ภาวะสองรูปแบบทางเพศและวงรอบการสืบพันธุ์ในรอบปีของตะพาบน้ำ

Amyda cartilaginea

นายนพดล กิตนะ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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SEXUAL DIMORPHISM AND ANNUAL REPRODUCTIVE CYCLE OF THE COMMON ASIATIC SOFTSHELL TURTLE *Amyda cartilaginea*

Mr. Noppadon Kitana

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Zoology
Department of Biology
Graduate School
Chulalongkorn University
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Thesis Title	Sexual dimorphism and annual reproductive cycle of the common asiatic softshell turtle *Amyda cartilaginea*
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วงรอบการสืบพันธุ์ในรอบปีพิจารณาจากการเปลี่ยนแปลงระดับฮอร์โมนเทสโทสเตอโรน อีสตราไดออล และโปรเจสเตอโรนในเลือด ซึ่งตรวจสอบด้วยเทคนิคเรดิโออิมมิวโนแอสเสย์ตามวิธีการขององค์การอนามัยโลก พบว่าตะพาบน้ำทั้งสองเพศมีวงรอบการสืบพันธุ์เป็นฤดูกาล โดยเพศผู้มีระดับเทสโทสเตอโรนสูงในช่วงก่อนฤดูวางไข่ ส่วนเพศเมียมีระดับอีสตราไดออลสูงในช่วงก่อนฤดูวางไข่ และมีระดับโปรเจสเตอโรนสูงทั้งในช่วงก่อนฤดูวางไข่ และในช่วงฤดูวางไข่ อีสตราไดออลและโปรเจสเตอโรนในเพศผู้ และเทสโทสเตอโรนในเพศเมียอยู่ในระดับที่สามารถตรวจสอบได้ตลอดทั้งปี แสดงให้เห็นถึงหน้าที่ของกลุ่มฮอร์โมนข้ามเพศในตะพาบน้ำ นอกจากนี้ยังพบว่าระดับอีสตราไดออลของทั้งสองเพศ และระดับโปรเจสเตอโรนของเพศผู้ สอดคล้องกับระดับอุณหภูมิของกรุงเทพมหานครอย่างมีนัยสำคัญ ($p<0.05$) จากผลการศึกษาแสดงให้เห็นว่าตะพาบน้ำมีวงรอบการสืบพันธุ์แบบก่อนผสมพันธุ์ โดยระดับฮอร์โมนเพศจะเพิ่มสูงในช่วงก่อนฤดูกาลสืบพันธุ์จากนั้นจะลดลงสู่ระดับต่ำตลอดทั้งปี

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

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NOPPADON KITANA: SEXUAL DIMORPHISM AND ANNUAL REPRODUCTIVE CYCLE OF THE COMMON ASIATIC SOFTSHELL TURTLE *Amyda cartilaginea*. THESIS ADVISOR: ASSOC. PROF. VITHAYA YODYINGYUAD, Ph.D. THESIS CO-ADVISOR: ASSIST. PROF. KUMTHORN THIRAKHUPT, Ph.D. 105 pp. ISBN 974-638-784-7.

Sexual dimorphism and annual reproductive cycles of mature male and female common Asiatic softshell turtles were studied in a captive population of *Amyda cartilaginea* maintained under semi-natural conditions at a temple pond at Prayurawongsawas temple, Bangkok, Thailand. The study was carried out from October 1996 to September 1997.

Sexual dimorphism was determined from mean comparison of parameters of morphological characters and regression analysis of plots of morphological characters. It was found that this softshell turtle species exhibited significant differences ($p<0.05$) in various parameters of morphological characters. Several of these sexually dimorphic traits are related to reproductive performance, including tail width, tail length, position of cloacal opening and size. The other traits might play important roles in decreasing intersexual competition for resources, including head size. These data indicated that *Amyda cartilaginea* is sexually dimorphic.

The annual reproductive cycle was investigated from changes in levels of plasma testosterone, estradiol and progesterone. These sex steroids were detected by radioimmunoassay according to the WHO matched reagent programme. It was found that male and female softshell turtles exhibited a seasonal reproductive cycle. The males displayed high levels of testosterone in prenesting period, while the females showed a prenesting peak of estradiol and high levels of progesterone during the prenesting and perinesting period. Detectable levels of heterologous sex steroids i.e. androgens in females and estrogens in males were apparent in both sexes indicating evidence for functions of heterologous sex steroids in this softshell turtle. Plasma estradiol levels in both sexes and plasma progesterone levels in the males were well correlated ($p<0.05$) with temperature of the Bangkok Metropolis area. The results suggest that softshell turtles exhibit a prenuptial reproductive cycle which results in rising plasma sex steroid levels prior to the mating season and decreasing to basal level at other periods of the year.

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต K. Noppadon

สาขาวิชา สัตววิทยา ลายมือชื่ออาจารย์ที่ปรึกษา Vithaya Yodyingyuad

ปีการศึกษา 2540 ลายมือชื่ออาจารย์ที่ปรึกษาร่วม K. Thirakhupt

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Chapter 1

Introduction

Conservation biology is a topic of increasing interest and concern as the world approaches the 21st century. Advantages of wildlife conservation include preservation of biodiversity, beneficial aspects on ecosystem balance and economic advantages from wise utilization of natural resources. Conservation management can take many forms, including habitat preservation and enhancement, translocation of individual animals to more suitable habitats, maintenance and breeding of animals in captivity and direct hormonal stimulation of breeding. Reproductive biology is believed to be one of the requirements bases for a successful conservation program (Wildt et al.., 1992; Edgar, 1993; Cockrem, 1997).

Thailand is one of a few countries world-wide with more than 25 species of turtles. A total of 28 species representing 26 genera in 6 families occur in Thailand. Some of these turtles could be developed to be of economic importance, such as softshell turtles of the family Trionychidae. But with ever-increasing habitat destruction, hunting and human settlement, Thailand is listed among 15 countries with the largest number of threatened species of reptiles (IUCN, 1996). Many species of turtles are disappearing and may become extinct before we have the chance to study and conserve them. These include the Impressed tortoise *Manouria impressa*, the Painted terrapin *Callagur borneoensis*, the Striped giant softshell turtle *Chitra chitra* and the Asian giant softshell turtle *Pelochelys cantorii* (Kumthorn Thirakhupt and van Dijk, 1994; Saowanee Sematong and Kumthorn Thirakhupt, 1994). Even the widely distributed species, the common Asiatic softshell turtle *Amyda cartilaginea*, is listed as a vulnerable species according to the IUCN red list of threatened animals (IUCN, 1996). Conservation efforts should include both habitat conservation and captive breeding programs which are based on an understanding of reproductive biology. At present this knowledge is still scarce for turtles in tropical regions including Thailand, leading to unsuccessful recovery programs.

There have been efforts to farm *Amyda cartilaginea* on a commercial basis in order to supply local demand for its meat. But economic profit is still low compared to farming an introduced species, *Pelodiscus sinensis*, which is mainly

cultured for export to Taiwan and Hong Kong. There were also attempts to develop a breeding program of *Amyda cartilaginea* by the National Inland Fisheries Institute, but the results were only moderately encouraging due to lack of a reproductive biology basis (Sujin Nukwan, Panu Tavarutmaneegul and Anusin Inkuan, 1995). Data concerning the reproductive biology of *Amyda cartilaginea* at present was mainly obtained by observation from natural populations (Wirot Nutaphand, 1979; van Dijk, 1992; Wirot Nutaphand, 1990; Meylan, Moll and van Dijk, 1995). The data is still scarce in most aspects, especially sexual dimorphism and the annual reproductive cycle.

Sexual dimorphism, one aspect of reproductive biology, is a condition in which the males and females in a species are different in morphological traits such as coloration, size or other features. Presumably the dimorphism in some species reflects factors important in social interactions, survival, or reproduction (Bury, 1979). Three major hypotheses have been proposed to explain sexual differences in organisms: 1) the female fecundity hypothesis: females are larger because larger body size is associated with increased number or size of eggs, 2) the competition avoidance hypothesis: differences in head and mouth size and differences in microhabitat usage result in decreased intersexual competition for resources, and 3) the sexual selection hypothesis: males are larger because large male size is favored in male-male disputes over breeding territories (Darwin, 1889; Slatkins, 1984; Shine, 1989, 1990). In *Amyda cartilaginea* males show longer and heavier tails which are important in mating performance. There was also a proposed difference in plastron color by Smith in 1931. While other sexually dimorphic characters have not been recorded (Wirot Nutaphand, 1979; van Dijk, 1992; Meylan, Moll and van Dijk, 1995).

