EFFECTS OF COMMONLY USED ANALGESIC DRUGS AND TRIGEMINAL NERVE FIBER PROPORTIONS IN THE SPEKE-HINGED TORTOISE (*Kinixys spekii*) AND THE MARSH TERRAPIN (*Pelomedusa subrufa*)

By

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other University.

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DEDICATION

To the entire Wambugu family, whose understanding, patience and prayers gave me the courage and motivation to pursue this work to the end!

"Feeling is the subjective side of consciousness, knowledge its objective side.

Will is the relation between the subjective and the objective"

John Dewey (1859-1952), US Philosopher and Educator,

(*Psychology, 1889*)

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure

LIST OF PLATES

APPENDICES:

LIST OF ABBREVIATIONS

5-HT:	5-Hydroxy-tryptamine
AMPA:	Alpha-amino-3-hydroxy-5-methyl-4-isoxalone propionate
AP_4	2-amino-4-phosphonobutyrate
ASA	Acetyl salicylic acid
ATP	Adenosin triphosphate
CCK	Colecystokinin
CGRP:	Calcitonin-gene related peptide
CNS:	Central nervous system
COX	Cyclo-oxygenaase
DEG/ENaC	Degenerin/epithelial sodium channel
DVR	Dorsal ventricular ridge
GABA:	Gamma amino-butyric acid
IASP:	International Association for the Study of Pain
Ico:	Intra-coelomically
IP_3	Inositide triphosphate
LSD	Least significant difference
LTP	Long-term potentiation
MGluR	Metabotropic Glutamatergic receptors
NA:	Noradrenaline
NaCl	Sodium chloride
NKA	Neurokinin A
NMDA:	N-Methyl-D-Aspartate
NO	Nitric oxide
NRM	Nucleus rhaphe magnus
NS	Nociceptor specific
NSAIDs:	Non-Steroidal-Anti-Inflammatory Drugs
PAG	Peri-aquiductal gray
PEMT	Pulsed electromagnetic therapy
PGH	Prostaglandin-H_2

S.E.M.	Standard error of the mean
SP	Substance P
STT	Spinal thalamic tract
TENS	Transcutaneous electrical nerve stimulation
TRP	Transient receptor potential
TRPA	Transient receptor potential ankyrin
TRPC	Transient receptor potential canonical
TRPM	Transient receptor potential melastatin
TRPML	Transient receptor potential mucolipin
TRPN (NOMPC)	Transient receptor potential-No mechanoreceptor potential C
TRPP	Transient receptor potential polycystin
TRPV:	Transient receptor potential vanilloid
VIP:	Vasoactive intestinal peptide
VP	Ventroposterior
VPI	Ventral postero-inferior
VPL	Ventral postero-lateral
VPM	Ventral postero-medial
VRI:	Vanilloid receptor channel-1
WDR	Wide dynamic range

ABSTRACT

This study was undertaken to explore the nociception and antinociception in the speke-hinged tortoise (*Kinixys spekii*) and the marsh terrapin (*Pelomedusa subrufa*). Four nociceptive tests, namely the formalin-, hot plate-, capsaicin instillation- and acetic acid instillation tests were adapted and used to study nociception and antinociceptive effects of commonly used analgesics. A histological survey of the fiber proportions in the sensory branches of the trigeminal nerve was also carried out to obtain more information on the nociceptive system of the animals.

Forty-two tortoises and thirty-four terrapins were used in the study. In the formalin test, 100 µL of 12.5% formalin was injected subcutaneously in the hind paw of the animals and the total time spent in pain scored. Both animal species demonstrated a monophasic pain response, characterized by full limb retraction or partial usage of the limb. Thermal stimuli were induced using a hot plate analgesia meter set at 60 °C. Both animals responded by lifting one of the paws and 'attempting to escape'. Tortoises showed a mean response latency of 53.95 ± 3.53 seconds while that of the terrapin was 41.28 ± 3.41 seconds. In the capsaicin instillation test, two drops of capsaicin were directly instilled into the eye and the duration of eye closure measured in blocks of five minutes for 30 minutes. Terrapins showed sensitivity to capsaicin but there was no capsaicin-desensitization effect after repeated application of capsaicin. In the acetic acid instillation test, two drops of 10% acetic acid were directly instilled into the eye and the duration of eye closure scored in blocks of five minutes for 30 minutes.

In the formalin and the hot plate tests, both morphine and pethidine showed dose dependent anti-nociceptive effects, which were naloxone reversible, in both animal species. Morphine at dosages less than 7.5 mg/kg and pethidine at dosages less than 20 mg/kg did not induce any significant antinociceptive effects. Acetylsalicylic acid, flunixin meglumin, dexamethasone and hydrocortisone at the dosages used did not show any antinociceptive activity in neither the formalin nor the hot plate tests in any of the animal species.

In the ophthalmic nerve of the tortoise and the terrapin, the proportion of nerve fibers with diameters measuring 0.5-5.5 μm was only 17.8 and 18.6 % respectively. In the maxillary branch of the trigeminal nerve, the proportion of nerve fibers with diameters measuring 0.5-5.5 μm was 20.7 and 27 % in the tortoise and the terrapin respectively. The results suggest that testudines have relatively few nociceptive fibers, which comprise 18-27 % of the sensory afferents in the trigeminal nerve sensory afferents.

In conclusion, testudines have a nociceptive system, which is responsive to opioid analgesia. The proportion of nociceptive afferents is low. It is postulated that in testudines the shell, which protects these animals, might be an alternative to a comprehensive nociceptive system.

CHAPTER ONE

1.0 INTRODUCTION

Pain is an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (Merskey and Bogduk, 1994). The ability to detect and respond to tissue damaging and aversive stimuli is of a fundamental biological importance in animals and reflects a major evolutionary step in the animal phylesis (Kavailers, 1988, 1989; Mun'oz *et al.,* 1997; Woolf and Salter, 2000; Lariviere *et al.,* 2002; Sneddon, 2004; Murakami and Kuratani, 2007, Butler, 2008). Nociception is the detection of noxious stimuli by the nervous system. It involves the detection of such injurious or potentially damaging stimuli by specialized sensory neurons, the nociceptors (Almeida *et al.,* 2004; Giordano, 2005). Nociception is an important sensory component of major fundamental and clinical relevance (Le Bars *et al.*, 2001; Craig, 2003). A comprehensive understanding of pain should facilitate the development of novel strategies for pain management.

Different animals respond differently to noxious stimuli. This variability is dependent on species, age, sex, developmental changes, geographical distribution, genetic makeup, season, time of the day as well as the environmental changes (Kavaliers, 1988; 1989; Millan, 1999; Le Bars *et al.*, 2001; Stasiak *et al.*, 2003). The variability of pain responses is less in laboratory-bred animals, such as rats and mice, than in wild caught animals. This is attributed to breeding for laboratory use, which has resulted in the production of animals that respond quite uniformly.

Nociceptive tests have been applied to a vast number of animals, mainly mammals where rats and mice are the most used subjects (Le Bars *et al.*, 2001). Non-mammalian subjects, such as the snail (Kavailers, 1989); fish (Sneddon, 2003; Ashley *et al.*, 2007), amphibians (Willenbring and Stevens, 1996), crocodiles (Kanui *et al.,* 1990), and birds (Gentle, 1992) have also been studied. From the perspective of the evolution of sensory function in

vertebrates, the study of sensory systems in lower vertebrates is of great interest (Kavailers, 1988; Northcutt, 2002; Sneddon, 2004; Butler, 2008). The usage of different animal species in nociceptive studies has provided information on the species-differences and phylogenies of pain mechanisms (Stasiak *et al.*, 2003; Sneddon, 2004; Paul-Murphy *et al.,* 2004). However, there is little information on the mechanism of action of analgesic agents in reptiles (Read, 2004; Sladky *et al.,* 2007). Recognizing pain in reptiles, especially in testudines, is always a big challenge to owners and healthcare providers and analgesia is often not provided (Bennett, 1998; Read, 2004). Research regarding pain and its assessment, response to analgesics, and drug pharmacokinetics in testudines is needed (Read, 2004). Though reptiles display a wide range of behaviors when stimulated by potentially injurious stimuli, few nociceptive tests have been applied in reptiles (Kanui *et al.*, 1990; Sladky *et al.,* 2007).

1.1 TESTUDINES

The speke-hinged tortoise *(Kinixys spekii)* and the marsh terrapin *(Pelomedusa subrufa)* belong to the order testudinidae (Lee, 2001; Zug *et al.*, 2001). Testudines are a unique group of reptiles in that they are among the armored reptiles and have very long lifespans. Moreover, despite having existed on earth since prehistoric times, they have undergone little or no evolutionary change (Lee, 1997, 2001). The first testudines are believed to have existed during the early Triassic period (Mesozoic era) about 200 million years ago (Lee, 1997; Cao *et al.,* 2000; Zug *et al.*, 2001).

Whether testudines are anapsids or diapsid is still unclear (Lee, 1997, 2001; Rieppel and Reisz, 1999; Rieppel, 2000; Cao *et al.,* 2000; Zug *et al.*, 2001; Rest *et al.,* 2003). They have been classified as anapsid reptiles, among which all the other members are extinct (Lee, 1997, 2001). Anapsids were among the first vertebrates to evolutionary transit from water-breathing to air-breathing and have skulls with no temporal openings (Lee, 1997, 2001; Zug *et al.*, 2001). All other extant amniotes (egg-laying vertebrates) have skulls with temporal openings, although in mammals the opening has become the zygomatic arch (Lee, 1997, 2001). However, molecular phylogenetic studies have suggested that the anapsid-like skull of

testudines might be due to reversion rather than to anapsid descent (Cao *et al.,* 2000; Rieppel and Reisz, 1999; Rieppel, 2000; Rest *et al.,* 2003).

The extant members of the order testudinata are divided into sub-orders cryptodira and pleurodira, based on the movement or retraction patterns of the neck (Zug *et al.*, 2001). *Kinixys spekii* belongs to cryptodira group, which can retract the neck posteriorly into a medial slot within the body cavity. Cryptodira have a characteristic flexible articulation of the pelvic girdle and their jaw closure mechanism has an articulation on the trochlear surface of the otic capsule (Zug *et al.*, 2001). *Pelomedusa subrufa* is a member of the pleurodira group, which retracts the head and neck by laying it to the side (left or right) (Zug *et al.*, 2001). Pleurodirans have the pelvic girdle fused to the plastron and have a jaw closure mechanism with an articulation on the trochlear surface of the pterygoid (Zug *et al.*, 2001).

1.1.1 The speke-hinged tortoise

The speke-hinged tortoise is a small brownish-hinged tortoise, whose carapace is domed with a flat dorsal surface and sloping sides (Plate i). Its' anterior and posterior marginals are not flared. The center of each carapacial scute is tan to yellowish brown surrounded by dark brown or black hexagonal rings. The interscute space is yellowish. The plastron is yellow to tan with black radiations. Its head is brown, black, yellow or tan and the limbs and tail are grayish brown (Hailey and Coulson, 1996). It has a slightly domed shell at the back. The tail has a terminal spine and end in a claw like tubercle. Males have concave plastra and long thicker tails, while females have flat plastra and short tails. Hinge-back tortoise (genus *Kinixys)* is the only living tortoise with a movable hinge, which has developed across the back of the carapace (Hailey and Coulson, 1996; Hailey, 1998). This broad band of flexible connective tissue is located between the 4^{th}-5^{th} costals and the 7^{th}-8th peripherals in adults. This movable hinge provides protection by closing off the tortoise's hind legs and tail and it may assist in egg laying and respiration (Hailey and Coulson, 1996). Speke hinge-back tortoises have 5 claws in the fore foot and 4 in each rear foot. All hinge-backs are omnivorous (Hailey, 1998; Zug *et al.*, 2001).

Speke-hinged tortoises occur in Kenya southwards to the Republic of South Africa. *Kinixys spekii* lives in open woodlands in arid climates (Hailey, 1998; Hailey, *et al.*, 1998; Luiselli, 2003, 2005). During dry periods, it aestivates in the muddy bottoms of drying water holes and other available hiding places (Hailey and Coulson, 1996).

1.1.2 The marsh terrapin

The Marsh Terrapin (*Pelomedusa subrufa*) is also referred to as the African helmeted turtle. It has brown to olive carapace, which is oval, broad, and rather flattened dorsally (Plate ii) (Rödel, 1999). The carapace may be smooth or slightly serrated posteriorly, and the interscute seam is yellowish brown. The plastron is usually colored yellow to cream. No carapacial hinge is present. Adult males have concave plastra with a narrower posterior lobe, and long, thick tails. Females have somewhat broader carapace, flat plastra, and shorter tails. The marsh terrapin is semi-aquatic, and has webbed feet.

The marsh terrapin is widely distributed in tropical and subtropical Africa. All *Pelomedusae* are carnivorous and feed on a variety of insects, earthworms, crustaceans, snails, fish, amphibians, small reptiles, birds, and mammals (Rödel, 1999).

Plate i: The speke-hinged tortoise: Note the dome-shaped carapace and the hinge (arrow). The center of each scute is yellowish and is surrounded by dark brown rings.

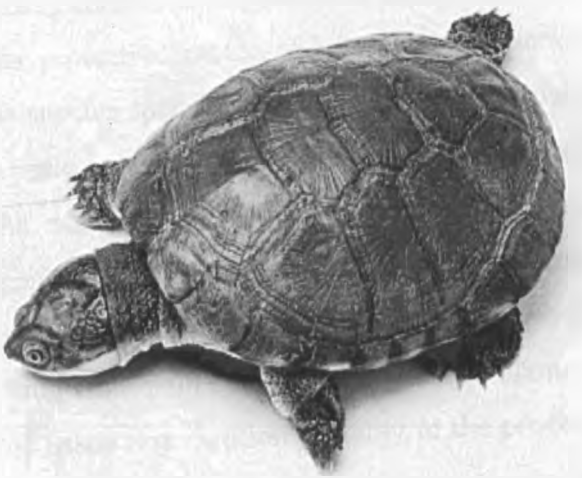

Plate ii: The marsh terrapin: Note the broad head, webbed feet and flattened carapace.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 HISTORY OF PAIN AND PAIN TERMINOLOGIES

Despite the knowledge of its existence since prehistoric times, a proper definition of pain has been lacking (Mersky and Bogduk, 1994). Pain is a perception, with both physiological and emotional components. Pain constitutes an alarm that ultimately has the role of helping to protect the organism: it both triggers reactions and induces learned avoidance behaviors, which may decrease whatever is causing the pain and, as a result, may limit the (potentially) damaging consequences (Millan, 1999; Le Bars, *et al.*, 2001). At the beginning of the twentieth century, Sherrington developed this concept and introduced the term *nociception* (from the Latin *nocere*, "to harm") (Le Bars *et al.*, 2001).

In non-human subjects, pain is defined as "an aversive sensory experience caused by actual or potential injury that elicits protective motor and vegetative reactions, resulting in learned avoidance and may modify species specific behavior, including social behavior (Millan, 1999; Le Bars *et al.*, 2001). The basic concept is that pain/nociception has at least three functions: to warn the individual of the existence of real tissue damage; to warn the individual of the probability that tissue damage is about to occur by realizing that a stimulus has the potential to cause such damage; and to warn a social group of danger as soon as it exists for any one of its members. Behaviors resulting from pain can facilitate other fundamental biological functions, such as the maintenance of tissue regeneration (notably in the processes of inflammation and healing). Moreover, animals with deficits in their nociceptive system have shorter lifespan, and even minor injuries can lead to catastrophic consequences (Caterina *et al.*, 2000; Le Bars *et al.*, 2001). In humans, nociceptive deficits are associated with autism and low survival success rates (Kapasi, *et al.*, 1992).

Merskey and Bogduk (1994) have also defined other commonly used terminologies in the field of pain research. These include hyperalgesia, analgesia, hypoalgesia, pain tolerance level, pain threshold, hyperesthesia and allodynia. Hyperalgesia is an increased response to painful stimulus. It is usually associated to inflammation or tissue injury. Hyperalgesia can be induced by heat, exposure to ultraviolet radiation or injection of hyperagesic substances such as prostaglandins, histamine, bradykinin, capsaicin, etc, into the skin.

Allodynia is a pathological condition in which pain sensation is elicited by a stimulus that is normally non-painful. It is due to activity in non-nociceptive, fast conducting, thinly myelinated A-beta afferents, which evoke pain following inflammation or nerve injury. Pain threshold is defined as the first barely perceptible pain to appear in an instructed subject under a given condition of stimulation. In humans, it is usually revealed by a verbal expression and measured in terms of lowest intensity of stimulus that will evoke it. In animals, reflex responses to presumed pain are used to measure pain threshold. These include the more obvious signs as lameness, biting and scratching at an irritation site, or obscure signs, such as inappetance, lassitude and dysuria.

2.2 MECHANISMS OF PAIN

2.2.1 Peripheral Mechanisms of Pain

The transduction of noxious stimuli is a function of nociceptors. All nociceptors are free nerve endings that have their cell bodies in the dorsal root ganglia or the trigeminal ganglia (Almeida *et al.,* 2004). The naked nerve endings can be directly activated by strong mechanical, thermal or chemical stimuli. Nociceptors can be sensitized, and can also be activated by tissue injury, inflammation, ischemia and low pH (Almeida *et al.,* 2004). Their membranes have receptors for opiates, gamma amino-butyric acid (GABA), local anesthetics, capsaicin and various algogens (Giordano, 2005).

Electrophysiological and neuroanatomical techniques reveal that two types of peripheral nerve axons are involved in pain transmission. These are the thinly myelinated A-delta and the unmyelinated C-axons (Millan, 1999; Almeida *et al.,* 2004; Giordano, 2005). C-type fibers have a diameter range of 0.3-4.5 μm and a conduction velocity of 0.5–2.0 m/s whereas A-δ fibers range from 1-5 μm in diameter and a conduction velocity of 12–30 m/s. The A-beta fibers are myelinated, with a diameter of approximately 10 μm and a conduction velocity of 30–100 m/s, and do not propagate noxious potentials in normal situations. However, the A-beta fibers are fundamental in the painful circuitry because they participate in the mechanisms of segmental suppression (Djouhri *et* al., 1998; Lawson, 2002; Djouhri and Lawson, 2004).

Based on the sensory modalities, there are four types of nociceptors. These are thermonociceptors, mechanonociceptors, chemonociceptors and polymodal nociceptors. Thermonociceptors are activated by noxious heat or cold (temperatures above 45°C and below 10 °C) (Tominaga and Caterina, 2004). Thermonociceptors contain the transient receptor potential (TRP) protein that detects heat, especially heat associated with inflammation (Stucky *et al.,* 2001). Capsaicin also acts on neuronally expressed transient receptor potential vanilloid 1 (TRPV1) (Montell *et al.,* 2002; Tominaga and Caterina, 2004). TRP channels are activated and regulated by a wide variety of stimuli and are expressed widely throughout the body. They are encoded by at least 33 channel subunit genes divided into seven sub-families: TRPC (canonical), TRPV (vanilloid), TRPA (ankyrin), TRPM (melastatin), TRPP (polycystin), TRPML (mucolipin) and TRPN (no mechanoreceptor potential C) (Tominaga and Caterina, 2004; Moran *et al.,* 2004; Nilius *et al.,* 2005; 2007). All TRP members form cation-selective channels, and are assembled as homo- or heterotetramers (Nilius *et al.,* 2005). TRPV1 is activated by noxious heat, acidic pH and capsaicin; TRPV2 is activated by noxious heat, TRPV3 and TRPV4 by warm, and TRPA1 and TRPM by noxious cold (Tominaga and Caterina, 2004).

Mechanociceptors transduce extreme pressure and mechanical deformation (McCarter and Levine, 2006). The mechanisms of mechanotransduction are poorly understood (Costa *et al.,* 2004). They may involve changes in ion permeability, due to the opening of mechanically sensitive channels in the neuronal membrane (McCarter and Levine, 2006). These nociceptors are nonselective to cations and permeate organic anions. They have a significant calcium conductance, and do not permeate chloride or sulfate anions (McCarter and Levine, 2006). The two leading candidate channel families in mammalian mechanotransduction are the degenerin/epithelial sodium channel (DEG/ENaC) family and the transient receptor potential (TRP) family (Costa *et al.,* 2004; McCarter and Levine, 2006).

Chemonociceptors respond to increased levels of endogenous and exogenous chemicals. Chemicals that activate these nociceptors include ions, arachidonic acids derivatives, metabolites, kinins, amines, cytokines, acetylcholine, amino acids, nitric oxides (NO), neuropeptides, opioids, ATP, and adenosine (McCarter and Levine, 2006). These endogenous compounds activate the nociceptive terminals through second messenger systems (McCarter and Levine, 2006). Most of these endogenous chemicals bind to G-proteins and induce the opening of the ligand-gated membrane channels (McCarter and Levine, 2006). The concentration of the chemical(s) determines the degree of depolarization in the nociceptive terminals and the frequency of nerve impulses generated (McCarter and Levine, 2006).

In most vertebrates, the majority of sensory afferents are Polymodal nociceptors, which respond to multiple types of noxious stimuli (Almeida *et al.,* 2004). They are optimally responsive to at least three distinct forms of stimuli, including thermal, mechanical and chemical stimuli; hence, the term 'polymodal nociceptor' (Millan, 1999; Almeida *et al.*, 2004). Both A-delta and C-polymodal nociceptors can undergo sensitization, leading to hyperalgesia (Lynn, 1994; Almeida *et al.*, 2004).

The other class of nociceptors is the silent nociceptors, which do not usually respond to noxious stimuli, but can become active following inflammation or sensitization by nerve

growth factor and algogenic substances (Almeida *et al.,* 2004; Costa *et al.*, 2004). They are high threshold stretch sensitive neurons in the viscera (Almeida *et al.,* 2004; Costa *et al.*, 2004). Because of the high depolarization threshold, these neurons are silent unless they are subjected to a severe deformation. Following sensitization, they become responsive and discharge vigorously, even during ordinary visceral distension. Silent nociceptors may play a role in mechanical "referred" pain and allodynia through viscerosomatic convergence in the spinal cord (Garrison *et al.,* 1992; Palecek *et al.*, 2002; Frøkjær *et al.,* 2005).

Nociceptors are also classified based on location. These include cutaneous, muscular, articular and visceral nociceptors. Cutaneous nociceptors occur in the skin. The majority of these nociceptors are A-δ and C-polymodal nociceptors (Lynn 1994; Almeida *et al.,* 2004). Less frequent are the nociceptors activated by mechanical and cold stimuli (Almeida *et al.,* 2004). They originate from small nervous-stems that, when approaching the epidermis, lose their myelin and ramify into extensive plexuses (Almeida *et al.*, 2004).

During hyperalgesia, the nociceptors become sensitized, leading to reduction of pain thresholds and in some cases, spontaneous activity (Campbell and Meyer, 2006).The hyperalgesia that occurs at the site of injury is primary hyperalgesia, while the hyperalgesia felt in the area, surrounding the injury is secondary hyperalgesia. Primary hyperalgesia to heat stimuli is believed to be mediated by sensitization of peripheral C and A-δ nociceptors (Campbell and Meyer, 2006; Kim, *et al.,* 2008). Secondary hyperalgesia is due to sensitization of neurons in the central nervous system caused by discharges of nociceptors (Urban and Gebhart, 1999, Dougherty, 2003; Campbell and Meyer, 2006). When stimulated, nociceptors release a variety of excitatory amino acids and other peptides like substance P (SP), calcitonin gene-relatetd peptide (CGRP), neurokinin-A (NK-A) and vasoactive intestinal peptide (VIP) in the CNS, which sensitize nociceptors and cause central sensitization (Sorkin and McAdoo 1993; Urban and Gebhart, 1999, Giordano, 2005). The N-methyl-D-aspartate (NMDA) receptors are essential for the development of centrally mediated hyperalgesia (Dolan and

Nolan, 2000). Activation of these receptors results in the production of a number of intracellular second messengers, such as nitric oxide and prostaglandins, which are also implicated in the development of hyperalgesia (Campbell and Meyer, 2006).

2.2.2 Spinal Mechanisms of Pain

2.2.2.1 Dorsal Horn Neurons and the Gate Control Theory

The cell bodies of nociceptive neurons are located in the dorsal root ganglion (DRG) or the trigeminal ganglia. All the primary afferent nociceptors converge on and synaptically excite neurons of the dorsal horn (the spinal cord) and medulla (trigeminal nucleus) (Millan, 1999). There are three main types of neurons in the dorsal horn (Price, 1999). These are the projection neurons, excitatory interneurons and inhibitory interneurons. The projection neurons relay nociceptive information to the brain while the excitatory interneurons relay information to the projection neurons, other interneurons and motor neurons, which mediate spinal reflexes. The inhibitory interneurons modulate the transmission of nociceptive information (Price, 1999).

Although the ability to experience pain is vital and essential for the survival of an animal, it is essential for the organism to be able to control and modulate the pain sensation. Painful stimuli transmitted to the spinal cord are modulated at the level of dorsal horn by the dorsal horn neurons. Melzack and Wall (1965) proposed the 'gate control theory' and since then, the theory has been widely revised (Melzack, 1999; Giordano, 2005). This theory proposes that the nociceptive information reaching the spinal cord is modulated by the non-nociceptive input reaching the spinal cord. The activation of A-beta low threshold mechanosensitive wide dynamic range (WDR) neurons inhibits the nociceptive activity of the nociceptor specific (NS) neurons by activating inhibitory interneurons in the spinal cord (Melzack and Wall, 1965; Melzack, 1999; Millan, 1999; Giordano, 2005). The gate control theory also explains how some pain treatment modalities such as massage and acupuncture alleviate pain.

