.

Table 4: Optical density values from an ELISA of 1 prepurified extract of mouse brain where fractions were collected at each minute during RP-HPLC of, as well as the OD values obtained for peaks 1, 2 and 3 for purified extract of one and two mouse brains respectively. Optical densities above non-specific background staining are shaded grey.

Time (minutes)	OD	Time (minutes)	OD
0	-0.008	8	-0.001
1	-0.013	9	0.001
2	-0.005	10	0.006
3	-0.013	11	-0.013
4	-0.005	12	-0.012
5	-0.008	13	-0.005
6	-0.001	14	-0.014
7	-0.012		

Optical densities (OD) of peak material			
One brain	**OD**	**Two brains**	**OD**
Peak 1	0.020	Peak 1	0.013
Peak 2	0.017	Peak 2	0.016
Peak 3	0.008	Peak 3	0.031

Note: Fractions were plated singly; minute 0 includes material collected between 0 and 1 minute. Primary antibody-678 (1:250) was used and optical density was measured at 405 nm 20 minutes after the substrate had been applied, using the immunoreactivity of PBS as a reference. Negative readings indicate no immunoreactivity.

3.3.3.2. Trials with mammalian tissue: naked mole rat

Although experiments for the purification of GnRH from the mouse brain were inconclusive, an attempt was made to extract GnRH from the brain of the naked mole rat. Naked mole rat pituitary and hypothalamus samples from a total of four naked mole rat brains were used in this experiment as GnRH is located in these regions as shown by the immunocytochemical part of this study. These brain regions were homogenised, prepurified and passed over the RP-HPLC column (2 brains were processed at a time and then pooled). Peak material was collected at each minute for the entire duration of the HPLC run (Fig. 27), using the established RP-HPLC method (Section 2.6.2.2.3). The fluorescence chromatograms of brain material from the mouse and naked mole rat have several similarities. Both have two distinct peaks which elute at 7 and 8 minutes, neither of which are immunoreactive when analysed for the presence of GnRH by ELISA (Table 5), as well as poorly resolved peaks between 10 and 15 minutes. When examining the UV chromatogram (of naked mole rat tissue), likely candidates for GnRH (based on retention time) are three well resolved peaks between 10 and 15 minutes. In naked mole rat tissue, a major peak elutes at — 14 minutes (— 28 % acetonitrile) and two minor peaks elute at — 11 minutes (— 26 % acetonitrile) and —12 minutes (— 26.5 % acetonitrile), which correspond (in terms of retention time) to peaks 2 and 3 from mouse tissue (Fig. 26). It is important to note that these peaks are not visible by fluorescence detection, indicating that they do not contain a tryptophan. In the fluorescence chromatogram of naked mole rat tissue, the resolution of the peaks that elute between 10 and 15 minutes was poor. The size of the peaks in the latter half of the chromatogram were also very small, indicative of a small amount of peptide material. The equivalent of three hypothalami and pituitaries were retained for analysis by mass spectrometry, while the remaining peak material was used to test for GnRH immunoreactivity. Some fractions showed very weak immunoreactivity (Table 5) at minutes 3, 7, 8, 10, 12 and 13. The highest immunoreactivity corresponds to a small peak at 7 minutes, which is only just visible in the UV chromatogram, and not at all in the fluorescence chromatogram due to the poor resolution of the peaks. While faint immunoreactivity was detected in the naked mole rat and mouse brain extracts by ELISA, the corresponding amount of GnRH was small and lie below the threshold which is detectable by mass spectrometry.

Therefore, due to lack of material, GnRH remained undetected in samples of mouse and naked mole rat tissue.

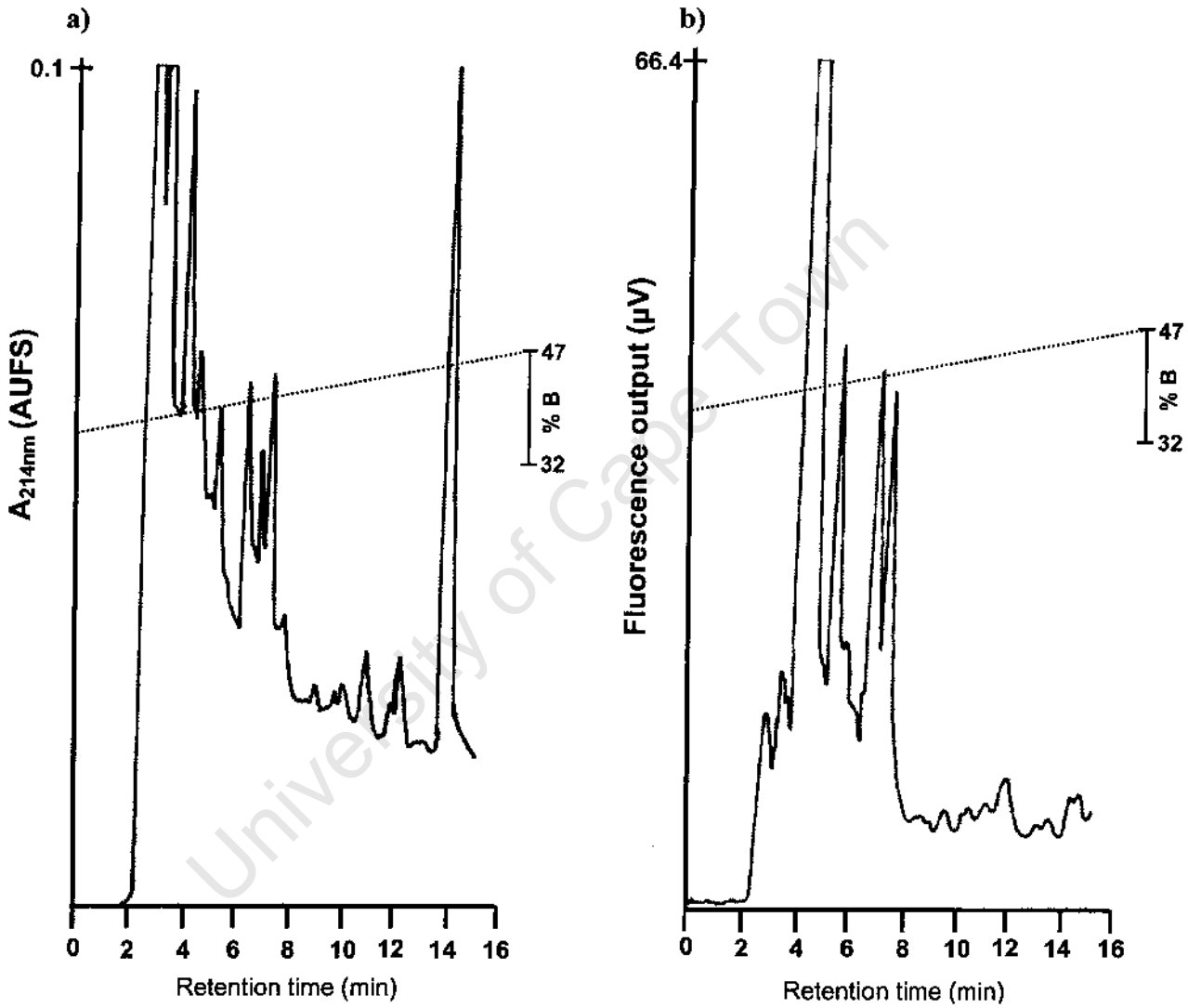


Figure 27: UV absorbance (a), measured at 214 nm in AUFS (absorption units full scale) and fluorescence (b) measured at excitation 276 nm/emission 350 nm, profiles of prepurified extract from the hypothalami/pituitaries of 4 naked mole rats. Gradient and solvents as in Figure 21.

Table 5: The absorbance readings of fractions collected at each minute during RP-HPLC of prepurified extract of 1 naked mole rat hypothalamus/pituitary. See also Table 4 for methodological details.

