# Nociception and Antinociception in the Speke's Hingeback Tortoise (*Kinixys spekii*): Involvement of Cholinergic Mechanisms

**Joakim Dahlin**
Supervisors: Klas S.P. Abelson, Stanley N. Wambugu, Titus I. Kanui.

A collaboration between:
The Department of Neuroscience, Biomedical Centre, Uppsala University, Uppsala, Sweden
The Department of Animal Physiology and Anatomy, University of Nairobi, Nairobi, Kenya

## Abstract

Tortoises are widely used as domestic animals and therefore their pain mechanisms have to be understood. They are also interesting to study from a comparative biology point of view. In the present study, a method called the suspended formalin test – a variant of the normal formalin test – has been developed to study the pain in these animals. This method makes it possible to understand the pain response and to test different analgesic drugs in tortoises. In contrary to the formalin test in other animals, the study suggests that tortoises do not show differentiable acute and inflammatory pain phases. The muscarinic acetylcholine receptor agonist oxotremorine is shown to have an analgesic effect in doses of 200 µg/(kg bw) and 100 µg/(kg bw) on tortoises, and the higher dose in combination with the antagonist atropine 2.5 mg/(kg bw) increases the pain threshold even further. The nicotinic acetylcholine receptor agonist epibatidine did not affect the pain threshold in the doses studied, though it has an effect on the activity of the animal. The epibatidine also induces salivation, suggesting that it affects the muscarinic acetylcholine receptors. In summary, the pain mechanisms in tortoises are different from mammals, and therefore the tortoises have to be treated with customized analgesic drugs when pain is imminent.

## Introduction

The ability to detect a noxious event and experience pain is fundamental for survival of both animals and humans. When physical injury is imminent to a body part, pain can serve as a warning signal to avoid unnecessary stress to the certain area. Furthermore, pain can operate as a forewarning to escape or evade a possible danger. The International Association for the Study of Pain (IASP) defines pain as "An unpleasant and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. (…) Pain is always subjective. (…)" (IASP, 1979). Even though acute pain in many instances is vital, the ability to control and alleviate the sensation is important. The antinociceptive drugs and treatments available today are not always suitable or sufficient to alleviate persisting pain, and in some cases, they imply numerous adverse effects. Thus, research has to be done to further understand the physiological background of pain, as well as the modulation of pain in order to develop new more efficient pharmaceuticals.

Little research has been done on the study of pain in the order Chelonia – i.e. tortoises, terrapins and turtles – even though they are widely housed as domestic animals, moreover studied under laboratory and zoological conditions. To be able to determine and improve

the well-being of a Chelonian as well as for conservation purposes, more information about the perception of pain in these animals has to be attained. In addition, studying the biology of pain in non-mammalian species is an important stage for a further understanding of the development of the nociceptive system.

In the search for antinociceptive drugs, the role of acetylcholine and cholinergic receptors – subdivided into muscarinic and nicotinic receptors – has been evaluated during the last decades. In rodents, Abelson & Höglund (2002b) showed that an increase of spinal acetylcholine after intravenous administration of the antinociceptive muscarinic receptor agonist oxotremorine appears to be associated with antinociception. In contrary, atropine, which is a muscarinic receptor antagonist, appears to lower the acetylcholine and cause hyperalgesia (Abelson & Höglund, 2002b). However, the results are not unanimous, Ghelardini *et al* (1990) showed that atropine induces analgesia in doses ranging from 1 to 100 μg/kg and hyperalgesia when 5 mg/kg was administered. Furthermore, other antinociceptive drugs such as α2-adrenergic receptor agonists (Abelson & Höglund, 2004), lidocaine (Abelson & Höglund, 2002a), epibatidine (Kommalage & Höglund, 2004), serotonergic and γ-amino butyric acid (GABA) receptor agonists (Kommalage & Höglund, 2005a; Kommalage & Höglund, 2005b) increase the spinal acetylcholine release in rats. The connection between acetylcholine release and antinociception has also been studied in various other species such as sheep (Bouaziz *et al*, 1995; Bouaziz *et al*, 1996), cats (Yaksh *et al*, 1985) and humans (Flodmark & Wramner, 1945; Lambert & Appadu, 1995). Due to the fact that spinal acetylcholine appears to play an important role in the pain modulation at the spinal level, it has to be further investigated.

Systemic administration of the muscarinic agonist oxotremorine has been shown to provide antinociception in rats (Abelson & Höglund, 2002b; Capone *et al*, 1999). Adverse effects, such as salivation and tremors, are also well documented (Wang *et al,* 2004). Hence, the antinociceptive effects of oxotremorine will be evaluated in this study. Moreover, recently, it was shown that the opioid morphine produced analgesic effects in the red-eared slider turtle (Sladky *et al*, 2007). Though, epibatidine – acting through the nicotinic (Bonhaus *et al*, 1995) and partially through the muscarinic (Kommalage & Höglund, 2004) acetylcholine receptors – has been suggested to be a more potent analgesic agent than morphine (Spande *et al*, 1992; Sullivan *et al*, 1994). Therefore, the analgesic effect of epibatidine will be investigated.

It has been shown that oxotremorine induces salivation in rats and mice, and that the effect can be inhibited by the muscarinic antagonist atropine (Ögren *et al*, 1985). However, epibatidine has been suggested to not induce salivation in mice (Ellis *et al*, 1999). Though, (-)-epibatidine has analogs, 2-exo{5-(3-methyl-1,2,4-oxadiazolyl)}-[2.2.1.]-7-azabicycloheptane and 2-exo{5-(3-amino-1,2,4-oxadiazolyl)}-[2.2.1.]-7-azabicycloheptane denoted CMI-936 and CMI-1145 respectively, that act through the muscarinic acetylcholine receptors and induce salivation (Ellis *et al*, 1999). Furthermore, since Kommalage & Höglund (2004) suggests that epibatidine partly act through the muscarinic acetylcholine receptors, this fact will be considered when studying salivation after intracoelomic injection of the drugs into the tortoises.

The formalin test has previously been used to assess the pain response caused by injection of dilute formalin into the paw of the animal (Dubuisson & Dennis, 1977). In rats, the pain response caused by formalin, can be subdivided into a first immediate acute phase and a second inflammatory response. This first phase is c-fiber mediated and transpires during the

first five minutes, followed by an almost silent response up to the 20-30th minute (Hunskaar & Hole, 1986). Then, a second chronic phase occurs, caused by a peripheral inflammation and central sensitization. The formalin test has been used when assessing the analgesic effects of both oxotremorine (Capone *et al*, 1999) and epibatidine (Curzon *et al*, 1998) in rats.