The annual reproductive cycle is another important feature of reproductive biology contributing to the mode of reproduction and temporal changes in fertility of organisms. With the advent of sensitive methods for hormone measurement, investigations of hormonal profiles have become powerful tools for the study of reproductive cycles. In chelonian species, reproductive cycle in term of hormonal profiles have been studied in some freshwater species, including *Chelydra serpentina*, *Chrysemys picta* and *Sternotherus odoratus* (Callard et al., 1978; Lewis, Mahmoud and Klicka, 1979; Licht, 1982; Licht, Breitenbach and Congdon, 1985, Mahmoud and Licht, 1997), some tortoises including *Geochelone* spp, *Gopherus agassizii* and *Testudo* spp (Rostal et al., 1993; Casares et al., 1994), and some

marine turtles including *Caretta caretta*, *Chelonia mydas*, *Dermochelys coriacea*, *Lepidochelys kempi* and *Lepidochelys olivacea* (Licht et al., 1979; Licht, Rainey and Cliffton, 1980; Licht et al., 1982; Licht, Wood and Wood, 1985; Wibbels et al., 1990; Guilette et al., 1991; Wibbels et al., 1992; Rostal et al., 1996, 1997; Whittier, *Corrie and Limpus, 1997; Rostal et al., 1998).* In the temperate zone, turtles show a distinct seasonal reproductive cycle. Males show typical spring mating behavior and autumn spermatogenesis; both are under major control of testosterone. Females show autumn vitellogenesis and oocyte maturation, spring mating, followed by nesting behavior. In females, testosterone plays a role as a precursor for estradiol synthesis, while estrogen levels correlate with vitellogenesis and progesterone has an important role in the ovulatory process. In general only a few studies concern the reproductive cycle of softshell turtles, while most of the aforementioned studies of turtle hormonal profiles were performed in temperate zone and thus are unlikely to be applicable to tropical turtles due to climatic differences.

A study of *Amyda cartilaginea* for sexual dimorphism, in order to properly identify sex from secondary sex features and other morphological traits, and annual reproductive cycle, in order to monitor changes in fertility year-round by detecting plasma sex steroids profile, could form a useful basis for both endangered turtle recovery programs and economic animal development programs in the future.

Objectives

1. To study sexual dimorphism, in order to properly identify sex from secondary sex features and other morphological traits.

2. To study the annual reproductive cycle of mature male and female *Amyda cartilaginea* by detecting changes in plasma sex steroids profile year-round.

Anticipated benefit

To obtain basic knowledge about sexual dimorphism and the annual reproductive cycle of mature male and female *Amyda cartilaginea* in a tropical region. This could be used for endangered turtle recovery programs and economic animals development programs in the future.

Chapter 2

Literature Review

2.1 Softshell turtle

General cladistic classification of softshell turtles is:

Kingdom Animalia
Phylum Chordata
Subphylum Vertebrata
Class Reptilia
Order Chelonia (Testudines)
Suborder Cryptodira
Family Trionychidae

Phyletic relationship of softshell turtles and other living families of turtles is shown in figure 2-1.

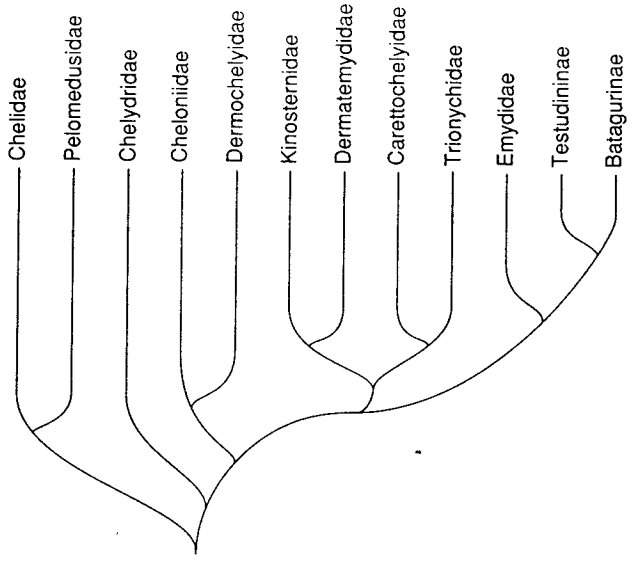

Figure 2-1 Dendrogram of presumed phyletic relationships among the living families of turtles (Zug, 1993).

The softshell turtle of the family Trionychidae is an old and very distinctive group of aquatic Testudines. In nearly all other families of turtles the body is surrounded by a bony shell, formed from dermal bone with participation of the ribs and certain bones of the pectoral girdle, cover by large epidermal scutes. In Trionychids the bony shell is strongly reduced and large area are formed by cartilage. The dorsal carapace lacks the ring of peripheral bones (except *Lissemys*), and costal plates are short with the rib ends projecting out of them. The ventral plastron are reduced to a trellislike frame as in sea turtles. The carapace and plastron are connected only by cartilaginous and ligamentous connective tissue and there is no bony connection as occurs in other turtles. This construction allows considerable volume changes inside shell, necessitated by flexion and retraction of the long neck and large head. The surface of the shell is a thick, leatherly skin with no hint of epidermal scutes. This specialized flexible shell is reported to originated with contribution to three aspects of softshell turtle behavior i.e. to facilitate greater swimming speed by allowing longer strokes of the legs, to increase burrowing efficiency, and to allow increased speed and range of striking and snapping at preys and predators (Pritchard, 1979; van Dijk, 1992; Zug, 1993).

Head of softshell turtles typically ends in a protruding snorkellike snout. Trionychids can lie hidden on the bottom and extend their neck until only the tip of the snout projects above the water surface. They lack cloacal bursae and hence do not indulge in anal respiration. Air breathing occurs via the tube-nose, and is supplemented by pharyngeal respiration. It is possible that they conduct a fair amount of underwater respiration through the skin (Pritchard, 1979; van Dijk, 1992; Zug, 1993).

All species of softshell turtle are predominantly carnivorous. Preys are captured by both foraging and ambush. Juveniles eat small invertebrate preys and with growth tackle ever larger prey. Several species develop broad crushing surfaces on their jaws as a result of feeding predominantly on freshwater mussels and snails. Carrion is eaten whenever encountered e.g. the large softshell turtles are the main agents of disposal of all the incompletely cremated corpses and dead cattle that are entrusted to the great rivers of India. (van Dijk, 1992; Zug, 1993)

The distribution of the family is roughly similar to that of the Emydidae. Softshell turtles are abundant in temperate eastern North America and tropical Southeast Asia. They are also found on the more western islands of

Indonesia, one species is found in the Middle East, and there are several in Africa (Pritchard, 1979).

The living members of the family are universally accepted to form two natural monophyletic within a monophyletic Trionychidae. The subfamily Cyclanorbinae is characterized by cutaneous femoral valves which cover the hind limbs when withdrawn, and various skeletal features. Cyclanorbines include only three genera(four species), and show no apparent differences in general behavior and ecology from the Trionychines. The subfamily Trionychinae is characterized by the absence of peripheral bones, a very short bridge between carapace and plastron, a greatly reduced plastron, fusion of hyo- and hypoplastral bones occurring in old adults, and other less obvious osteological features. The Trionychines, although geographically more widespread, are not particularly diverse(18 species); their greatest diversity is in southern Asia and southeastern North America (van Dijk, 1992; Zug, 1993; Webb, 1997).