Time (minutes)	OD reading	Time (minutes)	OD reading
0	-0.004	8	0.003
1	-0.013	9	-0.016
2	-0.013	10	0.025
3	0.016	11	-0.015
4	-0.014	12	0.020
5	-0.008	13	0.015
6	-0.024	14	-0.016
7	0.036		

CHAPTER FOUR: DISCUSSION

4. Introduction

Social behaviours are the result of complex evolutionary adaptations of brain morphology, connectivity and chemistry (Insel & Young, 2000). For example, in reproductive suppression in vertebrates, behavioural cues (such as courtship displays and the release of pheromones) act at varying levels on the hypothalamic-pituitary-gonadal axis (Rissman *et al,* 1997), disrupting or altering the hormonal cascade that controls reproductive processes. Generally, in social animals, reproductive success relies heavily on social status, with the most dominant individuals producing more offspring than individuals lower down in the dominance hierarchy (Faulkes *et al,* 1991). Naked mole rats are eusocial and are well known for their unique breeding regime and bizarre social structure, yet the physiological mechanisms underlying these features are poorly understood. Renewed interest in neuropeptides has permitted the study of behaviour at a molecular and cellular level as most neuropeptides and their receptors are located in discrete regions in the brain and typically function as neuromodulators with slower, long lasting effects. Neuropeptide systems are also known to show noteworthy plasticity during development and in response to fluctuations in the hormonal milieu (Insel & Young, 2000). In vertebrates, the hypophyseal-portal system is important for regulating a host of physiological processes which are mediated by neuropeptide hormones. Reproduction is one of such processes which occurs as a result of the delicate interplay between species-specific copulatory behaviour (such as lordosis and genital nuzzling in *H. glaber)* and physiology. The dynamic nature of reproductive condition is facilitated by the GnRH neuronal system (Rissman *et al, 1997).* Since *H. glaber* have an unusual social setup, the current study serves as a comparison between the GnRH systems of naked mole rats at different levels of the social hierarchy.

4.1. Body and brain morphometrics

The body size and mass of female naked mole rats is closely linked to their breeding and social status (Jarvis *et al,* 1991). In the current study, high ranking and reproductively active females were significantly larger and heavier than low ranking non-breeders (Fig. 9), results which corroborate other studies (Jarvis *et* al, 1991). Subordinates also have a prepuberty like body form and hormonal milieu (Faulkes *et al,* 1991). However, since subordinates are not sterile, they are capable of climbing up the reproductive hierarchy (provided that suppression is released) and therefore can change from this prepuberty like state to one where reproductive activation is accompanied by increased body size (Faulkes *et al,* 1991). From the current study, it appears that these morphological alterations are not discrete but rather form a continuum i.e. as the subordinate reproductive system gradually responds to modifications in the internal hormonal state, their morphology changes accordingly which eventually gives rise to the morphology observed in a breeding female.

The brain is the organ which is central to driving these drastic morphological changes and therefore one would expect that the changes in behaviour, body size and neuroendocrine functioning observed in *H. glaber,* would result because of alterations in brain morphology. The current study examined whether the differences observed in somatic morphology persist in the brain, by measuring the entire brain as well as brain areas that are known to be involved in reproductive function, namely the hypothalamus and pituitary. Observations from the current study reveal that the gross anatomy of the naked mole rat brain is similar to that of the mouse brain with the major external features (such as the brain stem, hypothalamus, cerebral hemispheres and cerebellum) in analogous positions. Another interesting observation is that while naked mole rats are blind, they still possess a residual optic chiasm. In 2006, Xiao *et al* published the first stereotaxic atlas of a naked mole rat brain. A total of 15 animals (sex not specified) aged between 2 and 24 years were used to construct the atlas, and the figures presented in that publication represent sections from the brain of a male naked mole rat, aged 2 years. Subsequently, Xiao published a report which stated that there was no difference in brain length between naked mole rats of different ages or body masses, although he does not report on differences between males and females

or breeders and non-breeders (Xiao, 2007). The results generated in the current study on female specimens are consistent with Xiao's report that brain length does not differ between naked mole rats of different ages or body masses. In addition, my study shows that brain width, hypothalamus width and hypothalamus length of breeding and non-breeding females do not differ, despite significant variations in body mass, body length and breeding status.

The current study reveals that pituitary size and shape however, differ in female naked mole rats depending on breeding status (Fig. 12, 13). The pituitary gland of the queen is large and bulbous with clearly defined anterior and posterior regions and is densely surrounded by connective tissue, while the subordinate pituitary gland tends to be smaller, particularly in the posterior region. Interestingly, one subordinate in the current study was semi-perforate, indicating some level of reproductive activation. She was also the longest and heaviest subordinate, had the largest pituitary and one of the highest degrees of GnRH immunoreactivity of the subordinate group. This observation shows that the changes in reproductive physiology of subordinate females (such that she would begin to become perforate) are accompanied by an increase in overall body and pituitary gland size. Therefore, from the current study, it seems that these morphological characteristics are not discrete, but rather form a continuum of reproductive activation which parallels the reproductive hierarchy (although this should be more rigorously investigated with an increased sample size, as well as by comparing hormonal state and body morphometrics).

The enlarged posterior pituitary of breeding females, relative to the smaller posterior pituitary of subordinates (observed in the current study) may be as a result of increased demand for the neuropeptides vasopressin and oxytocin. These neuropeptide hormones are small (9 amino acids long), similar in structure and both are produced by neurons in the hypothalamus and stored and released into the blood from the posterior pituitary (Cheung & Lustig, 2007). Vasopressin is principally involved in osmoregulation but has also been implicated in pair bonding, parental and dominance-subordinance behaviour (Bielsky & Young, 2004; Rosen *et al,* 2007). Immunocytochemical studies of the hypothalamic and brain stem regions of naked mole rat brains (Rosen *et al,* 2007) found no sexual dimorphism of the numbers of vasopressin cells when comparing male and female naked mole rats. However,

significant differences were observed when comparing breeding and non-breeding males and females, with greater numbers of vasopressin cells in the hypothalamus of breeders of both sexes. Oxytocin is involved primarily in the milk-ejection reflex during lactation, as well as facilitating contractions during parturition (Cheung & Lustig, 2007). It has also been implicated in the modulation of social behaviours, such as pair bonding, and sexual behaviour (Bielsky & Young, 2004; Holmes *et al,* 2009). Another immunocytochemical study by Rosen *et al* (2008) investigated oxytocin in the naked mole rat hypothalamus and showed that, as with vasopressin, there was no sexual dimorphism in the distribution of oxytocin when comparing subordinate males and females. However, the study neither examined the pituitary of the naked mole rat, nor explored the distribution of oxytocin in the brains of breeding females. The current study is one of the first to comment on pituitary size in naked mole rats.

In other species, changes in social or breeding status have been associated with alterations in the morphology of brain cells, particularly of GnRH neurons. For example, the size of GnRH neurons in African cichlids *(Haplochromis burtoni)* increases in sexually mature females as well as in dominant males with larger territories (Rissman *et al,* 1997). Immunocytochemical studies on the GnRH cells of female musk shrews *(Suncus murinus)* show that the number and size of the cells increase after female musk shrews have been exposed to males (Dellovade & Rissman, 1994; Dellovade *et al,* 1995). In this species, the females are spontaneous ovulators and mating activates ovulation, hence the increase in GnRH cell size and number. Little research has focussed on the comparative brain morphology of breeding and non-breeding naked mole rats. However, a study conducted by Holmes *et al* (2007) used sections of naked mole rat brain, stained with thionin, to compare the size of specific brain nuclei (aggregation of nerve cells), as well as the number and size of cells contained within those nuclei, between breeding and non-breeding males and females. They demonstrated that the ventromedial nucleus of the hypothalamus, which is known to be key in reproductive behaviour in other mammals (lesions to this area eliminate the reproductive behaviour, lordosis; Malsbury *et al,* 1977), contains more cells in breeders than in non-breeders, although the identity of the cell types is unknown. From results obtained in the current study, there seems to be little difference in overall hypothalamus size, however, from the work described above, there is clear evidence of plasticity of the neurosecretory cells in this region of the

naked mole rat brain. GnRH neurons typically reside in the hypothalamus, and therefore evidence of neural plasticity (i.e. the ability of neurons to change their size, morphology, number or organisation) concerning other neuropeptides in this region captured the interest of my study.

4.2. GnRH in *H. glaber* and other mole rats

4.2.1. Immunocytochemical studies: method development

The current study is one of the first studies to identify GnRH in the brains of naked mole rats and to conduct a comparison between the GnRH neuron system of breeding and non-breeding females using immunocytochemistry. From an ethical and biological perspective, brain tissue is valuable as it involves sacrificing animals for experimental material thereby depleting a stock which is not quickly or easily replenished. Obtaining brain tissue of breeding *H. glaber* females is particularly difficult as queens are long lived and therefore the number of available specimens is limited, unless one has access to a large number of colonies. Another ethical consideration is that by simply removing a breeding female from a colony, severe social instability can result which may lead to the (unnecessary and brutal) deaths of other naked mole rats in the colony. Taking these considerations into account, the immunocytochemical methodology, in the current study, was first applied and optimised with non-mammalian tissues.