The aim of this study is to develop a method for studying pain in tortoises, analyze the pain response of the formalin test and investigate if analgesic drugs used for mammals that act on muscarinic and nicotinic acetylcholine receptors – oxotremorine and epibatidine – can alleviate pain in reptiles as well. Additionally, if oxotremorine is analgesic, the effect after blocking the muscarinic receptors with the antagonist atropine, as well as blocking the opioid receptors with naloxone will be studied. Moreover, if epibatidine shows an analgesic effect, the result of blocking the nicotinic receptors with mecamylamine and the opioid receptors with naloxone will be investigated. In connection with the formalin test, any changes in the tortoises' physiology – explicitly occurrence of salivation, urination and defecation– will be evaluated.

## Materials and methods

All experiments were approved by the Kenyan Wildlife Society, KWS, before they were conducted.

### *Animals*

Twenty-four animals of Speke's Hinge-Back tortoise, *Kinixys spekii*, were used in the tests. They were collected from Machakos district, South-East of Nairobi, Kenya. The age ranged from 7 to 30 years on an estimate and the weights of the animals were 666 ± 22 g (S.E.M.). The tortoises were housed in metallic cages measuring 1.25 m × 0.9 m × 0.58 m with 22 cm of soil and stones from the Kenyan nature. Each cage contained a bigger stone as enrichment. The tortoises were kept under 12 hours of natural light and 12 hours of darkness (6.30 am – 6.30 pm), high relative humidity and temperature 22-28°C. The tortoises were labeled with a permanent marker on the scutes on the carapace and the plastron. All tortoises were bathed, washed and weighed weekly.

Food was provided three to four times per week for six hours, and consisted of fresh, thinly sliced carrots, cabbages, tomatoes and Kikuyu grass (*Pennisetum clandestinum*). Tap water, which was changed daily, was provided *ad libitum*.

### *Experimental setup*

All tortoises were acclimatized to the new environment for one month before experimentation. During this period and during the experiments the tortoises were handled daily.

Preliminary tests revealed that intradermal injection of formalin was unreliable since the formalin had a tendency to leak out of the solid dermis. Therefore, as done in the crocodile (Kanui *et al*, 1990), a subcutaneous injection of formalin was administered. To use the formalin test where the animal was free to move appeared inadequate since the animal in many cases was passive, completely retracted into the shell, and consequently it was not possible to score any pain behaviors. Thus, the animal was suspended with a thin cotton

string (ARTCCOT, CT-613) around the shell and the string attached to a metallic stand. The tortoises were suspended horizontally and the head and the limbs were allowed to move freely according to figure 8 (appendix).

When in pain the tortoises showed the following behaviors while in motion and they were given a pain score with 0 as minimum and 5 as maximum:

| | |
|---|---|
| Moving all limbs except the injected limb. | 5 |
| Moving all limbs, but only the injected limb carefully. | 2.5 |
| Moving all limbs normally. | 0 |

While not in motion the following behaviors were observed and scored as followed:

| | |
|---|---|
| The injected limb was completely retracted. | 5 |
| The injected limb was almost completely retracted. | 3.75 |
| The injected limb was half-out. | 2.5 |
| The injected limb was almost completely out. | 1.25 |
| The injected limb was completely out. | 0 |

Each position of the foot is depicted in figure 9 to 13 in appendix. The point in time when a behavior was changed was recorded with the given behavior. Also, behaviors such as urination, defecation, salivation and any behavior deviating from the ordinary were documented. Preliminary test suggested that 60 minutes was an appropriate time for studying the pain of the animals. The initiation of the observation period ($t = 0$) was set to the point in time when the formalin injection was done.

The experiments were done between 10 am to no later than 17 pm. A resting period of nine days was given between two consecutive experiments in different paws and 24 days between experiments in a previously used paw.

*Drugs and injections*

The following drugs were used: Oxotremorine sesquifumurate salt (Sigma), (±)-Epibatidine dihydrochloride (Sigma), Atropine sulfate salt (Sigma), Naloxone hydrochloride dehydrate (Sigma), Formaldehyde 40 % (Merck). All drugs were dissolved in 0.9 % saline solution. The drugs, all except formalin, were injected intracoelomically under the loose skin of the tortoise 30 minutes prior to the injection of formalin in accordance with figure 6 in appendix. The dose volume was equivalent to 500 µl/(kg body weight) and injected with a 27 gauge needle (Terumo, Neolus). The control group was given an equivalent volume of 0.9 % saline solution. 5 minutes before formalin injection, the tortoise was suspended. Then the 10 % formal saline was injected with a 28½ gauge needle (U-100 Insulin Syringe) in a volume of 100 µl s.c. into one of the hind paws between digit IV and V (note that the first digit is absent in *Kinixys Spekii*) (Crumly & Sánchez-Villagra, 2004) as shown in figure 7 in appendix.

The drugs were administered in doses estimated from doses known to produce a significant effect in rats according to the formula

$$X_1 = X_2 \cdot \frac{W_1^{-0.25}}{W_2^{-0.25}}$$ (Hau & Poulsen, 1988)

where $X_1$ is the demanded dose in the tortoise, $X_2$ is the dose used in rats, $W_1$ is the weight of the tortoise, approximately 650 g, and $W_2$ is the weight of the rats, about 400 g. The doses determined were preferably set low, since the effects of the drugs have not been studied in tortoises. The doses of oxotremorine approximately corresponds to the average doses used by Abelson & Höglund (2002b) and the doses of atropine was established from Ghelardini *et al* (1990) and Abelson & Höglund (2002b). The oxotremorine doses tested in this study were 200 µg/(kg bw) and 100 µg/(kg bw) and the doses of the atropine were set to 2.5 mg/(kg bw) and 1.0 mg/(kg bw). The epibatidine doses were established from Seppä & Ahtee (2000) and selected to 5 µg/(kg bw) and 2.5 µg/(kg bw). 5 mg/(kg bw) and 2.5 mg/(kg bw) were set for naloxone based on the study of Fukazawa *et al* (2005). The dose of mecamylamine was related to the dose used in mice in the research by Yue *et al* (2007), however, the formula is changed to

$$X_1 = X_2 \cdot \frac{W_1^{-0.5}}{W_2^{-0.5}}$$ (Hau & Poulsen, 1988)

when the animal weights less than 100 g, in mice that is approximately 20 g. The doses 50 µg/(kg bw) and 100 µg/(kg bw) were therefore established.

The tests were blinded, and therefore, the person injecting and observing the tortoises, did not know which substance that was being injected.

*Statistics*

The data was analyzed with a one-way analysis of variance (ANOVA) with a two-sided Dunnett's post-hoc test using SPSS 11.5. When studying salivation, defecation and urination the two-sided Fisher's exact test was used. *P*-values lower than 0.05 were considered significant.