2.2.2.2 Dorsal Horn Neurotransmitters

Several chemicals are involved in the transmission of nociceptive information in the spinal cord (Basbaum, 1999; Giordano, 2005). Glutamate is the most abundant neurotransmitter in the nervous system and plays major role in nociceptive transmission in the dorsal horn, particularly through the NMDA receptors (Giordano, 2005). This neuromediator acts on two classes of receptors, the ionotropic and metabotropic receptors. The ionotropic receptors are divided according to the agonist: N-methyl-D-aspartate (NMDA), alpha-amino-3-hydroxy-5-methyl-4-isoxalone propionate (AMPA), kainate and 2-amino-4-phosphonobutyrate (AP4), (Giordano, 2005).

The NMDA receptor complex is a multimeric channel permeable to Na^+ and Ca^+, and is both ligand and voltage gated. At normal resting potential (-70 mV) Mg^{2+} blocks the ionophore of the NMDA receptor, and relieve of this blockade only occurs after a membrane depolarization of -30 mV. This depolarization occurs after prolonged activation of AMPA receptors by glutamate, leading to activation of NMDA receptors, which causes large prolonged depolarization associated with Ca^{2+} influx. This process underlies the medium- to long-term changes that occur in chronic pain states, including changes in peripheral receptive fields, induction of gene transcription and long-term potentiation (LTP) (Giordano, 2005). LTP involves enhancement in synaptic efficacy, a synaptic correlate of learning and memory in the hippocampus and cerebral cortex, and may play a role in the development of a cellular 'memory' for pain or enhanced responsiveness to noxious inputs (Ru-Rong *et al.*, 2003).

Nitric oxide (NO) is probably involved in positive feedback mechanism, acting in conjunction with presynaptic NMDA receptors to upregulate afferent input further, and thereby potentiates the excitatory effects of glutamate. In animal models of neuropathic pain, NO synthesis results in a decrease in the behavioral correlates of pain (Basbaum, 1999; Ru-Rong *et al.*, 2003).

The glutamatergic metabotropic receptors (mGluR) comprise of three groups (I-III) and at least eight subtypes, mGluR1- mGluR8. Group I mGluRs may play a modulatory role in

nociceptive processing, central sensitization and pain behavior (Budai and Larson, 1998). The role of mGluR II and III is less clear (Budai and Larson, 1998; Ru-Rong *et al.,* 2003). G protein-coupled receptor are activated by an external signal in the form of a ligand or other signal mediator, which creates a conformational change in the receptor, causing activation of a G protein (Bockaert Pin, 1999). AMPA receptors are ligand gated and permeate the selective entry of Na^+, resulting in short latency excitatory postsynaptic potentials (Giordano, 2005).

Nociceptive fibers also release a variety of neuropeptides such as substance P (SP), neurokinin A (NKA), calcitonin gene related peptide (CGRP), somatostatin (SST) and cholecystokinin (CCK) (Basbaum, 1999). Most of these neurotransmitters have excitatory effects on neurons. The release of SP, which co-exists with glutamate in primary afferents, occurs following cutaneous thermal, mechanical or chemical noxious stimuli and is potentiated by peripheral inflammation (Furst, 1999; Giordano, 2005). SP plays a modulatory role on the incoming nociceptive information, probably in conjunction with NKA and CGRP in the spinal dorsal horn (Milllan, 1999; Giordano, 2005).

The contribution of the various transmitters in the response to afferent nerve stimulation depends on the duration of stimulation (Ru-Rong *et al.,* 2003; Keeble and Brain, 2004). High frequency stimulation of C fibers initially produces a short latency (onset), and a short duration (millisecond) depolarization of transmission neurons and this is because of the action of glutamate on AMPA receptors (Giordano, 2005). This type of glutamate receptor operates a ligand-gated sodium channel. The AMPA receptor response is followed by a longer onset and a longer duration (seconds) response caused by the release of tachykinins and CGRP, which activate G-protein linked receptors. SP and NKA activate phospholipase C and Inositol triphosphate (IP_3) production (Basbaum, 1999; Ru-Rong *et al.,* 2003; Keeble and Brain, 2004). The neuronal responses to neuropeptides differ qualitatively according to the nature of the transduction mechanism to which their receptors are linked. In addition, neuropeptides are generally released at higher frequencies of nerve stimulation (5-40 Hz) than non-peptides (1-10 Hz), and that peptide transmitters tend to be depleted more quickly than non-peptides after

prolonged nerve stimulation (Giordano, 2005). This is because the synthesis of neuropeptides involves the assembly of amino acids on a mRNA template and occurs in the cell body of sensory neurons, followed by transport of the peptide to the terminal (Ru-Rong *et al.,* 2003).

2.2.2.3 Ascending Systems in the Spinal Cord

Nociceptive information ascends in the white matter of the spinal cord, through the second-order neurons. These neurons are of two physiological types i.e. nociceptive-specific neurons (NS) and the wide-dynamic range neurons (WDR). The nociceptive-specific fibers receive exclusive input from primary nociceptive afferents, whereas the wide-dynamic range neurons receive synaptic contacts from low thresh-hold mechanoreceptive primary afferents as well as from primary nociceptive afferents (Price, 1999; Price *et al.*, 2000; Almeida *et al.,* 2004). WDR and NS neurons cross over to the controlateral white matter of the spinal cord and then project to the thalamic ventroposterior lateral nucleus. There are several of these tracts, some short and others long traversing the entire spinal cord (Price, 1999; Melzack, 1999).

The spinothalamic tract (STT) is located in the ventrolateral quadrant of the spinal cord and the majority of the axons crosses locally and ascends contralaterally. Cells of origin of the STT mainly originate from Rexed's lamina I, IV and V, and terminate in the ventroposterior (VP) thalamic nuclei, in a somatotopic fashion (Price, 1999; Price *et al.*, 2000; Almeida *et al.,* 2004). Spinothalamic tract is very important in the transmission of signals associated with pain and temperature sensation.

Spinoreticular tract originates from the deep layers of the gray matter (laminae VI and VII) and ascends through the ventrolateral quadrant to terminate in reticular formation of the brainstem. One part of the tract terminates in several nuclei in pons and medulla, such as nucleus paragigantocellularis, nuclei reticularis, pontis caudalis and oralis; nucleus gigantocellularis and nucleus subcoeruleus. Another major termination is in the parabrachial region, including the locus coeruleus and the parabrachial nuclei (Willis and Westlund, 1997;

Basbaum *et al.*, 2005). This tract is involved in the motivationo-affective characteristics of pain, and in the activation of brain stem structures responsible for descending suppression (Price *et al.*, 2000; Almeida *et al.*, 2004).

The spinomesencephalic tract includes projections to different areas in the midbrain. Most axons project from layers I and V of the spinal cord. The tract primarily terminates in the superior colliculus and the periaquiductal grey (PAG) (Almeida *et al.*, 2004). Projections to the PAG mainly activate descending pain control networks, and are also involved in autonomic and somatomotor aspects of defense reaction. Nociceptive activity in the superior colliculus is thought to be involved in multisensory integration, behavioral reactions and orientation to pain (Basbaum *et al.*, 2005).

The postsynaptic dorsal column pathway mainly originates from laminae III and V. Although the vast majority of these fibers are non-nociceptive, some nociceptive neurons project through this tract. The tract is organized into two distinct pathways. One pathway is close to the midline of the spinal cord, originates from the lumbar-sacral region, while the other is at the junction of the gracile and cuneiform bundles, and originates from the thoracic column (Almeida *et al.*, 2004). The pathway is involved in visceral pain transmission (Willis and Westlund, 2001; Palecek, *et al.*, 2002).

The spino-brachial-amygdala system originates in laminae I and V of the dorsal horn, ascending in the dorsolateral funiculus. It projects to the parabrachial area of the pons, and from there to the amygdala. This system may normally be involved in fear and memory of pain, as well as in behavioral and autonomic reactions to noxious events, such as vocalization, flight, freezing, pupil dilation and cardiorespiratory responses (Basbaum, 2005; Millan, 1999; Almeida *et al.*, 2004).

2.2.3 The Role of Thalamus and the Cerebral Cortex in Nociception

The thalamus represents the main relay station for all sensory information destined to the cortex, and is involved in the reception, integration, and transfer of the nociceptive information. The different projections to its nuclei and from them to the cortex define the functional circuitry of pain processing (Millan, 1999; Almeida *et al.*, 2004). The lateral nuclear complex consists of the ventroposterolateral (VPL), ventroposteromedial (VPM) and ventroposteroinferior (VPI) nuclei. Neurons of the WDR type predominate in the VPL and VPM nuclei, and those of nociceptor specific neurons are found in the VPI nucleus (Almeida *et al.*, 2004; Basbaum *et al.,* 2005). They all respond to the thermal, chemical and mechanical stimuli.

The VPL nucleus is recognized as the main somatosensory relay, with convergence of noxious and innocuous stimuli of cutaneous, muscular, and articular origins (Almeida *et al.*, 2004). The VPM nucleus presents cell types and organization similar to the VPL nucleus, being similarly involved in the sensory- discriminative aspects of thermal, mechanical and chemical information (Almeida *et al.*, 2004; Basbaum *et al.,* 2005). The outputs from VPL nuclei ascend via thalamocortical afferents, which are third order neurons, to the somatosensory cortex (SI) where a conscious localization and characterization of pain occurs. Neurons from VPM nuclei are projected to the anterior cingulate gyrus, which is involved in the perception of suffering and emotional perception of pain. The secondary somatosensory cortex (SII) regions of the parietal cortex, the insular cortex and the medial frontal cortex have all been identified as regions activated by noxious stimuli from cutaneous and muscular tissue (Almeida *et al.*, 2004).

In birds and reptiles, the dorsal ventricular ridge (DVR) receives inputs from the thalamus, and is analogous to the mammalian cortex (Murakami and Kuratani, 2007; Abdel-Mannan, 2008; Butler, 2008). The dorsal ventricular ridge has three cell layers, unlike the mammalian six cell layered neocortex (Abdel-Mannan, 2008; Butler, 2008). It is believed that the DVR has transformed into part of the mammalian multilayered neocortex by relocating corresponding cell groups (Butler, 2008).

2.2.4 Descending Modulatory Systems in Control Nociception

Several brainstem regions are involved in the modulation of the nociceptive transmission through the descending inhibitory systems of the spinal cord (Millan 1999; Almeida *et al.*, 2004). These connections from the brainstem to the spinal cord can change or modify information that is coming from the peripheral nervous system to the brain. In this way, the brain can thus reduce pain, by a mechanism of descending analgesia. It uses feedback loops that involve several different nuclei in the brainstem reticular formation. Two important areas of the brainstem that are involved in reducing pain are the periaqueductal gray (PAG) and the nucleus raphe Magnus (NRM) (Basbaum, 1999; Millan, 1999; Basbaum *et al.,* 2005; Giordano, 2005).

The descending systems include corticospinal, the raphe spinal and the reticulospinal systems (Millan, 1999; Giordano, 2005). The cortical spinal cells terminate in the LIII-LVI or even in lamina VII in the cat and are absent in LI and LII. The influence of the cortical spinal pathway upon dorsal horn interneurons includes a prominent inhibition in LV and excitation in LVI, but no effect on LIV (Millan, 1999; Giordano, 2005). The raphe spinal system arises from midline raphe magnus of the brainstem and consists of the dorsal lateral funiculi, and terminates in LI, LII, LV and medial parts of LVI and VII. The parts of the dorsal horn, which receive inputs from the raphe magnus, are those parts concerned with nociceptors and which give rise to spinothalamic and spinoreticular tracts (Millan, 1999; Giordano, 2005).
Noradrenergic and serotonergic neurons are more diffuse but they mainly arise from locus coeruleus and the raphe nuclei respectively. The descending noradrenergic system terminates in the marginal layer, laminae II, IV, VI and the ventral horn (Price, 1999).

2.2.4.1 The Serotonergic system

The raphe nuclei are the major sources of serotonin (5-HT) in the CNS. Although not every raphe neuron contains 5-HT, Raphe neurons may also contain the monoamines noradrenaline and dopamine as well as the peptide transmitter, cholecystokinin (CCK) (Furst, 1999). There

are seven subtypes of serotonergic receptors, 5-HT $_{1-7}$ (Nelson, 2004). With the exception of the 5-HT_3 receptor which is a ligand gated ion channel, all other 5-HT receptors are G protein coupled seven transmembrane receptors that activate an intracellular second messenger cascade (Nelson, 2004; Nichols and Nichols, 2008). Descending serotonergic fibers to the spinal cord have been implicated in modulation of pain at the spinal level as well as modulation of general sensory and motor function.

Serotonergic fibers terminate on enkephalinergic interneurons of the intermediate gray of the spinal cord (Basbaum, 1999; Furst, 1999). They project to the presynaptic terminals of primary afferents, which mediate pain (A-δ and C fibers) and utilize substance P. The serotonergic input is excitatory to the enkephalinergic interneurons, which in turn are inhibitory on the primary afferents. Thus, raphe neurons can inhibit the flow of pain information from the periphery. Serotonergic fibers also terminate on presympathetic neurons in the intermediolateral cell column of the spinal cord (Furst, 1999). The raphe is inhibitory to these neurons.

Stimulation of the raphe nuclei produces a powerful analgesia and thus blocks pain transmission. Depletion of 5-HT by P-chlorophenylamine reduces stimulation-produced analgesia which is reversible by administration of 5-hydroxytryptophan, a 5-HT precursor (Furst, 1999; Millan, 1999; Giordano, 2005). However, serotonin may not be directly involved in the inhibition of pain transmission since serotonergic agonists do not have significant analgesic effects (Furst, 1999).

2.2.4.2 The Noradrenergic System

The noradrenergic system is associated with the locus coeruleus, which is located in the rostral pons in the floor of the rostral part of the fourth ventricle. It utilizes noradrenaline (NA) as its neurotransmitter and is the major source of noradrenaline supplying the CNS. Afferents from the locus coeruleus are widespread in both the ascending and descending directions. Descending noradrenergic neurons terminate in the spinal cord dorsal horn, where NA is released to inhibit nociceptive transmission (Furst, 1999). There are several receptor types (and subtypes) for NA ($\acute{\alpha}2_a$, $\acute{\alpha}2_b$ and $\acute{\alpha}2_c$) (Furst, 1999). Stimulation of spinal ά2 adrenoceptors

results in very potent antinociception, as seen after intrathecal administration of ά2 adrenoceptor agonists (Saunders and Limbeird, 1999; Giordano, 2005).

2.2.4.3 The GABA-ergic System

GABA-ergic interneurons are involved in tonic inhibition of nociceptive input. GABA normally plays an inhibitory role on the dopaminergic cells (Giordano, 2005). Opioids and endogenous opioid neurotransmitters activate the presynaptic opioid receptors on GABA-ergic neurons. This inhibits the release of GABA in the ventral tegmental area. Inhibition of GABA allows the dopaminergic neurons to fire more vigorously causing the release of extra dopamine in the nucleus accumbens. The two types of GABA receptors, the ligand-gated Cl^- channel ($GABA_A$) and the GTP-binding protein coupled receptor ($GABA_B$) are important in spinal antinociception. Activation of GABA-ergic interneurons reduces the release of excitatory neurotransmitters glutamate, SP and CGRP from primary nociceptive afferents (Furst, 1999; Giordano, 2005). The inhibitory effects of $GABA_A$ are preferentially through postsynaptic mechanisms, while those of $GABA_B$ are presynaptic inhibition through the suppression of the effects of excitatory amino acids from the primary nociceptive terminals (Giordano, 2005).

2.2.4.4 The Cholinergic System

Muscarinic receptors have been shown to be involved in spinal antinociceptive mechanisms interacting with the GABA-ergic system (Baba *et al*, 1998; Xu *et al*., 2000; Chen and Pan, 2003), opioidergic (Chen *et al.,* 2001), and adrenergic (Honda *et al.,* 2002) receptor systems. Nicotinic receptors are also involved in modulation of nociceptive information. Interactions of cholinergic with the serotonergic and adrenergic systems have been demonstrated (Li and Eisenach, 2002; Xu *et al*., 2000). Systemic morphine causes increased release of acetylcholine in the spinal cord (Chen and Pan, 2001). Intrathecal injection of the cholinergic receptor agonists or acetyl cholinesterase inhibitors produces antinociception in mammals (Chen and Pan, 2001). The spinal endogenous acetylcholine plays an important role in mediating the analgesic effect of systemic morphine through both muscarinic and nicotinic receptors (Chen and Pan, 2001).

2.3 THE TRIGEMINAL COMPLEX

The trigeminal complex consists of the three main branches of the trigeminal nerve (ophthalmic, maxillary and mandibular nerves), the trigeminal ganglion, and the four-brainstem nuclei of the trigeminal system (principle, mesenchephalic and spinal sensory nuclei, and a motor nucleus) (Dubbeldam *et al.*, 1995, 1998; Milllan; 1999; Sessle, 2000; Fried *et al.*, 2001). The trigeminal nerve carries information about touch, temperature, pain and proprioception originating from the head region (Lazarov, 2008). It is also referred to as the great sensory tract of the head region. It is the second largest cranial nerve after optic nerve, located just after the optic chiasma (Bronchu, 2000, Lutz *et al.*, 2002; Oduntan, 2005).

The trigeminal nerve arises from the lateral side of anterior end of medulla and passes infront of the optic capsule, and immediately enters into a large semi-lunar ganglion, the trigeminal ganglion in the dura mater. From there arises the three principal branches, ophthalmic, maxillary and mandibular nerves (Bronchu, 2000; Lutz *et al.*, 2002). The ophthalmic nerve is the first to branch and it advances some way within the dura, coursing anterolaterally alongside the frontal bones. The nerve then enters the orbit through the dorsal orbital fissure, in the orbital sphenoid (Bronchu, 2000). After branching of the ophthalmic nerve, the maxillo-mandibular nerve shortly courses anterolaterally and exits the neurocranuim through the foramina rotundum, in the sphenopalatine region. The maxillary nerve quits the mandibular branch and enters into the surrounding muscles advancing rostrally, while the mandibular branch courses ventrally (Bronchu, 2000). In general, the ophthalmic division, which is predominantly sensory, serves the skin of the upper parts of the head and parts of the nares. The maxillary division, also mainly sensory, innervates teeth and mucosa of the maxilla, the upper lip, lateral nose, maxillary sinus and nasopharynx. The mandibular division has both sensory and motor fibers, and innervates the mouth region and masticatory muscles (Fried *et al.*, 2001; Lazarov, 2008).

The trigeminal ganglion is analogous to the dorsal root ganglion of the spinal cord, with majority of the sensory afferents having their cell bodies in it (Lazarov, 2008). The trigeminal ganglion lies in the Merckel's cavity, posterolateral to the cavernous sinus, in the floor of the

neurocranium. The motor neurons (part of the mandibular branch) bypass this ganglion, and have their cell bodies located in the brainstem motor nuclei of the trigeminal complex (Lutz *et al.*, 2002; Lazarov, 2008). The central branches of the neurons located in the trigeminal ganglion enter the brain stem at the level of the pons and project in complex networks to the principal sensory and spinal trigeminal nucleus (Sessle, 2000). Via second-order neurons, the impulses are conveyed from here to the thalamus, and on towards the sensory cortex (Fried, *et al.*, 2001).

In mammals, the proportion of unmyelinated to myelinated fibers is much lower in trigeminal nerve compared to the spinal dorsal horn. This may reflect the importance of the trigeminal nerve in tactile exploration of the environment (Sessle, 2000). Several studies have focused on the trigeminal somatosensation, among which the proportion of fiber types has been investigated. For instance, in crotaline snakes, both myelinated and unmyelinated fibers are present in all the principle branches of the trigeminal nerve, with a predominance of unmyelinated fibers (54.8%) (Hisajima *et al.*, 2002). In the rainbow trout, the trigeminal nerve is composed of 4% unmyelinated fibers, 9% A-alpha, 53% A-beta and 33% A-δ fibers (Sneddon, 2002). The trigeminal nerve of agnathans lacks myelination whereas elasmobranches do not have unmyelinated fibers and lack nociceptors (Sneddon, 2004).

2.4 THE INDUCTION AND MEASUREMENT OF PAIN

The objective of pain researchers is to achieve a scientific understanding of the mechanisms involved and clinical control of pain. To achieve these goals, a valid, reliable, and flexible measurement technology must be availed (Edens and Gil, 1995). Pain can be evoked by many methods but there are four main kinds of noxious stimuli: thermal, electrical, mechanical and chemical stimulation (Svensson *et al.*, 1997; Millan, 1999; Le Bars *et al.*, 2001). Experimental pain originating from the skin has been more extensively studied because the skin offers greater accessibility to nociceptors than visceral structures.

The procedure selected for the induction of painful stimuli should be applicable to both humans and animals, sensitive to agents of low analgesic potency, cause minimal tissue

damage, easy to perform and detect pain end points, and it should provide a relationship between the intensity of stimulus and the intensity of pain experience. Most tests that are used to study pain in animals involve motor responses to nociceptive stimuli (Edens and Gil, 1995). These depend on an implicit hypothesis that there is a strong relationship between nociception and motor activity (Le Bars *et al.*, 2001). The stimuli should be quantifiable, reproducible and non-invasive. In addition, key in these behavioral tests is describing the behavioral parameters that are measured. This may involve defining the responses as a function of stimulus intensity.

Pain can be induced in two ways, namely short duration stimuli (phasic pain) or long duration stimuli (tonic pain). Phasic animal models of pain are the most commonly used. Generally, phasic animal models of pain involve short period of stimulation, have somatic rather than visceral sites of stimulation and mainly involve the measurement of threshold (Millan, 1999; Le Bars *et al.*, 2001). Tonic animal models of pain usually involve injection of irritant, algogenic material as the nociceptive stimuli. Tonic models quantify the behavioral responses observed after the application of a stimulus over time with a potency that is going to vary with time (Le Bars *et al.*, 2001).

2.4.1 Methods of Inducing Pain

2.4.1.1 Chemical Stimulation

Different types of algogenic chemicals have been used to induce pain. These include formalin, acetic acid, capsaicin, carrageenin, histamine, serotonin, acetylcholine, bradykinin and prostaglandins. The most commonly used chemical methods are the formalin and the writhing tests, where dilute solutions of formalin or acetic acid are used respectively (Le Bars, *et al.*, 2001). Chemical stimuli differ from other forms of stimuli in that it is progressive and of longer duration. Consequently, the stimuli do not lead to the typical reflexes produced by synchronized afferent nerve stimulation as in other forms of pain induction (Le Bars *et al.*,

2001; Liard *et al.*, 2001). Chemical stimulation leads to very stereotyped behavioral responses in rodents. The tests using chemical stimulation do not involve the measurement of the threshold but instead involve the measurement of a behavior in units of time, over a given observation period. Chemical nociceptive tests bear some resemblance to clinical pain (Liard *et al.*, 2001).

2.4.1.2 Thermal Stimulation

Thermal induction of pain is a common practice in nociceptive studies. The skin is usually the site of stimulation. The method is easy to perform. However, the method is limited by the manner in which it excites neurons. Depending on intensity, heat usually activates thermoreceptors alone, then thermoreceptors and nociceptors, and then nociceptors alone (Svensson *et al.*, 1997; Le Bars *et al.*, 2001). The source of nociceptive stimuli can be distant from its target (e.g. radiant heat from a lamp) or can be in direct contact with the skin (e.g. the hot plate test, tail immersion test). Contact thermodes also have the disadvantage of additionally stimulating touch receptors (Svensson *et al.*, 1997). Moreover, the rate of thermal transfer is dependent on the quality of the thermode-skin contact and thus on the pressure of heat application (Svensson *et al.*, 1997; Le Bars *et al.*, 2001). Examples of thermal nociceptive tests include the hot plate, tail flick, tail immersion tests.

2.4.1.3 Mechanical Stimulation

Mechanical stimuli are applied to the paw or tail of an animal, or may even involve the distension of a holoviscous organ (Liard *et al.*, 2001). The pressure applied can be constant or gradually increasing, and it is the pain threshold that is measured. The responses to mechanical stimuli are graded in relation to the intensity and/or duration of the stimulus, and can range from simple reflexes to more complex motor behaviors (Le Bars *et al.*, 2001; Liard *et al.*, 2001; Staahl and Drewes, 2004). Mechanical stimuli have the disadvantage of activating both low-threshold mechanoreceptors and nociceptors (Edens and Gil, 1995). It is also limited in its application in freely moving animals.

2.4.1.4 Electrical Stimulation

Electrical stimulation has the advantage of being quantifiable, reproducible, and noninvasive and of producing synchronized afferent signals. Though widely used, it does not resemble natural pain and may excite both nociceptive and non-nociceptive neurons simultaneously (Fan *et al.*, 1995; Svensson *et al.*, 1997; Le Bars *et al.*, 2001). In addition, electrical stimulation does not involve signal transduction as it completely short-circuits peripheral receptors, thus preventing any study of peripheral transduction mechanisms (Fan *et al.*, 1995; Svensson *et al.*, 1997). The electrical stimuli can be applied in a very brief and sudden fashion, which can result in synchronous excitation of multiple fiber types. This can affect the behavioral responses that are graded as a function of stimulus intensity- from spinal reflexes, through complex vocalizations, and up to very organized behaviors such as escape or aggression (Fan *et al.*, 1995; Le Bars *et al.*, 2001).

2.4.2 Behavioral Nociceptive Assays

Behavioral pain studies indirectly explore the sensory system of the experimental animals. A basic requirement is that the pain test should exclusively activate pain fibers and the animal gives a corresponding motor output that can be quantified. Some of the commonly used nociceptive tests include:

2.4.2.1 The Formalin Test

Formalin test is one of the most commonly used behavioral nociceptive assays. The test quantifies the behavioral responses to moderate cutaneous pain, induced by formalin injection. The formalin test was developed and used by Dubuisson and Dennis (1977) to rate pain in saline and morphine treated rats. They described in detail the behavior induced by formalin injection and developed a scheme for quantifying the pain related behaviors. The behaviors observed in rodents include elevating, shaking, licking or biting the injected paw or reducing the weight put on it.