Considering that the hypophyseal-portal system is imperative for the correct functioning of the reproductive system of vertebrates, and that it is functionally equivalent to the corpora cardiaca/corpora allata system in insects and X-organ/sinus gland complex in crustaceans (Fig. 1), immunocytochemistry was first optimised by using tissue from the eyestalks of./ *lalandii.* The antigen selected in this species was crustacean hyperglycaemic hormone (cHH) which is a neuropeptide hormone involved in glucose metabolism in crustaceans; it is produced in the X-organ and stored in the sinus gland of *J. lalandii* eye stalks (Marco *et al,* 1998). The results of the present study clearly show a strong immunoreaction to a cHH antibody in the sinus gland in the eyestalk, which confirms previous data that the sinus gland is the

storage site of this neuropeptide hormone (Marco & Gäde, 1999). It further demonstrated that the immunocytochemical procedure is ideal for use on neural tissue. The methodology was further refined for detecting GnRH in the hypothalamus of sexually mature mice. In the region of the hypothalamus, GnRH immunoreactivity in the mouse brain was found in the median eminence (Fig. 15), furthermore, a few neurons were located in the region of, and just posterior to, the optic chiasm as well as in the mediobasal hypothalamus (results not shown). This distribution of GnRH in this region of the brain is typical in mice (Douglas, 1976; Gill *et al,* 2008) and is likely to represent mGnRH as this is the only form that mice are currently known to possess (Somoza *et al,* 2002). Therefore, the use of mouse brain tissue as a control demonstrates that the methodology used in the current study was suitable for neuropeptide detection in rodent tissue. There was little background staining when using the selected antiserum (antibody-678, which binds to the N- and C- terminals of GnRH) and omission of the primary or secondary antibodies abolished all staining, indicating specificity of the antiserum for GnRH. Staining was also eliminated in tissue sections of mouse brain treated with preabsorbed antigen (GnRH) — antibody (678) complexes, thus reaffirming the specificity of antibody-678 for GnRH.

4.2.2. Immunocytochemical studies: naked mole rats

The sequence of the form of GnRH found in naked mole rats is currently unknown, and therefore the broad spectrum antibody-678 was used as it binds to the N- and C-terminals. Since these structures are highly conserved in all forms of GnRH studied to date (King *et al,* 1994, Millar *et al,* 2008, Fig. 2), it was ideal for this explorative type study. The advantage of using an antiserum capable of detecting a wide variety of GnRHs means that if the study species has an unknown or variant form of GnRH, such as in the naked mole rat, it can be detected even though overall structural identity may be unknown (provided that the terminal structures are conserved). However, a shortcoming of using this antiserum is that conclusions cannot be drawn about the different GnRH systems (i.e. GnRH I, II or III) directly. The current study measured a representative area (every 20th section) of GnRH-ir in the brains of naked mole rats and compared this between breeding and non-breeding females. This is the first study to identify GnRH in the brain of the naked mole rat, and while the exact form of

GnRH found in this species remains unknown, immunoreactivity was detected using antibody-678.

The distribution of GnRH in the naked mole rat brain was closely examined and is restricted exclusively to the caudal part of the hypothalamus, specifically the median eminence and the anterior pituitary in both queen and subordinate naked mole rats. No GnRH immunoreactivity was detected in the medial septum or the preoptic area. The number of sections containing immunoreactivity did not differ significantly between queens and subordinates. Queens, however, have a significantly larger area of immunoreactivity when compared to subordinates, suggesting that GnRH is inhibited in subordinates at the level of production.

In most mammals, GnRH neurons lie along the septo-preoptico-infundibular continuum, however there are interspecies differences with respect to the degree to which they are present caudal to the POA (Molteno *et al,* 2004; Johnson & Sidman, 1979). For example, GnRH neurons in the rat typically reside in the preoptic area and do not extend to the mediobasal hypothalamus (Merchenthaler *et al,* 1984) while GnRH cell bodies in the brain of the opossum *(Mondelphis domestica)* do not migrate into the preoptic area at all (Molteno *et al,* 2004). In the mink *(Mustela vision),* only a few GnRH cells reside in the preoptic area while the majority are found in the mediobasal hypothalamus (Ntoumi *et al,* 1994; Molteno *et al,* 2004). The variability of GnRH distribution along the septo-preoptico-infundibular continuum in other mammalian species may be associated with the migration of GnRH neurons from the olfactory placode. The detection of GnRH in the anterior part of the pituitary gland of naked mole rats in the current study is significant as it is an uncommon location for GnRH neurons to reside in the rodent. There have been few reports locating this neuropeptide hormone in the pituitary, however, GnRH immunoreactivity was detected by immunocytochemistry in the rat anterior pituitary (Bauer *et al,* 1981; Li *et al,* 1984) and GnRH mRNA has been isolated from both rat and human pituitary cells (Pagesy *et al,* 1992; Miller *et al,* 1996; Schirman-Hildesheim *et al,* 2005; Mongait *et al,* 2006). Studies on ewes suggest that there are two populations of GnRH neurons which regulate gonadotropin release (Lehman *et al,* 1997). Schirman-Hildesheim *et al* (2005) proposes that GnRH neurons in the pituitary prime the organ for the LH surge, while GnRH neurons in the hypothalamus control the more constant release of FSH. It

is not implausible to suggest that GnRH neurons reside in the pituitary as both GnRH neurons and the anterior pituitary are embryologically of ectodermal origin (Burgess *et al,* 2002).

It is important to note that there is no evidence of contamination of the tissue sections, such that the staining observed would be an artefact of the antiserum binding to the peptide-receptor complex rather than to the GnRH contained within the neurons. The GnRH antiserum used in this study has been well characterised and is known to bind to the terminal ends of the peptide (King *et al,* 1994). The termini are also the binding site for the receptor interactions (Millar, 2005) and therefore would not be free to bind to both the receptor and the antiserum. Staining was also abolished in tissue sections of naked mole rat brain treated with preabsorbed antigen (GnRH) — antibody (678) complexes. While the form of GnRH found in naked mole rats remains unknown, there is reason to believe that it is not mGnRH. Interestingly, a pilot immunocytochemical study on naked mole rat tissue (conducted in the laboratory of Dr Marco) showed no immunoreactivity in brain sections incubated with an antiserum specific for mGnRH (antibody-1076, a polyclonal raised in rabbits which binds to amino acids 4 to 7), while adjacent sections incubated with the broad spectrum antibody-678 showed clear immunoreactivity. When antibody-1076 was applied to mouse brain, which is known to contain mGnRH, staining was clear in the median eminence as expected (results not shown). This implies that the form of GnRH found in *H. glaber* is not mGnRH, but a variant with structural differences in the region of amino acids 4 to 7 (Caitlin Smith & Heather Marco, unpublished observation). Most mammals studied to date possess mGnRH, except for the guinea pig and the capybara which have the structural variant gpGnRH (Montaner *et al,* 1999; Grove-Strawser *et al,* 2002). A study by Kalamatianos *et al* (2005) revealed that a partial cDNA sequence of the GnRH precursor in Highveld mole rats *(Cryptomys hottentotus pretoriae),* of the same suborder as naked mole rats, has the highest degree of identity (86 %) with that of the guinea pig GnRH precursor mRNA. Like the naked mole rat, the Highveld mole rat is a social, subterranean rodent (van der Walt *et al,* 2001). Because of the relatively close phylogenetic relationship between naked mole rat, Highveld mole rat and guinea pig, it is highly possible that naked mole rats do not possess mGnRH but rather a variant that is similar to gpGnRH, although this remains to be elucidated. gpGnRH differs from mGnRH at both positions 7 (in the region

where antibody-1076 binds) and position 2 (in the region where antibody-678 binds). Therefore it is important to note that if the form of GnRH found in naked mole rats does in fact have a different amino acid structure to mGnRH, then the affinity of antibody-678 for the GnRH antigen may be diminished if the variation lies within the terminal structures.