## Results

*The suspended formalin test, test I.*

The pain score was studied in blocks of 10 minutes using a time average of each interval. Figure 1 shows the pain score of the suspended formalin test using 10 % formal saline injected subcutaneously into the hind paw of the animal and 0.9 % physiological saline injected intracoelomically 30 minutes before. The second interval shows a significantly lower pain score than during the first ten minutes. The pain score of the third interval is significantly lower than both the first and the second interval. Hereafter, the pain score of one interval is not significantly different from the next, though the pain scores after the 20-30 minute block are all significantly different from the first and the second interval.

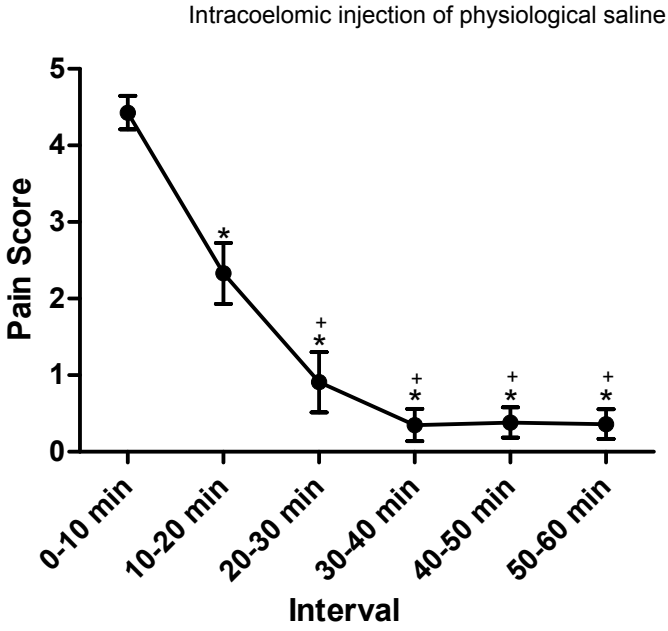


Fig. 1. The suspended formalin test performed in tortoises (n = 12). Minimum pain = 0. Maximum pain = 5. The pain score is a mean value ± S.E.M. in blocks of 10 minutes up to 60 minutes after formalin 10 % injection. * represents P<0.00001 from the first interval according to Dunnett's post-hoc test. + represents P<0.0001 compared to the second interval according to Dunnett's post-hoc test.

*The suspended formalin test with possible analgesic drugs, test I*

The suspended formalin test was performed together with an intracoelomic administration of oxotremorine in doses of 200 µg/(kg bw) and 100 µg/(kg bw), and epibatidine in doses of 5 µg/(kg bw) and 2.5 µg/(kg bw) according to figure 2. They are compared to a negative control, where each animal was injected with 0.9 % physiological saline. The pain scores of the oxotremorine treated groups of both concentrations are significantly lower than the control in the first ten minute interval. Thereafter, there is no significant difference. In the epibatidine treated group, a trend can be seen that the drug causes some analgesia, though the difference with the control is not significant during the first interval. The pain score of the epibatidine 5 µg/(kg bw) treated group is higher in the sixth interval compared to the control.

Figure 3a shows the time integral of the pain score for each drug and concentration during the entire experiment. There was no significant difference between the control group given physiological saline and any of the oxotremorine and epibatidine treated groups. Figure 3b indicates the activity of the animal, defined as the time when the animal was moving in the air. The movement of the animals was clearly reduced in the oxotremorine and epibatidine treated groups compared to the control group injected with physiological saline.

After injection of 200 µg/(kg bw) and 100 µg/(kg bw) oxotremorine and 5 µg/(kg bw) and 2.5 µg/(kg bw) epibatidine respectively, some tortoises began salivating. This occurred in a significantly higher number of cases, according to Fisher's exact test, than in the control group treated with physiological saline where salivation was not present in any case. The 200 µg/(kg bw) oxotremorine treated group defecated in four out of six cases and the control in two out of twelve, suggesting a tendency that defecation is stimulated by oxotremorine, though this was not significant ($P = 0.11$). No conclusions could be drawn from the occurrence of urination.

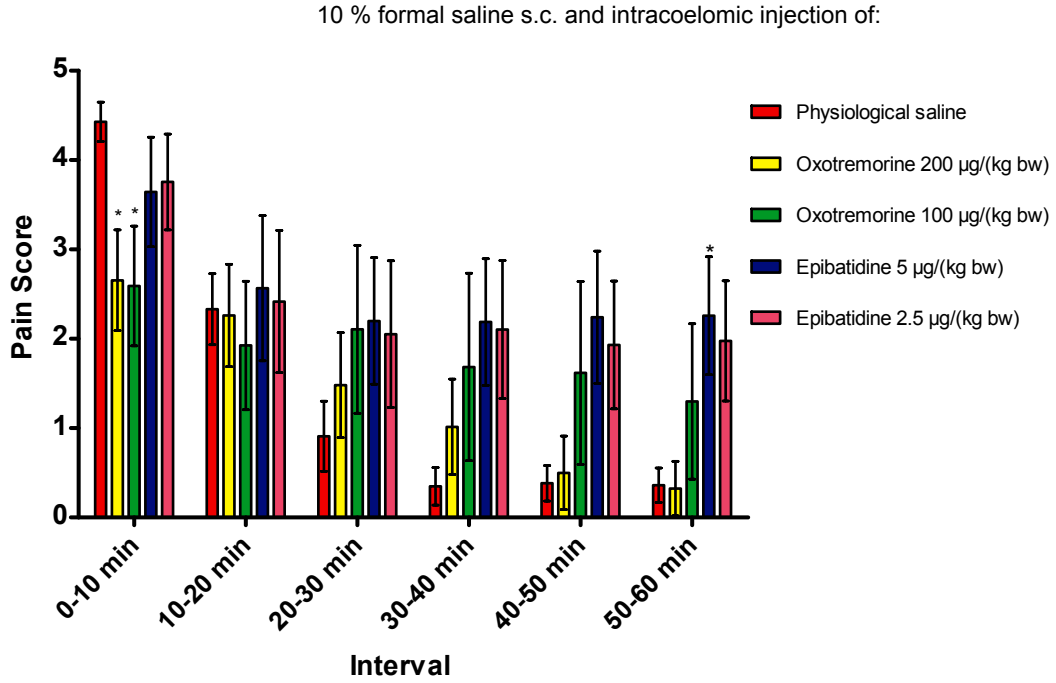


Fig. 2. The diagram illustrates the drugs' effect on pain score in the suspended formalin test. The pain score is indicated as a mean value ± S.E.M. The control consists of a group treated with physiological saline 0.9 % , number of experiments n = 12. The drugs tested are oxotremorine 200 µg/(kg bw), n = 6. Oxotremorine 100 µg/(kg bw), n = 6. Epibatidine 5 µg/(kg bw), n = 6. Epibatidine 2.5 µg/(kg bw), n = 6. * represents P<0.05 from the control according to Dunnett's post-hoc test.