Formalin is the aqueous solution of 37% (w/w) formaldehyde (CH_2=CO) in water. Various concentrations and volumes have been used, ranging from 0.5- 15% (Le Bars *et al.*, 2001). The commonly adopted volumes are in the range of 5-150 µL. The site of injection of formalin is also important. The most commonly used location is the dorsal surface of the paw. Different concentrations may have different effects on the pain responses elicited. Low concentrations (0.002-0.2%) only induce the first phase of pain, whereas concentrations more than 1% formalin causes both early and late phases of pain (Le Bars *et al.*, 2001; Lee and Jeong, 2002; Oyadeyi *et al.*, 2007).

Formalin test induces two distinctive phases of nociceptive behavior in rodents (Le Bars *et al.*, 2001). This behavior consists of an initial phase, occurring about three minutes after the injection, and then after a quiescent period, a second phase between the 20th and 30th minutes. The intensities of these behaviors are dependent on the concentration of formalin that is administered (Rosland *et al.*, 1990). The first phase results essentially due to direct stimulation of nociceptors, whereas the second involves a period of sensitization during which inflammatory phenomena occur (Le Bars *et al.*, 2001; Capone and Aloisi, 2004). The involvement of NMDA receptors in the second phase of formalin test has also been suggested (Omote *et al.*, 2000; Capone and Aloisi, 2004). Injection of formalin sets up a cascade of events leading to the release of excitatory amino acid glutamate, which activates NMDA receptors in the spinal cord. The release of glutamate requires nitric acid (NO) which is believed to play a crucial role during prolonged nociception (Omote *et al.*, 2000; Capone and Aloisi, 2004). A number of factors, including the ambient temperature, sounds, odors, bright light, high atmospheric pressure, presence of moving objects, can influence behavioral responses in the formalin-test (Le Bars *et al.*, 2001).

There are several advantages the formalin test has over other tests. The pain stimulus bears some resemblance to most clinical pain. There is little or no restraint required during the experiment. The pain elicited by formalin nociception is continuous and enables a temporal nociceptive profile to be measured. It is very sensitive to mild analgesic effects of various

substances including those of anti-inflammatory drugs. However, most of the concentrations of formalin used cause long lasting histological and macroscopic changes such as depilation, scarring and ulceration (Le Bars *et al.,* 2001). Formalin test has been widely used in a number of animals including mice, rat, cat, monkey (Le Bars *et al.,* 2001), rabbits (Farabollini *et al.,* 1988), crocodiles (Kanui *et al.,* 1990), domestic fowls (Hughes and Sulka, 1991) and frogs (Oyadeyi *et al.*, 2007).

2.4.2.2 The Hot plate Test

The hot plate test is a commonly used phasic nociceptive test, which measures the responses to a brief noxious heat stimulus. Response latency is measured. Results that are more informative can be obtained by varying the temperatures of the hot plate from 50-59 °C. (Tjølsen *et al.*, 1991, 1992; Ding, *et al.*, 2005). In rodents, the pain behaviors observed on the hot plate include jumping, kicking, dancing, lifting of the foot, biting the foot/paws, and attempts to flee (Tjølsen *et al.*, 1992; Le Bars *et al.,* 2001). A plate heated to a constant temperature produces two behavioral components that can be measured in terms of their reaction times, namely paw licking and jumping. Both are considered supraspinally-integrated responses (Le Bars *et al.*, 2001; Ding, *et al.*, 2005). In juvenile crocodiles, the behaviors exhibited include lifting the toes, lifting the foot and attempts to escape (Kanui, *et al.*, 1990. The behaviors vary in intensity and severity depending on, among other factors, the hot plate surface temperature, ambient temperature and skin temperature (Le Bars *et al.,* 2001). To avoid tissue damage on the paws of animals, a temperature limit or cut-off time, or both is chosen as the experimental end-point (Tjølsen *et al.*, 1992).

2.4.2.3 The Capsaicin Instillation Test

In this test, very low concentrations of capsaicin dissolved in a vehicle are topically instilled into the eye and pain response assessed (Kanui *et al.*, 1990; Farazifard *et al.,* 2005). Capsaicin is prepared as a 1% solution by diluting it in 10% ethanol, 10% tween 80 and 80% saline. Further dilutions are made with saline to formulate concentrations in the range of 10^{-1} to 10^{-10}. Eye protection responses like blinking, blepharospasms, wiping, rubbing, head shaking and

eyeball movements are scored. The test has been applied in different animals and yields reliable results

2.4.2.4 The Chemically Induced Writhing Test

The intraperitoneal administration of agents that irritate serous membranes provokes a very stereotyped behavior in the rodents, which is characterized by abdominal contractions, movements of the body as a whole (particularly of the hind paws), twisting of dorsoabdominal muscles, and a reduction in motor activity and motor incoordination (Laird *et al.*, 2001). The test is sometimes called the abdominal contortion test, the abdominal constriction response, or the stretching test, but more commonly referred to as the "writhing test" (Le Bars *et al.*, 2001; Laird *et al.*, 2001). Several chemical irritants have been used, e.g. Zymosan, acetic acid, phenylquinone, bradykinin and acetylcholine, dilute hydrochloric acid, adenosine triphosphate, potassium chloride and tryptamine (Laird *et al.*, 2001).

Although sensitive to weak analgesics, the writhing test lacks specificity, and may show positive results with a wide range of analgesic substances as well as with material with no analgesic activity like adrenergic blockers, antihistamines, muscle relaxants, monoamine oxidase inhibitors, and neuroleptics (Le Bars *et al.*, 2001; Laird *et al.*, 2001). Thus, a positive result with this test does not necessarily mean that there is analgesic activity. The test is good for screening new analgesic materials. Though of poor sensitivity, the writhing test is sensitive and predictive (Le Bars et al., 2001; Laird et al., 2001). The test is simple to perform and is possible to quantify the response, and to correlate the variable with the stimulus intensity within a reasonable range.

2.4.2.5 Adjuvant Induced Arthritis

This is a chronic pain model where the stimulus is inflammatory reaction caused by the injected material. Intradermal injection of *Mycobacterium butyricum* with Freund's adjuvant into the tails of rats induces polyarthritis (Le Bars *et al.*, 2001; Yu *et al.*, 2002). The polyarthritis induced is similar to various inflammatory conditions, and results from the test

are predictive of analgesic and anti-inflammatory activity of the test substance. Scoring is based on behavioral responses (such as vocalization upon manipulation of the tibio-tarsal joint) and paw edema (paw volume) (Yu *et al.*, 2002). Simultaneous measurements of paw edema and vocalization have been used to separate the anti-inflammatory effects from the antinociceptive activity of drugs (Le Bars *et al.*, 2001). Other substances used include urate crystals and carrageenin, but these are related to models of chronic inflammatory pain. The disadvantage is that animals suffer from an immunological condition (induced by *Mycobacterium butyricum*), which does not necessarily reflect the nociceptive effects of this material. Several modifications of this test have been developed and applied in different animal species.

2.4.2.6 The Tail Flick Test

The tail flick test is a phasic nociceptive test that measures tail reflex response latencies after radiant heat stimulation or after immersing the tail in hot/cold water. Radiant thermal stimulus is more commonly used form of stimulation, and tail flick withdrawal responses are measured as the tail flick response latencies (Ding, *et al.*, 2005). The two variants of the tail flick test only differ in the surface area of the skin where the pain invoking stimuli is applied (Keefe *et al*, 1991; Le Bars *et al.*, 2001).

The tail flick test has the advantages of being simple to perform and the test displays minimal inter-animal variability. The tail flick response is a spinally integrated nociceptive reflex, and not disrupted by spinalization. The test is a mainly used for screening analgesic drugs and in studies of spinal mechanisms of nociception (Keefe *et al*, 1991; Le Bars *et al.*, 2001). The test also allows for repeated testing without conditioning effects. The skin temperature greatly influences the test results and therefore, in screening analgesics that lowers skin temperature there is prolonged response latency (Le Bars *et al.*, 2001). Modifications of the test have been developed and applied to different animal species (Sladky *et al.*, 2007). The test gives reliable results and can be performed with relative ease in a wide range of species.

2.4.2.7 The Yeast or Carrageenin Induced Hyperalgesia

The test involves intradermal injection of an irritant material such as yeast, croton oil, or carrageenin injected into the hind paw of the animal (Yu *et al.*, 2002). This causes inflammatory changes and hyperalgesia. Pain is then quantified by applying pressure on the inflamed paw by means of a metal cylinder and the pressure (mmHg) at which the animal begins to vocalize or struggle is recorded. The contralateral paw is used as the positive control. The test has been used to distinguish between drugs acting in the CNS and locally at the site of inflammation. It is also sensitive to narcotic as well as non-narcotic analgesics (Yu *et al.*, 2002).

2.5 METHODS OF RELIEVING PAIN

The ultimate goal of pain research is to ensure a proper control of pain. For proper control of pain, several things need to be put into consideration. These include the cause of pain, its site, type and mechanism, its intensity and its probable duration, nature of the disease causing the pain, treatment methods locally available and practicable under the circumstances, and complications that may develop consequent to each method of treatment.

There are very many techniques used in the treatment of pain. These include analgesic agents (narcotic and non-narcotics), transcutaneous electrical nerve stimulation (TENS), nerve blockade, thermotherapy, magnetic therapy and capsaicin.

2.5.1 Narcotic Drugs

Narcotics (opioids) are some of the most potent therapeutic agents used to relieve pain. Morphine is the prototype opiate analgesic drug. Other opioids commonly used include meperidine, pethidine, codeine, fentanyl, methadone, dextromethophan and methadone (Price, 1999). Opioids are very effective in alleviating both acute and chronic pain. Their main disadvantage is the occurrence of side effects. Tolerance and addiction are associated with prolonged use of opioids. Tolerance is because of adaptive changes in multiple neural systems following prolonged use of opioids. These changes include functional uncoupling between

opioid receptors and their effectors, leading to adaptations in many intracellular messenger pathways (Price, 1999).

The analgesic effects of opioids are mediated through opioid receptor binding (mu, kappa and delta). The μ, δ, or κ receptors are found on inhibitory interneurons in the CNS (Carlson *et al.*, 2004). Opioid drugs cause analgesia by reducing neuronal excitability and by influencing the release of neurotransmitters. These effects are mediated through the inhibition of voltage sensitive calcium channels and the increase of potassium conductance (Carlson *et al.*, 2004).

2.5.2 Non-Narcotic Analgesic Drugs

Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutic agents in pain management (Sethna, 1999). These drugs are used as first line analgesic agents. Unlike narcotic analgesics, NSAIDs are not characterized by tolerance, physical and psychological dependence. They also have good antipyretic and antihyperalgesic activity. However, they also have some side effects, like gastrointestinal irritation, and the analgesia induced is limited by the ceiling effect (Sethna, 1999).

NSAIDs exert their effects through inhibition of cyclooxygenase enzyme. Cyclooxygenase catalyses the formation of prostaglandins, prostacyclin and thromboxane by converting arachidonic acid to prostaglandin-H_2 (PGH_2), the precursor of the series-2 prostanoids. The prostaglandins are not tissue irritants, but enhance pain and inflammation by inducing the release of mediators of inflammation (Vane and Botting 1987). NSAIDs are classified into cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2) inhibitors based on enzyme cyclooxygenase (COX) isomer they preferentially inhibit. Most COX-1 act peripherally as non-selective inhibitors of the enzyme cyclooxygenase, inhibiting both the COX-1 and COX-2 isoenzymes. COX-2 inhibitors mediate their actions through selective inhibition of cyclooxygenase-2 isoenzyme (Deeks *et al.*, 2002). The central antihyperalgesic effect of NSAIDs is also due to the inhibition of spinal cyclooxygenase activity (Malmberg and Yaksh, 1992). Flunixin and possibly other NSAIDs may also reduce pain through centrally mediated

mechanisms involving alpha-2 adrenoceptors and mu-opioids receptors (Chambers *et al.*, 1995).

Long-term use of NSAIDs usually results in side effects in the patient. Most of the side effects are due to cyclooxygesae-1 inhibition. The use of COX-2 inhibitors augments some of those side effects (Deeks *et al.*, 2002). The effects of NSAIDs as analgesics in reptiles has not been fully established (Hernandez-Divers, 2006, Maticic *et al.*, 2007).

Steroidal anti-inflammatory drugs are also widely used in the management of painful inflammatory conditions. The mechanisms of action of this group of drugs are through the inhibition of phospholipase A_2. This leads to a decreased production of the inflammatory mediators.

2.5.3 Transcutaneous Electrical Nerve Stimulation (TENS) and Acupuncture

Transcutaneous electrical nerve stimulation (TENS) is a non-invasive pain relief method involving electrical stimulation to the skin, at various frequencies, intensities and pulse durations (Wright and Sluka, 2001). These frequencies can be broadly classified as high frequency (>50 Hz), low frequency (<10 Hz), or burst TENS (bursts of high-intensity stimulation administered at a much lower frequency) (Wright and Sluka, 2001; Sluka and Walsh, 2003). High frequency TENS can inhibit both primary and secondary hyperalgesia whereas low frequency TENS only inhibits secondary hyperalgesia (King and Sluka, 2001).

Two different theories explaining the mechanisms of action of TENS in pain relief have been proposed (Sluka and Chandran, 2002; Han, 2003; Han, 2004). The principle behind high-frequency TENS is the gate control theory of pain, where stimulation of large-diameter afferent fibers inhibits the transmission of nociceptive impulses carried by small-diameter fibers from reaching higher brain centers (Han, 2004). The other probable mechanism is by release of endogenous opioids and other neurotransmitters that inhibit nociceptive transmission. This opioid-mediated inhibition could be segmental or supraspinal. Both high- and low-frequency TENS reduce dorsal horn neuron responsiveness to noxious stimuli in normal animals (Sluka and Chandran, 2002; Han, 2003). Peripheral alpha-2 adrenoceptors also

contribute, in part, to TENS antihyperalgesia (King, *et al.,* 2005). The analgesic efficacy of TENS is however controversial (Brosseau, *et al.*, 2002).

Acupuncture involves the insertion of needles into designated acupuncture points, and subsequently the needles are manually manipulated or electrical current is administered via the needles. When electrical current is administered via the needles, it is similar to TENS in terms of parameters and mechanisms, and it is more effective than manual acupuncture. Like TENS, the acupuncture analgesia is the result of physiological and neuropharmacological processes induced by afferent inputs excited by acupuncture (Wright and Sluka, 2001; Lundeberg and Stener-Victorin, 2002; Han, 2003; Han, 2004; Kawakita and Okada, 2006).

Acupuncture excites receptors or nerve fibers in the stimulated tissue, which are also physiologically activated by strong muscle contractions, and the effects on certain organ functions are similar to those obtained by protracted exercise. Acupuncture produces rhythmic discharges in nerve fibers, and causes the release of endogenous neurotransmitters including opioids, monoamines, oxytocin and other neuropeptides (SP, CGRP) important in the control of sensory, affective and cognitive elements of pain (Lundeberg and Stener-Victorin, 2002; Han. 2003, 2004).

2.5.4 Nerve Blockade

A variety of neural blockade techniques have been used to provide effective and safe analgesia. These include local infiltration of anesthetics, epidural block or nerve sectioning. These techniques are particularly of importance in the relieve of severe and relentless chronic pain, often associated with reflex dystrophy, causalgias and neuropathic pain (Capdevila, *et al.*, 1999; Price, 1999; Marchettini *et al.,* 2000). Nerve blockade can be used as a diagnostic and/or prognostic measure. Diagnostic blocks are performed to obtain information on the mechanism of pain in the individual and to determine the pain pathways. Prognostic blocks are used to predict the effects of neurosurgical section and thus facilitate proper selection of patients.

Therapeutic blocks produce analgesia by interrupting pain pathways and abnormal reflex phenomena and by producing vasodilatation. One of the nerve block techniques is the injection or infiltration of local anesthetic or neurolytic agents into the affected part of the

body. This method of treatment has been applied to treat cancer pain, arthritis, musculoskeletal pain and neuralgias. The local anesthetic can be applied into incision sites, nerve or nerve plexuses, or into neuraxis (Capdevila, *et al.*, 1999; Price, 1999; Marchettini *et al.,* 2000).

Another neurological procedure involves the sectioning of nerves. Sectioning of both sensory and motor nerves provides numbness in the affected area. Sympathetic nerves are involved pain transmission. Sympathetic ganglia contain nerves that transmit nociceptive information to various parts of the body, including to the heart. Blockade of the afferent outflow will result in pain relief, reduction in stress and anxiety. Several varieties of sympatholytic procedures are available for pain relief (Capdevila, *et al.*, 1999; Price, 1999; Marchettini *et al.,* 2000).

Other neural blockade techniques that have been used to relieve pain include posterior rhizotomy, chordotomy and bulbar tractotomy, lobotomy and thalamotomy. These techniques, together with peripheral nerve blocks, are useful in relieving intense pain provoked by deep breathing, coughing or moving a body part affected by the surgery. Although nerve blocks are useful in relieving pain, they are in most cases accompanied by adverse side effects. Peripheral nerve block may cause permanent nerve injury either by direct traumatic needle contact or through nerve compression. However, sensory motor deficits may result from nerve injuries (Marchettini *et al.,* 2000). Moreover, sympathetic blocks may result in adverse side effects such as impotence, orthostatic hypotension and paraplegia.

2.5.5 Thermotherapy

Thermal modalities (heat or cold) have also been used to treat painful conditions. The mechanism of this form of analgesia is not clear but a vasodilatory hypothesis has been suggested (Sluka *et al.,* 1999). Heat induces some vasodilatory effects, which lead to an increase in the removal of waste products and increased oxygen supply in the tissues. There is also an increase in the removal of inflammatory compounds, which are known to activate and sensitize nociceptive afferents. The use of cold/cryoanalgesia works by decreasing skin temperature and blood flow. This results in slowing of peripheral nerve conduction velocity and therefore, fewer signals reach the CNS (Sluka *et al.,* 1999). The efficacy of thermotherapy in pain treatment is however questionable (Robinson, *et al.*, 2002).

2.5.6 Magnetic Therapy

Magnetic therapy or magnotherapy is a form of alternative medicine involving static magnetic fields for pain management. Static magnetic fields are produced by permanent magnets, positioned at the vicinity of the painful body regions (Pittler *et al.*, 2007). The common form of application is by use of pulsed electromagnetic therapy (PEMT), which has been widely used to counteract pain resulting from various inflammatory conditions (Pipetone and Scott, 2001; Pittler *et al.*, 2007). However, the optimal modes of magnetic field administration and efficacy remain intensely controversial (Pipetone and Scott, 2001; Brown *et al.*, 2002). In addition, the mode of action of magnetic pulses in pain alleviation is not clearly understood (Winemiller, 2003).

Magnets produce energy in the form of magnetic fields. Two main types of magnets exist: static or permanent magnets, in which the magnetic field is generated by the spin of electrons within the material itself, and electromagnets, in which a magnetic field is generated when an electric current is applied. Most magnets that are marketed to consumers for health purposes are static magnets of various strengths, typically between 30 and 500 mT. Magnets have been incorporated into arm and leg wraps, mattress pads, necklaces, shoe inserts and bracelets (Pittler *et al.*, 2007). However, evidence for the scientific principles or biological mechanisms to support such claims is limited. According to one proposed mechanism, nociceptive C-fibers have a lower threshold potential, and magnetic fields selectively attenuate neuronal depolarization by shifting the membrane resting potential (Lednev, 1991). Another theory suggests that magnetic fields promote an increase in blood flow through the skin and the subcutaneous and muscular tissues, which reduces the pain (Trock, 2000)

2.5.7 Capsaicin

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) is the active component of chili peppers, which are plants belonging to the genus *Capsicum*. Together with its related compounds, the capsaicinoids, capsaicin causes irritation and produces a burning sensation in most mammals and subsequently an inactivation of the sensory neurons (Park *et al.*, 2008). Capsaicin is currently used in topical ointments for relieve of various forms of chronic pain including post-

herpetic neuralgia, trigeminal neuralgia, diabetic neuropathy, osteoarthritis and cluster headaches (Kim *et al.*, 2008).

Capsaicin acts through its specific receptor, the transient receptor potential vanilloid 1 (TRPV1) on the polymodal nociceptors, the majority of which contain neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP) (Benham *et al.*, 2003; Tominaga and Caterina, 2004; Kim *et al.*, 2008). Repeated administration of capsaicin produces a desensitization effect due to the depletion of the neurotransmitters and inactivation of sensory neurons (Kim *et al.*, 2008)

2.5.8 Other Substances/Methods Used for Pain Treatment

Various other substances/methods have been used to treat pain. These include anticonvulsants, antidepressants, NMDA-antagonists, capsaicin and some physical therapy methods like exercise and stress. Exercise-induced analgesia is partly due to an increase in beta-endorphin levels (Sluka and Walsh, 2003). Stress-induced analgesia may also be mediated via endogenous opioids (Andrea *et al.*, 2005; Kurrikoff *et al.*, 2008). Stress induced analgesia may be caused by stressors such as surgery, anticipation of pain, chronic pain, chronic stressful states, labor and childbirth (Price *et al.*, 2000).

Anticonvulsants such as carbamazepine have been reported to produce effective analgesia especially for neuropathic pain syndromes (Dickenson and Chapman, 2000; Kim *et al.*, 2008). Similarly, antidepressants and NMDA- receptor antagonists relieve both acute and chronic pain syndromes.

2.6 HYPOTHESES

It was hypothesized that:

i. The formalin-, hot plate-, capsaicin instillation- and acetic acid instillation tests can be applied in testudines and used to test the antinociceptive effects of commonly used analgesic drugs.

ii. The proportion of nociceptive afferents in the sensory branches of the trigeminal nerve is low in testudines.

2.7 RESEARCH OBJECTIVES

The general objective of this study was to explore the nociceptive system of testudines. The specific objectives of this study were:

i. To study nociception in the speke-hinged tortoise and the marsh terrapin, using the formalin, hot plate, capsaicin instillation and acetic acid tests or their modifications.

ii. To determine the antinociceptive effects of morphine, pethidine, ASA, flunixin, dexamethasone and hydrocortisone using the formalin and the hot plate tests.

iii. To estimate the fiber proportions in the sensory branches of the trigeminal nerve.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 ANIMALS

Thirty-seven adult speke-hinged tortoises (20 females and 17 males) and twenty-nine marsh terrapins (14 females and 14 males) were used in the behavioral nociceptive tests. They were sourced from Machakos and Makueni districts respectively, about 60-80 Km South-East of Nairobi, Kenya, from a professional licensed animal dealer. The animals were transported in well-ventilated cages by road to the Department of Veterinary Anatomy and Physiology, University of Nairobi where the investigations were carried out. Body weight, sex and plastron length of each animal was recorded. For identification purposes, the animals were marked on the carapace with specific numerals using a marker pen.

The animals were housed in a well-ventilated room, with translucent windows. Tortoises were kept in metallic cages, measuring 1.25 × 0.9 × 0.6 M. The cages were filled with sand and stones up to a depth of 30 cm. Each cage had at most 20 tortoises. Cabbages, carrots, tomatoes and kikuyu grass (*Pennisetum cladestinum)* were fed to the animals twice a week. The animals were provided with as much feed as they could consume, and the remains removed after six hours of feeding. Fecal debri were also removed. Drinking water was provided in small dishes, at two points in each tank. The dishes were cleaned after every other day. The animals were bathed in a basin filled with ten liters of water at least once a week.

Terrapins were kept in two tanks, similar in size to those used for the tortoises. The tanks had sand covering a depth of 30 cm and a centrally located plastic basin. The basin was filled with 20 liters of tap water and stones placed on one side to allow for easy entry and exit. They were fed on sliced raw meat and minced meat, which was placed in a separate basin half-filled with water. The animals were allowed to feed for approximately six hours, at which time there was no observable feeding behavior. Feeding was done at least once a week. The water basins were cleaned twice a week and refilled with clean water.

The health status of the animals was monitored using general signs like change in behavior and physical examination of the skin, mucous membranes and body excrements. Both tortoises and terrapins were habituated to the laboratory for at least one month before the start of experiments. During this period, they were handled daily. Occasionally, the animals were placed in wire mesh cages measuring 60 × 40 × 27 cm, that were eventually used as observation cages.

3.2 NOCICEPTIVE TESTING

Four nociceptive tests were used. These were the formalin, hot plate, capsaicin instillation and acetic acid eye instillation tests. Before experimentation, tortoises were brushed with a soft brush to remove soil and sand. Terrapins in the sand burrows were retrieved, brushed and put in a shallow water basin for five minutes. They were transferred to wire-mesh cages for a further 60 minutes to facilitate arousal and drying of the skin.

A randomized block design was used based on the differential body weights of the animals. Based on preliminary studies, animal reuse was restricted to at least four weeks, and this did not seem to affect the behavioral pain score nor the health of the animals. Blinding was done by a separate person and the observer was not aware of what had been injected into the animal.The experiments were always performed at a room temperature of 25-28 ^{o}C, and between 9 a.m. and 4 p.m. The experimental room used had minimal disturbance. The animals were timid and shy, and to obtain unambiguous results, a quiet environment was necessary. All the experimental procedures were carried out under controlled laboratory conditions, in accordance with the guidelines the set forth by the American Physiological society (2002). During the entire experimental period, the animals remained in good health and even gained weight.