4.2.3. A comparison of the GnRH system in Bathyergids

Within the Bathyergid family there is a continuum of sociality from species that are solitary to ones that are highly social and to date, the GnRH systems of a number of these mole rat species have been fairly well examined, although the amino acid sequence of the GnRHs found in these species has not been elucidated (Table 6). The solitary species breed seasonally and are reproductively constrained by ecological factors, such as rainfall and food availability, rather than by social interactions (Oosthuizen & Bennett, 2007). The Cape mole rat *(G. capensis)* and the Cape dune mole rat *(B. suillus)* are two solitary mole rat species that have been comprehensively studied. Both display a high degree of aggression towards conspecifics (Hart *et al,* 2008) and will only tolerate other individuals in their tunnels during the breeding season (Oosthuizen & Bennett, 2007). During the non-breeding season, there is a significant decrease in the levels of LH in both species (FSH was not investigated), which results in gonadal regression in the Cape mole rat, a feature which is yet to be investigated in the Cape dune mole rat (Oosthuizen & Bennett, 2007; Hart *et al,* 2008). A neuroanatomical investigation on the brain of the Cape dune mole (Table 6) rat by Hart *et al* (2008) revealed that GnRH I neurons were located in the region of the medial septum, preoptic area and the mediobasal hypothalamus. GnRH processes were found in dense aggregations in the OVLT (which lies anterior to the hypothalamus) and the external zone of the median eminence. Cape mole rats have a similar distribution of GnRH I neurons with immunoreactivity (using a rabbit anti-GnRH I primary antiserum) detected in the medial septum and preoptic area, however only a few ir-cells were located in the caudal part of the mediobasal hypothalamus (Table 6). GnRH I processes were also found in the OVLT, in the regions surrounding the suprachiasmatic nucleus as well as in the lateral margins of the median eminence where they were particularly dense (Oosthuizen *et al,* 2008).

Table 6: A comparison of the brain regions with GnRH immunoreactivity in mole rats.

Common name	Solitary/social	Brain region							Reference
		MBH	ME	MS	POA	OVLT	ACN	Pituitary	
Cape dune mole rat	Solitary	✓	✓	✓	✓	✓	x	x	Hart *et al* (2008)
Cape mole rat	Solitary	✓	✓	✓	✓	✓	x	x	Oosthuizen *et al* (2008)
Natal mole rat	Social	✓	✓	✓	✓	✓	x	x	Oosthuizen *et al* (2008)
Common mole rat	Social	✓	✓	✓	✓	✓	x	x	Du Toit *et al* (2005)
Highveld mole rat	Social	✓	✓	✓	✓	✓	✓	x	Du Toit *et al* (2005)
Damaraland mole rat	Social	✓	✓	✓	✓	✓	x	x	Molteno *et al* (2004)
Naked mole rat	Eusocial	✓	✓	x	x	x	x	✓	Current study
Mouse	Solitary	✓	✓	✓	✓	✓	✓	x	Gross & Baker (1976)

Abbreviations

ACN	arcuate nucleus	MS	medial septum,
MBH	mediobasal hypothalamus	OVLT	organum vasculosum of the lamina terminalis
ME	median eminence	POA	preoptic area
✓	GnRH present in this region	x	GnRH absent in this region

Social mole rat species do not only contend with ecological constraints, but the community structure of all social mole rats is such that reproduction is monopolised by a single female and one to three breeding males (Faulkes & Bennett, 2001), providing an additional avenue through which reproductive suppression can act. Colony sizes can vary from just a few individuals, as in the Mashona mole rats, to several hundred like *H. glaber*. Individuals of all mole rat species (barring naked mole rats) use incest avoidance as means of controlling reproduction (Faulkes & Bennett, 2001). Suppression of reproduction in subordinate Mashona mole rats *(C. darlingi)*, the Giant Zambian mole rat *(Cryptomys mechowi)* and the common mole rat *(Cryptomys hottentotus hottentotus)* is mediated exclusively by behavioural means (Bennett *et al,* 1997; Bennett *et al,* 2000; Sharff *et al,* 2001; Faulkes & Bennett, 2001). The Damaraland *(C. damarensis)* and Highveld *(Cryptomus hottentotus pretoriae)* mole rats are inhibited from reproducing by behavioural and physiological means, while the naked mole rats are suppressed entirely by inhibition of ovulation (Bennett *et al,* 1996; du Toit *et al,* 2006; Bennett *et al,* 2007). Highveld mole rats have decreased follicular activity and the concentration of circulating LH (FSH not investigated) in this species is significantly lower in non-breeding females in comparison to breeding females. Research by van der Walt *et al* (2001) show that subordinates of this species have decreased pituitary sensitivity when subjected to doses of synthetic GnRH, however, the author does not disclose the type of GnRH used in their study. The partial cDNA sequence of the GnRH precursor in Highveld mole rats shows the highest degree of identity with gpGnRH precursor mRNA (Kalamatianos *et al,* 2005), therefore, it is likely that this is the form of GnRH found in the Highveld mole rat. If so, it is imperative that gpGnRH be used for biological assays in this species rather than mGnRH. Damaraland mole rats *(C. damarensis)* are perhaps the most interesting as in terms of social structure and habitat, they are very similar to naked mole rats. *C. damarensis* are highly social, subterranean bathyergids and their reproduction is limited to a single breeding pair. Behaviourally, Damaraland mole rats are reproductively suppressed by incest avoidance; however, there is an additional physiological inhibition of ovulation in subordinate females (Faulkes & Bennett, 2001). Subordinate Damaraland mole rat females have very low concentrations of circulating LH (4.3 ± 0.64 mi.u./ml, Bennett *et al,* 1996) as well as a decreased LH release in response to low dose (2 µg) GnRH challenges (FSH not

investigated). Urinary progesterone levels in this species are also low, indicating that not only is there down-regulation of GnRH receptors in the subordinate pituitary, but the decreased LH release is sufficient to block ovulation (Bennett *et al,* 1996). Interestingly, GnRH neurons of subordinate Damaraland mole rats have a higher hormone content (measured by radioimmunoassay using extracts of mole rat brain (n = 10) and antibody-678; Molteno *et al,* 2004) than the GnRH neurons of breeders and the authors conclude that that the block to ovulation is a downstream effect of impaired GnRH release rather than decreased GnRH production.

The block to ovulation observed in subordinate naked mole rats is also a result of depleted plasma LH. The concentration of LH in subordinates is significantly lower than in breeding females (Faulkes *et al,* 1990b) suggesting some disruption of the hypothalamic-pituitary-gonadal axis. There is also reason to suspect that the subordinate pituitary is desensitised to GnRH as injections of 0.1 µg of synthetic peptide produced lower LH responses in subordinates (1.3f 0.2 to 2.9 ± 0.5 mi.u/ml) than it did in queens (3.4 ± 0.8 to 9.6 ± 2.0 mi.u./ml). Changes in pituitary responsiveness to synthetic GnRH have been observed in rats where pituitary receptor concentrations parallel the hormonal fluctuations of the oestrus cycle (Smith, 1984; Fox & Smith, 1985; Schirman-Hildesheim *et al,* 2005).

To date, neuroanatomical studies using immunocytochemistry have been conducted on the brains of four social mole rat species (Table 6), namely the Natal mole rat *(C. hottentotus natalensis),* the Common mole rat *(C. hottentotus hottentotus),* Highveld mole rat *(C. pretoriae)* and the Damaraland mole rat *(C. damarensis).* In all four of these species, GnRH neurons were found in the region of the medial septum and preoptic area. Ir-neurons were also located in or around the OVLT. The distribution of cells in the hypothalamus differed slightly between the different species. In the Highveld mole rat, cells were found in the mediobasal hypothalamus, particularly in the region of the arcuate nucleus, while in the Common, Natal and Damaraland mole rats only few or no cells were found in this region (Molteno *et al,* 2004; Oosthuizen *et al,* 2008; du Toit *et al,* 2006). GnRH neurons and processes were also detected in the subfornical organ (found on the dorsal part of the third ventricle) with dense aggregations of processes in or around the OVLT and a scattering of immunoreactivity in the suprachiasmatic nucleus. In the highveld and the common

mole rat, the only two social species which breed seasonally, GnRH neurons were detected in the arcuate nucleus (du Toit *et al,* 2006). In all four species, the GnRH neurons project their processes to the external zone of the median eminence. In the common, Highveld and Damaraland mole rats these aggregations of projections occur in the lateral edges of the median eminence, while the processes in the natal mole rat lie across the breadth of the median eminence (Molteno *et al,* 2004; Oosthuizen *et al,* 2008; du Toit *et al,* 2006).