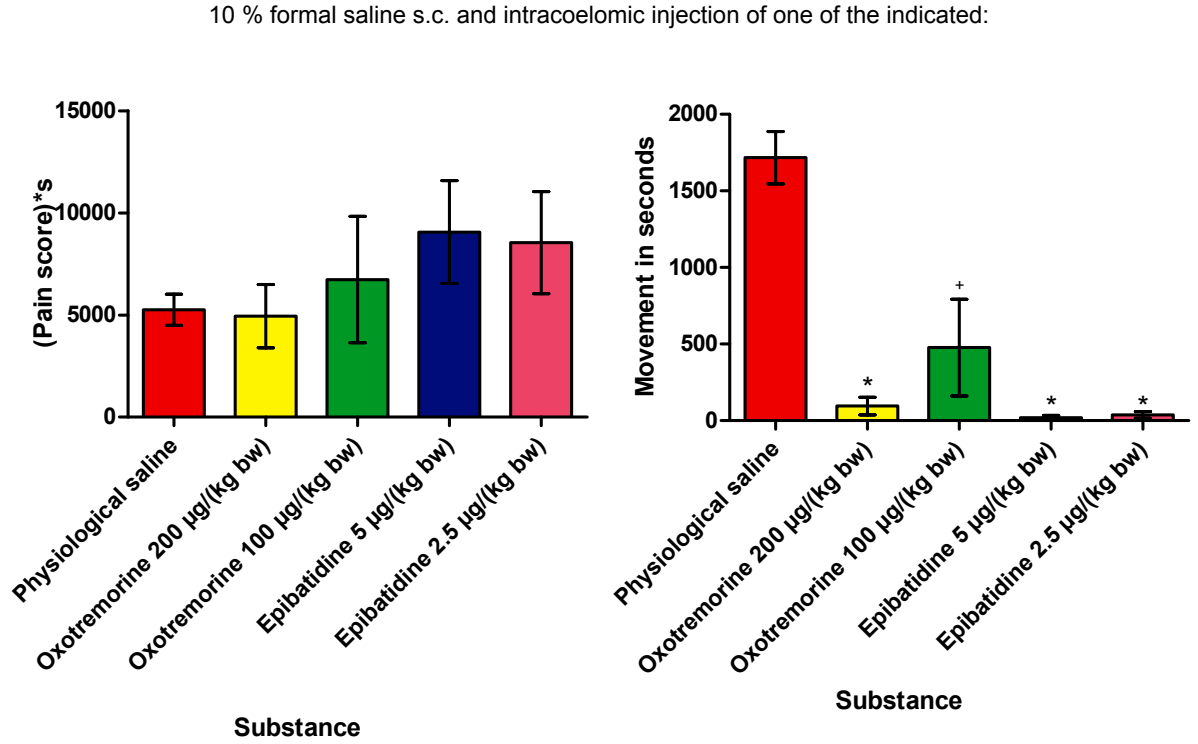


Fig. 3a. The drugs' effect on the time integral of the pain score in the suspended formalin test. The time integral is indicated as the mean of the product of the pain score and the number of seconds ± S.E.M. There is no significant difference between the drugs used, determined by Dunnett's post-hoc test. 3b. The drugs effect on the mean movement in seconds ± S.E.M. * represents P<0.00005 from the control group with physiological saline as determined by Dunnett's post-hoc test.

*The suspended formalin test with an analgesic drug and a possible antagonist, test II*

The suspended formalin test was done with oxotremorine 200 µg/(kg bw) to provide analgesia, together with a drug blocking either the muscarinic or opioid receptors, namely atropine and naloxone respectively. The particular concentration of oxotremorine was chosen since it provided the most considerable effect on lowering the pain and movement. Together with the oxotremorine, atropine was administered in a dose of 2.5 mg/(kg bw) and 1 mg/(kg bw), and naloxone was given in doses of 5 mg/(kg bw) and 2.5 mg/(kg bw). Oxotremorine with 0.9 % physiological saline was used as control. During the second interval, atropine caused analgesia compared to the control group according to figure 4. Furthermore, there was a clear trend that atropine induced analgesia in both concentrations during the entire experiment.

The total pain was lowered considerably in the higher dose of the atropine treated group. However, none of the substances gave a significant variation from the control ($P = 0.12$) (fig. 5a). Nevertheless, the atropine 2.5 mg/(kg bw) treated group reversed the effect of oxotremorine considering the activity of the animal, i.e. the movement in seconds raised significantly, whereas the lower concentration of the atropine and the naloxone did not provide any different results from the control group (fig. 5b). Noteworthy, the 2.5 mg/(kg bw) atropine treated group did neither salivate, defecate nor urinate during any of the trials, even though this was not significantly different from the saline control. Considering the heavy salivation in some of the tests, any animal treated with oxotremorine hereafter was given atropine in a dose depending on the severity of the effects of the oxotremorine to improve recovery. The effects were reversed within 30 minutes in all cases. Atropine in a dose of 1 mg/(kg bw) and naloxone in any of the doses tested did not show any reversing effects.