3.2.1 The Formalin Test

12.5% formalin was prepared from a stock solution of 100% formalin (w\w), which contains 37% formaldehyde in water and a stabilizer (10% methanol) in saline. The animals were gently lifted up, and using a micro-liter syringe and a 26-gauge needle, 100 µl of 12.5% formalin was injected subcutaneously into the inter-claw space of the hind limb. The choice of

volume and concentration used was based on preliminary experiments. In the control group, 100µl of saline (0.9% NaCl in water) was injected in a similar manner to that of formalin. Immediately after the formalin or saline injection, the animal was placed in the observation chamber and scoring started immediately. The total time spent in the behavior full limb retraction and/or partial limb usage was measured over a 30-minute observation period.

3.2.2 The Hot Plate Test

Pain was induced using an IITC Inc. Model 35D Analgesia meter. The copper plate (27 × 29 cm), which was always kept clean, was enclosed by 30 × 30 × 30 cm lidded Perspex box. A digital thermometer (Termoektro, a/s, Type: 2105; Serial no. 1296; Sensor: cu-cuNi; Range ¯ 100/+400) was used to determine the hot plate surface temperature. The temperature of the hot plate was set at 60 °C. Lower temperatures did not induce clearly quantifiable nocifensive responses.

The animal was placed in an acclimation chamber (30 × 30 × 30 cm lidded Perspex box) for five minutes before being put on the hot plate. This ensured that the animals did not retract their limbs into the shell once placed on the hot plate. However, animals that retracted the limbs on placement to the hot plate were removed and the experiment discontinued. Animals that did not show any nocifensive responses by the fifth minute were removed from the hot plate to avoid tissue injury. Testing was done three times in each animal at intervals of 60 minutes and the mean response latency recorded.

3.2.3 The Capsaicin Instillation Test

Capsaicin (98%) (Sigma, U.S.A.) was used to make a 1% stock solution of capsaicin using a vehicle (10% ethanol, 10% tween 80 and 80% of saline). Further dilutions were made using saline. Two drops of capsaicin (10^{-3} mg/ml) was instilled directly into the cornea. The control animals were instilled with the capsaicin vehicle. The eye-protective responses (blinking, wiping, rubbing and head shaking) were recorded. The animals were restrained by a suspension technique, whereby a fine rope was closely tied around the shell, and fixed onto a tripod stand (Plate iii). The animal remained positioned on the stand, in front of the experimenter. This method of restraint provided for easier scoring since testudines are very

timid and resort to head and limb retraction when molested, and the animals showed no apparent discomfort. The animals were acclimatized to this form of restraint for at least four weeks. The duration of eye closure was measured in blocks of 5 minutes for 30 minutes.

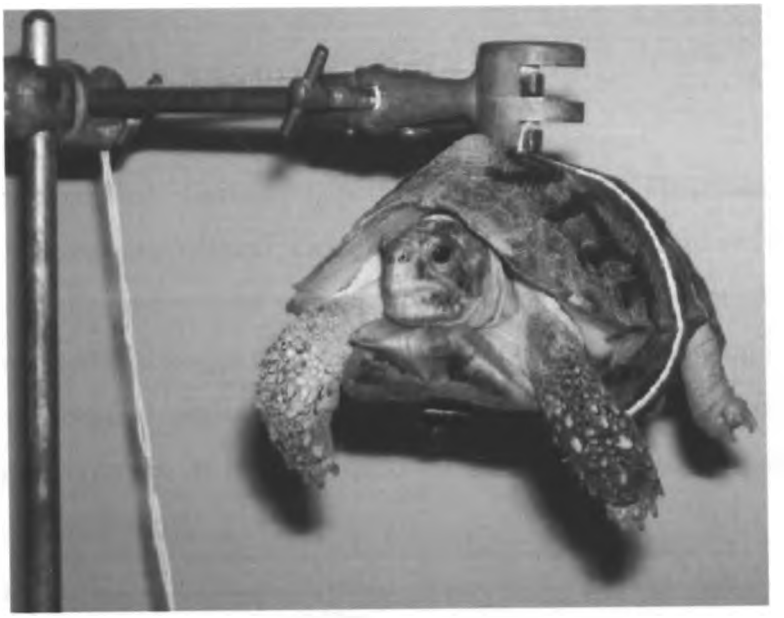

Plate iii: A suspended tortoise demonstrating the 'suspended animal technique'.

3.2.4 The Acetic Acid instillation test

Glacial acetic acid (98%) was diluted into a concentration of 10% using distilled water. Intra-ceolomic acetic acid injection did not produce any quantifiable pain responses and ocular instillation of the same material was used in this test. Two drops of 10% acetic acid were instilled into the eye of a suspended animal and the ensuing nocifensive behaviors scored in five-minute intervals for 30 minutes. The duration of eye closure was measured.

3.3 ANTINOCICEPTIVE TESTING

3.3.1 Effects of Opioids

Morphine (Sigma International, Germany), pethidine (Martindale Pharmaceuticals, Essex, U.K.) and naloxone (Sigma International, Germany) were used. Morphine and pethidine were diluted in saline; while naloxone powder was dissolved in saline to form a stock solution of 25 mg/ml. Morphine was used in dosages of 5, 7.5, 10 or 20 (mg/kg) intracoelomically (ICo), 30 minutes before the nociceptive testing. Pethidine was used in the dosages of 10, 20 or 50 (mg/kg). In the control animals, an equal volume of saline was injected intracoelomic, 30 minutes before nociceptive testing.

To investigate whether the antinociceptive effects of morphine and pethidine were reversible by opioid receptor blockade, naloxone was co-administered with morphine or pethidine. Naloxone was administered at dose rates of 2.5 or 5 mg/kg, while morphine and pethidine were administered at dosages of 10 and 50 mg/kg respectively. The drugs were administered 30 minutes before the nociceptive testing.

3.3.2 Effects of Non-Steroidal and Steroidal Anti-inflammatory Drugs

Lysine acetylsalicylic acid (ASA) (Caplin Point Laboratories, Madras, India) and flunixin (Sigma International, Germany) were the two NSAIDs used in the study. ASA was diluted in saline. Flunixin powder was dissolved in saline to form a stock solution of 25 mg/ml, and further dilution made using saline. ASA was used at dose rates of 100 or 200 mg/kg while those of flunixin were 10, 50 or 100 mg/kg. The drugs were injected ICo, 30 minutes before the nociceptive testing. The control animals were injected with an equal volume of saline, ICo.

For steroidal anti-inflammatory drugs, dexamethasone (Martindale Pharmaceuticals, Essex, U.K.) and hydrocortisone sodium succinate (Sinochem Mingbo ltd., P. R. China) were diluted in saline. Dexamethasone at 10, 20 or 50 mg/kg and hydrocortisone at 100 or 200 mg/kg were injected ICo, 30 minutes before the nociceptive testing. The control animals were injected with an equal volume of saline, ICo.

3.4 FIBER PROPORTIONS IN THE SENSORY BRANCHES OF THE TRIGEMINAL NERVE OF TESTUDINES

3.4.1 Tissue Processing

Five speke-hinged tortoises and five marsh terrapins were used in the histological experiments. The animals were anesthetized with 600 mg sodium-pentobarbitone, intracoelomicaly (Euthanase®, Centaur labs) (Gartrell and Kirk, 2005). The anesthetic depth was assessed using the paw and corneal reflex, and deep anesthesia occurred after 30-45 minutes. The animal was put on dorsal recumbency and the heart exposed by removing the plastron, using a bone cutter. The carapacio-plastral junction was cut on both sides and the soft tissues freed from the plastron using a scalpel blade. The animals were intracardially perfused with 250 ml of heparinised saline (2,500 I.U) for about 30 minutes, to remove blood. This was followed by intra-cardial perfusion with 250 ml of fixative solution containing 2.5% glutaldehyde in 0.1M phosphate buffer. After detaching the head, the muscles in the head were carefully removed and the braincase removed dorso-laterally. The trigeminal nerve and ganglia were exposed by elevating the brain, and the preparation put in the same fixative solution for at least four hours. About 2 mm long nerve sections of the ophthalmic and maxillary nerve branches were cut, and left overnight in 0.01M phosphate buffer, at 4 °C.

Approximately 15 hours later, the nerve sections were placed in 1% osmium tetroxide in 0.1M phosphate buffer for 4 hours, at room temperature. After washing with saline, the tissues were dehydrated with ascending concentrations of ethanol, cleared with propylene oxide, infiltrated and embedded in epoxy-resin mixture. The blocks containing the nerves were trimmed and 1-µm thick sections cut using a microtome. The sections were mounted on glass slides, stained with 1% Toluidine blue, and viewed at a magnification of ×1000 using a light microscope (Feirabend *et al.*, 1998).

3.4.2 Fiber Calibration and Estimation of the Fiber Proportions

The tissue slides were projected on a televised screen, which enlarged the images by a further ×1.8. A 5 cm long calibrated polythene tape was used to measure the nerve diameters (Fig. 16 a). One division was equivalent to 1.8 μm in length. Sampling of the counting fields was done using the forbidden line rule with a counting frame (Fig. 16 b). Five fields were randomly sampled in each fiber specimen and counting done on six square grids. Since there were no myelinated fibers, the axons were categorized into three groups based on vertebrate fiber types (Sneddon, 2002, 2004). Basically, the fibers were categorized into small-, medium- and large-diameter fibers, with diameter ranges of 0.5-5.5 μm (A-δ range), 5.6-10 μm (A-beta range) and 10-26 μm (A-alpha range) respectively.

3.5 STATISTICAL ANALYSIS

All values were expressed as mean ± standard error of mean (S.E.M.). The data was analyzed with one-way ANOVA and two-sided Dunnett's post hoc test using SPSS version 12.0.1. In the estimation of sensory fiber composition, the sample proportions were analyzed for percentage of each fiber type, and sample proportions compared using the Least Significant Difference (LSD) subsequent to ANOVA. P-values lower than 0.05 were considered significant.

CHAPTER FOUR

4.0 RESULTS

The mean weight of the tortoises was 570.19 ± 35.05 g, with a range of 212-1030 g. They had a mean plastron length of 13.25 ± 0.3 cm (range 10-170). Terrapins had a mean weight of 361.9 ± 40.96g (70-1000 g), and had mean plastron length of 11.33 ± 0.41 cm (6.7-15.1 cm).

4.1 NOCICEPTION IN THE SPEKE-HINGED TORTOISE AND THE MARSH TERRAPIN

4.1.1 The Formalin Test

The formalin injection immediately induced behavioral pain responses, in both animal species. These were 'complete limb retraction' and 'carefully using the limb'. In the behavior 'complete limb retraction', the animals completely retracted the whole limb from the surface of observation cage, and walked using three legs. In the behavior 'carefully using the limb,' the animals walked by a limping gait, with the injected limb bearing less weight. Occasionally, some animals became quiescent, whereby they retracted the head and all the limbs. In such cases, the experiments were discontinued. Other notable behaviours displayed by the animals were frequent urination, defecation and hypermotility. In the Speke-hinged tortoise, the mean time spent in nocifensive behavior after subcutaneous injection of formalin was 8.3 ± 1 minutes, while that of the controls was 0.69 ± 0.3 minutes. In the formalin injected group, the time spent in nocifensive behavior was significantly greater than that of the saline injected group ($P < 0.001$, Fig. 1a).

In the marsh terrapin, the time spent in nocifensive behavior was 9.4 ± 2.4 minutes, while that of the controls was 0.49 ± 0.3 minutes. As in the speke-hinged tortoise, the time spent in nocifensive behavior after formalin injection was statistically significant ($P < 0.001$, Fig. 1b).

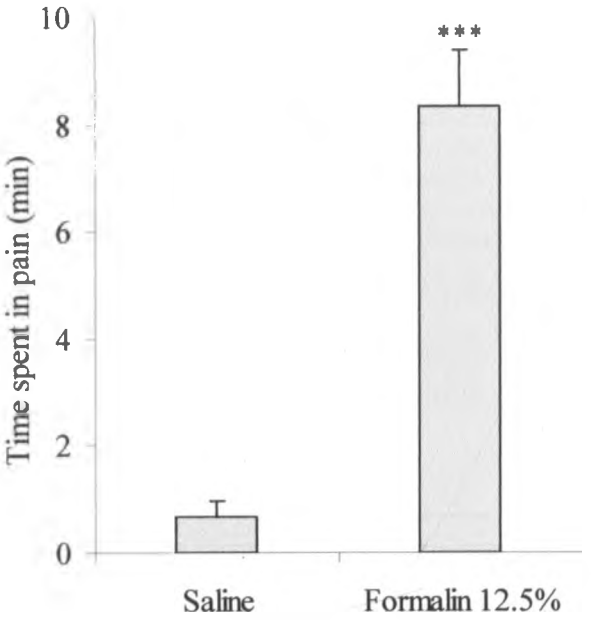

Fig. 1a: Effects of subcutaneous injection of saline or formalin 12.5% in the Speke-hinged tortoise (n = 16 in the formalin treated group and 7 in the control group). Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. *** denotes $P < 0.001$.

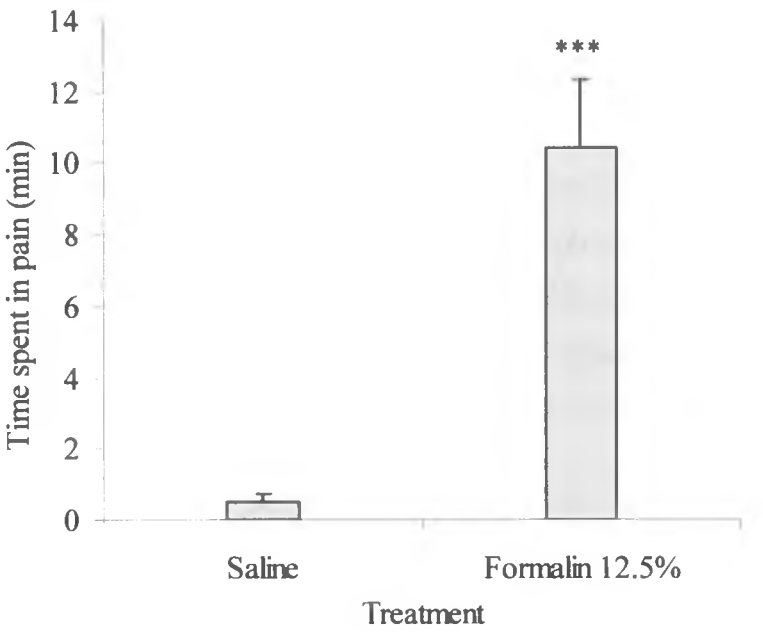

Fig. 1b: Effects of subcutaneous injection of saline or formalin injection in the marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M and n = 7. *** denotes $P < 0.001$.

4.1.2 The Hot Plate Test

To establish the base line thermal response latencies, the animals were placed on a hot plate adjusted at different surface temperatures, and a hot plate surface temperature of 60 ^{o}C was chosen. Lower temperatures did not induce a clearly quantifiable behavioral response nor escape behaviuor. In the speke-hinged tortoise, the mean hot plate response latencies for the three tests were 46.14 ± 4.04, 56.57 ± 4.22 and 59.14 ± 3.30 seconds, respectively. The average of these three consecutive tests was calculated as the mean response latency for each animal. In this test, the mean response latency was 53.95 ± 3.53 seconds. The response latencies of the first, second and third testing were not statistically different from the mean response latencies ($P > 0.05$, Fig. 2a).

In the marsh terrapin, the hot plate response latencies after three tests in each animal were 35.17 ± 3.77, 36.33 ± 3.23 and 52.33 ± 8.66 seconds respectively. The mean response latency of the three consecutive tests was 41.28 ± 3.41 seconds. Although the response latency for the third test was slightly higher, there were no statistically significant differences between the response latencies for the first, second and third testing and those of the mean response latency ($P > 0.05$, Fig. 2b).

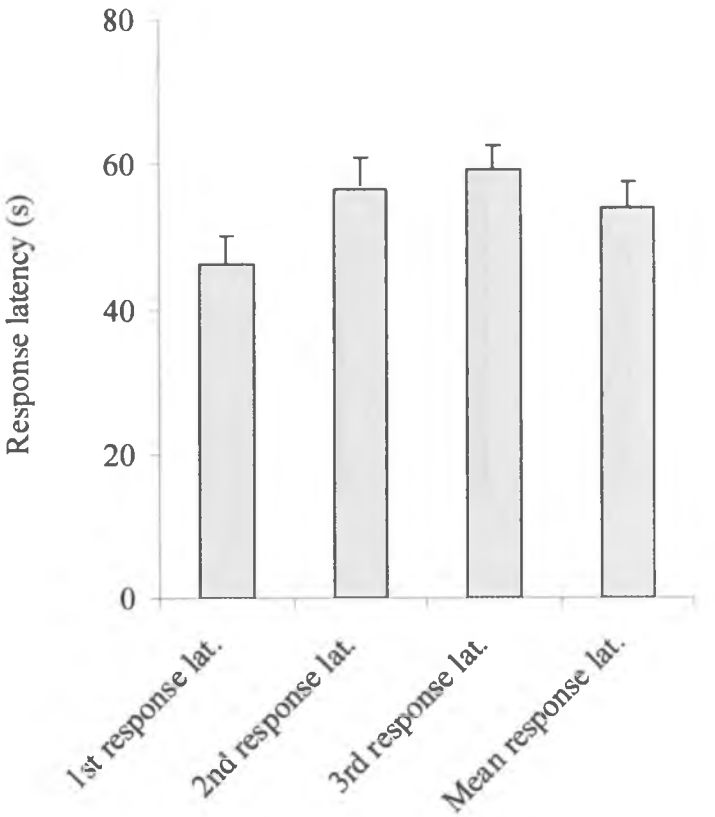

Fig. 2a: Hot Plate response latencies in the speke-hinged tortoise. Bars represent means ± S.E.M, n = 7, Lat. denotes latency.

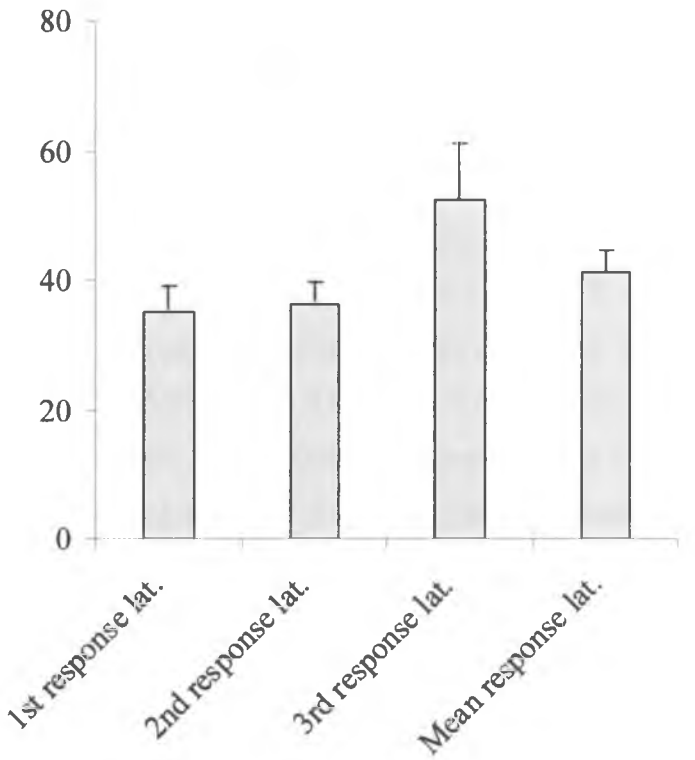

Fig. 2b: Hot Plate response latencies in the marsh terrapin. Bars represent means ± S.E.M., n = 6, Lat. denotes latency.

4.1.3 Capsaicin Instillation Test (In the Marsh Terrapin)

Instillation of two drops of capsaicin 10^{-3} g/ml in the marsh terrapin induced a number of eye protective responses such as blinking, blepharospasms, eye closure, rubbing, head shaking and sometimes head retraction. The most common nocifensive behavior was eye closure, which was scored in 5-minute intervals for 30 minutes. The total duration of eye closure in the 30-minute observation period was 625 seconds (Fig. 3a). In five-minute time blocks, the mean duration of eye closure was 240.67, 215.53, 81.62, 37.98, 15.89 and 23.41 seconds, respectively. In the control group, the duration of eye closure in 30 minute observation period in blocks of 5 minutes was 62.97, 10.80, 0.98, 0.61, 0.44 and 0.87 seconds respectively, with a cumulative total of 83 seconds. The effects of capsaicin were statistically significant in the first, second and third time blocks ($P < 0.05$, Fig. 3a). The method was however limited by retraction of the head into the shell.

In another set of experiments, the phenomenon of capsaicin desensitization was evaluated. Two drops of capsaicin 10^{-3} were instilled into the eye, daily for six consecutive days. The desensitizing effects of repeated capsaicin application were not observed ($P > 0.05$, Fig. 3b). The control group had lower pain scores over same period of treatment, with no statistically significant differences.

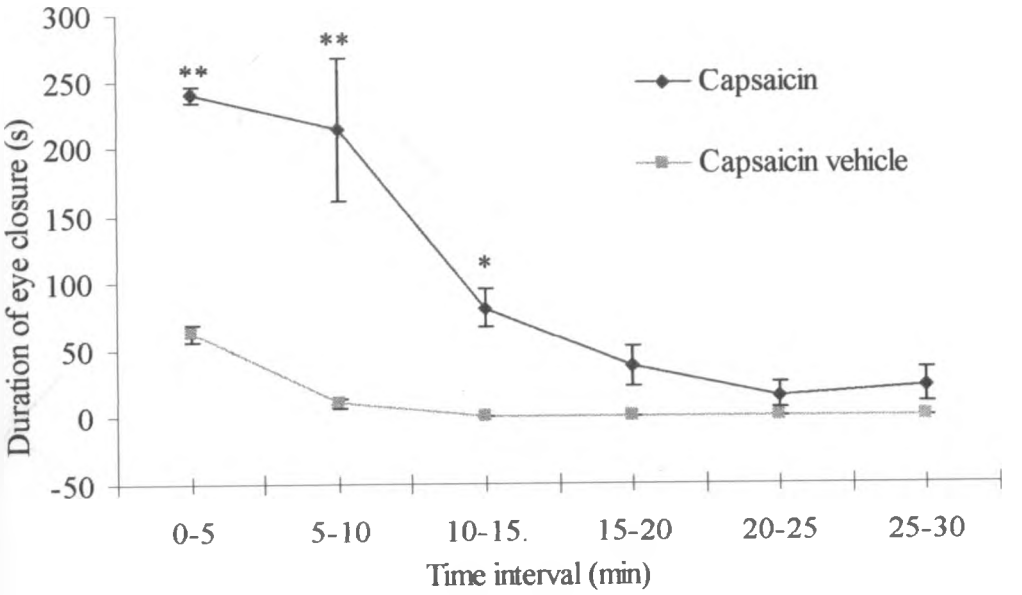

Fig. 3a: Time course of mean duration of eye closure, in blocks of 5-minutes, after capsaicin (10^{-3} g/ml) or vehicle instillation into the eye, in the marsh terrapin. Values are presented as means ± S.E.M. and n = 6-7. The duration of eye closure was statistically significant during the first 15 minutes. * and ** denotes $P < 0.05$ and $P < 0.01$ respectively.

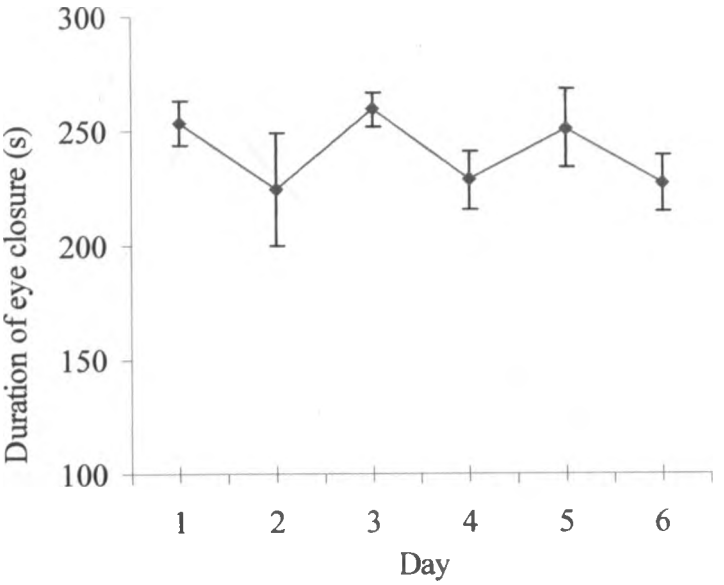

Fig. 3b: Mean duration of eye closure during the first 5-minutes following capsaicin 10^{-3} g/ml instillation in the marsh terrapin, for five consecutive days. No desensitization was induced by repeated capsaicin instillation. Values are presented as means ± S.E.M. and n = 7.

4.1.4 Acetic Acid Instillation Test

In the speke-hinged tortoise, the mean duration of eye closure in 5 minute blocks in the 30 minute observation period was 169.87 ± 17.0, 54.5 ± 22.4, 22.2 ± 14.0, 2.3 ± 0.8, 4.1 ± 2.2 and 5.9 ± 4.7 seconds respectively. In the control group, the mean durations of eye closure in 5-minute time blocks in the 30 minute observation period was 0.6 ± 0.3, 0.1 ± 0.1, 0.2 ± 0.1, 0.4 ± 0.2, 0.5 ± 0.4 and 0.3 ± 0.1 seconds respectively. The nocifensive responses were statistically significant in the first 10 minutes ($P < 0.05$, Fig. 4a). Like the casaicin instillation test, the method was limited by the retraction of the head into the shell.