2.2 *Amyda cartilaginea*

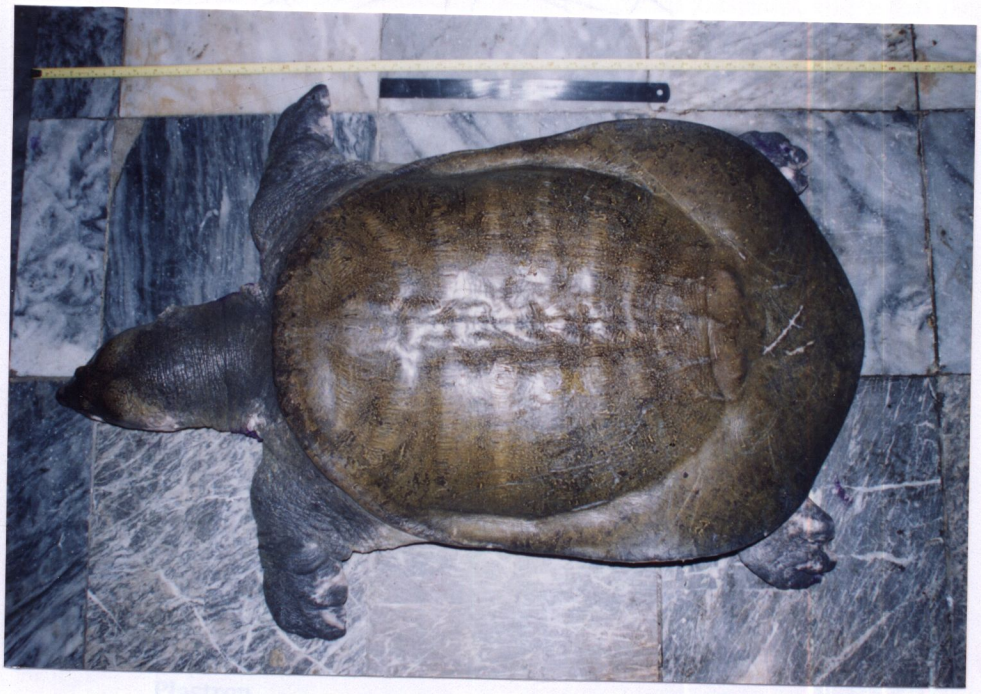

Figure 2-2 *Amyda cartilaginea* (sampled specimen BP0051096).

Biology and status of *Amyda cartilaginea* have been currently reviewed by Meylan, Moll and van Dijk (1995). The followings are partly extracted from their work.

A. cartilaginea is a large softshell turtle with a bony disc length to 403 mm and a total carapace length up to 830 mm and weight up to 35 kg or in exceptional cases over 70 kg. This species has long epiplastra in contact or narrowly separarated from each other on the midline, one neural between the first pair of costals, and a strong median ridge on the symphysis. Carapace and plastron of *A. cartilaginea* is illustrated in figure 2-3.

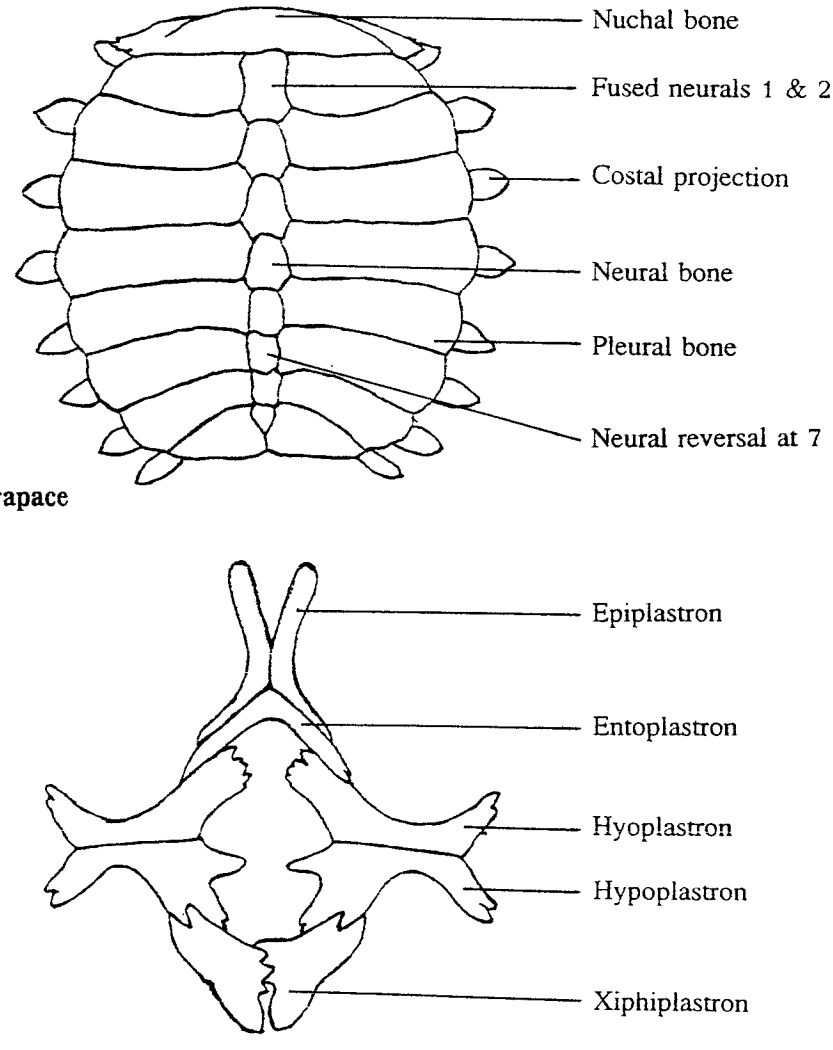

Figure 2-3 Carapace and plastron of *Amyda cartilaginea* (Modified from Wachira Kitimasak, 1996).

This softshell turtle species is found in southern and eastern Myanmar, possibly extending to extreme southwestern Yunnan, all of Thailand and Cambodia, the Mekong drainage area of Laos, central and possibly south Vietnam, the Malay Peninsula and the continental shelf islands of Sumatra, Banka, Java, Balitung, Lombok, and Borneo. Its suggested occurrences in northern Vietnam, the Molluccas or Timor remain unconfirmed. The distribution map of this species is shown in figure 2-4. The occurrence on the western Thailand which was not presented in this figure should be noted (Kumthorn Thirakhupt and van Dijk, 1994).

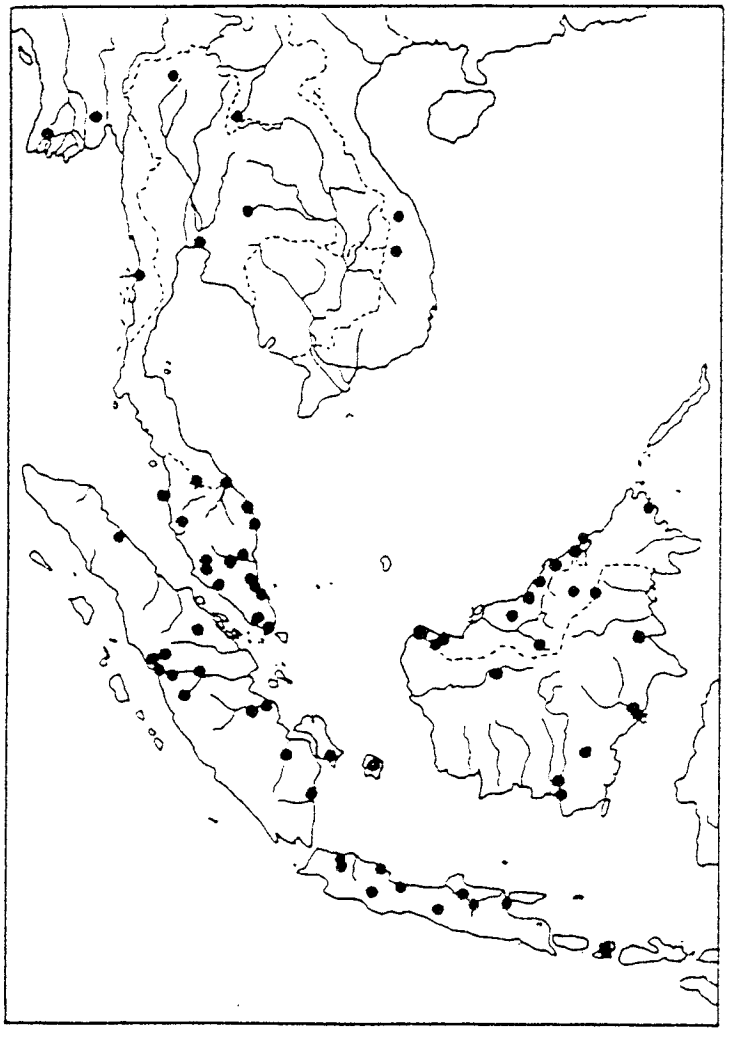

Figure 2-4 Distribution of *Amyda cartilaginea* (Iverson, 1992).

A. cartilaginea appears to be very generalised in habitat preferences. In northern and western Thailand, it inhabits quiet areas of streams up to altitude of 400-600 m. The species is quite susceptible to death by dehydration, limiting its occurrence in seasonal water bodies. It appears most abundant in lowland lentic

waters such as swamps, ponds and oxbow lakes adjacent to large rivers. In Thailand, where these areas were long ago converted to agricultural land and settlement areas, the species inhabits rivers, reservoirs, ponds, canals and ditches.

Little objective data are available about activity cycles; the animals spend long periods buried in bottom substrate and can be active at any time of day or night, although activity seems to peak at dawn and dusk. Basking was observed once in Bangkok. Wirot Nutaphand (1979) wrote that the animals like to spend long periods buried ashore, but these may be animals burying themselves in shallow water before water levels dropped.