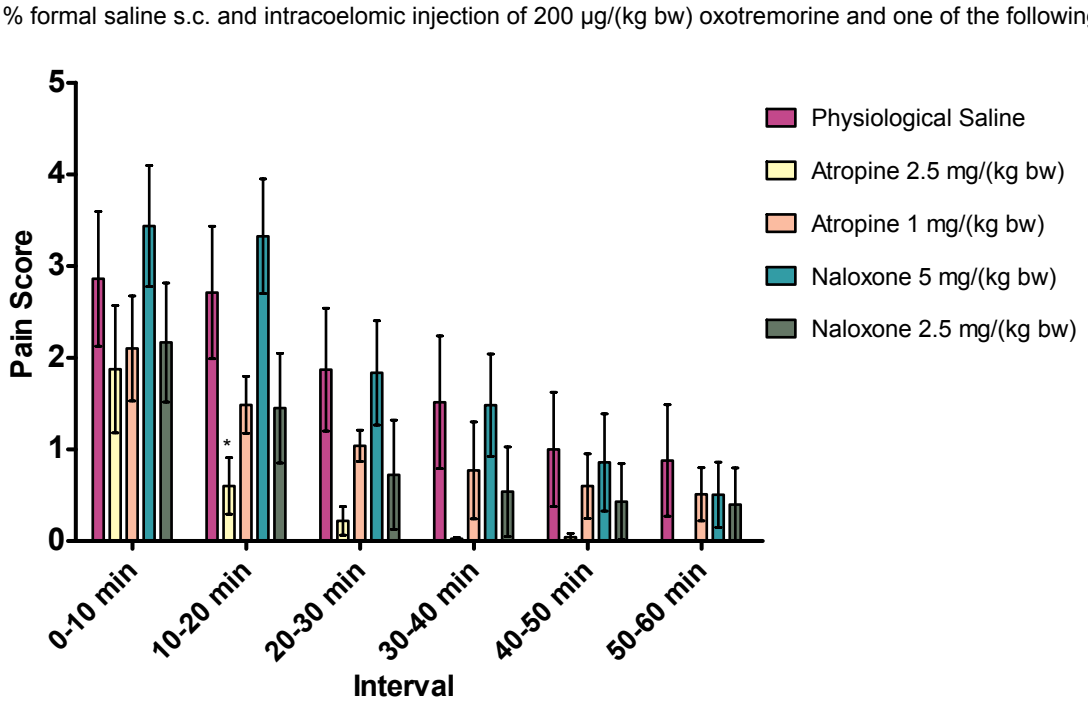


Fig. 4. The diagram illustrates the effect of 200 µg/(kg bw) oxotremorine and its antagonists on pain score in the suspended formalin test. The pain score is a mean value ± S.E.M. The control consists of a group treated with the oxotremorine and physiological saline 0.9 %, number of experiments n = 6. The antagonists used were: Atropine 2.5 mg/(kg bw), n = 6. Atropine 1 mg/(kg bw), n = 6. Naloxone 5 mg/(kg bw), n = 6. Naloxone 2.5 mg/(kg bw), n = 6.* represents $P<0.05$ from the control according to Dunnett's post-hoc test.

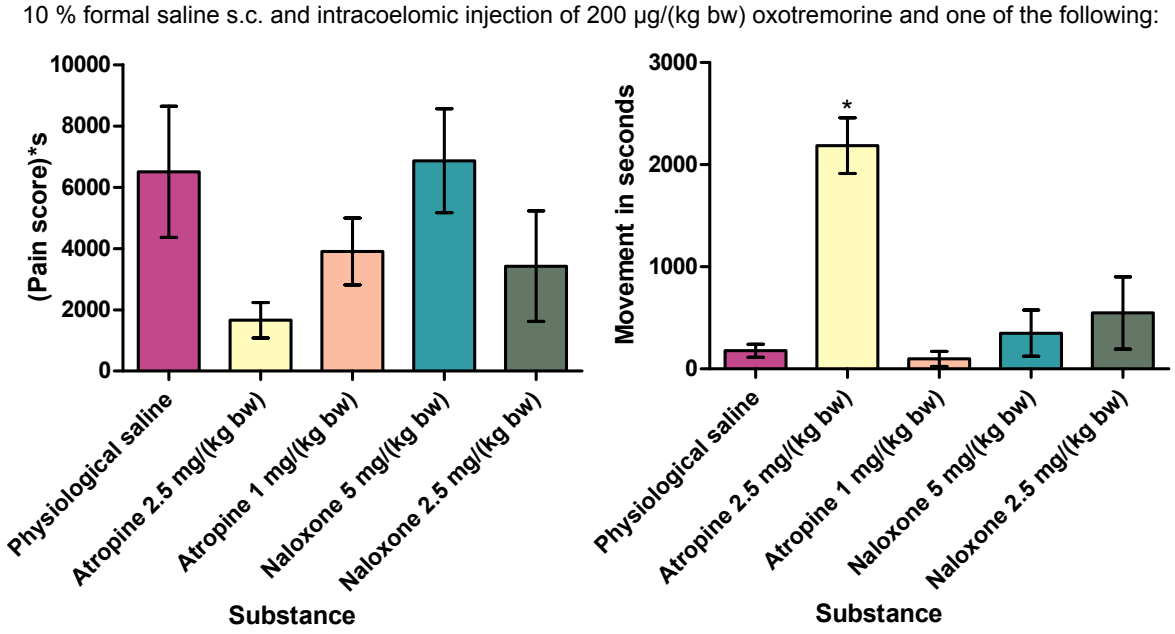


Fig. 5a. The antagonists' effect on the time integral of the pain score in the suspended formalin test with 200 µg/(kg bw) oxotremorine. The time integral is indicated as the mean of the product between the pain score and the number of seconds ± S.E.M. There is no significant difference between the antagonists used, determined by Dunnett's post-hoc test. 5b. The antagonists' effect on the mean movement in seconds ± S.E.M. * represents $P<0.00005$ from the control group with physiological saline as determined by Dunnett's post-hoc test.

## Discussion

The present study showed that the formalin test with suspended animals seems to be a good method for assessing pain in tortoises. The suspended formalin test in tortoises showed no indication of increased pain after the diminishing of the first acute phase. Though, this first and only phase dominated during the first 10 minutes and lasted for approximately 20 minutes. The second inflammatory phase – starting around minute 20-30 – that is evident in rats and mice was not detectable in tortoises. Nonetheless, it remains to be investigated if the inflammatory phase coincides with the acute, if the inflammation is not sufficiently severe to cause pain, or if the inflammatory phase actually does not exist.

The suspended formalin test with muscarinic and nicotinic acetylcholine receptor agonists showed that oxotremorine provided analgesia, predominantly during the first ten minutes, with no significant difference thereafter. Whether the short duration was due to too early administration of the drug, short half-life of the drug in the body or because of the efficiency of the substance is presently unclear. Epibatidine also showed a slight but not significant decrease of pain score during the first ten minutes. However, the pain score during the late phase of the experiments were higher than the control. Why epibatidine had this effect is still unknown. Since the epibatidine did not provide analgesia, the antagonists of the drug were not tested.

To test whether the drugs had any effects other than pain related on the animals, the movement in seconds was recorded. In the first test, it was shown that the drugs oxotremorine and epibatidine had a tranquilizing effect compared to the control. Moreover, they induced salivation. The fact that epibatidine induced salivation in tortoises further suggests that

epibatidine act partly through the muscarinic acetylcholine receptor, even though the salivatory effects are opposite to the effects seen in mice (Ellis *et al*, 1999). This might depend on evolutionary structure of the muscarinic and nicotinic acetylcholine receptors or the dose administered in mice. However, this has to be studied further.

In the second test, the high dose of atropine reversed the effect of the 200 μg/(kg bw) oxotremorine concerning movement, taking the values back to normal. The 2.5 mg/(kg bw) atropine also completely inhibited secretion of any body fluid, in other words it completely prevented salivation, urination and defecation in all treated tortoises, though this could not be shown to be significantly different from the physiological saline treated control group according to Fisher's exact test. Naloxone on the other hand did not reverse any effects of the oxotremorine.