In the marsh terrapin, the mean duration of eye closure in 5 minute intervals in the 30 minute observation period was 257.8 ± 10.9, 179.4 ± 8.2, 92.2 ± 25.1, 40.1 ± 29.4, 25.3 ± 21.6 and 3.0 ± 1.4 seconds respectively. In the control group, the respective mean durations of eye closure were 0.2 ± 0.1, 0.2 ± 0.1, 0.1 ± 0.1, 0.1 ± 0.1, 0.1 ± 0.1 and 0.2 ± 0.1. The effects of acetic acid were statistically significant in the first 15 minutes ($P < 0.05$, Fig. 4b).

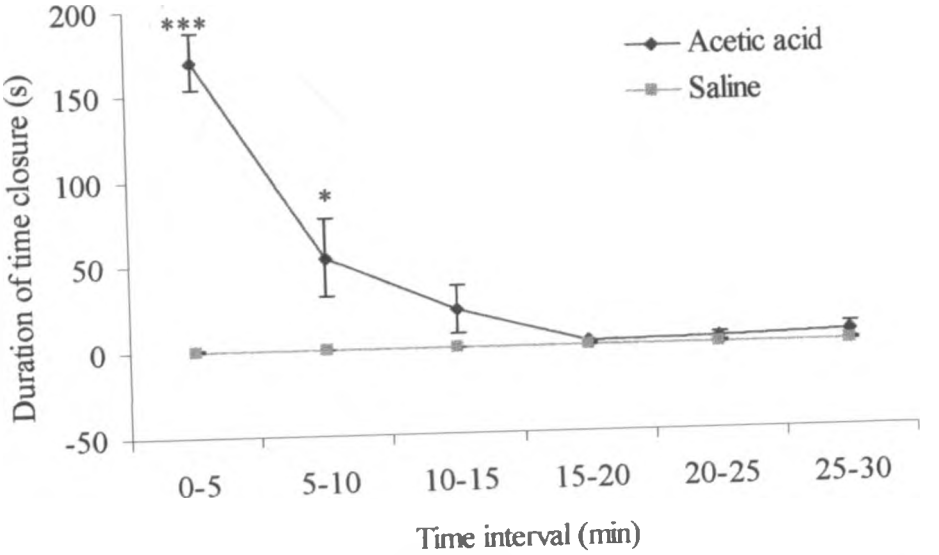

Fig. 4a: Time course of mean duration of eye closure, in blocks of 5 minutes, after acetic acid (10%) or saline instillation into the eye in the speke-hinged tortoise. Values are presented as means ± S.E.M. and n = 10. * and *** denotes $P < 0.05$ and $P < 0.001$ respectively.

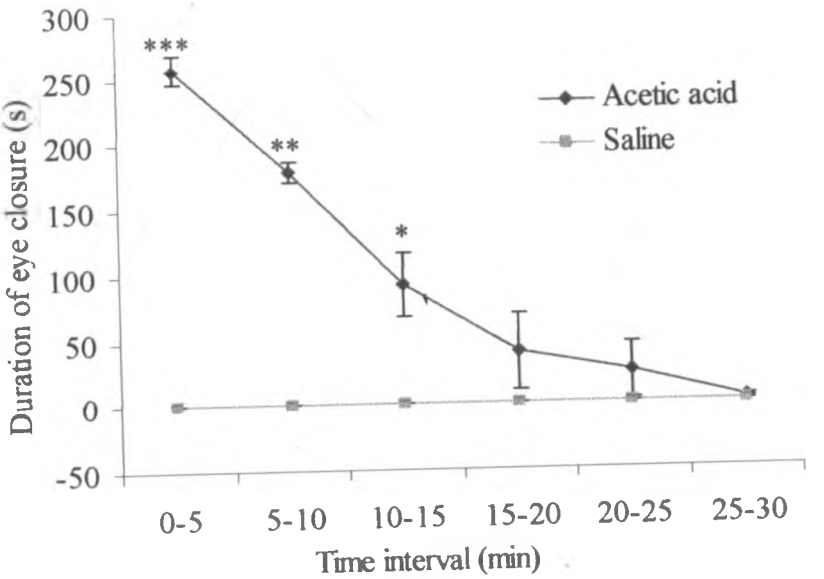

Fig. 4b: Time course of mean duration of eye closure, in blocks of 5 minutes, after acetic acid (10%) or saline instillation into the eye in the marsh terrapin. Values are presented as means ± S.E.M. and n = 10. *, ** and *** denotes $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively.

4.2 EFFECTS OF ANALGESIC DRUGS IN THE SPEKE-HINGED TORTOISE

4.2.1 The Formalin Test

4.2.1.1 Effects of Morphine, Pethidine and Naloxone

Morphine was used in dosages of 5, 7.5, 10 and 20 (mg/kg), based on preliminary studies, and the resultant times spent in nocifensive behavior were 14.63 ± 1.15, 9.83 ± 1.08, 6.72 ± 0.37 and 5.67 ± 0.33 minutes, respectively. In the control group, the time spent in nocifensive behavior was 13.09 ± 1.02 minutes. On multiple comparisons of the different treatment means, the effects of morphine 7.5, 10 and 20 (mg/kg) were statistically significant ($P < 0.05$, Fig. 5a). The effects of morphine 5 (mg/kg) were not statistically different from the control group ($P > 0.05$; Fig. 5a).

To test whether naloxone could reverse the effects of morphine, morphine 10 (mg/kg) was chosen. The mean times spent in nocifensive behavior after combined administration of morphine 10 mg/kg and saline, morphine 10 mg/kg and naloxone 2.5, and morphine 10 mg/kg and naloxone 5 mg/kg were 7.78 ± 0.81, 13.50 ± 1.43 and 17 ± 2.27 minutes, respectively. Naloxone at either dosage significantly inhibited the morphine-induced antinociception ($P < 0.05$, Fig. 5b).

Pethidine was administered in dosages of 10, 20 and 50 mg/kg, based on preliminary studies. The ensuing times spent in nocifensive behavior were 16.17 ± 0.70, 12.42 ± 1.11 and 11.67 ± 0.92 minutes, respectively. In the control group, nocifensive responses lasted 17.0 ± 1.81 minutes. The effects of pethidine 20 and 50 (mg/kg) were statistically significant ($P < 0.05$; Fig. 5c).

To test whether naloxone could reverse the effects of pethidine, a dosage of 50 (mg/kg) was chosen. The mean times spent in nocifensive behavior after combined administration of pethidine 50 (mg/kg) and saline, pethidine 50 and naloxone 2.5 mg/kg, and pethidine 50 and naloxone 5 mg/kg. The resultant times spent in nocifensive behavior were 11.72 ± 1.37, 12.33 ± 0.95 and 18.5 ± 1.65 minutes, respectively. The effects of naloxone 5 mg/kg were statistically significant on pethidine-induced antinociception ($P < 0.01$, Fig. 5d).

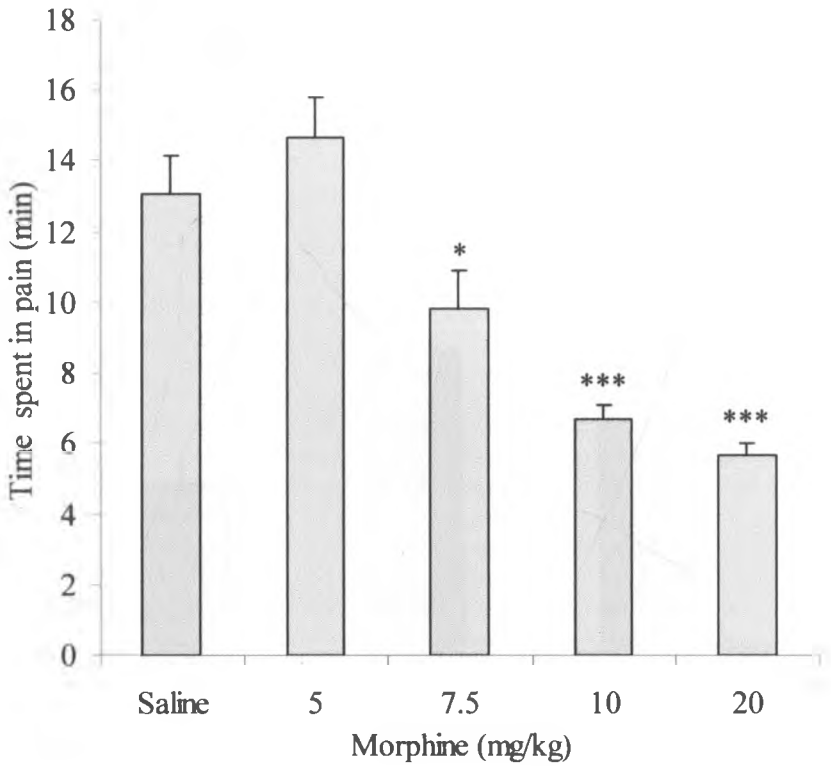

Fig. 5a: Effects of intracoelomic administration of saline or morphine (5, 7.5, 10 or 20 mg/kg), in the formalin test, in the speke-hinged tortoise. Bars represent means ± S.E.M., n = 7-9 in each group and treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. * and *** denotes $P < 0.05$ and $P < 0.001$ respectively.

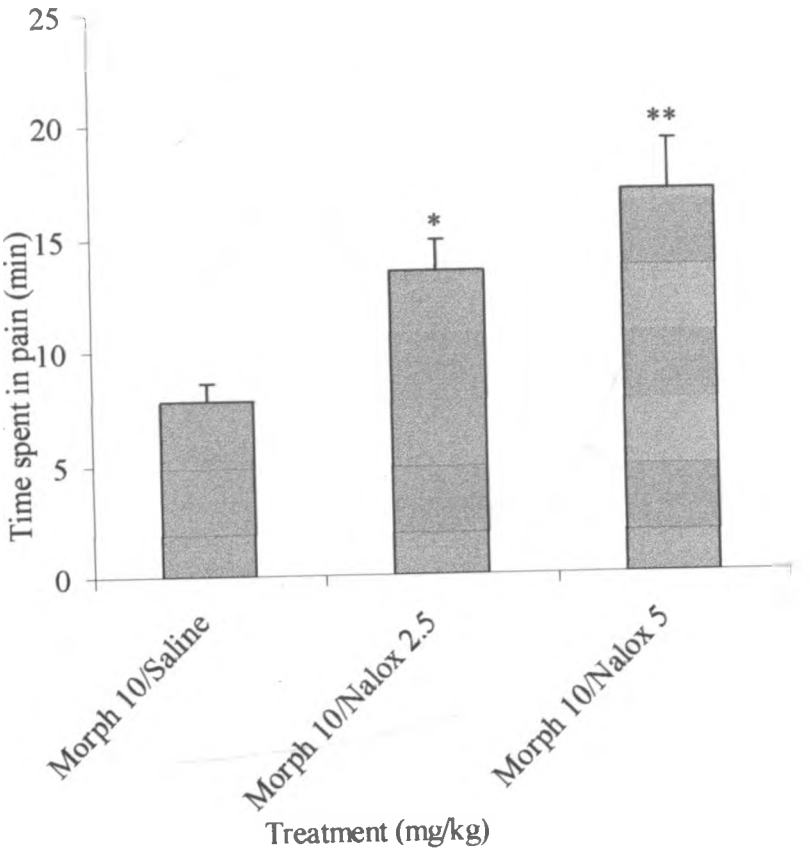

Fig. 5b: Effects intracoelomic co-administration of morphine and naloxone in formalin test in the speke-hinged tortoise. Treatment groups were morphine 10 mg/kg and saline (Morph 10/Saline), morphine 10 mg/kg and naloxone 2.5 mg/kg (Morph 10/Nalox 2.5), and morphine 10 with naloxone 5 mg/kg (Morph 10/Nalox 5). Bars represent means ± S.E.M. and n = 6-7. Treatment means were compared using the Dunnett's test (2-sided). * and ** denotes $P < 0.05$ and $P < 0.01$ respectively.

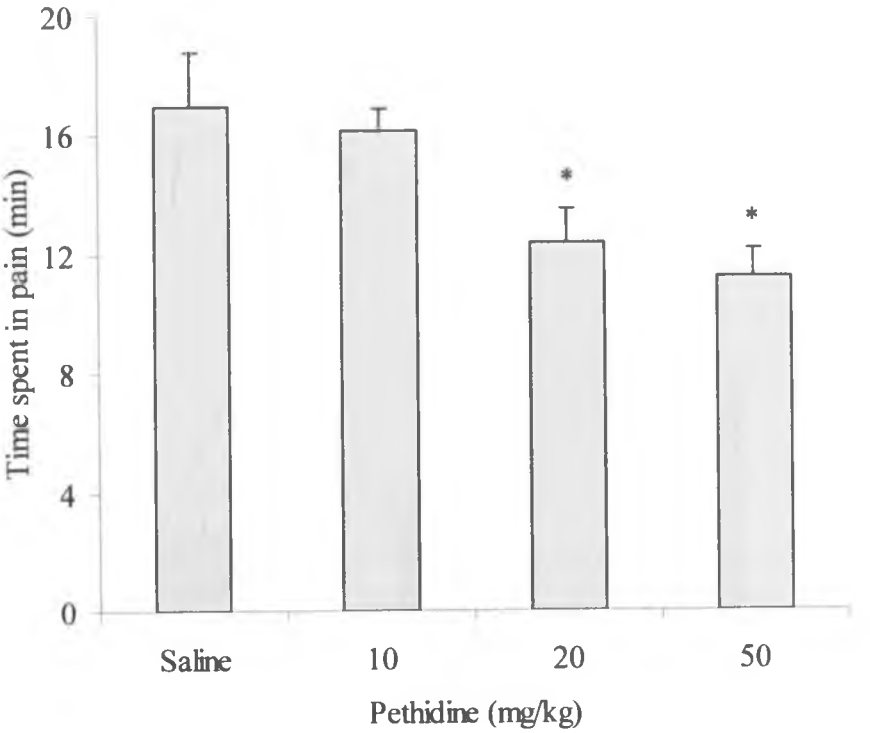

Fig. 5c: Effects of intracoelomic administration of saline or pethidine (10, 20 and 50 mg/kg) in the formalin test in the speke-hinged tortoise. Bars represent means ± S.E.M. and n = 6. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. * denotes $P < 0.05$.

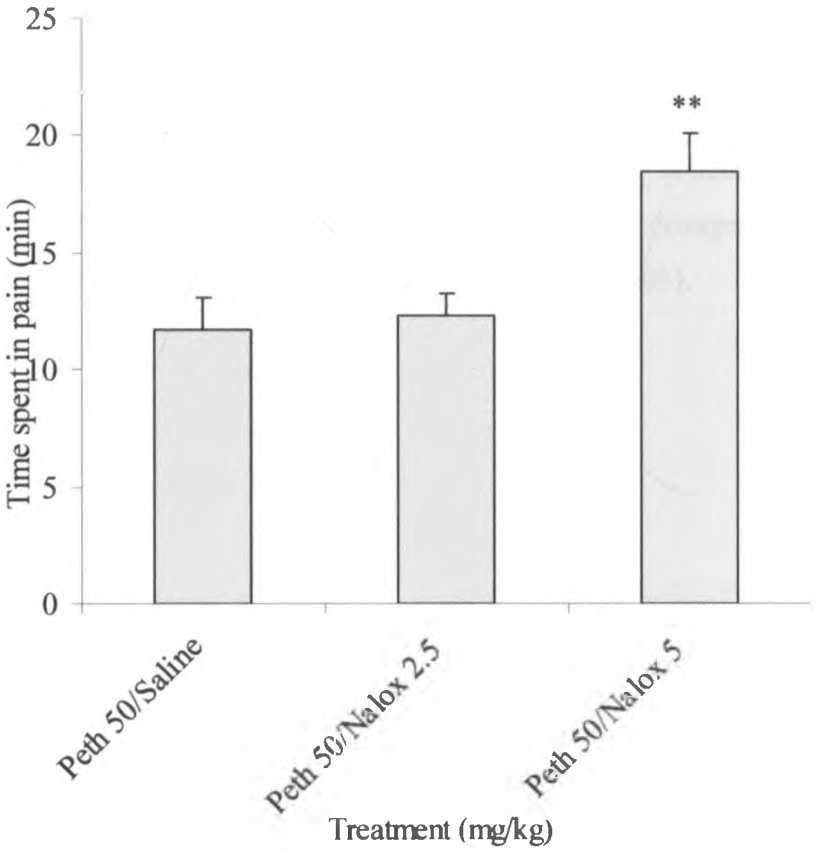

Fig. 5d: Effects of intracoelomic co-administration of pethidine and naloxone: pethidine 50 mg/kg and saline (Peth 50/Saline), pethidine 50 mg/kg and naloxone 2.5 mg/kg (Peth 50/Nalox 2.5), or pethidine 50 mg/kg and naloxone 5 mg/kg (Peth 50/Nalox 5) in the formalin test, in the speke-hinged tortoise. Bars represent means ± S.E.M. and n = 6-7. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. ** denotes $P < 0.01$.

4.2.1.2 Effects of ASA and Flunixin

ASA (100 or 200 mg/kg) did not cause any significant reduction in time spent in nocifensive behavior in the formalin test ($P > 0.05$, Fig. 6a). The mean time spent in nocifensive behavior after ICo administration of ASA 100 or 200 mg/kg was 18.67 ± 1.52 and 17.5 ± 1.65 minutes, respectively, while that of controls was 17.0 ± 1.81 minutes.

Flunixin was used in dosages of 50 or 100 mg/kg. The time spent in nocifensive behavior was 14.67 ± 1.6 and 15.67 ± 1.15 minutes, respectively. None of the dosages caused a significant decrease in the time spent in nocifensive behavior ($P > 0.05$, Fig. 6b).

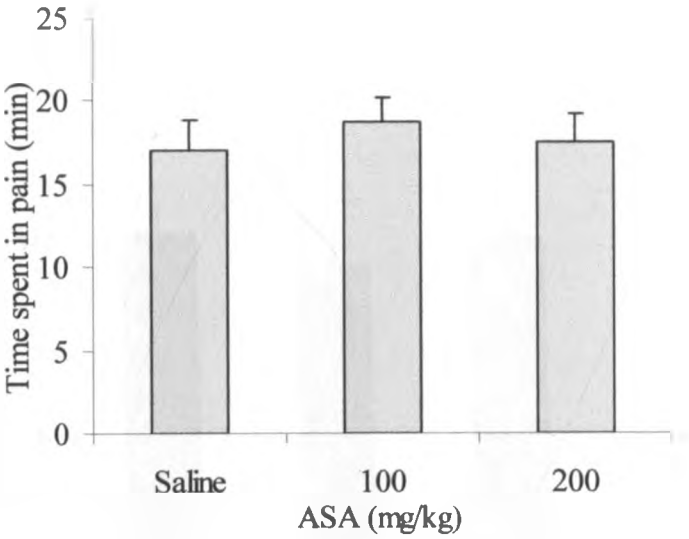

Fig. 6a: Effects of intracoelomic administered saline or ASA (100 or 200 mg/kg) on the mean time spent in nocifensive behavior in the formalin test in speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

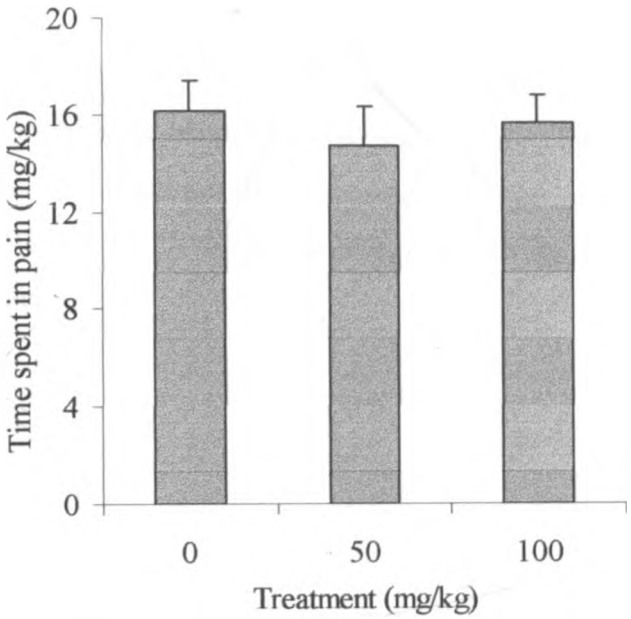

Fig. 6b: Effects of intracoelomic administration of saline or flunixin (50 or 100 mg/kg) in the formalin test in speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.2.1.3 Effects of Dexamethasone and Hydrocortisone

Dexamethasone 20 or 50 (mg/kg) did not cause any statistically significant decrease in time spent in nocifensive behavior in the speke-hinged tortoise ($P > 0.05$, Fig. 6c). The mean time spent in nocifensive behavior after ICo administration of dexamethasone 20 or 50 mg/kg was 17.33 ± 1.38 and 18.67 ± 1.8 minutes, respectively. In the control group, the time spent in pain was 15.5 ± 1.77 minutes.

Hydrocortisone (100 or 200 mg/kg) did not cause any statistically significant reduction in the time spent in nocifensive behavior in the speke-hinged tortoise ($P > 0.05$, Fig. 6d). The time spent in nocifensive behavior after ICo administration of hydrocortisone 100 or 200 mg/kg was 15.0 ± 1.95 and 15.17 ± 1.72 minutes, respectively, while that of the control group was 17.0 ± 1.81 minutes.

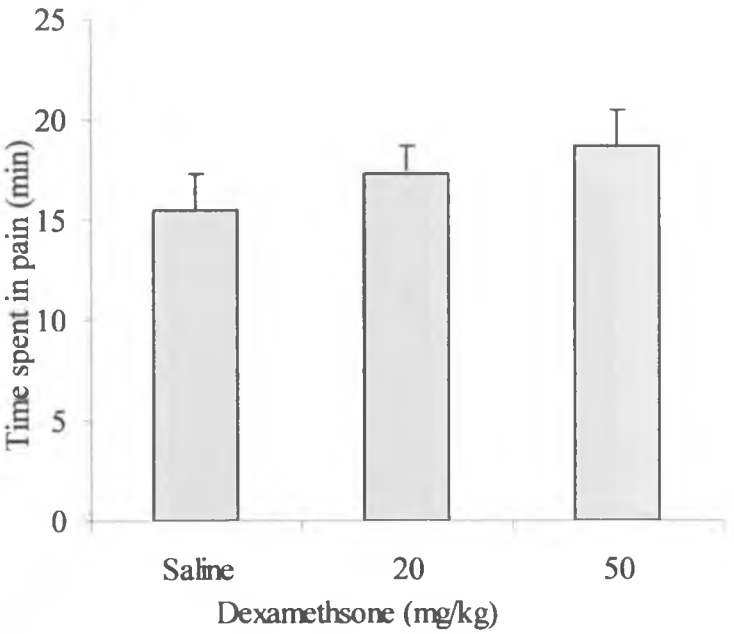

Fig. 6c: Effects of intracoelomic administration of saline or dexamethasone (20 or 50 mg/kg) in the formalin test in the speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

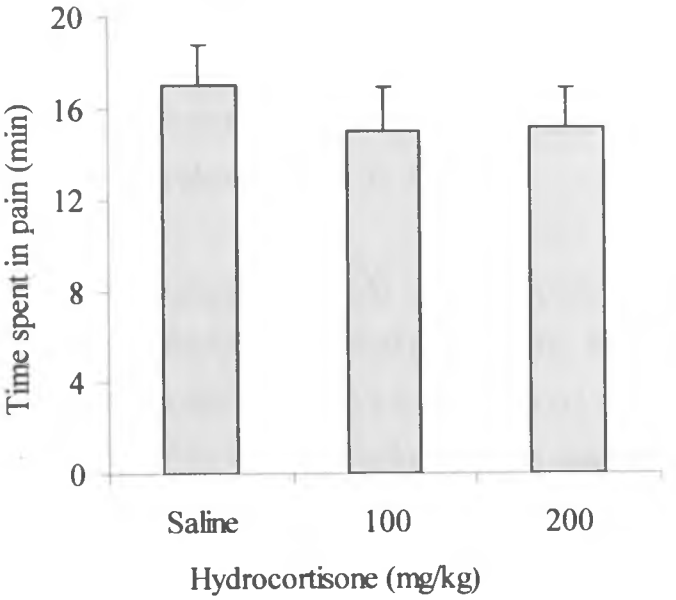

Fig. 6d: Effects of intracoelomic administration of saline or hydrocortisone (100 or 200 mg/kg) in the formalin test in speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.2.2 Hot Plate Test

4.2.2.1 Effects of Morphine, Pethidine and Naloxone

The hot plate base line latencies (controls) were determined by administering saline. The mean hot plate response latencies after ICo administration of morphine 5, 7.5, 10 and 20 mg/kg were 45.83 ± 1.39, 78.11 ± 12.42, 209.7 ± 14.2 and 192.7 ± 14.8 seconds, respectively, while that of the control group was 58.0 ± 3.4 seconds. The effects of morphine 10 and 20 mg/kg were statistically significant ($P < 0.05$). The effects of morphine 5 and 7.5 mg/kg were not statistically different from the controls ($P > 0.05$, Fig. 7a).

The effects of naloxone on morphine-induced antinociception were also investigated. The mean response latencies after morphine 10 mg/kg and saline, morphine 10 and naloxone 2.5 mg/kg, and morphine 10 mg/kg and naloxone 5 mg/kg were 69.28 ± 4, 72.83 ± 5.42 and 54.39 ± 2.98 seconds, respectively. Naloxone at 5 mg/kg caused a statistically significant reversal of the effects of morphine 10 mg/kg ($P < 0.01$, Fig. 7b).

The mean response latency after ICo administration of pethidine at dosages 10, 20 or 50 mg/kg were 54.28 ± 4.37, 71.67 ± 5.77 and 203.78 ± 9.76 seconds, respectively. The control group had a mean of 46.56 ± 2.12 seconds. On multiple comparisons of the different treatment means, the effects of pethidine 20 and 50 (mg/kg) were statistically significant ($P < 0.05$, Fig. 7c). Pethidine 10 mg/kg did not cause statistically significant increase in the response latency ($P > 0.05$).