4.2.4. GnRH and ovariectomy

Typically, ovariectomy ceases the synthesis and release of estrogens because part of the negative feedback loop is removed and GnRH release is initially promoted resulting in decreased GnRH content of the median eminence (Clarke & Pompolo, 2005). The processes thereafter are somewhat vague as different research groups report diverse results. For example, in rat brains, Roberts *et al* (1989) showed that proGnRH gene expression (i.e. GnRH mRNA) and GnRH content (measured by radioimmunoassay) of the hypothalamus was lowest nine days after ovariectomy, Kelly *et al* (1989) found no effect of ovariectomy on proGnRH levels (of both gene expression and gene product) in the hypothalamus of rats and Toranzo *et al* (1989) reported a 25 % increase in proGnRH mRNA (tested by *in situ* hybridisation) after ovariectomy. These varied results may be a consequence of variability within the methodologies used, such as length of time that animal was ovariectomised or the stage of the oestrous cycle when the animals were killed. In the present study, the naked mole rat queens (n = 2) had been ovariectomised for at least 5 years. Even though the exact stage of their oestrus cycle was unknown when they were killed, no difference was observed between the relative amounts of GnRH found in their brains compared to the brains of queens who were actively breeding. Recent studies suggest that social mole rats display a unique independence from gonadal hormones (Holmes *et al,* 2009). For example, sexual behaviour in Damaraland mole rats persists robustly in both males and females for at least five months following gonadectomy (Holmes *et al,* 2009). Therefore, the results from the present study suggest that, while there is little doubt that steroid hormones play a significant role in maintaining the cyclical nature of oestrus, it is likely that their direct action on GnRH production is limited.

4.2.5. Proposed mechanisms for GnRH plasticity observed in naked mole rats

4.2.5.1. Neurogenesis

At a cellular level, the differences in GnRH production observed between breeding and non-breeding naked mole rats could be the result of one of two scenarios. Either there are a finite number of neurons that increase or decrease their synthesis of the peptide depending on breeding status, or there is recruitment of new GnRH neurons at the time of puberty when a subordinate becomes reproductively active. In the current study, there is some evidence of cell recruitment, which can be seen in the drastic change in size of the pituitary. However, whether this is due to neurogenesis (development of new neurons in the adult brain, Gould & Gross, 2002) or rather the development of non-neural tissues, such as support cells, remains to be investigated. Neurogenesis in the region of the lateral ventricles and the dentate gyms is not uncommon in adult mammals, although development of new neurons outside of these regions is very rare (Abrous *et al,* 2005; Ming & Song, 2005). Holmes *et al* (2007) provide evidence for neural remodelling in naked mole rats by showing that breeders have more cells than subordinates in specific brain regions involved in reproduction. This demonstrates that naked mole rats do have the potential to produce new neurons. Nevertheless, the addition of new cells (either neural or non-neural) to the region of the hypothalamus and pituitary of adults remains uncommon (Holmes *et al,* 2007).

4.2.5.2. Embryological development and the vomeronasal organ

The vomeronasal organ (VNO) is an accessory olfactory sense organ, found in the nose, which is critical for mediating socio-sexual behaviours in many mammals, particularly rodents, through the detection of chemical signals (Takami, 2002; Smith *et al,* 2007). Typically, the VNO is stimulated by various olfactory signals which fuel the secretion of GnRH and the subsequent release of LH which increases the intensity of sexual behaviours (Døving & Trotier, 1998; Aujard *et al,* 2005). GnRH neurons are also embryologically linked to the VNO as both are derived from olfactory epithelium; GnRH neurons are known to migrate from the olfactory placode, through the VNO to their final destination, the hypothalamus (Takami, 2002). Generally,

disruption of GnRH neurons during embryological development can have dire effects on the GnRH neuronal system and thus affect the reproductive capabilities of sexually mature individuals (Ebling, 2005). Because the olfactory and GnRH systems are linked, if GnRH secretion is deficient because of defective neuron migration from the olfactory placode to the hypothalamus, this is often accompanied by defects to the olfactory system.

During postnatal development, the VNO is influenced by gonadal hormones, and gonadectomy or reduced steroid hormone secretion drastically diminishes the growth of the organ (Segovia & Guillamón, 1982). Removal or impairment of the development of the VNO in various species produces changes in sexual behaviours and aggression, and is also implicated in delayed puberty (Smith *et al,* 2007). Subordinate naked mole rats are sexually and behaviourally monomorphic (i.e. males and females are sexually and behaviourally the same) and appear to be in a state of suspended puberty. The VNO of *H. glaber* is also proportionally smaller than in other rodents and is unusual as it also shows no postnatal growth, suggesting impaired function (Smith *et al,* 2007). Interestingly, while little research has been conducted on the gonadal hormones of female naked mole rats, subordinate males have lower levels of testosterone than breeding males (Holmes *et al,* 2009), which may influence sexual and VNO development. One may argue that prior studies show that the olfactory system has no role in the reproduction of *H. glaber*, however, while urinary pheromones have been ruled out as mediators of reproductive suppression in this species, it still remains possible that other volatile chemicals of a different origin may be involved. For example, sex specific peptides are secreted by the lacrymal glands of mice which stimulate the vomeronasal organ and therefore activate GnRH secretion (Kimoto *et al,* 2005). When naked mole rats encounter one another, they are known to sniff the facial region of their conspecific, which is suggestive of some odoriferous chemical used for identification (Holmes *et al,* 2009). Since the VNO and the GnRH system are so intimately linked, there may be some inhibition of GnRH neuron migration or alternatively, an inhibition of the chemical cues required for the onset of puberty (because of VNO malfunction) which, mediated by reduced gonadal hormone secretion, could cause the socially-induced infertility of this species.

4.2.5.3. Stress and the reproductive axis

When an organism experiences a stressful situation (such as an aggressive attack), the hypothalamic-pituitary-adrenal axis (HPA) axis is activated which leads to peripheral and behavioural changes that increase the chance of the organism's survival by enhancing the ability to adjust homeostatically (Kalantaridou *et al,* 2004). Energy (in the form of nutrients and oxygen) is diverted away from non-essential, long term functions such as reproduction, growth and feeding, to the central nervous system and muscles in order to cope with the stressful situation (Dobson & Smith, 2000). There are two key hormone types that are involved in stress physiology: endogenous opioids act to decrease pain, while glucocorticoids, such as cortisol, work to restore homeostasis. Both suppress reproduction by inhibiting GnRH neuron function (Reeder & Kramer, 2005). Naked mole rats live in a high stress environment and are faced daily with aggressive social interactions. Work by Tuomi (2006) shows that endogenous opioids have little effect on naked mole rat reproductive physiology while chronically elevated levels of cortisol are more likely to play a role in reproductive suppression, however the mechanisms still remain unclear. When a stressful situation arises, corticotropin-releasing hormone (CRH) is released from the hypothalamus, which in turn stimulates the release of adrenocorticotrophic hormone (ACTH) from the pituitary which then stimulates the release of cortisol from the adrenal cortex (Kalantaridou *et al,* 2004). There are multilevel interactions between the HPA and the hypothalamic-pituitary-gonadal axis (i.e. inhibition of hormone secretion from the hypothalamus, pituitary and gonads) which involves different organs and inhibitory hormones. For example, chronic stress-mediated suppression of the reproductive system may facilitate delayed puberty (Marchlewska-Koj, 1997) and glucocorticoids have also been linked to GnIH whereby acute and chronic stress increases expression of the inhibitory peptide. Glucocorticoid receptors have also been found on over 50 % of GnIH neurons in the rodent brain, indicating a strong and direct association of stress with GnIH (Kirby *et al,* 2009). Therefore stress is a very likely link between aggressive behaviour and reproductive suppression (Kalantaridou *et al,* 2004).

4.2.5.4. Delayed puberty

While reproductive activity only occurs when an organism is sexually mature, GnRH neurons begin their development well before then. In most mammals, the GnRH system is active for a short while at birth as it begins to facilitate the development of the male and female reproductive systems; it is then arrested until puberty (Rissman *et al,* 1997, Ebling, 2005). The hormonal and morphological appearance of subordinate naked mole rats seems to be one of suspended or delayed puberty and the physiological suppression seems to be maintained by aggressive contact with the queen (Clarke & Faulkes, 2001). Her aggression levels vary towards different individuals, and she is most hostile towards the higher ranking colony members, especially if they are not closely related to her (Clarke & Faulkes, 1997; Clarke & Faulkes, 2001). This is because they are the biggest threat to her dominant position. Ovarian activation is identified in naked mole rat females as an increase in plasma progesterone which is indicative of the development of corpora lutea that only arise if ovulation has occurred (Margulis *et al,* 1995). Because the subordinates are not sterile, they are capable of becoming reproductively active at any time and therefore, the queen needs to actively maintain the suppression by aggressive means (Margulis *et al,* 1995). How this aggression translates to delayed puberty is unknown, but it is interesting to note that naked mole rat pups are subjected to significant levels of aggression (17 % to 31 % of all interactions with the breeding males and female) shortly after they are born (Faulkes *et al,* 1991). One could postulate that the queen is aggressive towards pups because they have active GnRH systems that need to be suppressed.