A. cartilaginea is reported to feed on a variety of aquatic arthropods, molluscs, fish and amphibians. It is also reported as opportunistic omnivore eating fruits and seeds.

This softshell turtle species is a popular food animal and is widely and intensively hunted. At present live animals, frozen whole or butchered animals, and prepared softshell turtle curry are all available in the Bangkok weekend market. Several upmarket restaurants serve softshell turtles dishes; for chauvinistic reasons, wild *A. cartilaginea* is preferred over cultured Chinese softshell turtle. Its curio value and perceived wholesome effects sustain a demand for softshell turtles for consumption. In rural areas, softshell turtles and other wild animals remain an important protein source for local people. Several softshell turtle populations are in decline, yet the species still appears moderately abundant because several populations occur inside protected areas.

Internationally, this species was listed as a threatened species (vulnerable) according to the IUCN red list of threatened animals (1996). It is not listed on any CITES appendix. In Thailand it is protected animal under the Wild Animal Protect Act of 1992.

2.3 Sexual dimorphism

Sexual dimorphism is a condition in which the males and females in a species are different in morphological traits such as coloration, size or other features. Presumably the dimorphism in some species reflects factors important in social interactions, survival, or reproduction (Bury, 1979).

Three major hypotheses have been proposed to explain sexual differences in organisms: 1) the female fecundity hypothesis: females are larger because larger body size is associated with increased number or size of eggs, 2) the competition avoidance hypothesis: differences in head and mouth size and differences in microhabitat usage result in decreased intersexual competition for resources, and 3) the sexual selection hypothesis: males are larger because large male size is favored in male-male disputes over breeding territories (Darwin, 1889; Slatkins, 1984; Shine, 1989, 1990).

Graham (1979) reviewed and provided a compilation of criteria for sex determination of selected turtle species as followings.

1. *Chelydra serpentina*: preanal tail length of male is greater than 86 % of posterior plastral lobe length while female is less than 86 %. Anal opening of male is posterior to rear carapacial margin while female is anterior.
2. *Sternotherus odoratus*: male has a small patch of scales on inner hindleg surface while female is relatively smooth. Male has longer and stouter tail ending in a blunt nail. Male has broader skin area between plastral seems.
3. *Clemmys insculpta*: preanal tail length of male is twice as long as that of female. Male has concave plastron and prominent forelimb scales while female has flat and reduced.
4. *Terrapene carolina*: male usually larger in plastron length an has red iris while female has yellow-brown. Posterior plastral lobe is concave in male and flattened in female. Male has longer and stouter tail and shorter, stronger and curve hindlimb nails.
5. *Malaclemys terrapin*: male is smaller with more point behind carapace. Anal opening of male is posterior to rear carapacial margin while female is anterior. Head shape of male is dorsal outline narrower and more pointed.
6. *Graptemys barbouri*: male is smaller with longer and thicker tail. Anal opening of male is posterior to rear carapacial margin while female is anterior.
7. *Chrysemys picta*: male is smaller in shell dimensions with elongated foreclaws. Anal opening of male is posterior to rear carapacial margin while female is anterior.

8. *Deirochelys reticularia*: male is smaller with longer and thicker tail. Anal opening of male is posterior to carapacial margin while female is anterior.
9. *Gopherus agassizi*: male is larger than female with longer gular projections and longer and thicker tail. Plastron of male is concave while female is flattened.
10. *Chelonia mydas*: male carapace is taperig more posteriorly while female is more rounded posteriorly. Rear plastral lobe of male is narrower. Tail of male is vertically prehensile, tipped with a heavy nail, and extends far beyond rear of carapace while female is barely reach rear edge of carapace.
11. *Trionyx spiniferus*: male seem to be smaller with longer and thicker tail with anal opening near tip. Carapace pattern of male retains the juvenile dark ocelli or white dots while female loses juvenile pattern and develop mottled lichenlike markings on the carapace.

Berry and Shine (1980) reviewed data on sexual size dimorphism, reproductive behavior, and habitat types in turtles and reported that patterns of sexual size dimorphism are correlated with habitat type and male mating strategy that:

1. In most terrestrial species, males engage in combat with each other. Males typically grow larger than females.
2. In semiaquatic and bottom-walking aquatic species, male combat is less common, but males often forcibly inseminate females. As in terrestrial species, males are usually larger than females.
3. In truly aquatic species, male combat and forcible insemination are rare. Instead, males utilize elaborate precoital displays, and female choice is highly important. Males are usually smaller than females.

In term of sexual selection theory, males are larger than females when large male size evolves as an adaptation to increase success in male combat, or to enable forcible insemination of females. Incontrast, males are usually smaller than females where small size in males evolves to increase mobility, or because selection for increased fecundity may result in increased female size (Berry and Shine, 1980).

Gibbons and Lovich (1990) investigated sexual dimorphism in turtles with emphasis on *Trachemys scripta* and found that females attain larger body sizes than males in all populations. Other reported sexually dimorphic traits are longer precloacal length, elongated foreclaws, longer snout and melanism in males.

Lambert (1993) studied sexual dimorphism of *Geochelone sulcata* in Mali, and found that sexual dimorphism is more strongly marked by gular extension which is according to males combat. There is a significant correlation between rear aperture diameter and carapace length in male, but not in female.

Dunaway (1994) investigated morphometric variations between male and female *Terrapene carolina* in Alabama, and indicated that the posterior portion of the carapace of females is higher than the anterior portion, while in males, the anterior portion is higher than the posterior portion.

Mushinsky, Wilson and McCoy (1994) studied growth and sexual dimorphism of *Gopherus polyphemus* in central florida and proposed that the best indicator of sex is the size, shape and depth of plastral concavity. While anal notch and anal width are also dimorphic but less distinct. This study also mentioned diminished sexual dimorphism due to rapid growth and abrupt attainment of sexual maturity in this population.

Lambert (1995) investigated sexual dimorphism of *Geochelone pardalis* in Somaliland and showed that males tend to be more elongated and slightly narrower carapace. Rear aperture diameter decreases exponentially in male but increases exponentially in female.

In *Amyda cartilaginea* males show longer heavier tails and seems to reach larger sizes than females. Smith (1931 cited in Meylan, Moll and van Dijk, 1995) wrote that the plastron is white in males and grey in females. While other sexually dimorphic characters have not been recorded (Wirot Nutaphand, 1979; van Dijk, 1992; Meylan, Moll and van Dijk, 1995).

2.4 Annual reproductive cycle

General reproductive biology of turtle base on macro- and microscopic changes of gonad have been thoroughly reviewed by Moll (1979). In

temperate zone, patterns of reproductive cycle of males and females could be summarized as followings.

Males: Spermatogenesis begins in the spring, peaks in late summer, and ends in the fall as spermatozoa leave the testes to overwinter in epididymis. Spring is the peak period for breeding in most species, although fall and continuous breeding may also occur. Endocrine cells activity peaks during gametogenic quiescence, rapidly decreases as spermatogenesis is renewed, and is minimal at the peaks of spermiogenesis.

Females: Generally, females have an annual cycle but in some species cycles of 2 to 4 years are also evident. The female cycle consists of 4 phases including 1) follicular enlargement resulting from vitellogenesis begins in late summer or fall until completion of the second phase in spring; 2) ovulation and intra-uterine period; 3) nesting period - nesting seasons commonly fall between late April and late July but in lower latitude, nesting often begins earlier and extends over a greater period; and 4) latent period.

Reproductive cycle of turtles based on endocrine patterns have been reviewed by Licht (1982) with emphatic commentary that the reproductive cycle depends upon responses to pituitary hormones, and the gonads are also important endocrine organs secreting a series of steroid hormones. Hence detailed information on the nature and levels of circulating pituitary and sex hormones is critical for understanding the true functional status of the reproductive system and the mechanisms regulating reproductive cycles.

Endocrinology of reproductive cycle in reptiles have been reviewed in various aspects by many authors in the Tenth International Symposium on Comparative Endocrinology (Norris and Jones, 1987). The followings are some parts of their works.

Whittier and Crews (1987) reviewed patterns and control of seasonal reproductive cycle in reptiles and suggested that timing of reproduction in a population is determined by 1) when the most offspring survive and 2) when parents, often females, are capable of energetically supporting the production of viable young at the least cost to themselves. Not all aspects of reproduction, including gonadal growth, sex steroid hormone secretion and sexual behavior, are expressed at the same time. In most species that have been studied with respect to the environmental cues and physiological mechanisms influencing reproduction,

these three events are functionally associated. However, many species have a dissociated reproductive tactic in which gonadal activity and sexual behavior are expressed at different times. Other species exhibit a constant reproductive pattern in which gonads are maintained in a near steady state and sexual behavior is expressed in response to particular environmental cues. Diagram of the three aforementioned reproductive patterns is shown in figure 2-5.