The effects of naloxone on the effects of pethidine (50 mg/kg) were also evaluated. The treatment combinations used were pethidine 50 (mg/kg) and saline, pethidine 50 and naloxone 2.5 mg/kg, and pethidine 50 mg/kg and naloxone 5 mg/kg. The mean response latencies after these treatment combinations were 97.33 ± 12.41, 68.39 ± 7.3 and 48.11 ± 2.06 seconds, respectively. On multiple comparisons of the different treatment means, naloxone at either dosage significantly reversed the effects of pethidine 50 mg/kg ($P < 0.05$, Fig. 7d).

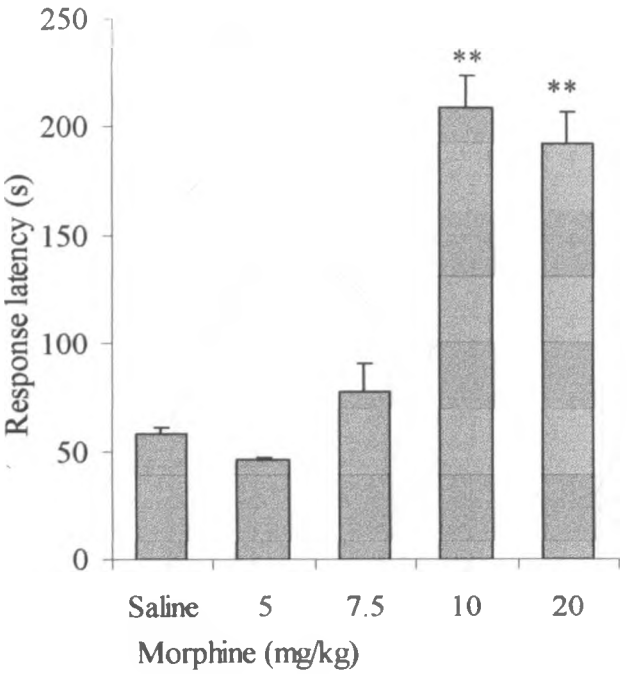

Fig 7a: Effects of intracoelomic administration of saline or morphine (5, 7.5, 10 or 20 mg/kg) in the hot plate nociception in speke-hinged tortoise. Treatment means were analyzed using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6. ** denotes $P < 0.01$.

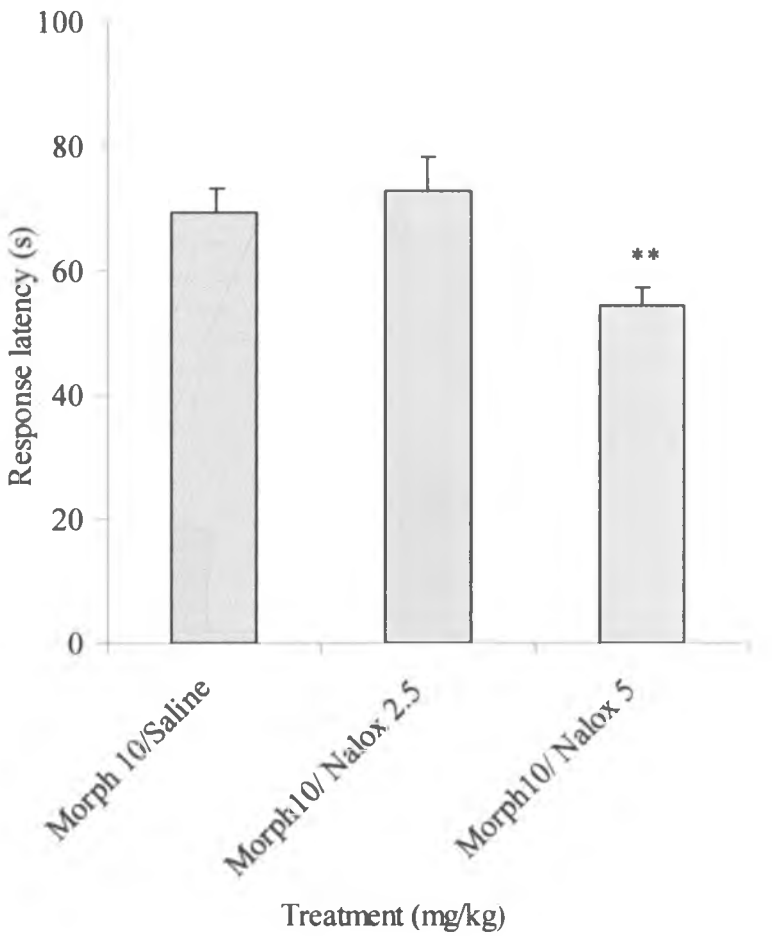

Fig. 7b: Effects of intracoelomic co-administration of morphine 10 mg/kg and saline (Morph 10/Saline), morphine 10 mg/kg and naloxone 2.5 mg/kg (Morph 10/Nalox 2.5) and morphine 10 mg/kg and naloxone 5 mg/kg (Morph 10/Nalox 5) combinations in the hot plate test in speke-hinged tortoise. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6. ** denotes $P < 0.01$.

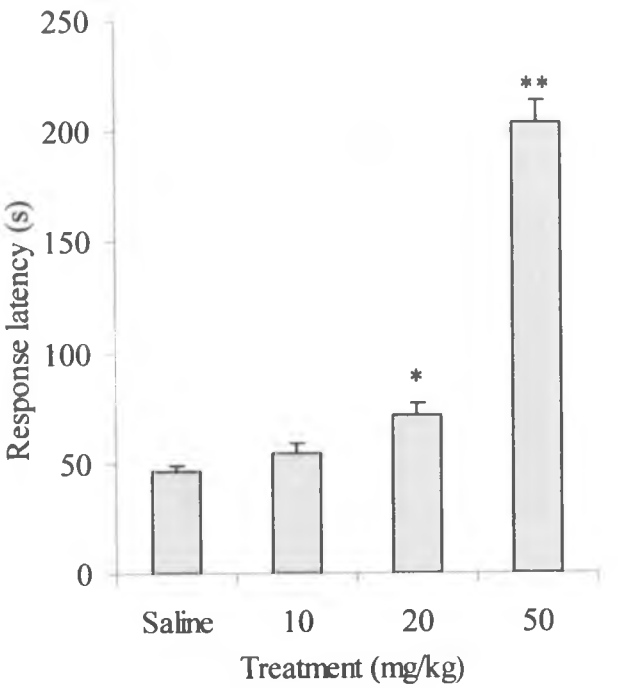

Fig. 7c: Effects of intracoelomic administration of saline or pethidine (10, 20 and 50 mg/kg) in the hot plate test in the speke-hinged tortoise. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6. * and ** denotes $P < 0.05$ and $P < 0.01$ respectively.

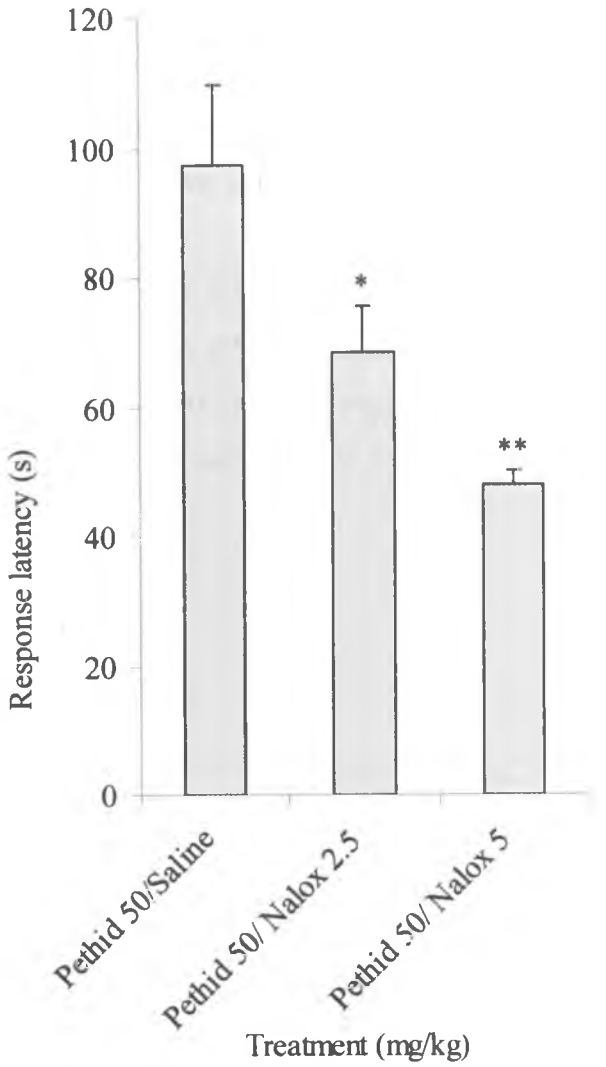

Fig. 7d: Effects of intracoelomic co-administration of pethidine 50 mg/kg and saline (Peth 50/Saline), pethidine 50 and naloxone 2.5 mg/kg (Peth 50/Nalox 2.5) and pethidine 50 mg/kg and naloxone 5 mg/kg (Peth 50/Nalox 5), combinations in the speke-hinged tortoise, in the hot plate response latencies. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6-7. * and ** denotes $P < 0.05$ and $P < 0.01$ respectively.

4.2.2.2 Effects of Acetylsalicylic Acid and Flunixin

Acetylsalicylic acid (100 and 200) did not cause any statistically significant change in the mean response latency in the speke-hinged tortoise ($P > 0.05$, Fig. 8a). The mean response latencies after ICo administration of ASA 100 or 200 mg/kg was 52.06 ± 2.94 and 52.56 ± 2.59 seconds, respectively, while that of the controls was 46.56 ± 2.11 seconds.

Flunixin (50 and 100 mg/kg) did not cause any statistically effects in the mean hot plate response latencies ($P > 0.05$, Fig. 8b). The mean hot plate response latencies after ICo administration of Flunixin 50 or 100 mg/kg was 47 ± 2.78 and 63.1 ± 6.4 seconds, respectively, compared to the control, 52.28 ± 2.67 seconds.

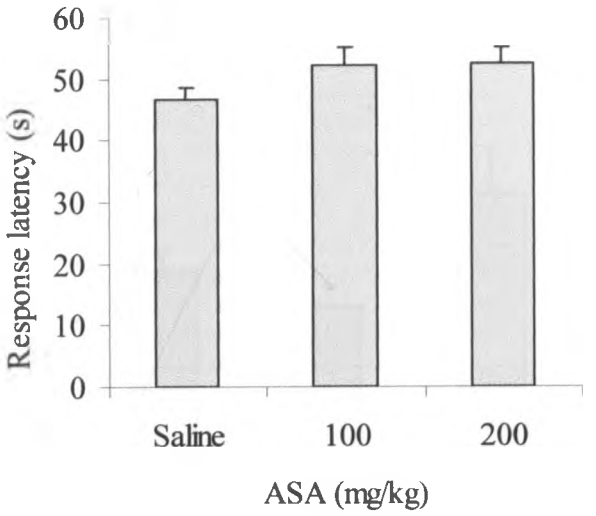

Fig. 8a: Effects of intracoelomic administration of saline or ASA (100 or 200 mg/kg) in the hot plate test in the speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

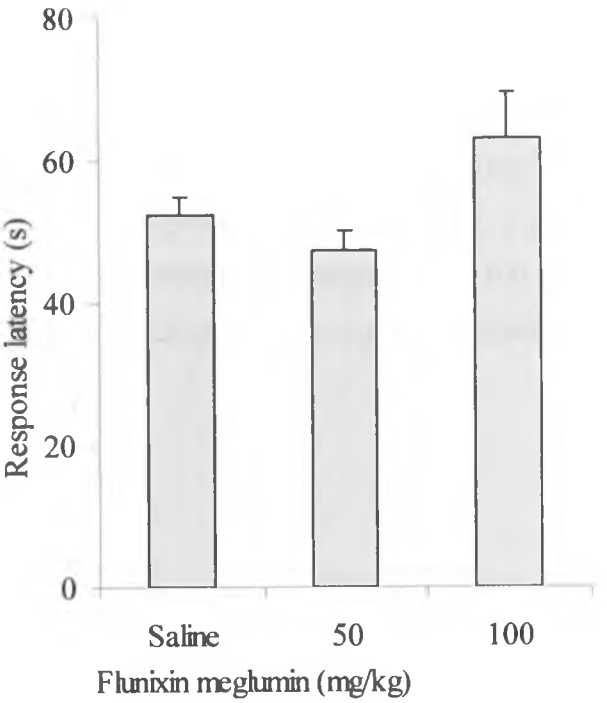

Fig. 8b: Effects of intracoelomic administration of saline or flunixin (50 or 100 mg/kg) on the hot plate nociception in the speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.2.2.3 Dexamethasone and Hydrocortisone

Dexamethasone at dosages of 20 or 50 (mg/kg) did not cause any statistically significant difference in the mean response latencies in the speke-hinged tortoise in the hot plate nociception ($P > 0.05$, Fig. 8c). The mean response latencies after ICo administration of dexamethasone 20 or 50 (mg/kg) were 45.78 ± 2.65 and 62.39 ± 6.8 seconds, respectively, while that of the control group was 58 ± 3.4 seconds.

Hydrocortisone at either dosages of 100 or 200 (mg/kg) did not cause any statistically significant change in the response latency ($P > 0.05$, Fig. 8d). The mean hot plate response latencies after ICo administration of hydrocortisone 100 or 200 (mg/kg) were 63 ± 4.69 and 54.11 ± 5.93 seconds, respectively, while that of the control group was 58 ± 3.4 seconds.

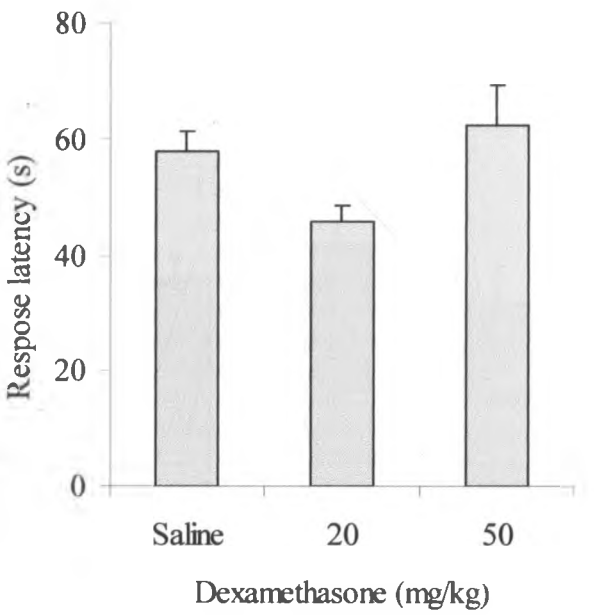

Fig. 8c: Effects of intracoelomic administration of saline or dexamethasone (20 or 50 mg/kg) in the hot test in the speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

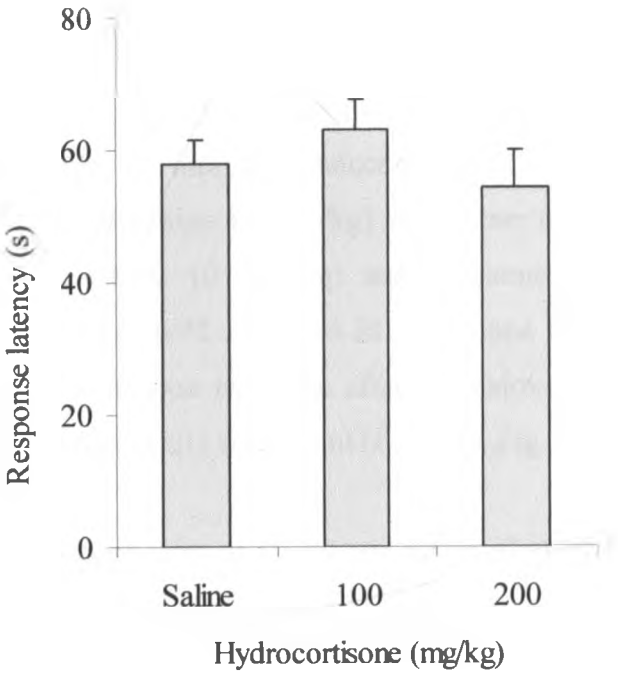

Fig. 8d: Effects of intracoelomic administration of saline or hydrocortisone (100 or 200 mg/kg) in the hot plate test in the speke-hinged tortoise. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.3 EFFECTS OF ANALGESIC DRUGS IN THE MARSH TERRAPIN

4.3.1 The Formalin Test

4.3.1.1 Effects of Morphine, Pethidine and Naloxone

Morphine was used in dosages of 5, 7.5, 10 and 20 (mg/kg). The mean times spent in nocifensive behavior after ICo administration of morphine were 10.8 ± 0.6, 10.5 ± 1, 8.5 ± 0.9 and 6.7 ± 0.4 minutes respectively. In the control group, the mean time spent in pain was 12.3 ± 1.3 minutes. Multiple comparison tests showed that the effects of morphine 10 and 20 (mg/kg) were statistically significant ($P < 0.05$, Fig. 9a). Morphine 7.5 mg/kg induced a slight but insignificant decrease in the time spent in nocifensive behavior ($P > 0.05$).

The effects of naloxone on morphine-induced antinociception were also investigated. The treatment groups were morphine 10 (mg/kg) and saline, morphine 10 (mg/kg) and naloxone 2.5 (mg/kg), and morphine 10 (mg/kg) and naloxone 5 (mg/kg). The times spent in nocifensive responses were 6.72 ± 0.37, 6.18 ± 0.59 and 12.59 ± 0.85 minutes, respectively. Following multiple comparison tests, the effect of naloxone 5 mg/kg on morphine induced antinociception were statistically significant ($P < 0.05$, Fig. 9b).

Pethidine was used at dose rates of 10, 20 and 50 (mg/kg), based on preliminary studies. The mean times spent in nocifensive behavior were 11.62 ± 0.61, 8.17 ± 0.83 and 7.83 ± 0.6 minutes, respectively, while that of the controls was 12.33 ± 1.26 minutes. The effects of pethidine 20 and 50 (mg/kg) were statistically significant ($P < 0.05$, Fig. 9c).

To investigate whether the antinociceptive effects of pethidine could be reversed by naloxone, pethidine 50 (mg/kg) was co-administered with naloxone 2.5 or 5 (mg/kg). The drug combinations used were as follows: pethidine 50 (mg/kg) and saline, pethidine 50 (mg/kg) and naloxone 2.5 (mg/kg), pethidine 50 and naloxone 5 (mg/kg), and naloxone 5 (mg/kg) and saline. The mean times spent in nocifensive behavior were 7.63 ± 0.77, 8.83 ± 0.83 and 10 ± 1.29 minutes, respectively. Multiple comparisons of the different treatment groups showed that the effects of naloxone at either dosage were not statistically significant ($P > 0.05$, Fig. 9d).

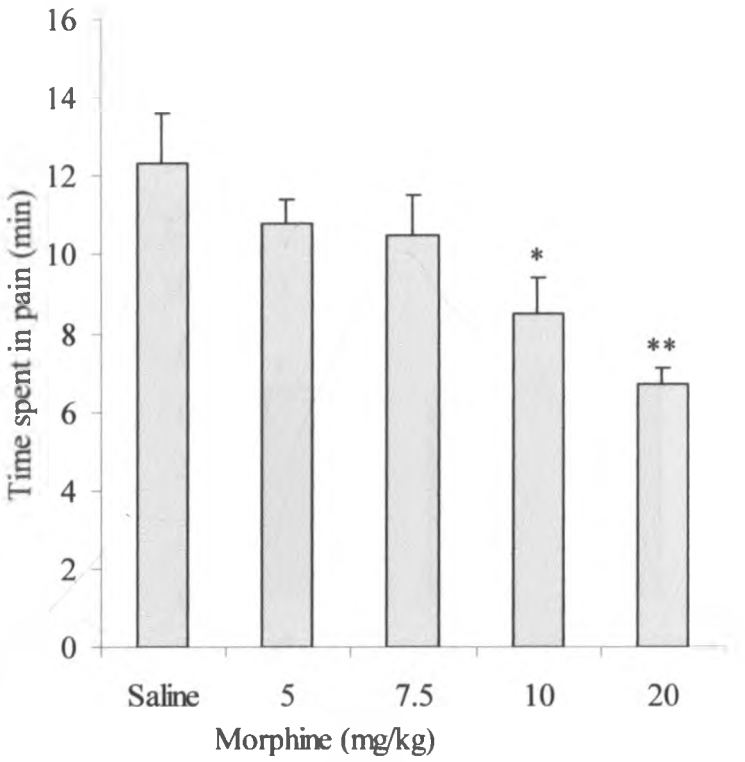

Fig. 9a: Effects of intracoelomic administration of saline or morphine (5, 7.5, 10 or 20 mg/kg) in the formalin test, in the marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6-7. * and ** denotes $P < 0.05$ and $P < 0.01$ respectively.

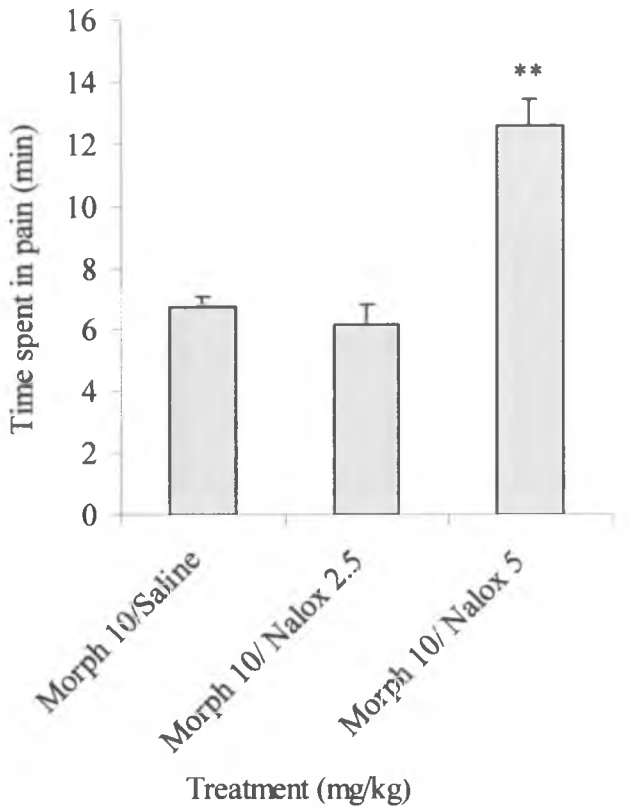

Fig. 9b: Effects of intracoelomic co-administration of morphine (10 mg/kg) and saline (Morph 10/Saline), morphine 10 mg/kg and naloxone 2.5 mg/kg (Morph 10/Nalox 2.5) and morphine 10 mg/kg and naloxone 5 mg/kg (Morph 10/Nalox 5) in the formalin test in marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6. ** denotes $P < 0.01$.

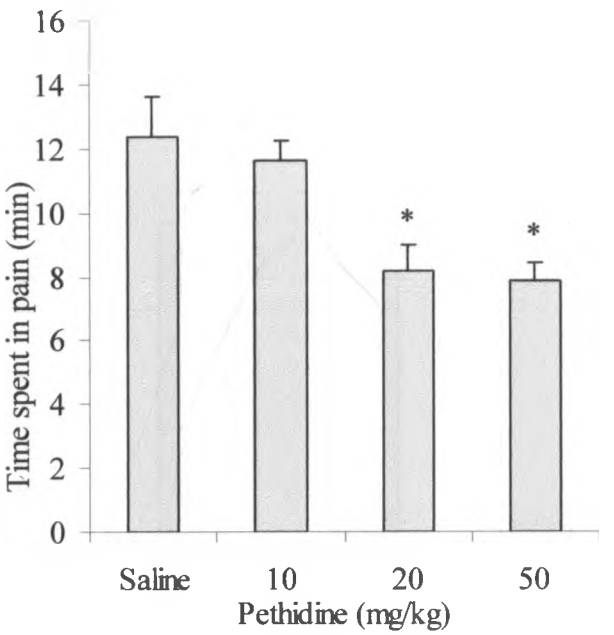

Fig. 9c: Effects of intracoelomic administration of saline or pethidine (10, 20 and 50 mg/kg) in the formalin test, in the marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 8-6. * denotes P< 0.05.

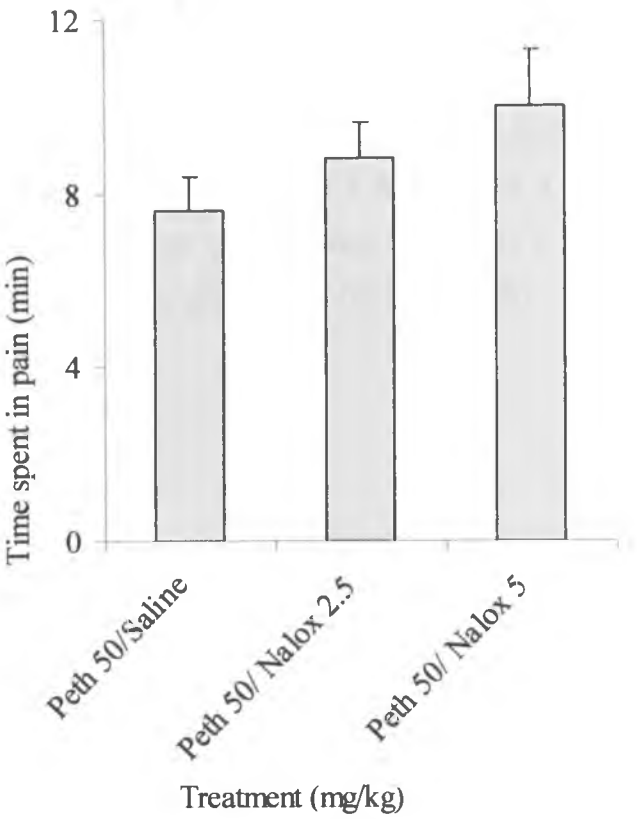

Fig. 9d: Effects of intracoelomic co-administration of pethidine 50 (mg/kg) and saline (Peth 50/Saline), pethidine 50 mg/kg and naloxone 2.5 mg/kg (Peth 50/Nalox 2.5), or pethidine 50 mg/kg and naloxone 5 mg/kg (Peth 50/Nalox 5) in the formalin test in the in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6\text{-}7$.