Note that GnRH production and release in the non-breeding females of *H. glaber* is not entirely eliminated as a) the hormone has been detected in the current study using immunocytochemistry and b) there must be some circulating GnRH, albeit a very low concentration, to stimulate the limited release of LH that has been detected in subordinate naked mole rat blood (Faulkes *et al,* 1990b). While the amount of hormone stored and the rate at which it is released in the different breeding groups has not been quantified, theoretically, the suppressed level of GnRH in the subordinate brain may be insufficient to maintain high levels of gonadotropin production, the

effects of which can be seen in the low amount of circulating LH, lack of ovulation and the presence of underdeveloped ovaries in subordinates. It is still unclear as to what mechanism underpins this finding, however, it is important to note that the GnRH system is a plastic one. In vertebrate females, there are daily fluctuations in GnRH production and secretion based on the position of the oestrus cycle. Perhaps the most significant changes in the GnRH system in mammals occur at puberty. In terms of neuroendocrinology, puberty is defined as the reactivation of GnRH secretion and maturation of the reproductive system (Ebling, 2005; Foster *et al,* 2006). At puberty, the GnRH system is reactivated and the release of the hormone changes from a low level, irregular pattern to more distinct pulses (Clarkson & Herbison, 2006; Foster *et al,* 2006). At present, it is unclear exactly how puberty in mammals is initiated, however, a host of different cues (such as nutrient status and stress level) have been identified as key players in this change between breeding and non-breeding state (Ebling, 2005). The GnRH requirements for the onset of puberty and the ability to reproduce are also quite different. Herbison *et al* (2008), by combining immunocytochemistry and fertility testing (i.e. number of offspring) showed that a mere 70 GnRH producing neurons are required to spark puberty in male and female mice, while 200 GnRH neurons are required in female mice in order to display normal fertility. Currently, the number of GnRH neurons found in the naked mole rat is unknown. However, if the decreased amount of GnRH-ir observed in the subordinate brain (i.e. an immunoreactive area of 57.56 mm^2 ± 36.91 mm^2 in subordinate brains vs. 111.17 mm^2 ± 36.91 mm^2 in queen brains) in the current study is proportional to the number of GnRH cells, this may explain why some subordinate naked mole rats show signs of reproductive activation, but are unable to reproduce.

While it seems clear that the block to ovulation in subordinate naked mole rats is related to the decreased amount of GnRH present in their brains, it is unlikely that GnRH is the sole cause of impaired gonadotropin secretion and overall reproductive suppression. Most current literature focuses on individual aspects of this highly complex, yet organised system (Kalra *et al,* 1997). However, *in vivo,* the reproductive axis (particularly GnRH neurons) receives many different neural and chemical inputs, allowing it to monitor the external environment and adjust reproductive function accordingly. GnRH synthesis and release (both during and after puberty) is mediated by a large number of different factors. For example, kisspeptin is a likely candidate

for reproductive suppression in naked mole rats as it is directly involved in stimulating GnRH to trigger the onset of puberty (Seminara & Crowley, 2008). On the other hand, GnIH has been identified as a GnRH antagonist which may enforce suppression of GnRH in naked mole rat subordinates. Since larger individuals are more dominant and therefore more likely to attempt a take over of the reproductive position, energy balance and growth are therefore two other important cues (mediated by NPY and growth factors) that may be involved in reproductive activation or suppression (Simerly, 2005). Neurotransmitters, such as melatonin, dopamine and y-aminobutyric acid (GABA) (Genazzani *et al,* 2000; Ebling, 2005) have also been implicated in the control of GnRH neurons and aid in the integration of signals from various brain areas. There is such a wide array of factors that may influence GnRH and sexual maturity that future studies would benefit from a multi-layered approach to investigate suppression of reproduction in naked mole rats.

4.3. Neuropeptides: an ancient means of cellular communication

Secretion of chemical factors is an ancient form of cellular communication and hormones are secreted by even the simplest of creatures, such as sponges and protists (Hartenstein, 2006). It is therefore of little surprise that insects and crustaceans share a number of structurally homologous peptides, a product of their common ancestry (Gäde & Marco, 2006). The adipokinetic hormone/red pigment-concentrating hormone (AKH/RPCH) family of peptides contains structurally related peptides that have a diversity of biological functions in these two arthropod taxa (Gade & Marco, 2006). Peptides of the AKH family (including Cam-HrTH factors) are involved in the mobilisation of lipids and carbohydrates for metabolism in insects (Goldsworthy & Mordue, 1989), while RPCH mediates colour change in crustaceans by concentrating red pigment in specialised chromatophores to produce a "blanched" or lighter appearance (Gäde & Marco, 2006). Interestingly, the peptides of the AKH/RPCH family also share a degree of structural homology with GnRH namely peptide length (not for all variants), the pGlu at the N terminal and a C terminal amidation. Moreover, a specific AKH has recently been identified as the ligand for the GnRHR in *Drosophila melanogaster* and *Bombyx mori* (Staubli *et al,* 2002). Lindemann *et al* (2009) also show that an AKH-GnRH related peptide can modulate egg-laying in

Caenorhabditis elegans by acting as a ligand for an orphan *C. elegans* G-protein coupled receptor (Ce-GnRHR) which displays homologies between both AKH and GnRH receptors. These data suggest that an AKH/GnRH signalling system arose before the divergence of protostomes and deuterostomes (Lindemans ***et al,*** 2009). Vertebrates and invertebrates diverged 500 million years ago (Hartenstein, 2006) and therefore it is remarkable to consider the structural similarities of neuropeptides and neuropeptide systems that have persisted for so long over evolutionary time. Thus, the current study included pilot efforts to extract neuropeptides from mammalian tissue, with the ultimate aim of sequencing GnRH from ***H. glaber,*** but also to serve as a comparison with invertebrate neuropeptide systems.

4.3.1. Extraction and purification of neuropeptides from H. glaber: pilot study

Although naked mole rat tissue was not abundantly available in the current study, attempts were made to optimise methods for the successful extraction and purification of neuropeptides from the brain of ***H. glaber,*** for the future aim of isolating and sequencing GnRH from this species. Practice with relevant methods were obtained by making and processing crude extracts from insect neural tissues, viz. corpora cardiaca from the stick insect, *Carausius morosus,* and from mouse brain, and by using synthetic mGnRH. A RP-HPLC solvent gradient for successful chromatographic separation of C. *morosus* neuropeptides (namely Cam-HrTH I and II) already existed and incorporated the use of a fluorescence detector to enhance detection of peptides containing tryptophan (Gale, 1984; Gäde, 1990). Synthetic mGnRH was used to design an efficient RP-HPLC gradient for use with mouse and naked mole rat brain extracts, and since mGnRH contains a tryptophan at position 3 which can emit a fluorescence signal, fluorescence and UV detectors were both used to monitor peak material. Synthetic mGnRH consistently eluted at 43 — 44 % B (25 % acetonitrile) and Cam-HrTH I and II eluted at 47 — 48 % B (28 % acetonitrile) and 49 — 50 % B (30 % acetonitrile), respectively.

In the current study, method optimisation using synthetic mGnRH indicated that there was little loss of peptide during the homogenisation or prepurification procedures.

However, as extracts of mouse brain were applied to the RP-HPLC column, chromatographic separation of mGnRH was unconvincing. When prepurified extracts of maximally 2 mouse brains at a time were passed over the RP-HPLC column, three small peaks (1, 2 and 3) resulted which broadly corresponded to the approximate elution time of mGnRH (Fig. 21, 26). The most likely candidate for mGnRH (i.e. with a retention time of — 12 minutes) would be the poorly-resolved peak 2. In an ELISA, however, GnRH-ir could not be obtained even though twice the amount of peak material was used. It could be argued that too little GnRH is present after RP-HPLC for detection by ELISA; the current study revealed that synthetic mGnRH can easily be detected by ELISA in amounts of less than 0.5 pmol i.e. in much smaller peaks than represented in Figure 26.