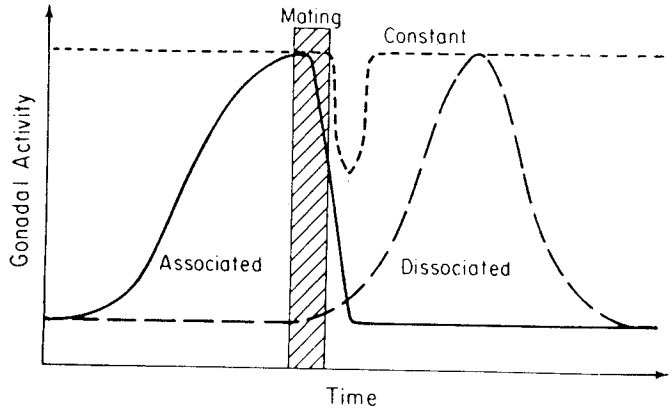

Figure 2-5 Three reproductive patterns exhibited in vertebrates (Whittier and Crews, 1987).

Many species of snakes and freshwater turtles exhibit dissociated reproductive cycles in which gonadal activity and sexual behavior peak at different times of year.

Lofts (1987) reviewed testicular function and stated that in male reptiles testosterone has been identified in the plasma of many species while androstenedione and epitestosterone have also been isolated from testicular tissue of some species. According to histochemical, radioimmunological and ultrastructural techniques the interstitial Leydig cells are identified as the main loci for androgen synthesis. A peak in plasma testosterone levels have been recorded before mating in turtles. In postnuptial species including turtles there is an uncoupling of the spring plasma testosterone peak from the spermatogenetic cycle. Sertoli cells are suggested to be loci for intratubular steroids secretion. Furthermore, production of tubular androgens varies seasonally and shows maximum synthesis at times when there is also a high level of spermatogenetic activity.

Chieffi and Pierantoni (1987) reviewed regualtion of ovarian steroidogenesis and suggested that in female turtle, testosterone seems to play a role primarily as a precursor for estradiol synthesis, estrogen titers correlate well with vitellogenesis, while progesterone appears to have an important role in the ovulatory process.

Ho (1987) reviewed endocrinology of vitellogenesis and stated that estrogen is the primary stimulus to vitellogenesis in female reptiles including turtles.

Nagahama (1987) reviewed endocrine control of oocyte maturation and reported the involvement of progesterone in the process of oocyte maturation in female turtles.

Xavier (1987) reviewed functional morphology and regulation of the corpus luteum and showed that female reptiles exhibit fully developed corpora lutea and function as endocrine glands by secreting progesterone. In some turtles corpora lutea begin to regress before oviposition resulting in plasma progesterone rapidly declines to basal level after a preovulatory or ovulatory surge.

Moore (1987) reviewed regulation of reproductive behaviors and stated that in general testicular steroid hormones (testosterone, dihydrotestosterone and estradiol) activate male reproductive behaviors and ovarian hormones (estradiol and progesterone) activate female reproductive behaviors. Although there is some evolutionary conservatism in the control of reproductive behavior of vertebrates, species specificity is also evident in some reptiles.

Reproductive cycle of turtles in term of endocrinology have been studied in many species of turtles in the followings families.

Family Emydidae (freshwater turtle)

Callard et al. (1978) investigated annual ovarian cycle of *Chrysemys picta* and reported that the females display marked changes in three plasma steroids (testosterone, estradiol and progesterone) during the periovulatory period, increasing sharply prior to or around the time of ovulation during mid-May to mid-June, and falling sharply there after. There are also smaller peaks of testosterone and estradiol during fall period of ovarian recrudescence.

Lewis, Mahmoud and Klicka (1979) studied seasonal changes in plasma progesterone and estradiol in female *Chelydra serpentina* and showed that among four stages of annual ovarian cycle (hibernation, preovulation, postovulation and vitellogenesis) the highest progesterone level is in luteal stage in June, while estradiol is highest in May immediately before ovulation.

Licht, Breitenbach and Congdon (1985) studied seasonal cycle in testicular activity in *Chrysemys picta* under natural conditions and found that plasma testosterone levels exhibit biphasic cycle with peak in spring and fall. Plasma testosterone is lowest on the day that aimals emerge from hibernation at the end of March, then increase rapidly to a transient peak that persist for 2 weeks. Plasma testosterone rise again in September before the onset of hibernation and shortly after the late summer peak in spermatogenic recrudescence.

Mahmoud et al. (1985) investigated ultrastructural changes in testes of *Chelydra serpentina* in relation to plasma testosterone and reported that testosterone level is highest in May and October (mating) and relatively low during the rest of the year. Leydig cells are found to be potentially active throughout the year while Sertoli cells are active only during spermatogenesis from May to October. It was suggested that testosterone of Leydig cells origin is concerned mainly with mating behavior and that of Sertoli cells origin with spermatogenesis and maturation of sperm.

Mahmoud and Licht (1997) studied seasonal gonadal cycle in natural population of *Chelydra serpentina* and reported that in the females testosterone, estradiol and progesterone are highly correlated with follicular growth and vitellogenesis. There is a significant increase in estradiol and progesterone as the ovulation occur. In the males testosterone is significantly correlated with testicular growth and spermiation. Courtship and mating behavior are observed in spring, summer and fall.

Family Testudinidae (tortoise)

Casares et al. (1993) measured faecal steroids in *Geochelone elephantopus*, *G. gigantea*, *Testudo graeca*, and *T. hermanni* and found that from August to September, estrone, estradiol and testosterone rise simultaneously in 3 of 6 *Testudo* spp males, while estrone increases during the follicular development in 4

of 6 *Testudo* spp females and 2 of these show simultaneous surges of both estrone and estradiol. From March to May, estrone and testosterone increase in the 4 *Geochelone* spp females. While estradiol surges in 2 *G. gigantea* females between April and May and 1 animal shows a further surge in October.

Rostal et al. (1994) studied seasonal reproductive cycle of *Gopherus agassizii* under semi-natural conditions and reported that male tortoises display a significant rise in plasma testosterone during the summer from May to August which continue into the fall mating period, then decline prior to hibernation. Upon emergence from hibernation in April testosterone levels are significant reduced during the spring mating period (April to May). Female tortoises also show significant rise in testosterone from July to October prior to hibernation. This increase coincides with the onset of fall mating period. The female testosterone levels are highest following emergence from hibernation prior to ovulation in April and May.

Family Cheloniidae (sea turtle):

Licht et al. (1979) investigated serum steroids associated with breeding activity in captive *Chelonia mydas* and found that in the males, androgen (testosterone and dihydrotestosterone) levels are lower during mating than in the prebreeding season. In the females, estrogen (estradiol and estrone) levels drop while testosterone peaks in the mating season and boths remain relatively low throughout the nesting season, while progesterone continue to rise progressively during the prebreeding and mating season and upto the time of nesting.

Licht, Rainey and Cliffton (1980) studied serum steroids associated with breeding activities in natural populations of *Chelonia mydas* and showed that the mating males display high testosterone level but not much differ from prebreeding males. In the females, there is no different in testosterone level between mating and nesting females but the level drop significantly in the first day following nesting. Progesterone is also similar between mating and nesting females but shows an elevation in 1-2 days postnesting.

Licht et al. (1982) studied changes in steroids in female *Lepidochelys olivacea* and found that progesterone levels markedly increase within a day after

oviposition and then return to near baseline level within 2 to 3 days, while testosterone and estradiol levels show little changes in periovulatory period.

Licht, Wood and Wood (1985) investigated annual and diurnal cycles in plasma testosterone in captive male *Chelonia mydas* and found that testosterone is at nadir in September to November and increase to a peak in April, then begin to decline coincidently with the onset of mating behavior which is peaked in May-June. Spermatogenesis and androgen secretion are not uncoupled hence suggesting that *C. mydas* does not exhibit postnuptial testicular cycle which is typical in many temperate zone turtle. Furthermore it was found that plasma hormones are relative stable over a 24 hours period.