4.3.1.2 Effects of Acetylsalicylic Acid and Flunixin

Acetylsalicylic was administered in dosages of 100 or 200 mg/kg. The mean times spent in nocifensive behavior were 13.83 ± 1.64 and 14.17 ± 1.6 minutes, respectively, while that of the control group was 12.43 ± 1.26 minutes. None of these dosages caused statistically significant effects ($P > 0.05$, Fig. 10a).

Flunixin was administered in dosages of 10, 50 or 100 mg/kg. The corresponding mean times spent in nocifensive behavior were 15.33 ± 0.99, 13 ± 1.44 and 15.67 ± 1.89, minutes respectively, while that for the control group was 12.43 ± 1.26 minutes. None of the dosages caused statistically significant effects ($P > 0.05$, Fig. 10b).

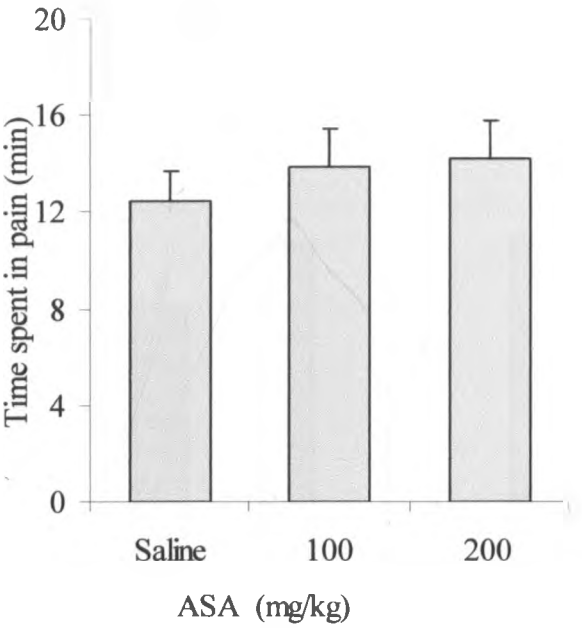

Fig. 10a: Effects of intracoelomic administration of saline or ASA (100 or 200 mg/kg) in the formalin test in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

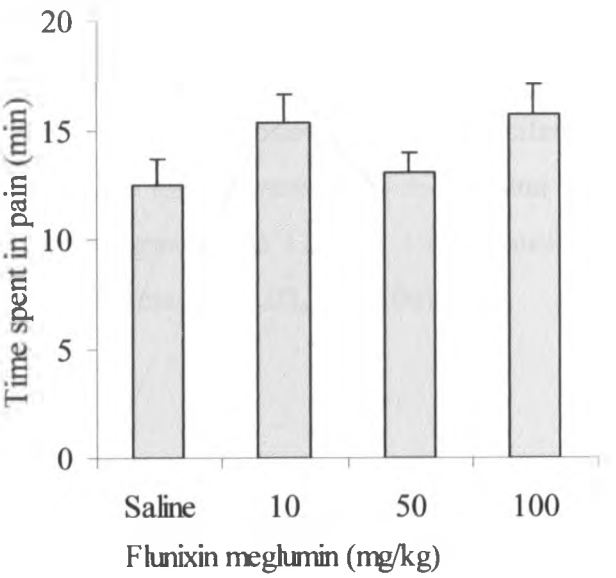

Fig. 10b: Effects of intracoelomic administration of saline or flunixin (10, 50 and 100 mg/kg) in the formalin test, in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.3.1.3 Effects of Dexamethasone and Hydrocortisone

Dexamethasone was administered, in dosages of 10, 20 and 50 mg/kg. The resultant mean times spent in nocifensive behavior were 13.17 ± 1.01, 13.17 ± 1.17 and 11.83 ±1.25 minutes, while that of the control was 12.43 ± 1.26 minutes. None of the dosages used caused statistically significant effects on the mean time spent in nocifensive behavior in the formalin test ($P > 0.05$, Fig. 10c).

Hydrocortisone was similarly administered in dosages of 100 or 200 (mg/kg), based on preliminary studies. The mean times spent in nocifensive behavior administration of hydrocortisone 100 or 200 mg/kg were 14.17 ± 2.73 and 12.5 ± 0.96 minutes, respectively, while that of the control group was 12.43 ± 1.26 minutes. None of the dosages induced a statistically significant effects ($P > 0.05$, Fig. 10d).

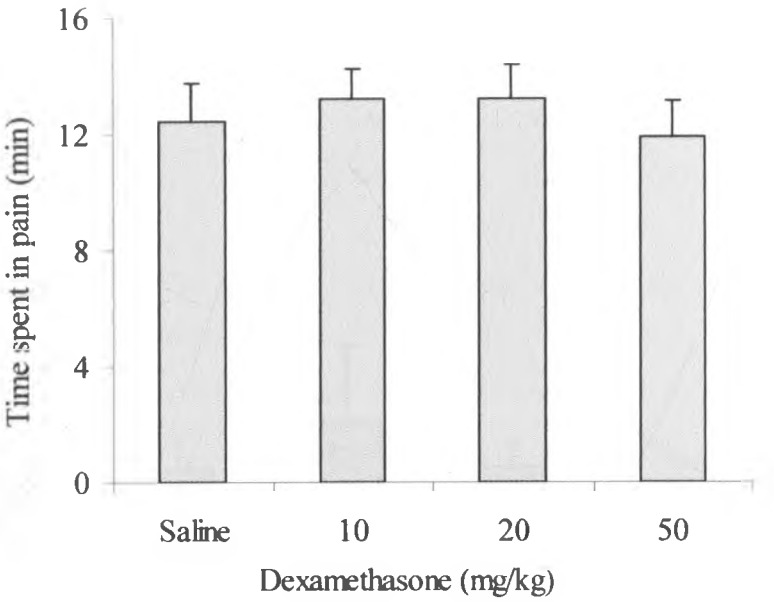

Fig. 10c: Effects of intracoelomic administration of saline or dexamethasone (10, 20 or 50 mg/kg) in the formalin test, in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

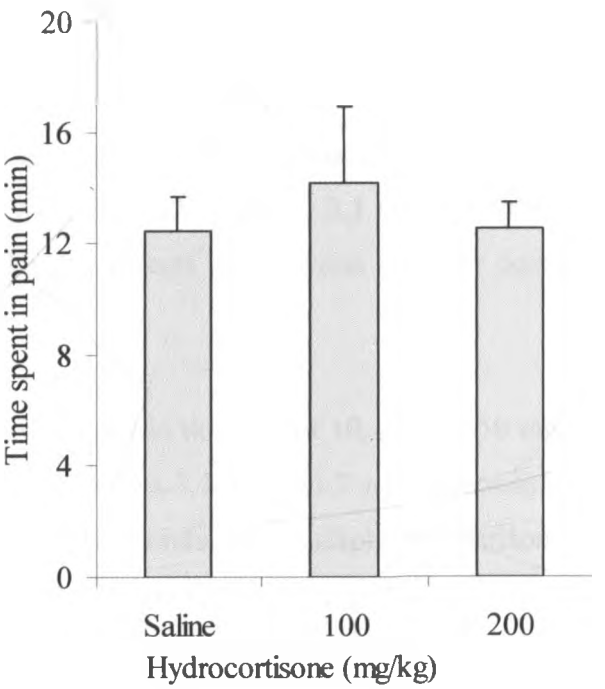

Fig. 10d: Effects of intracoelomic administration of saline or hydrocortisone (100 or 200 mg/kg) in the formalin test, in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.3.2 The Hot Plate Test

4.3.2.1 Effects of Morphine, Pethidine and Naloxone

The hot plate base line latencies for each of the experiments were determined by administering saline. Morphine was administered in dosages of 5, 7.5, 10 or 20 mg/kg, ICo, and the mean hot plate response latencies were 54.5 ± 2.7, 59.7 ± 6.3, 176.6 ± 217 and 182.4 ± 19.1 seconds, respectively. In the control group, the mean response latency was 44.4 ± 3.3 seconds. The effects of morphine 10 and 20 (mg/kg) were statistically significant ($P < 0.01$, Fig. 11a).

To test whether the effects of morphine were reversible by naloxone on the hot plate test, the following drug combinations were used: morphine 10 (mg/kg) and saline, morphine 10 and naloxone 2.5 mg/kg, and morphine 10 and naloxone 5 mg/kg. The resultant mean response latencies were 70.1 ±2.1, 44.5 ± 3.1 and 53.1 ± 4.1 minutes respectively. Statistical analysis showed that the effects of naloxone at either dosage were statistically significant ($P < 0.05$, Fig. 11b).

Pethidine was used in dosages of 10, 20 and 50 mg/kg. The resultant mean response latencies were 57.4 ± 2, 59.8 ± 3.1 and 121.7 ± 8.1 seconds respectively, while that of the control group was 44.4 ± 3.3 seconds. On multiple comparisons, the effects of pethidine 50 mg/kg were statistically significant ($P < 0.01$, Fig. 11c).

To test for reversal of the effects of pethidine by naloxone, a dosage of pethidine 50 mg/kg was chosen. The mean hot plate response latencies after ICo administration of pethidine 50 mg/kg and saline, pethidine 50 mg/kg and naloxone 2.5 mg/kg, pethidine 50 mg/kg and naloxone 5 mg/kg, and naloxone 5 mg/kg and saline were 98.6 ± 5.5, 85.3 ± 10, 71.8 ± 5.4 and 49.3 ± 2.1 seconds respectively. On multiple comparisons of the different treatment means, naloxone at the high dose (5 mg/kg) induced statistically significant effects on pethidine (50 mg/kg) induced antinociception ($P < 0.05$, Fig. 11d).

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To test for reversal of the effects of pethidine by naloxone, a dosage of pethidine 50 mg/kg was chosen. The mean hot plate response latencies after ICo administration of pethidine 50 mg/kg and saline, pethidine 50 mg/kg and naloxone 2.5 mg/kg, pethidine 50 mg/kg and naloxone 5 mg/kg, and naloxone 5 mg/kg and saline were 98.6 ± 5.5, 85.3 ± 10, 71.8 ± 5.4 and 49.3 ± 2.1 seconds respectively. On multiple comparisons of the different treatment means, naloxone at the high dose (5 mg/kg) induced statistically significant effects on pethidine (50 mg/kg) induced antinociception ($P < 0.05$, Fig. 11d).

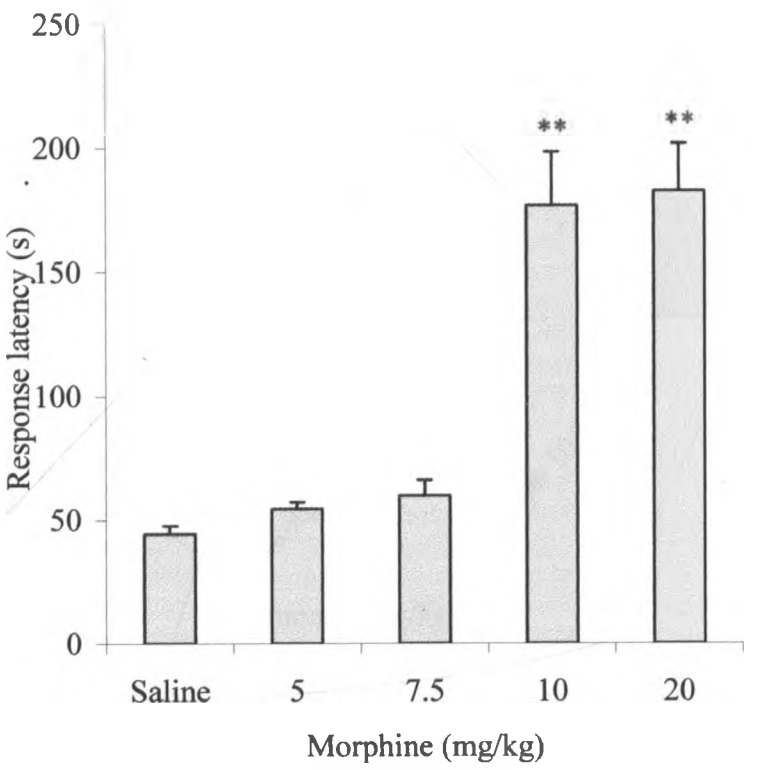

Fig. 11a: Effects of intracoelomic administration of saline or morphine (5, 7.5, 10 and 20 mg/kg) in the hot plate test, in the marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6-7. ** denotes $P < 0.01$.

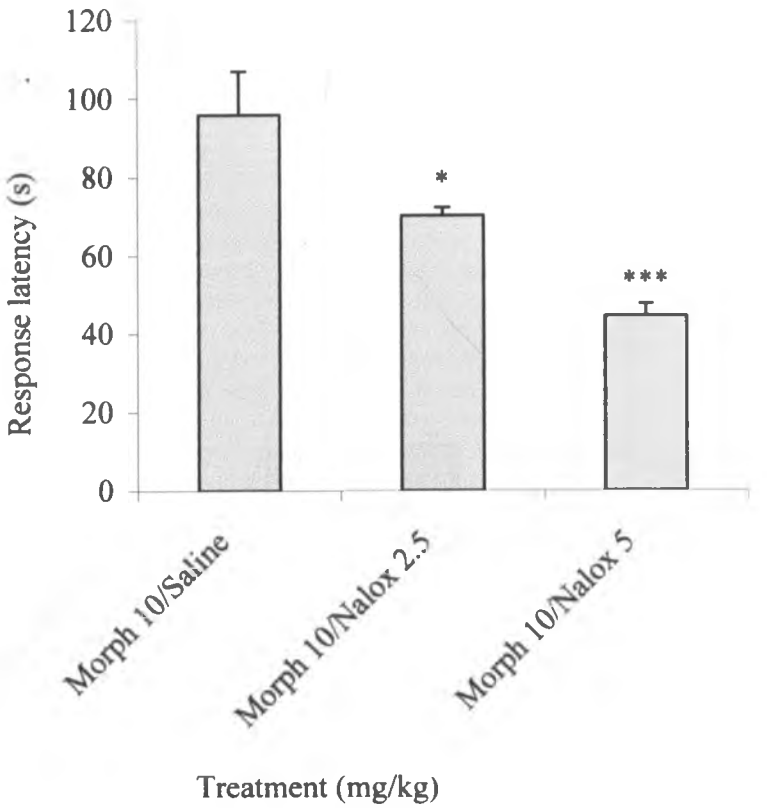

Fig. 11b: Effects of intracoelomic co-administration of morphine 10 mg/kg and saline (Morph 10/Saline), morphine 10 mg/kg and naloxone 2.5 mg/kg (Morph 10/Nalox 2.5), and morphine 10 and naloxone 5 mg/kg (Morph 10/Nalox 5), in the hot plate test in the marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6-8. * and ***denotes $P < 0.05$ and $P < 0.001$ respectively.

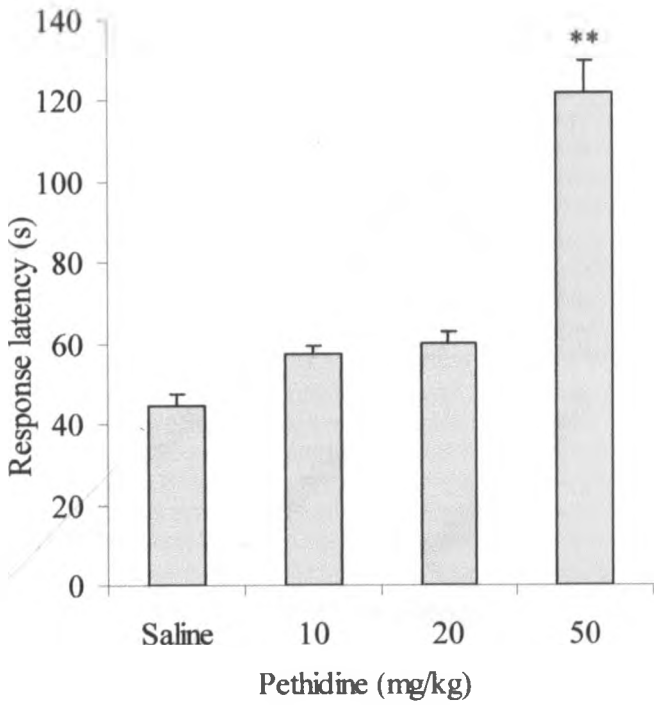

Fig. 11c: Effects of intracoelomic administration of saline or pethidine (10, 20 or 50 mg/kg) on the hot plate test in the marsh terrapin. Treatment means were compared using Dunnett's t, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6. ** denotes $P < 0.01$.

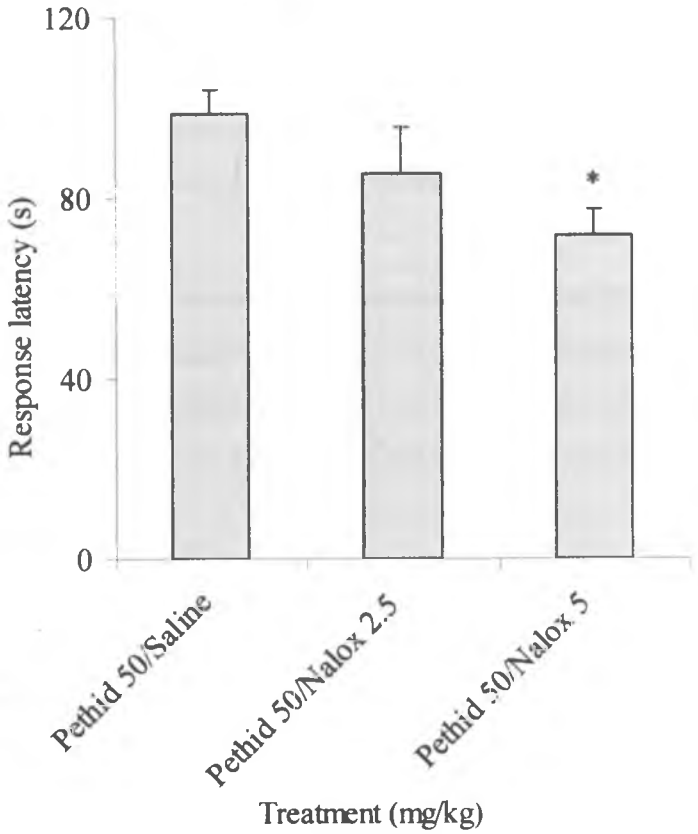

Fig. 11d: Effects of intracoelomic co-administration of pethidine 50 mg/kg and saline (Peth 50/Saline), pethidine 50 mg/kg and naloxone 2.5 mg/kg (Peth 50/Nalox 2.5) or pethidine 50 mg/kg and naloxone 5 mg/kg (Peth 50/Nalox 5) in the hot plate test in the marsh terrapin. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. Bars represent means ± S.E.M. and n = 6-7. * denotes $P < 0.05$.

4.3.2.2 Effects of Acetylsalicylic Acid and Flunixin

Acetylsalicylic acid at either dosage used did not cause any statistically significant change in the mean response latency in the marsh terrapin ($P > 0.05$, Fig. 12a). The mean response latencies after ICo administration of ASA 100 or 200 mg/kg were 44.06 ± 1.53 and 54.11 ± 4.44 seconds, respectively, while that of the control group was 50.61 ± 3.47 seconds.

Flunixin at either dosage used did not cause any statistically significant change in the mean hot plate response latencies ($P > 0.05$, Fig. 12b). The mean hot plate response latencies after ICo administration of flunixin 50 or 100 mg/kg were 47.1 ± 2.69 and 61.48 ± 6.84 seconds, respectively, compared to the controls, 55.05 ± 3.97 seconds.

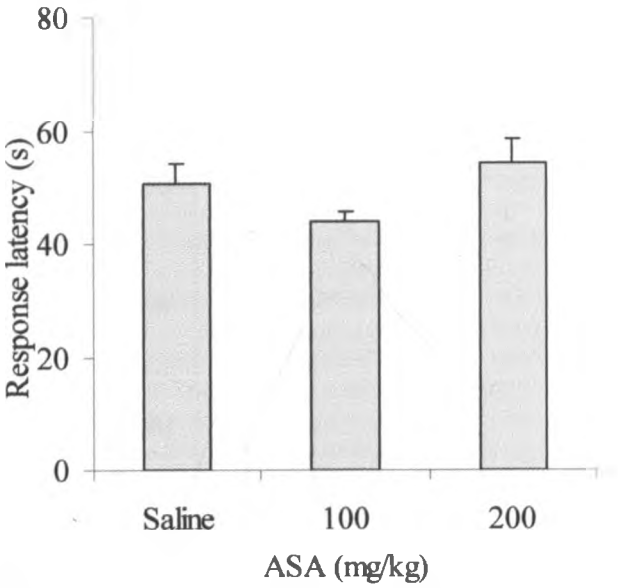

Fig. 12a: Effects of intracoelomic administration of saline or ASA (100 or 200 mg/kg) in the hot plate response latency, in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

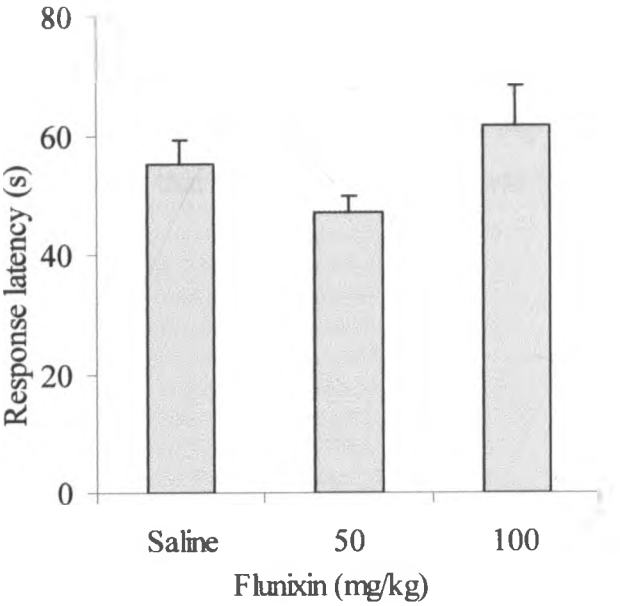

Fig. 12b: Effects of intracoelomic administration of saline or flunixin (50 or 100 mg/kg) in the hot plate, in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.3.2.3 Effects of Dexamethasone and Hydrocortisone

Dexamethasone (10, 20 or 50 mg/kg) did not cause any statistically significant change in the mean response latencies, in the marsh terrapin ($P > 0.05$, Fig. 12c). The mean response latencies after ICo administration of dexamethasone 10, 20 or 50 mg/kg were 48.3 ± 1.9, 49.4 ± 3.7 and 71.3 ± 12.2 seconds, respectively, while that of the control was 53.3 ± 2.4 seconds.

Hydrocortisone at either dosage of 100 or 200 mg/kg did not cause any statistically significant increase in the response latency ($P > 0.05$, Fig. 12d). The mean hot plate response latencies after ICo administration of hydrocortisone 100 or 200 mg/kg were 40.72 ± 2.39 and 63 ± 7.91 seconds, respectively, while that of the control group was 53.28 ± 2.39 seconds.

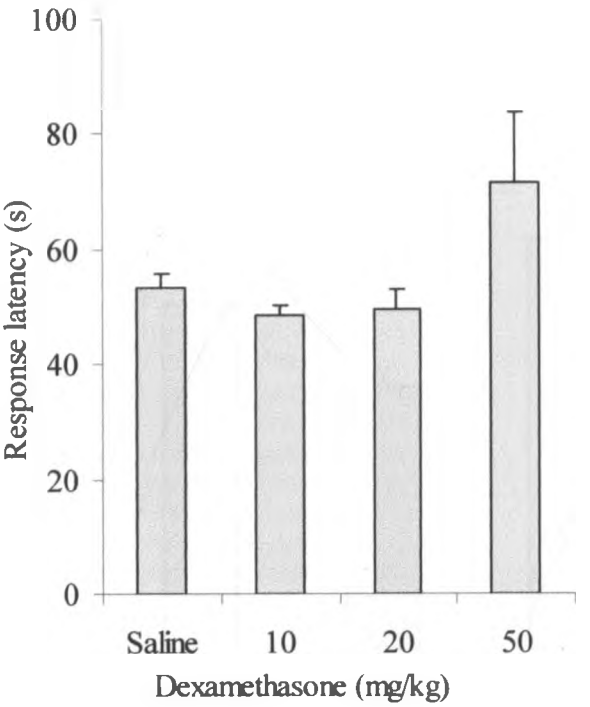

Fig. 12c: Effects of intracoelomic administration of saline and dexamethasone (10, 20 or 50 mg/kg) in the hot plate response test in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

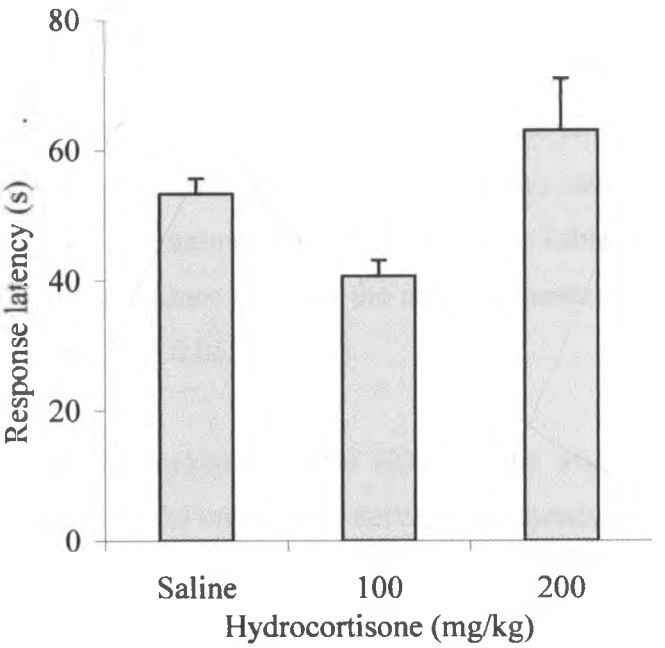

Fig. 12d: Effects of intracoelomic administration of saline or hydrocortisone (100 or 200 mg/kg) in the hot plate response test in the marsh terrapin. Treatment means were analyzed using ANOVA and the level of significance set at $P < 0.05$, and $n = 6$.