In the suspended formalin test with an antagonist to the muscarinic receptor agonist oxotremorine, atropine administration led to significantly lower pain threshold than the group treated with only oxotremorine and saline solution during the interval 10-20 minutes, and apparently, the mean pain score was lower during the whole experiment. This was in accordance with Ghelardini *et al* (1990) who saw that atropine in low doses produced analgesia in rats. Conversely, it was opposite to the results of Abelson & Höglund, who indicated that atropine produced hyperalgesia in rats in both high and low doses (2002). This might suggest that different strains, species and animals belonging to different classes respond differently to the atropine treatment.

The total pain during the test is defined as the time integral of the pain score i.e. the product of pain score and the time in seconds that the experiments is performed. The total pain in the first set of experiments was not significantly different between the different substances. In the second set, it could be suggested that the atropine treated group showed less pain than the control and the higher dose provided more analgesia, nevertheless the differences were not significant.

The variability of the pain response between different animals was considerable, probably since the tortoises were collected from the wild, thus they were of different age, sex and size. Consequently, significant differences between different trials were hard to achieve, suggesting that bred tortoises could be a good alternative to reduce the variations and therefore attaining more consistent results.

In summary, the suspended formalin test may be used to assess pain and evaluate analgesic drugs in tortoises. The tortoises did not show the characteristic biphasic nociceptive response seen in numerous mammals. Among the drugs tested in the tortoises, the muscarinic agonist oxotremorine 200 μg/(kg bw) in combination with the muscarinic antagonist atropine 2.5 mg/(kg bw) provided the greatest increase in pain threshold. The epibatidine did not provide analgesia in the doses administered. Nonetheless, the occurrences of salivation after injection of epibatidine might suggest that the acetylcholine receptors in tortoises are different from those in mammals, though this needs to be further investigated. This study not only presents information about how to test analgesic drug candidates customized for tortoises, but also provides more insight into the understanding of the nociceptive system in general.

## References


Abelson, K.S.P. & A.U. Höglund: Intravenously administered lidocaine in therapeutic doses increases the intraspinal release of acetylcholine in rats. *Neurosci. Lett.* 2002a, **317**, 93-96.

Abelson, K.S.P., & A.U. Höglund: Intravenously administered oxotremorine and atropine, in doses known to affect pain threshold, affect the intraspinal release of acetylcholine in rats. *Pharmacology & Toxicology* 2002b, **90**, 187-192.

Abelson, K.S.P., & A.U. Höglund: The effects of the $\alpha_2$-adrenergic receptor agonists clonidine and rilmenidine, and antagonists yohimbine and efaroxan, on the spinal cholinergic receptor system in the rat. *Basic Clin. Pharmacol. Toxicol.* 2004, **94**, 153-160.

Bonhaus, D. W., K. R. Bley, C. A. Broka, D. J. Fontana, E. Leung, R. Lewis, A. Shieh & E. H. Wong: Characterization of the electrophysiological, biochemical and behavioral actions of depibatidine. *J. Pharmacol. Exp. Therap.* 1995, **272**, 1199–1203.

Bouaziz, H., C. Tong & J.C. Eisenach: Post-operative analgesia from intrathecal neostigmine in sheep. *Anesth. Analg.* 1995, **80**, 1140-1144.

Bouaziz, H., C. Tong, Y. Yoon, D.D. Hood and J.C. Eisenach: Intravenous opioids stimulate norepinephrine and acetylcholine release in spinal cord dorsal horn: Systematic studies in sheep and an observation in a human. *Anesthesiology* 1996, **84**, 143-154.

Capone, F., A.M. Aloisi, C. Giancarlo, P. Sacardote & F. Pavone: Oxotremorine-induved modifications of the behavioural and neuroendocrine responses to formalin pain in male rats. *Bran Res.* 1999, **830**, 292-300.

Curzon, P., A.L. Nikkel, A.W. Bannon, S.P. Arneric & M.W. Decker: Differences between the antinociceptive effects of the cholinergic channel activators A-85380 and (±)-epibatidine in rats. *J. Pharmacol. Exp. Ther.* 1998, **287**, 847-853.

Crumly, R.S & M.R. Sánchez-Villagra: Patterns of variation in the phalangeal formulae of land tortoises (Testudinidae): Developmental constraint, size and phylogenetic history. *Journal of experimental zoology* 2004, **302**, 134-146.

Dubuisson, D. & S.G. Dennis: The formalin test: A quantative study of the analgesic effects of morphine, meperidine, and brain stem stimulation in rats and cats. *Pain* 1977, **4**, 161-174.

Ellis J.L., D. Harman, J. Gonzalez, M.L. Spera, R Liu, T.Y. Shen, D.M. Wypij & F Zuo: Development of muscarinic analgesics derived from epibatidine: role of the M4 receptor subtype. *J. Pharmacol. Exp. Ther.* 1999, **288**, 1143-1150.

Flodmark, S. & T. Wramner: The analgesic action of morphine, eserine and prostigmine studied by a modified Handy-Wolff Goodell method. *Acta physiol. scand.* 1945, **9**, 88-96.

Fukazawa Y., T. Maeda, W. Hamabe, K. Kumamoto, Y. Gao, C. Yamamoto, M. Ozaki & S. Kishioka: Activation of spinal anti-analgesic system following electroacupuncture stimulation

in rats. *J. Pharmacol. Sci.* 2005, **99**, 408-414.

Gheraldini, C., P. Malmberg-Aiello, A. Giotti, M. Malcangio & A. Bartolini: Investigation into atropine-induced antinociception. *Brit. J. Pharmacol.* 1990, **101**, 49-54.

Hau, P. & O.M. Poulsen: Doses for laboratory animals based on metabolic rate. *Scand. J. Lab. Anim. Sci.* 1988, **15**, 81-83.

Hunskaar, S. & K. Hole. The formalin test in mice: dissociation between inflammatory and non-inflammatory pain. *Pain* 1987, **30**, 103-114.

IASP: Pain terms: a list with definitions and notes on usage. Recommended by an IASP subcommittee on taxonomy. *Pain* 1979, **6**, 249-252.

Kanui T.I., K. Hole & J.O. Miaron: Nociception in crocodiles: Capsaicin instillation, formalin and hot plate tests. *Zoo. Sci.* 1990, **7**, 537-54.

Kommalage, M. & A.U. Höglund: (±) Epibatidine increases acetylcholine release partly through an action on muscarinic receptors. *Basic & Clinical Pharmacology & Toxicology* 2004, **94**, 238–244.

Kommalage, M. & A.U. Höglund: Involvement of spinal GABA receptors in the regulation of intraspinal acetylcholine release. *Eur. J. Pharmacol.* 2005a, **525**, 69-73.