Wibbels (1990) studied seasonal changes in gonadal steroids in *Caretta caretta* and reported that the males show a prenuptial spermatogenic cycle coincident with increased concentrations of testosterone which is high during migration and mating period. In the females, serum estradiol levels increase approximately 4-6 weeks before migration to mating and nesting area suggesting a period of increased vitellogenesis, and then decrease 1-2 weeks prior to migration. Serum testosterone, estradiol and progesterone are elevated during nesting if turtles will nest again, while during the last nesting all steroids are low.

Guilette et al. (1991) investigated changes in plasma estradiol and progesterone during natural oviposition in *Caretta caretta* and reported that plasma steroids concentrations do not vary significantly during nesting with mean concentration of estradiol at 255 pg/ml and progesterone at 395 pg/ml.

Wibbels et al. (1992) studied levels of serum steroids in natural populations of *Chelonia mydas* and *Caretta caretta* and found that after nesting serum progesterone peak within 20 to 50 hours while testosterone decline and estradiol concentrations show no significant changes. The dynamics of progesterone and testosterone concentrations are consistent with the hypothesis that these hormones facilitate specific physiological events during ovulation and egg production.

Whittier, Corrie and Limpus (1997) investigated plasma steroids profiles in nesting *Caretta caretta* and showed that circulating levels of estradiol are mostly undetectable while testosterone and progesterone profiles in the nesting

females are associated with the individual turtles' progression through successive nesting episode, with a marked decline in all steroids by the last nesting episode of the season.

Rostal et al. (1997) studied nesting physiology of *Lepidochelys kempi* and reported that female serum testosterone and estradiol decline over the course of nesting season while progesterone levels do not fluctuate over the course of nesting season. It was found that serum testosterone is a useful prediction of nesting periodicity in *Lepidochelys kempi*.

Rostal et al. (1998) investigated seasonal reproductive cycle of *Lepidochelys kempi* under semi-natural conditions and reported that the males show a prenuptial rise in serum testosterone 4 to 5 months before mating period then sharply decline during mating period in March. The females also show prenuptial rise in serum testosterone and estradiol 4-6 months prior to mating period and decline during the mating period in April to July. The elevated estradiol correspond with the period of vitellogenesis. It was suggested that *Lepidochelys kempi* displays a distinct seasonal reproductive cycle in captivity.

Family Dermochelyidae (leatherback turtle)

Rostal et al. (1996) investigated reproductive physiology of female *Dermochelys coriacea* and reported that plasma testosterone and estradiol levels correlate well with reproductive conditions while there is no correlation between plasma progesterone level and reproductive conditions. Testosterone declines from 2245 pg/ml at the beginning of nesting cycle to 318 pg/ml at the end of the nesting cycle while estradiol decline from 53.30 pg/ml to 16.50 pg/ml in a similar manner. Endocrine and ovarian patterns of *Dermochelys coriacea* are similar to sea turtle of the family Cheloniidae.

Family Trionychidae (softshell turtle)

Licht (1982) studied reproductive cycle of male *Trionyx sinensis* (or *Pelodiscus sinensis* at present) and reported that testosterone is undetectable through most of the year (especially in spring when Leydig cells appear active and breeding is presumed to occur), and plasma testosterone only increases for a brief period in late summer after testes are fully recrudesced.

Sarkar et al. (1996) investigated photothermal effects on ovarian growth and function of *Lissemys punctata punctata*. Although this study was not an direct investigation for seasonal reproductive cycle, it provided experimental data that high temperature have a triggering role on ovarian growth and secretion of estrogen at the early preparatory phase, but once the ovarian function sets in, high temperature seems to have a regressive rather than stimulatory effect on ovary.

In *Amyda cartilaginea*, only general reproductive biology data are available. Wirot Nutaphand (1979) reported that under favorable conditions females can mature in 20 months, they will lay three or four clutches per year, clutch size varying from 7 to 30 eggs. The number of eggs per clutch increase from 6-10 in smaller females to 20-30 in larger ones.

The nesting season of *A. carlilaginea* coincides with the dry and hot seasons in Thailand, from February to July with a peak in March and April. The nesting occurs in the late afternoon or evening, in damp sandy area close to the water. (Sujin Nukwan, Panu Tavarutmaneegul and Anusin Inkuan, 1995; Wichase Khonsue, 1993; Wachira Kitimasak, 1996; Wirot Nutaphand, 1979). The incubation period can be up to 135-140 days, according to Bourret (1941 cited in Meylan, Moll and van Dijk, 1995). Wachira Kitimasak (1996) found that at room temperature in Bangkok hatchling period ranged between 74 and 95 days.

Chapter 3

Materials and Methods

3.1 Subjects

A captive population of *Amyda cartilaginea* was maintained in a turtle pond at Prayurawongsawas temple, Bangkok, Thailand under semi-natural conditions. Most of these softshell turtles were previously wild-caught and then brought alive to be liberated in the pond by merit-making people according to the Buddhist faith. The softshell turtles were adapted to the pond conditions and could perform natural habits including nesting behavior (van Dijk, 1992; Wichase Khonsue, 1993; Wachira Kitimasak, 1996). Figure 3-1 shows general appearance of the pond.

Figure 3-1 General view of turtle pond at Prayurawongsawas temple, Bangkok, Thailand.

The pond is 42 x 48 m. in size and contains a small island with nesting sand in the middle. Depth ranges from 0 m at the nesting area to a maximum depth of 3 m. The nesting area is available year-round. The softshell turtles are exposed to natural photoperiod and weather conditions. Freshwater from

the nearby Chao Phraya river is circulated through the pond every week. Mean air temperature of the Bangkok area was reported to ranged from 26.3 °C in December 1996 to 31.1 °C in May 1997. Mean relative humidity varied from 61 % in December 1996 to 76 % in September 1997. Mean daily rainfall ranged from 0 mm in December 1996 and January 1997 to 12.0 mm in September 1997. Mean daily sunshine duration ranged between 3.1 hours in July 1997 and 8.6 hours in March 1997. A monthly climatic data of Bangkok area is charted in Figure 3-2.

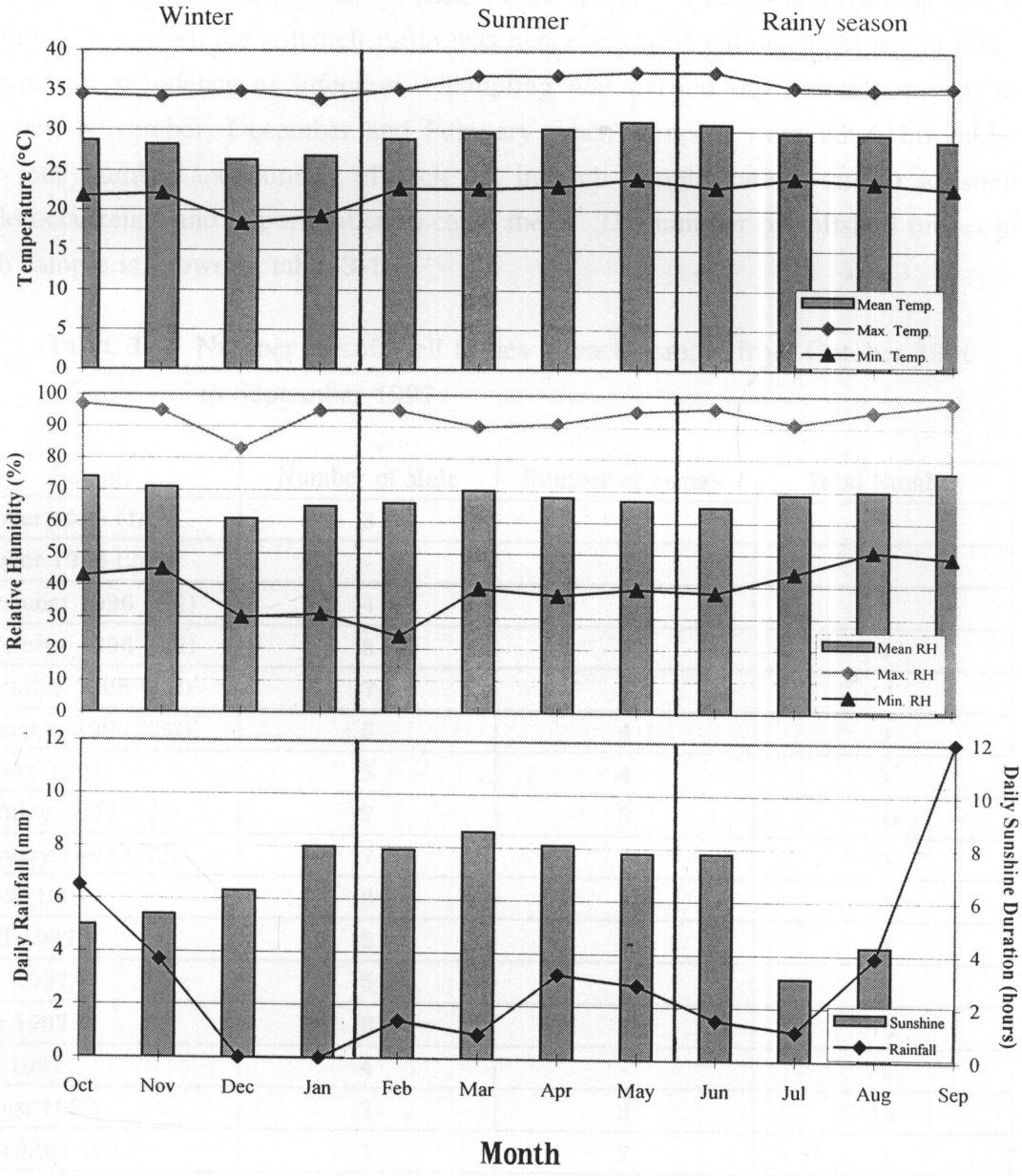

Figure 3-2 Climatic data of Bangkok Metropolis area during October 1996 to September 1997 (Meteorological Department, 1997).