4.4 NERVE FIBER PROPORTIONS IN THE SENSORY BRANCHES OF THE TRIGEMINAL NERVE IN TESTUDINES

To estimate the fiber proportions in the sensory afferents of testudines, the right ophthalmic and the right maxillary nerves were selected in both the speke-hinged tortoise and the marsh terrapin. The two branches of the trigeminal nerve were selected because both contain sensory afferent neurons, with no motor components. The ophthalmic nerve bundle had a mean diameter of 0.40 ± 0.02 mm in the tortoise and 0.45 ± 0.04 mm in the marsh terrapin. The maxillary nerve measured 0.92 ± 0.37 mm in the tortoise and 0.59 ± 0.05 mm in the marsh terrapin. There was no significant difference between the mean diameters of the ophthalmic bundle in both species of animals used. ($P > 0.05$, Table 1). Similarly, there was no statistically significant difference between the mean diameters of the maxillary bundle in the two animal species used ($P > 0.05$, Table 1).

There were no observable naked fibers in either of the trigeminal nerve branches in both animal species (Fig. 13). In the ophthalmic nerve of the tortoise, the proportion of nerve fibers measuring less than 5.5, 5.6-10 and more than 10 μm in diameter was 13.4 ± 1, 43.0 ± 2.1 and 18.9 ± 1.3 fibers respectively (total count = 2257, Table 2). In the ophthalmic nerve of the terrapin, the respective proportion of fibers was 14.4 ± 1.9, 42.1 ± 2.6 and 20.8 ± 1.2 respectively (total count = 2320, Table 2). On multiple comparisons of the different fiber proportions, there were no statistically significant differences between the nerve fiber proportions in the ophthalmic nerve in the two animal species ($P > 0.05$, Fig. 14). Nerve fibers measuring 5.5-10 μm constituted the largest proportion of fibers in either nerve branches in both the tortoise and the terrapin. Neurons with diameters less than 5.6 μm in diameter comprised 17.8% of all the nerve fibers in the tortoise and 18.7% in the terrapin (Table 2).

In the maxillary nerve of the tortoise, the proportion of fibers with diameters measuring less than 5.5, 5.6-10 and more than 10 μm was 17.5 ± 1.8, 51.4 ± 2.4 and 15.6 ± 2.5 fibers respectively (n = 3; total count = 1521), while in the terrapin it was 23.5 ± 1.6, 49.3 ± 2.2 and 14.2 respectively (total count = 2087, Table 2). On multiple comparisons of the different fiber

proportions, there were no statistically significant differences between the fiber proportions in the maxillary nerve branch in the tortoise and the terrapin ($P > 0.05$, Fig. 14). Like the ophthalmic nerve, nerve fibers measuring 5.6-10 μm in diameter constituted the majority of nerve fibers in the sensory branches of the trigeminal nerve in both animal species. Nerve fibers with diameters measuring less than 5.5 μm constituted 20.7% and 27.0% in the tortoise and the terrapin respectively (Table 2). The proportion of nerve fibers measuring less than 5.5 μm was significantly higher in the maxillary nerve of the marsh terrapin than in that of the tortoise ($P < 0.05$, Fig. 14).

Table 1: The diameter (mm) of the ophthalmic and the ophthalmic nerve branches of the trigeminal nerve in the speke-hinged tortoise and the marsh terrapin

Animal species	Nerve branch	largest diameter	Count (N)
Tortoise	Ophthalmic	0.40 ± 0.02	5
	Maxillary	0.92 ± 0.37	3
Terrapin	Ophthalmic	0.45 ± 0.04	5
	Maxillary	0.59 ± 0.05	5

Table 2: The relative proportions and percentages of the different fiber types in the ophthalmic and the maxillary branches of the trigeminal nerve in the tortoise and the terrapin.

Nerve fiber category and diameter		**< 5.5 µm**	**5.6-10 µm**	**> 10 µm**
Tortoise ophthalmic nerve (n = 5)	Mean	13.4	43.0	18.9
	S.E.M.	1.0	2.1	1.3
	%	17.8	57.1	25.1
Terrapin ophthalmic nerve (n = 5)	Mean	14.4	42.1	20.8
	S.E.M.	1.9	2.6	1.2
	%	18.7	54.5	26.9
Tortoise maxillary nerve (n = 3)	Mean	17.5	51.4	15.6
	S.E.M.	1.8	2.4	2.5
	%	20.7	60.8	18.5
Terrapin maxillary nerve (n = 4)	Mean	23.5	49.3	14.2
	S.E.M.	1.6	2.2	1.1
	%	27.0	56.7	16.3

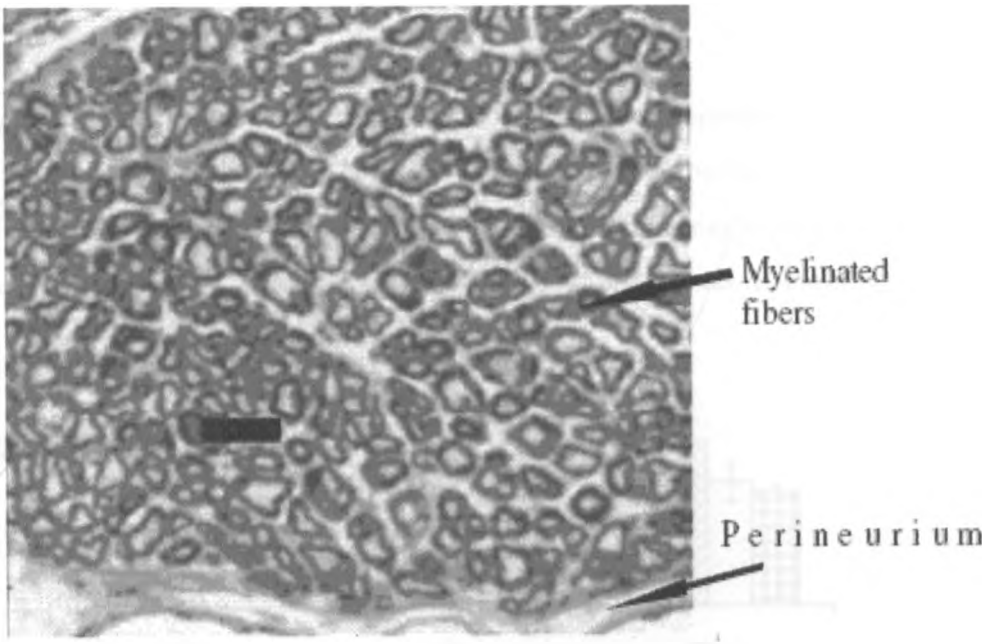

Fig. 13: A section of the ophthalmic branch of the trigeminal nerve in the marsh terrapin (×400, scale bar = 5 μm).

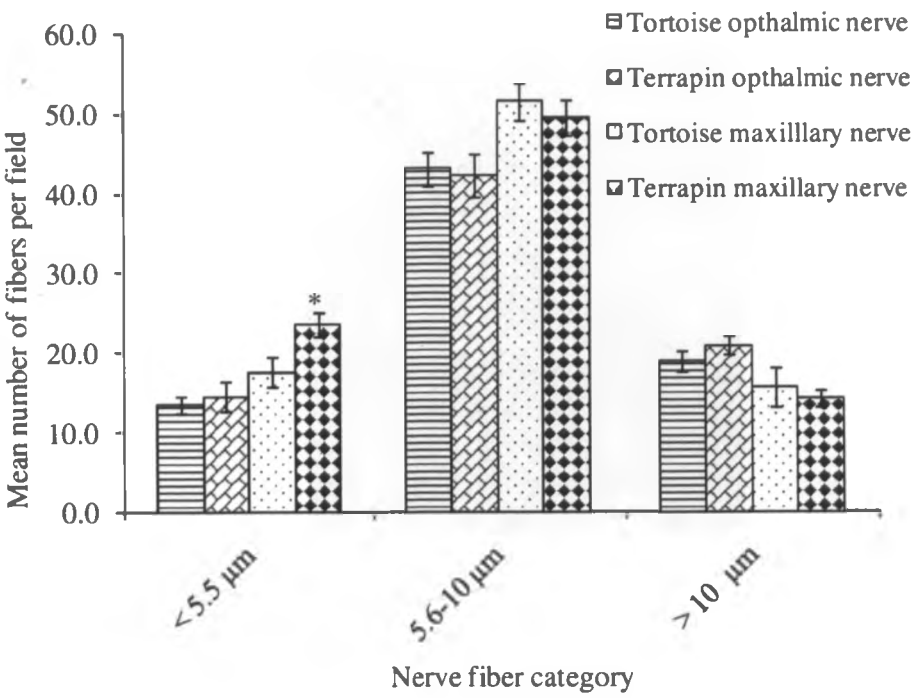

Fig. 14: The distribution of fiber types in the ophthalmic and the maxillary branches of the trigeminal nerve in the tortoise and the terrapin. Bars represent mean ± S.E.M. and n = 3-5. * denotes $P < 0.05$.

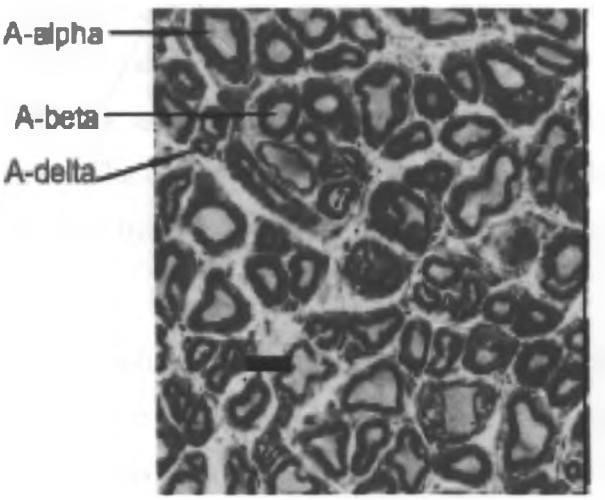

Fig. 15: A section of the maxillary branch of the trigeminal nerve in the marsh terrapin showing the presence of small, medium and large diameter myelinated fibers (× 1000, scale bar = 2 μm).

CHAPTER FIVE

5.0 DISCUSSION AND CONCLUSIONS

5.1 BEHAVIORAL NOCICEPTIVE TESTING

Behavioral tests of nociception are commonly used in studies on pain, but only few tests have been applied in testudines (Sladky *et al.*, 2007). Unlike in mammalian subjects, only few methods are available for studying nociception in testudines (Bennett, 1998; Read, 2004; Sladky *et al.*, 2007). One of the limiting factors in studies of nociception in testudines is the presence of the shell, which limits the number of possible sites of stimuli application. Moreover, testudines retract their head and limbs into the shell when molested. The present study showed that formalin and hot plate tests are reliable methods for studying pain mechanisms in testudines. The capsaicin and the acetic acid instillation into the eye can also be performed, but are limited by the retraction of the head into the shell.

5.1.1 The Formalin Test

The formalin test has been applied in various animals at varying concentrations and volumes. In the present study, the volume of 100 µL of 12.5% was chosen based on preliminary studies. The inter-animal variability was high but insignificant. Pain threshold has been shown to vary from one animal to the other based on age, sex, body weight, variability of skin temperature and individual's genetic make-up among others (Kavailers, 1988; 1989; Hoffmann *et al.*, 1998). Perhaps the differences could be minimized if the animals are captive bred and the period of handling during habituation prolonged. High concentrations of formalin preferentially activate the A-delta nociceptive afferents, while low concentrations preferentially activate the C-polymodal nociceptors (Rosland *et al.*, 1990). Lower concentrations of formalin did not induce clearly quantifiable pain responses. Probably, the threshold of their nociceptors is higher than that of most vertebrates or that their nociceptors are more inaccessible or fewer. The other possible explanation could be that testudines do not preferentially show pain until the stimulus is very invoking.

The animals showed monophasic pain behavior, which lasted for approximately nine minutes. In mammals and frogs, the formalin-induced nociception is characterized by both first and second phase of pain responses (Tjolsen, *et al.*, 1992; Oyadeyi, 2007). The second phase is probably due to stimulation of nociceptors by histamine, bradykinin and other inflammatory mediators, and requires a stronger stimulus than first phase (Le Bars *et al.*, 2001). The current results indicate that like crocodiles, testudines do not show the second phase of pain in the formalin test (Kanui *et al.*, 1990). This is probably due to the absence of inflammatory mediators or that the inflammatory process of reptiles is different from that of mammals.

5.1.2 The Hot Plate Test

From these results, the hot plate test is a reliable nociceptive test in testudines. However, in testudines, the test is limited by retraction of the head and limbs into the shell, urination and defecation, leading to a success rate of about 80%. The test has some advantages over other pain models, including rapid application and decay of the noxious stimuli, instant latency quantification, and unambiguous behavior after stimulus application (Le Bars *et al.*, 2001). Compared to hot plate tests in rodents, the thermal stimulus (60 °C) used was relatively high. This could be due to the presence of scales, which increase the interface between the hot plate and the nociceptors located at the dermo-epidermal junction or that testudine nociceptors have a high thermal threshold (Le Bars *et al.*, 2001; Sladky *et al.*, 2007). Different temperatures are thought to preferentially excite different types of nociceptors. Low temperatures preferentially activating C fibers and higher ones activate A-delta fibers (Le Bars *et al.*, 2001).

In this test, testing was done three times in each animal within an interval of one hour to minimize the inter-animal differences (Kanui *et al.*, 1990). However, repeated thermal stimulation can lead to thermal hypersensitivity, which was not observed in the present study (Ding *et al.*, 2005). In both animals, there was a slight but insignificant increase in response latencies for the third test. Using radiant heat stimulation, Sladky *et al.*, (2007) reported the first nociceptive test in testudines. Their test resembles the hot plate test used in this study in that they are both phasic thermal tests, and the variable measured is the response latency.

However, the surface area of the body stimulated is larger in the hot plate test because the animal is in direct contact with the hot surface unlike the radiant heat. The response latency to initial lifting of the foot may be used as a measure of pain threshold in testudines. The attempt to escape behavior occurred after a prolonged duration, well above the pain threshold. Response latency to initial lifting of the foot was scored because unlike the escape latency, it is easier to notice, its end-point is more consistent and has lower risk of damaging the paws of the test animal (Le Bars *et al.*, 2001).

5.1.3 The Capsaicin Instillation Test

Animal models of nociception involving intraopthalmic instillation of algogenic materials have been shown to be reliable tests of pain (Farazifard *et al.*, 2005; Kanui *et al.*, 1990). The capsaicin instillation test is limited in its application in testudines due to head retraction. The results indicate that the marsh terrapin is sensitive to low concentrations of capsaicin. Some animals like birds and the naked mole rats do not show pain behavior upon exposure to capsaicin (Park *et al.*, 2008). Like crocodiles, the marsh terrapin showed no decrease in sensitivity to capsaicin after repeated exposure to the irritant (Kanui *et al.*, 1990). Capsaicin desensitization is thought to be due to depletion of neurotransmitters such as SP and CGRP in the primary nociceptive afferents (Kim *et al.*, 2008; Park *et al.*, 2008). In mammalian systems, the majority of nociceptors are C polymodal nociceptors, and preferentially express TRPV1 receptors, which are gated by capsaicin, noxious temperature and protons (Benham *et al.*, 2003; Almeida *et al.*, 2004; Tominaga and Caterina, 2004; Kim *et al.*, 2008; Park *et al.*, 2008). Capsaicin is specifically neurotoxic to the C-polymodal nociceptors, and this is the basis for its desensitizing effects observed after repeated exposure (Kim *et al*, 2008). The absence of capsaicin desensitization could be attributed to either lack or depletion of neuropeptides in the primary nociceptive afferents, unusual spinal cord organization or lack of peptidergic fibers (Park *et al.*, 2008).

5.1.4 The Acetic Acid Instillation Test

Application of acetic acid has been performed in many ways including intraperitoneal injection (the writhing test), intradermal injection and intranasal instillation. Unlike in rodents, ICo injection of acetic acid at different volumes and concentration did not induce any quantifiable behavior in both the speke-hinged tortoise and the marsh terrapin. Though it could not be established why, it is probable that the visceral nociceptors in testudines are not sensitive to acetic acid. The topical application of the acid was developed and used in a concentration of 10%. The substance caused instant eye protective responses characterized by scratching, rubbing and eye closure, which lasted for about ten minutes. The test was however limited by retraction of the head into the shell.

5.2 EFFECTS OF OPIOIDS

In both species of testudines, the opioids used were antinociceptive at higher doses in both the formalin and hot plate tests. The results indicate that both the formalin and the hot plate tests may be useful nociceptive tests for studying effects of analgesic drugs in the speck-hinged tortoise and the marsh terrapin. The results showed that the animals are sensitive to opioid analgesics, but at relatively high dosages. The results suggest that morphine may be used for analgesia at dosages of 7.5 mg/kg and above. However, morphine at 7.5 mg/kg was only effective in the formalin test in the speke-hinged tortoise (Fig. 5a). Compared to a report on red-eared slider turtles, morphine at as low as 1.5 mg/kg showed statistically significant antinociception on radiant heat stimulation (Sladky *et al.*, 2007).

The difference in the antinociceptive dosages in the two tests used could be due the type of nociception and the scoring criteria. Morphine has been shown to be more potent in the radiant heat stimulation than in the hot plate test and has lowest potency in the formalin test (Morgan *et al.*, 2006). However, in our studies, morphine was more potent with the formalin test than with the hot plate test. In the present study, morphine at dosages lower than 7.5 mg/kg did not induce any statistically significant effects in both tests. Higher dosages had dose-dependent

antinociceptive effects. Pethidine also induced antinociception at relatively high dosages. Compared to the juvenile crocodiles where pethidine at 2 mg/kg had statistically significant antinociceptive effects, dosages as high as 10 mg/kg in either animal studied had no statistically significant effects. The results suggest that opioids can be useful in pain management in testudines, but at relatively high doses. The opioid receptor gene family is highly conserved across multiple vertebrate orders including bovids, chicken, bullfrogs, fish and elasmobrachs (Li *et al.*, 1996; Sneddon, 2004), but there is limited information on opioid receptors distribution in reptiles. The δ opioid receptors are more abundant than the μ opioid receptors in the central nervous system of aquatic turtles (Xia and Haddad, 2001).

Both morphine and pethidine are mu-agonists and the relatively high antinociceptive dosages may suggest the presence of fewer mu receptors in testudines compared to other animals. Further studied on the antinociceptive effects of opioid selective drugs could reveal the role of mu, kappa or delta opioid receptors in pain regulation in testudines. A study of the relative distribution of the opioid receptor subtypes in the nervous system of testudines could also shed some light on the organization of the opioidergic system of the animals. Naloxone, a non-selective opioid receptor blocker, showed significant inhibition of opioid induced antinociception in both animal species.

5.3 EFFECTS OF ANTI-INFLAMMATORY DRUGS

Although commonly used as analgesic drugs, ASA and flunixin did not show any statistically significant effects on the formalin or the hot plate tests. This suggests that the inflammatory process in testudines is absent or different from that in other vertebrates. The *N*-methyl glucamine salt of [2 (2-methyl-3-trifluoromethyl-amino) nicotinic acid], known as flunixin meglumine, has been shown to posses anti-inflammatory, anti-endotoxic and analgesic properties in sheep and horses (Chambers *et al.*, 1995; Welsh and Nolan, 1995). Its mechanism of action is based on inhibition of cyclo-oxygenase, which is involved in the production of pro-inflammatory mediators (Chambers *et al.*, 1995). Flunixin also inhibits pain through central mechanisms, involving μ-opioid and α2 adrenergic receptors (Chambers *et al.*,

1995). To the best of our knowledge, the effects of ASA and flunixin on nociception in testudines have not been reported. Higher dosages of either of these drugs caused general weakness and debility, which lasted for about seven days.

Like NSAIDs, the steroidal anti-inflammatory drugs, dexamethasone and hydrocortisone, had no statistically significant effects in the formalin and hot plate tests. On the basis of the above findings, it appears that NSAIDs and steroidal anti-inflammatory drugs have no value in the management of clinical pain in testudines. More investigations are required to examine the effects NSAIDs and steroidal anti-inflammatory drugs in a wide variety of reptiles, particularly testudines.

5.4 FIBER PROPORTIONS IN THE SENSORY BRANCHES OF THE TRIGEMINAL NERVE IN TESTUDINES

The trigeminal nerve is responsible for much of the somatosensory innervation of the face and head in vertebrates including man (Sessle, 2000 Sneddon, 2002; Lazarov, 2008). In mammals, birds, fishes and amphibians, unmyelinated fibers are distributed throughout the nerve (Sneddon, 2002; 2004), and comprise 4% in the trout, 50% in mammals, 5% in the frog and 0.7-1.2% in the stingray (Sneddon, 2002; Hamamoto and Simone, 2003). Histological studies have suggested the absence of umyelinated fibers in elasmobranches (Sneddon, 2002; 2004). In the present study, unmyelinated fibers were not seen in either of the nerve branches studied. However, the magnification at which the analysis was done could have limited the identification of such fibers, particularly if they are present in low numbers. Nerve fibers with diameters in the range for A-delta fibers were present in all the branches studied. The proportion of the small myelinated fibers in these sensory afferents was approximately 18-26%.

The majority of neurons in the trigeminal divisions of both animals were in diameter range of 5.5-10 μm. These could be equivalent to the A-beta fibers. The high proportion could have

evolved to facilitate tactile exploration of the environment. In mammals, the proportion of unmyelinated to myelinated fibers is usually lower in the trigeminal nerve compared to the spinal nerves, reflecting the importance of the trigeminal nerve in tactile exploration of the environment (Sessle, 2000). To obtain more information on the fiber types and proportions of nociceptive afferents in both the craniofacial and somatic afferents of testudines a detailed study is recommended. Electron microscopic studies are required to confirm the absence of unmyelinated fibers in the sensory branches of the trigeminal nerve, and further explore the fiber types in the spinal dorsal root ganglia. Electrophysiological studies can reveal the types of nociceptors, based on the differential conduction velocities and their roles in pain mechanisms. In addition, the distribution of opioid receptor subtypes and their involvement in pain regulation needs to be explored.

5.5 CONCLUSIONS

It is concluded that the formalin and the hot plate tests are reliable tests for studying nociceptive mechanisms in testudines. The nociceptive tests involving topical application of algogenic materials into the eye can also be used, but are limited by the retraction of the head into the shell. Like crocodiles, the marsh terrapin does not show a decrease in sensitivity to capsaicin after repeated capsaicin treatment. Testudines are responsive to opioid analgesia. In both the Speke-hinged tortoise and the marsh terrapin, the proportion of sensory afferents with diameters in range of nociceptive fibers in the trigeminal nerve is low.

Based on these findings, it is postulated that testudines possess a nociceptive system, but the proportion of their nociceptive afferents may be low. Being among the earliest vertebrates to evolutionary transit from living in water to living in the land, the shell might have been the main organ of protection against aversive environmental stimuli. The shell may thus be an alternative to a comprehensive nociceptive system.

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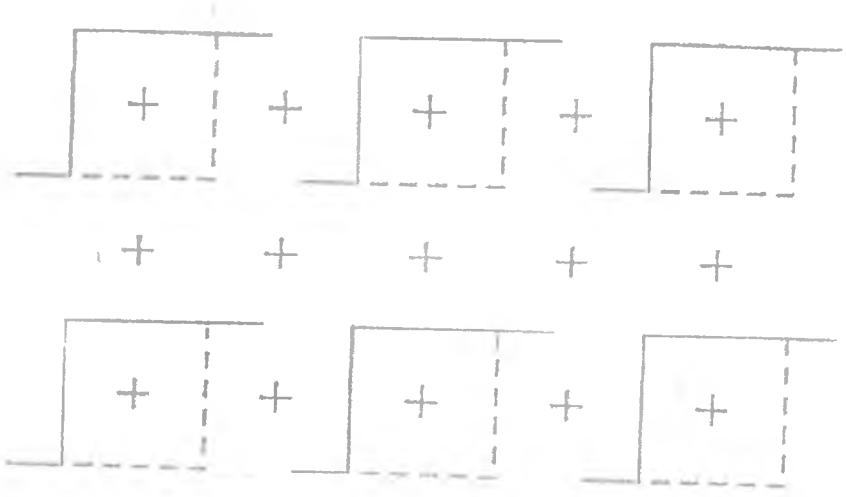
Appendix 1: The sampling grid



Appendix 2: The measuring tape