Kommalage, M. & A.U. Höglund: Involvement of spinal serotonin receptors in the regulation of intraspinal acetylcholine release. *Eur. J. Pharmacol.* 2005b, **509**, 127-134.

Lambert, D.G. & B.L. Appadu: Muscarinic receptor subtypes: Do they have a role in clinical anaesthesia? *Br. J. Anaesth.* 1995, **74**, 497-499.

Seppä, T., & L. Ahtee: Comparison of the effects of epibatidine and nicotine on the output of dopamine in the dorsal and ventral striatum of freely-moving rats. *Naunyn-Schmiedeberg's Arch. Pharmacol.* 2000, **362**, 444–447.

Sladky K.K., V. Miletic, J. Paul-Murphy, M.E. Kinney, R.K. Dallwig & S.M. Johnson: Analgesic efficacy and respiratory effects of butorphanol and morphine in turtles. *J. Am. Vet. Med. Assoc.* 2007, **230**, 1356-62.

Spande, T.F., H.M. Garraffo, M.W. Edward, H.J.C. Yeh, L. Pannel & J.W. Daly: Epibatidine: A novel (chloropyridyl)azabicycloheptane with potent analgesic activity from an Ecuadoran poison frog. *J. Amer. Chem. Soc.* 1992, **114**, 3475–3478.

Sullivan, J.P., M.W. Decker, J.D. Brioni, D. Donnelly Roberts, D.J. Anderson, A.W. Bannon, C.H. Kang, P. Adams, M. Piattoni Kaplan, M.J. Buckley, M. Gopalakrishnan, M. Williams & S.P. Arneric: (±)-Epibatidine elicits a diversity of in vitro and in vivo effects mediated by nicotinic acetylcholine receptors. *J. Pharmacol. Exp. Therap.* 1994, **271**, 624–631.

Wang, N., A. Orr-Urtreger, J. Chapman, R. Rabinowitz & A.D. Korczyn. Nicotinic acetylcholine receptor 5 subunits modulate oxotremorine-induced salivation and tremor. *J. Neurol. Sci.* 2004, **222**, 87–91.

Yaksh, T.L., R. Dirksen & G.J. Harty: Antinociceptive effects of intrathecally injected cholinomimetic drugs in the rat and cat. *Eur. J.Pharmacol.* 1985, **117**, 81-88.

Yue, C-Q., J. Ye, C-L. Li, R-T. Li & Q Sun: Antinociceptive effects of the novel spirocyclopiperazinium salt compound LXM-10 in mice. *Pharmacol. Biochem. Behav.* 2007, **86**, 643-650.

Ögren, S.O., S. Carlsson & T. BartFai: Serotonergic potentiation of musearinic agonist evoked tremor and salivation in rat and mouse. *Psychopharmacology* 1985, **86**, 258-264.

Appendix

Fig. 6. Shows an intracoelomic injection of a drug.

Fig. 7. Shows a subcutaneous injection of formalin

Fig. 8. Suspended tortoise

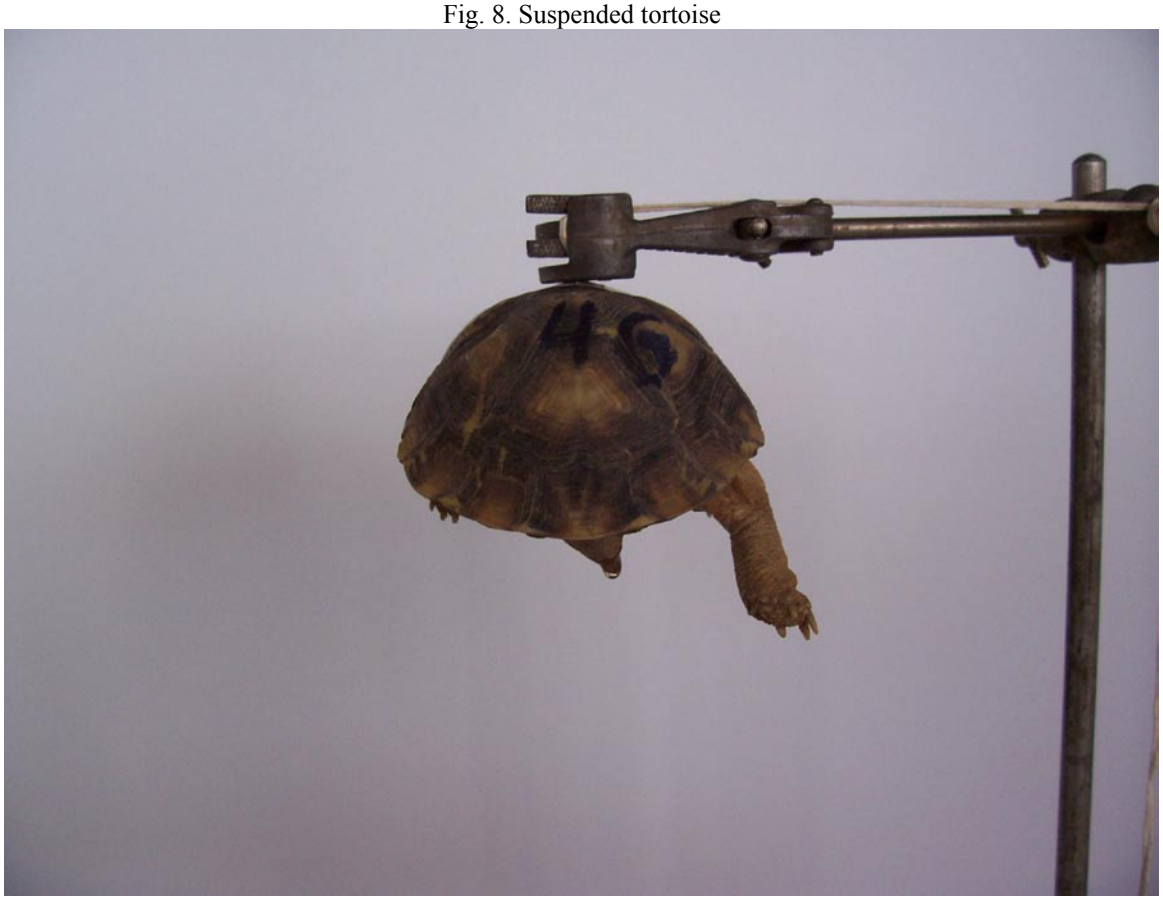

Fig. 9. Left rear limb completely retracted, right rear limb completely out.