* Total sunshine duration of September 1997 was not reported.

The softshell turtles can forage naturally for fish and carrion of dead turtles and are also fed with balls of fish by merit-making people.

During the one-year study period (October 1996 - September 1997), mature softshell turtles (according to Sujin Nukwan, Panu Tavarutmaneegul and Anusin Inkuan, 1995) were sampled from the pond with dip-nets during 3:00 pm - 6:00 pm. After measurements for morphological characters and blood collections, the softshell turtles were released back to the pond. Due to unfavorable water quality of the pond, the softshell turtle was hence released without marking in order to minimize incidence of infection. Sampling was carried out monthly except in October, November, December and February when samples were taken biweekly. The total number and number of each sex in each sample varied, due to softshell turtle occurrence and opportunities to catch them. The number of softshell turtles in each sample is shown in table 3-1.

Table 3-1 Number of softshell turtles in each sample from October 1996 to September 1997.

Sample	Number of Male	Number of Female	Total Number
October 1996 (1/2)	3	5	8
October 1996 (2/2)	-	5	5
November 1996 (1/2)	4	2	6
November 1996 (2/2)	6	3	9
December 1996 (1/2)	7	4	11
December 1996 (2/2)	5	4	9
January 1997	5	4	9
February 1997 (1/2)	7	3	10
February 1997 (2/2)	7	2	9
March 1997	6	3	9
April 1997	5	4	9
May 1997	5	3	8
June 1997	8	2	10
July 1997	4	-	4
August 1997	7	3	10
September 1997	1	7	8
Total	**80**	**54**	**134**

3.2 Sexual Dimorphism

The sampling softshell turtles were weighed with a commercial balance rated to 50 kg and measured for the following 18 morphological characters using a measuring tape or a vernier caliper. These parameters are illustrated in figure 3-3.

1. Carapace Length (CL): Curved maximum length of dorsal shell including posterior cartilaginous flap.
2. Carapace Width (CW): Straight maximum width of the dorsal shell including cartilaginous margin. The curved CW was abandoned to minimize error from irregular shell dome.
3. Bony Disc Length 1 (BDL1): Curved maximum length from nuchal tubercular ridge to the posterior of the dorsal bony shell.
4. Bony Disc Length 2 (BDL2): Curved maximum length of the dorsal bony shell. BDL2 represents the actual bony disc length but contains small error due to difficulty in locating the anterior end of bony disc.
5. Height (H): Maximum height when head is out.
6. Plastron Length 1 (PL1): Maximum length of the ventral bony shell from entoplastron to xiphiplastron.
7. Plastron Length 2 (PL2): Maximum length of the ventral bony shell from epiplastron to the xiphiplastron.
8. Plastron Width (PW): Width of the ventral shell measured between hyoplastron-hypoplastron conjunction including cartilaginous margin.
9. Plastron to Rear Margin of Carapace (PR): Length from end of the xiphiplastron to end of the posterior cartilaginous flap.
10. Plastron to Cloaca (PC): Length from end of the xiphiplastron to middle of cloacal opening.
11. Tailbase to Cloaca (TC): Length from tailbase to the middle of cloacal opening.
12. Cloaca to Tail Tip (CT): Length from the middle of cloacal opening to tail tip.
13. Tail Length (TL): Maximum length from the tail base to the tail tip.
14. Tail Width (TW): Maximum tail width.
15. Head Length without snout (HL): Length of head.
16. Head Length with snout (HLs): Length of head including snout.
17. Head Width (HW): Maximum width of head.
18. Head Height (HH): Maximum height of head.

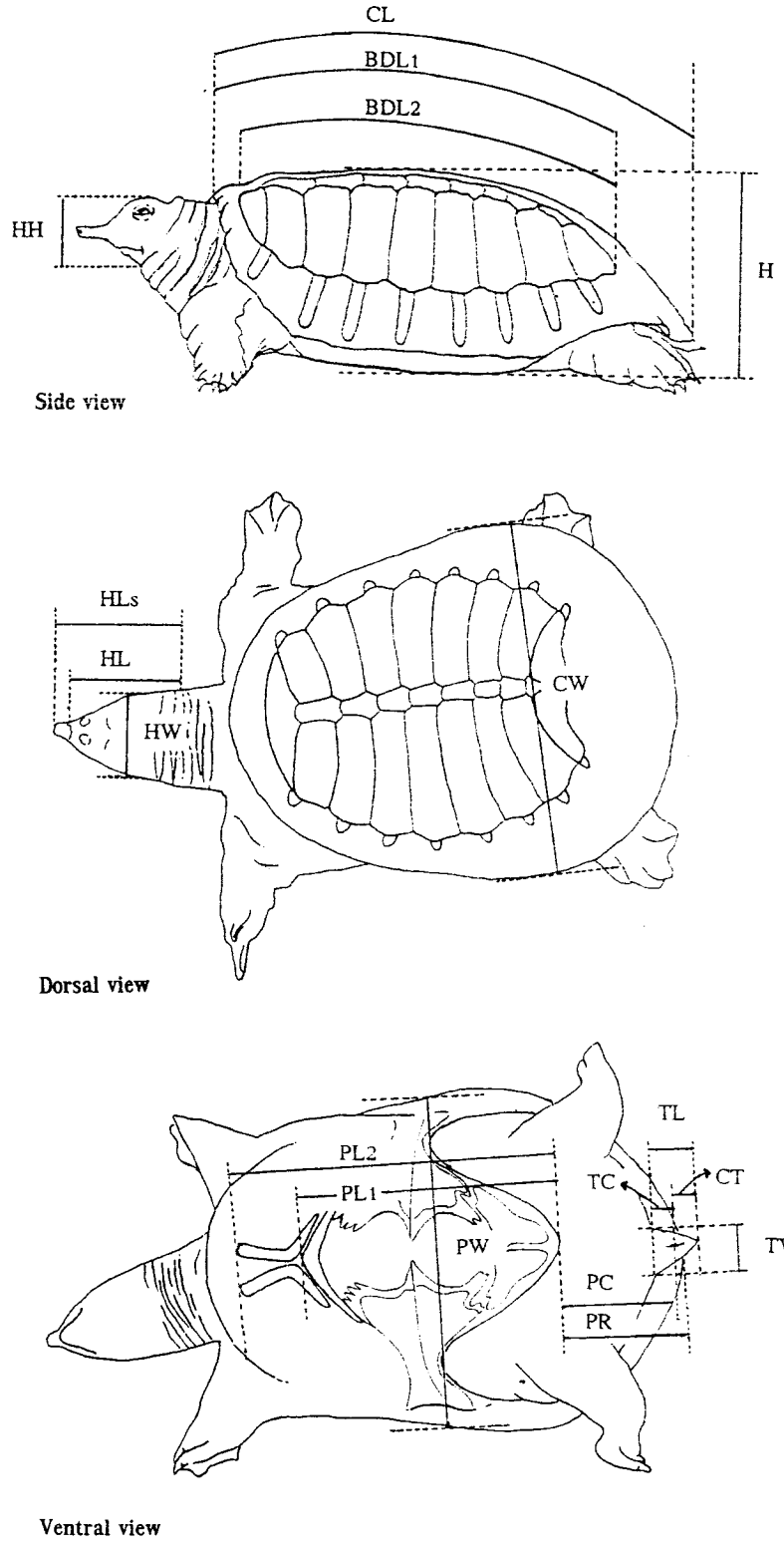

Figure 3-3 Eighteen morphological characters that were measured for the study of sexual dimorphism (side view was depicted from museum specimen: CUMZ (R) 1990-11-9-2, dorsal and ventral views were depicted from sampled specimen: BP1370997).