Crude extract of either 'A , 'A or 1 mouse brain were plated for ELISA to test for a dilution effect and compared with control extracts of the same amount of brain tissue mixed with 60 pmol synthetic mGnRH (Table 3). A positive control of 60 pmol synthetic mGnRH plated on its own had an OD reading of 0.325, while OD readings were much fainter (< 0.09) when 60 pmol synthetic mGnRH was plated together with mouse brain extract and even fainter still when mouse brain extract was plated on its own. It is clear that the ELISA methodology is sound as, in the current study, synthetic peptide can be detected in amounts as low as 0.1 pmol. The fact that there is a significant 'loss' of synthetic peptide when it is plated together with mouse extract suggests that there may be some endogenous factor, most likely an enzyme, which may break down the peptide. Enzymatic degradation can only be avoided with trials using specific enzyme inhibitors which are added before homogenisation. Other endogenous factors may either bind to the peptide, inhibiting its ability to bind to the plate or out-compete GnRH for binding sites on the ELISA plate, although this is much less likely than the enzymatic degradation scenario.

From the immunocytochemical part of the current study, it was established that the form of GnRH present in the naked mole rat brain is found in the hypothalamus and pituitary gland. Therefore, these two brain regions were targeted specifically. When RP-HPLC fractions of purified naked mole rat hypothalami and pituitaries were tested by ELISA, weak immunoreactivity was detected in several fractions of peak material. From the pilot study with synthetic mGnRH, it was clear that a minimum of 40 pmol

of GnRH was required in order to identify GnRH by mass spectrometric means. However, the amount of material (i.e. peak height) obtained from naked mole rat tissue was too little to analyse by mass spectrometry. Working with native neural preparations seems to be more complicated than with a purified substance. Because the RP-HPLC peaks are so poorly resolved, it stands to reason that there may be several substances co-eluting with GnRH and that GnRH is indeed, present in levels which are below the threshold which is detectable in the mouse and naked mole rat brain. While GnRH was positively identified in crude extracts from the brains of the Damaraland mole rats (using radioimmunoassays), it is important to note that the study by Molteno *et al* (2004) used ten animals in comparison to the four naked mole rats used in the current study. They also showed that subordinate Damaraland mole rats have a larger pool of GnRH than breeders and that GnRH is blocked at the level of release rather than production. From the immunocytochemical part of the current study, it was established that subordinates possess less GnRH than queens because of diminished GnRH production, therefore even less GnRH was available for extraction and detection. Alternatively, the complex milieu consists of enzymes which degrade the GnRH peptide, or "blocking factors" that prevent the GnRH ligand from binding to the GnRH antibody. At conclusion of the present study, there was insufficient *H. glaber* material to repeat the RP-HPLC, but the results of this pilot study will serve to improve future endeavours in this line of research.

4.3.2. RP-HPLC methodological considerations

4.3.2.1. Mammals versus insects

In the current study, neuropeptide (AKH/HrTH) extracts from the corpora cardiaca of only 2.5 adult stick insects were clearly separated and purified by chromatographic techniques (Fig. 20). The chromatographic separation of neuropeptides (GnRH) from the equivalent number of mouse or naked mole rat hypothalami/pituitary glands was unsuccessful, in the current study. A possible cause for this disparity may be linked to the overall structures of vertebrate and invertebrate neuroendocrine systems. Arthropods are an ancient lineage and are separated from mammals by 500 million years, therefore differences in their body plan are expected. However, homologous

genes of the invertebrate and vertebrate endocrine systems have been identified, as well as structural and functional similarities between vertebrate and invertebrate neuropeptides, indicative of an evolutionary link (Ottaviani *et al,* 2007). The nervous system of invertebrates is functionally similar to that of mammals, except much simpler in structure, therefore invertebrates (such as insects) are often the ideal choice of animal for neuroendocrine studies. Although their overall body size is typically smaller than mammals and they have fewer nerve cells (Gade, 1990), their neuroendocrine centres (including neurosecretory cells and neurohaemal organs) are large and conspicuous (Ottaviani, 2004). The neurosecretory system of insects has been well investigated as not only are insects abundantly available at a relatively cheaper cost, but they are short-lived and therefore grow and reproduce at a faster rate than mammal models such as the mouse or rat (Andre *et al,* 1989). Therefore, it is advantageous to use insect models for neuroendocrine studies.

Another point to consider is that insects have an open circulatory system, where haemolymph (equivalent to blood) fills the body cavity and bathes the body organs. Therefore, when hormones are released from neurohaemal sites, they do not flow directly to the target organs. The mammalian circulatory system is made up of a complex network of arteries and veins which transport hormones, nutrients and oxygen to and from specific organs and cells. When GnRH is released, it only has to travel a short distance from the hypothalamus, through the portal vasculature directly to the pituitary gland. The potency of hormones is such that only a small amount is required to elicit a significant response, which is amplified inside the target cell by signal transduction cascades (Cheung & Lustog, 2007). Since the portal vasculature is a direct line from the site where GnRH is produced to where it is released and there is no risk of loss or dilution, the peptide can be released in minute quantities.

4.3.2.2. GnRH in mammals: methodological considerations

GnRH has a reputation of being difficult to study as unlike other hormones, GnRH neurons have a scattered distribution (rather than confined to discrete nuclei) as well as paucity in cell number (Zhen *et al,* 1997). The total number of GnRH neurons in the brains of different species also varies; however, it appears to correlate with brain

and overall body size (Mellon & Newman, 1991). Large ungulates such as sheep or the springbok *Antidorcas marsupialis* have 1000 — 2000 GnRH neurons in their brain (Lehman *et al,* 1997; Robinson *et al,* 1997). Amongst the rodents that have been investigated, rats have approximately 1000 GnRH neurons (Wray & Hoffmann, 1986) while smaller rodents, such as mice, musk shrews, the Djungarian hamster and the Syrian hamster have approximately 600 (Herbison *et al,* 2008), 400 (Dellovade & Rissman, 1994; Dellovade *et al,* 1995), 400 (Yellon & Newman, 1991) and 700 (Jennes & Stumpf, 1980) neurons, respectively. Of the mole rat species studied to date, approximately 1500 GnRH neurons have been identified in the brains of the larger and heavier (— 915 g) Highveld and Cape Dune mole rats (du Toit *et al,* 2006; Hart *et al,* 2008). The smaller mole rat species have far fewer GnRH neurons, for example the Cape mole rat (— 180 g) has 420 (Oosthuizen *et al,* 2008), the Damaraland mole rat (— 140 g) has 650 (Molteno *et al,* 2004), the Natal mole rat (— 97 g) has 720 (Oosthuizen *et al,* 2008) and the Common mole rat (— 67 g) has 640 (du Toit *et al,* 2006). While the exact number of GnRH neurons found in the naked mole rat brain is unknown, this species is considerably smaller than the other mole rat species with a body mass of 30 — 60 g depending on breeding status, and therefore it is likely that GnRH cells in this species are only a few hundred, limiting the amount of GnRH available for extraction and detection. For the pilot study, there was too little tissue available to purify GnRH from only 4 naked mole rats. As seen in the immunocytochemical part of this study, the levels of GnRH in the brains of subordinate naked mole rats are significantly depressed in comparison to breeding individuals, which may hinder successful extraction, purification and identification of GnRH in this species.

The amount of synthetic mGnRH used as a control in the current study is not biologically representative, as *in vivo* GnRH is produced and released in much smaller quantities (— fmol). If one considers other studies that have used similar methods to extract and purify GnRH from different animals, most have used a large amount of tissue in order to successfully identify GnRH by chromatographic means. Table 7 is not an exhaustive account of all successful extractions of GnRH, but does give a general idea of the number of tissue samples required to extract GnRH from the brains (and/or pituitaries) of different species. As one can see, most studies use a large number of tissue samples. A handful of studies successfully extract and purify GnRH

from fewer brain samples, but these studies are typically conducted on specimens much larger (therefore containing more GnRH) than the naked mole rat, such as the capybara, cow, macaque and the human.

Another point to consider is that different structural analogs of GnRH share significant homology, however, variations in amino acid composition results in differences in hydrophobicity and ionisation which affect chromatographic retention time and charge state (Myers, 2007). The RP-HPLC solvent gradient designed in this study is quite 'tight' with synthetic mGnRH eluting towards the end of the run. Since the form of GnRH found in naked mole rats is unknown, its chromatographic retention and charge state are also unknown and it is possible that the form of GnRH in naked mole rats is more hydrophobic and therefore would elute outside the set solvent gradient. This is just one of several issues that must be addressed in future studies on GnRH in *H. glaber*.

Table 7: The number of brains used in studies for the successful extraction and purification of various forms of GnRH from different species using RP-HPLC (usually in conjunction with radioimmunoassay).