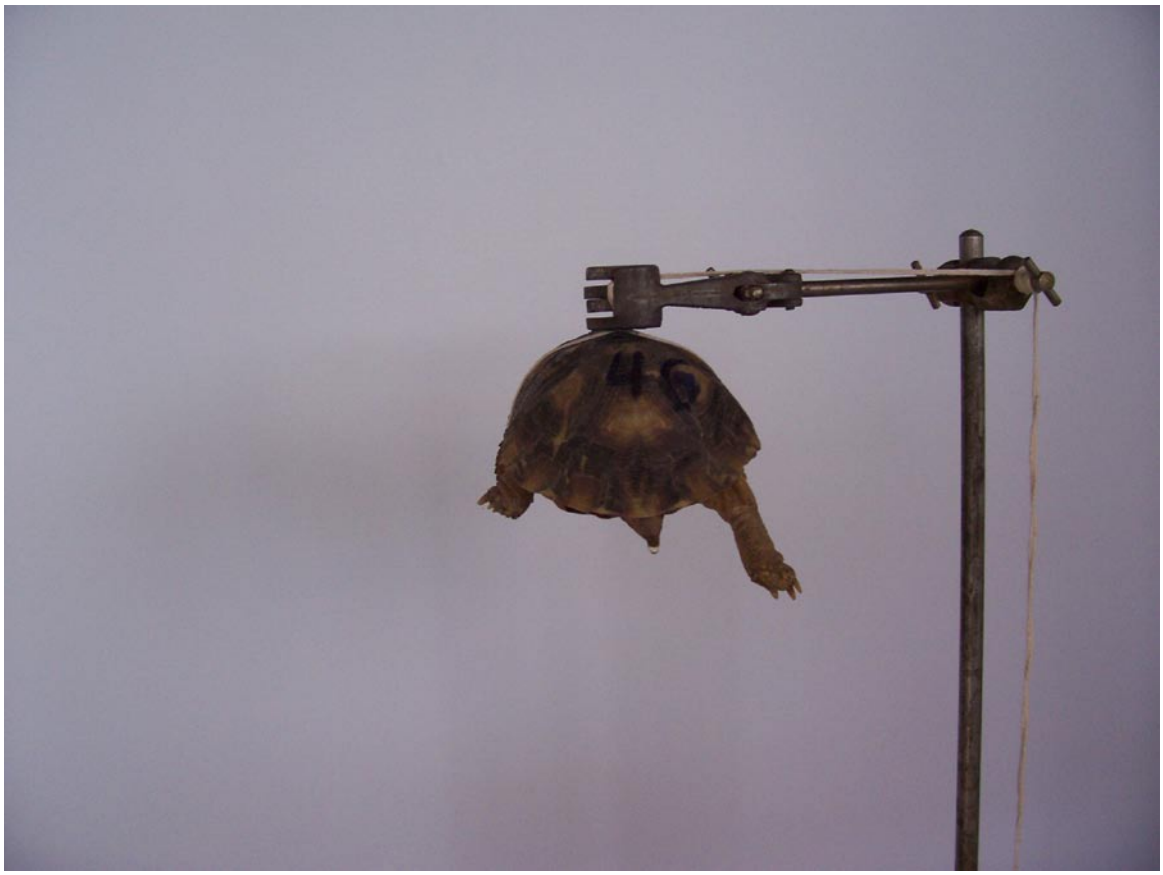

Fig. 10. Left hind limb almost completely retracted, right hind limb completely out.

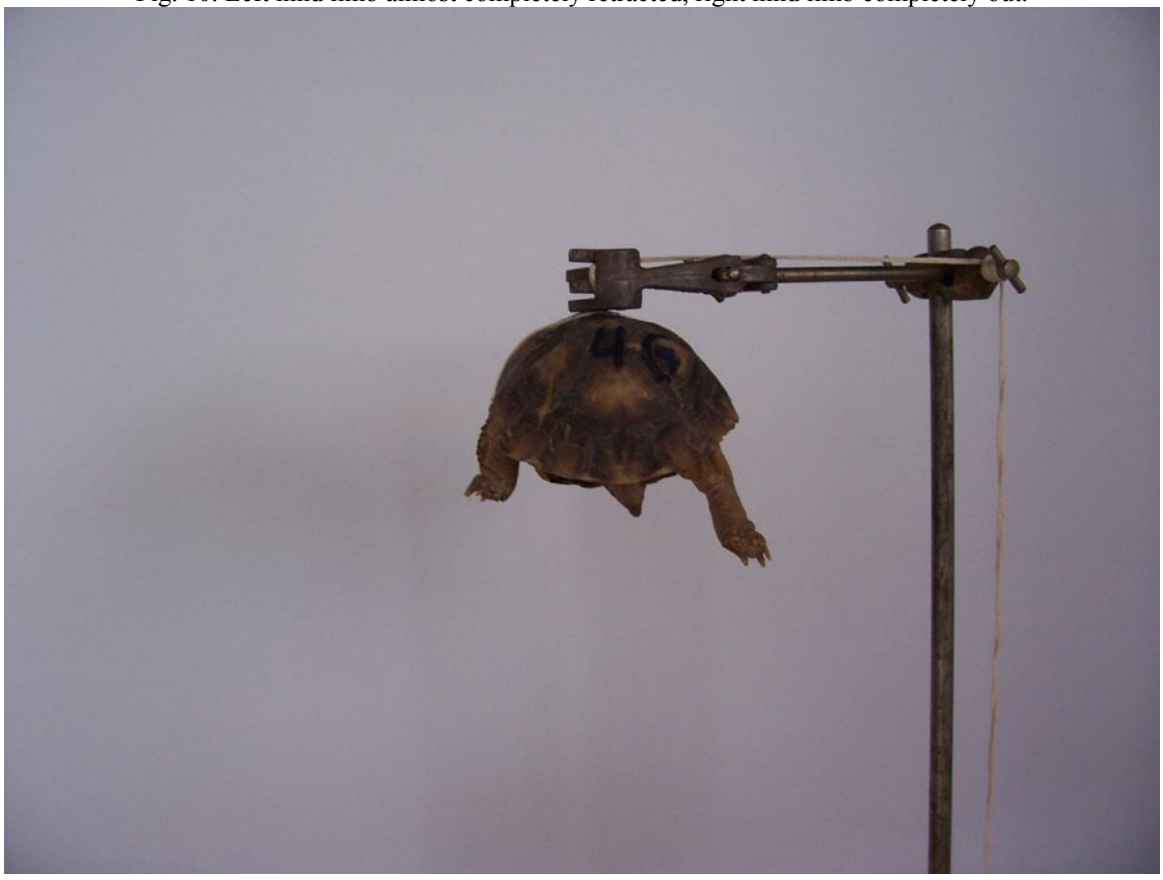

Fig. 11. Left hind limb half-out, right hind limb completely out.

Fig. 12. Left hind limb almost completely out, right hind limb completely out.

Fig. 13. All limbs completely out