Common name	Scientific name	Number of brains	Reference
Agnathans			
Atlantic hagfish	*Myxine glutinosa*	10	Sower *et al* (1995)
Lamprey	*Petromyzan marinus*	2150	Sower *et al* (1993)
Chondrichthyes			
Atlantic stingray	*Dasyatis sabina*	5	Forlano *et al* (2000)
Spotted dogfish	*Scyliorhinus canicula*	20	D'Antonio *et al* (1995)
Spiny dogfish	*Squalus acanthias*	450	Lovejoy *et al* (1992)
Osteichthyes			
White sucker fish	*Catostomus commersoni*	9	Robinson *et al* (2000)
Blackfin ice fish	*Chaenocephalus aceratus*	34	Miranda *et al* (1999)
Asiatic featherfin fish	*Chitala chitala*	11	O'Neill *et al* (1998)
Pacific herring	*Clupea harengus*	1200 + 190 (p)	Carolsfeld *et al* (2000)
Elephantnose fish	*Gnathonemus petersii*	10	O'Neill *et al* (1998)
Cichlid	*Haplochromis burtoni*	35 + 69 (p)	Powell *et al* (1995)
Pumpkin seed fish	*Lepomis gibbosus*	28	Powell *et al* (1995)
White croaker	*Micropogonias furnieri*	70	Montaner *et al* (2001a)
Marble rock cod	*Notothenia rossi*	39	Miranda *et al* (1999)
Pejerry	*Odontesthes bonariensis*	450	Montaner *et al* (2001b)
Pejerry	*Odontesthes bonariensis*	20 to 200	Stefano *et al* (1997)
Arawana	*Osteoglossum bicirrosum*	11	O'Neill *et al* (1998)
Red porgy	*Pagrus pagrus*	53	Montaner *et al* (2001a)
Butterfly fish	*Pantodon buchholzi*	11	O'Neill *et al* (1998)
Pacu	*Piaractus mesopotamicus*	33	Powell *et al* (1997)
Lungfish	*Protopterus annectens*	5 to 15	King *et al* (1995)
South Georgia ice fish	*Pseudochaenichthys georgianus*	13	Miranda *et al* (1999)
Gilthead sea bream	*Sparus aurata*	100 to 10 000 + 2500 (h)	Powell *et al* (1994)

Feather fin fish	*Xenomystus nigri*	11	O'Neill *et al* (1998)
Amphibian			
Common Argentine toad	*Bufo arenarum*	14	Miranda *et al* (1998)
Mexican tree frog	*Pachymedusa dacnicolor*	22	Iela *et al* (1996)
European Green frog	*Rana ridibunda*	1200	Conlon *et al* (1993)
Clawed toad	*Xenopus laevis*	62	King *et al* (1994)
Reptile			
Green Anole	*Anolis carolinensis*	21	Lescheid *et al* (1997a)
Tegu lizard	*Tupinambis teguixin*	6	Montaner *et al* (2000)
Aves			
Chicken	*Gallus gallus domesticus*	15 000 + 249 000 (h)	King & Millar (1982a) King & Millar (1982b)
Japanese quail	*Coturnix coturnix japonica*	50	Clerens *et al* (2003)
Rodent			
Capybara	*Hydrochaeris hydrochaeris*	3 to 5	Montaner *et al* (1998) Montaner *et al* (1999)
Rat	*Rattus norvegicus*	50	Chen *et al* (1998)
Damaraland mole rats	*Cryptomys damarensis*	10	Molteno *et al* (2004)
Ungulate			
Cow	species not specified	5 and 7	Yahalom *et al* (1999)
Primate			
Stumptail macaque	*Macaca speciosa*	3	Lescheid *et al* (1997b)
Rhesus macaque	*Macaca mulatta*	4	Lescheid *et al* (1997b)
Human	*Homo sapiens*	1 to 2	Chen *et al* (1998) Yaholm *et al* (1999)

In some studies, additional tissues were pooled with the brain tissue prior to homogenisation and extraction, and are indicated by (p) for pituitaries and (h) for hypothalalmi.

4.4. Conclusions and future considerations

This is the first study to identify GnRH in the brain of the naked mole rat and to compare the GnRH systems of breeding and non-breeding females using immunocytochemistry. The unusual reproductive regime of this eusocial species seems to be maintained by inhibition of GnRH production in subordinates. There are significant modifications to the overall size and mass when a subordinate becomes a queen, however, there is little dimorphism in brain morphology when comparing queens and subordinates, except for a significantly larger pituitary gland in queens.

While extraction of GnRH from naked mole rat tissue was ,unsuccessful, the pilot study highlighted the difficulties of extracting, purifying and identifying GnRH in mammalian tissues by chromatographic means. One of the major issues is the large amount of tissue required to extract sufficient quantities of peptide. Future studies would perhaps benefit more from molecular techniques, such as polymerase chain reaction (PCR) where only a small amount of RNA can be amplified, thus minimal naked mole rat tissue would be required. *In situ* hybridisation is another molecular technique requiring fewer source animals. This method can use radio labelled complementary RNA probes to mark RNA coding for the molecule in question. Molecular techniques also allow for quantitative measurement of the DNA or RNA molecules. Once the amino acid structure of GnRH in naked mole rats has been fully elucidated, synthetic peptide (of the same structure) can be manufactured, used in bioassays and a more accurate understanding of the biological significance of GnRH in naked mole rats can be gained. It is important, however, to study GnRH with a multi layered approach, where GnRH is considered in the context of other hormones (such as kisspeptin and GnIH).

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APPENDIX I

i. Colour balance

In digital photography, colour can be determined or analysed with two different models:

1) **Additive model (RGB): Red, green and blue make up the primary colours of light, and when combined they form white. The absence of light makes black. This is often the model used to visualise colour on a computer screen**

2) **Subtractive model (CMYK): Cyan, magenta and yellow when added together make black. The absence of these colours make white. This is the model that is often used when printing colour pictures as it is based on the light-absorbing properties of ink printed onto paper. Subtractive (CMYK) and additive (RGB) colours are complementary colours (Fig. 28).**

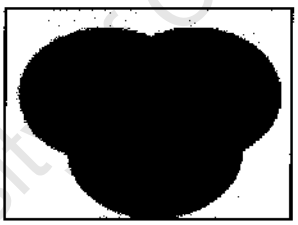

Figure 28: This diagram illustrates how the RGB and CMYK colour models are linked. R = red, G = green, B = blue, C = cyan, M = magenta, Y = yellow (adapted from Adobe Photoshop 7.0.1)

The colour balance command in Photoshop allows one to change the overall mixture of colours for generalised colour correction or image enhancement. There are three components to a photograph that one can alter to obtain the desired colour balance; shadows, midtones and highlights. Shadows are defined as the darker areas, highlights the lighter areas and midtones are the colours in between these two extremes. These three components are further divided into three scales of - 100 to + 100; comprised of RGB and CMYK components (Fig. 29). For example, an image with a high blue value will have a higher blue concentration. In the images of naked mole rat brain tissue, the rust colour in stained areas had a much higher yellow component than the background

stain, and could therefore be 'separated' out and enhanced by altering the colour balance. Photoshop was instructed to preserve luminosity as this allows for colour alteration, without changing the exposure, contrast and brightness settings.

Colour component (- 100)		Colour component (+ 100)	
Cyan	■ ⟷	■ Red	[S0; M0; H0]
Magenta	■ ⟷	■ Green	[S0; M45; H0]
Yellow	■ ⟷	■ Blue	[S-100; M-25; H+30]

Figure 29: The colour scales that one can change to alter colour balance. These colour scales can be applied to shadows, midtones and highlights. Values in square brackets are the colour settings that were used for this study for shadows (S), midtones (M) and highlights (H)

ii. Colour and pixel selection

Adobe Photoshop's magic wand tool was used to select stained areas based on their colour composition, instead of manually drawing the outline. This method gives a much more accurate selection as all pixels of a certain colour or colour range are selected. One can set the tolerance (a scale of 0 to 255) depending on the accuracy that one requires. For example, a tolerance of 0 will only allow the selection of pixels that have exactly the same RGB/CMYK colour composition. A higher tolerance allows for selection of a broader range of colours. For this study, the tolerance was low (30) so that unstained pixels were not included in the measurement of the stained area. In order to minimise the effects of background staining, pixels were only selected if the total ink was 50 % or greater. The total ink of each pixel represents the sum of the percentages of each CMYK component. The lower the percentage, the lighter the overall colour and visa versa.