Eighty male and fifty-four female softshell turtles were measured during the study period. Sexual dimorphism was analyzed according to the following two procedures.

1. Mean Comparison

In order to minimize size bias, the recorded morphological characters were transformed into relative quantity to carapace length (CL). The mean relative parameters were then compared between sexes using Student's t-test.

In addition to the aforementioned parameters, recorded morphological characters were tranformed to the following parameters: tail length beyond carapace margin, body outline, and cloacal position.

Tail length beyond carapacial margin was transformed from subtraction of plastron to rear margin of carapace from addition of plastron to cloaca and cloaca to tail tip {(PC+CT)-PR}. The results were transformed into relative numbers to carapace length and compared between sexes.

Body outline i.e. dorsal view of softshell turtle body such as oval, round, etc. was determined from subtraction of carapace width by plastron width (CW-PW) and then transformed into relative number to carapace length. The result of CW-PW represented difference between the maximum width of the dorsal shell and the width of the ventral shell measured between hyoplastron-hypoplastron conjunction which was hypothesized to be relatively constant position in most individuals. Higher number of CW-PW suggested the greater in posterior portion of softshell turtle body outline.

Cloacal position was determined from division of tailbase to cloaca with tail length (TC/TL). The number represented relative position of the cloacal opening. The lower number indicated relatively anterior position while higher number indicated relatively posterior position.

In every comparisons, probability of $p \leq 0.05$ was considered to be significantly different.

2. Regression Analysis

Every morphological characters of each sex was plotted against carapace length (CL), furthermore tailbase to cloaca was also plotted against tail length. The regression analysis were performed, and regression lines were tested for significance ($p \leq 0.05$) using Analysis of Variance (ANOVA). The slopes and elevations of the regression lines between sexes were compared using Student's t-test. Significant difference was considered from probability of $p \leq 0.05$.

General calculations were performed on computer by Microsoft Excel for Windows 95 version 7.0. Statistical analysis were performed on computer by SPSS for Windows release 7.0 except the comparisons for slopes and elevations of the regression line, which were hand-calculated following Zar (1984).

3.3 Annual Reproductive Cycle

After capture, the softshell turtles were allowed to acclimatize on land for 1 - 3 hours. They were weighed and then anaesthetized with ketamine hydrochloride at a dosage of 12.5 mg/kg intramuscularly. Five to ten millilitres of blood was withdrawed from the jugular vein of each individual with 38 mm 25 gauge needles and heparinized syringes. Blood samples were stored on ice up to 5 hours until cool centrifugation at 4°C, 1100 x g. Plasma samples were separated and frozen in small aliquots of 1.4 ml at -20°C until steroids assays.

Plasma samples were assayed for levels of testosterone (T), estradiol-17β (E_2) and progesterone (P) by radioimmunoassay procedures according to WHO matched reagent programme. The WHO assay method is designed to estimate steroid levels in human serum or plasma extracts. To quantify steroids in other species, it is advised to validate the assay method beforehand (Sufi, Donaldson and Jeffcoate, 1990). The validations included parallelism check for immunological similarity and reliability of the assay (Sukanya Werawatgoompa, 1982). The reliability of RIA is assessed by four main criteria: specificity, sensitivity, precision and accuracy (Chard, 1978, Thorell and Larson, 1978 both cited in Suchinda Malaivijitnond, 1994).

Parallelism:

Parallelism is interpreted as evidence of immunological similarity or as suggesting that observed immunoreactivity is not an assay artifact (Belfe, 1987; Kieffer and Malarkey, 1978; Kyle et al., 1987 cited in Kesorn Suwanprasert, 1991).

Softshell turtle plasma containing a high level of each steroid was diluted with hormone free plasma prior to assay (preparation of hormone free plasma is described in appendix II). The dilution curve was compared to the standard curve of human steroids. The parallelism was expressed as insignificant difference between slopes of the two regression lines (Sukanya Werawatgoompa, 1982; Zar, 1982).

Specificity:

Specificity is an ability of the assay to measure one specific compound and no other. The identifiable materials with physicochemical similarity to the ligand that can interfere with the assay by reacting directly with the binder are called cross-reacting materials. The most common way to present the specificity is to compare the amount of the cross-reacting materials under study which yields 50 percent inhibition of binding (x) with the amount of standard giving the same inhibition (y), and then express the potency of the cross-reacting materials as a percentage of the standard: Percent cross-reactivity = (y / x) x 100.

The cross reaction of WHO antiserum with other substances were tested by WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Sensitivity:

Sensitivity is defined as the minimal detection limit of an assay. It may refer to the least concentration of unlabelled ligand which can be distinguished from a sample containing no unlabelled ligand. It can be determined based on the confidence limits to the zero standard estimate. The sensitivity of an assay critically depend on the precision of the assay.

Blank test and standard were plotted together in a dose response curve of $\log_{10}$ dose against percent bound (% B/B0). The 95 % bound was determined as sensitivity of the assay.

Precision:

Precision, or reproducibility, is a measure of observed variation between repeated determination of the same sample. It is usually expressed as coefficient of variation. Intra-assay, or within assay, variation refers to precision within an individual assay. Inter-assay, or between assay, variation refers to precision from different sets of assays of the same sample. To monitor precision, the pooled samples should be set up and their concentrations should represent high, medium and low values in the assay. This provides an on-going check of precision at different parts of the standard curve.

Due to the limited amount of softshell turtle plasma, the pooled plasma was set up into two groups according to sex representing two different values in each assay. By the way it was found that the pooled plasma of each sex was subjected to contamination with plasma of other sex due to early miss-sexing. Thus the steroid levels in each pooled plasma did not represent the true value of each sex. It was actually valid only for monitoring the precision.

Accuracy:

Accuracy is the degree to which the estimate approximates the true value. It is expressed as a correlation coefficient between the determined and added values.

3.3.1 Testosterone assay

To perform radioimmunoassay for testosterone of softshell turtles, testosterone of the softshell turtles was allowed to compete with (1, 2, 6, 7-^{3}H) testosterone in binding to mouse antibody to testosterone-3CMO-BSA. Dextran-charcoal was utilized for separation of free from antibody-bound hormone. The following method procedures were according to WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Reagent preparation:

1. *Testosterone antiserum*

Testosterone antiserum was produced in mice by monoclonal antibody technique in response to testosterone-3CMO-BSA conjugate. It was provided in lyophilized form. The freeze dried antiserum is stable for several years if stored at 4°C. The content of one bottle of antiserum was reconstituted with 10

ml assay buffer solution, allowed to stand for 5-10 minutes and mixed before use with a final dilution of 1: 210,000 in assay tubes.

2. *Testosterone tracer*

Solution of (1, 2, 6, 7-^{3}H) testosterone that was provided in the concentration of 9.25 MBq (250 μCi) was transferred from sealed ampule to a 25 ml volumetric flask. The ampule was rinsed with a solution of toluene: ethanol (9:1 by volume), and the washings was added to the contents of the flask. The volume of the stock solution was made up to 25 ml with the toluene: ethanol (9:1) solution. The stock solution with concentration of 370 kBq or 10 μCi/ml was stored in a dark bottle at -20°C.

Working tracer was prepared as follows: 300 μl of the stock solution was transferred into a bottle and the solvent was allowed to evaporate. The residue was redissolved with 12 ml assay buffer solution. The bottle was allowed to stand for 30 minutes in order to resolve the tracer.

3. *Testosterone standard*

This was provided in ethanolic solution at a concentration of 220 nmol/l. An ampule and pipettes to be use were cooled to 4 °C. After the ampule was opened, aliquots of 3 or 4 x 100 μl were transferred to the 15 ml bottles. These aliquots were stored at 4°C until needed. When required, 10 ml assay buffer solution was added to the bottle and heated to 40°C in a water bath for 30 minutes. The solution was mixed vigorously and allowed to cool to room temperature before use. This solution (solution B) contained 2.2 nmol/l or 2,200 fmol/ml testosterone and was stable for 2-3 weeks when stored at 4°C. Upon assay, the solution B was serially diluted to obtain the following triplicate standard dose levels: 1100, 550, 275, 138, 69, 34 and 17 fmol/500 μl.

4. *Recovery tracer*

Recovery tracer was prepared to monitor the recovery of extraction. Three hundred microlitres of stock tracer was transferred to a vial, the solvent was allowed to evaporate and redissolved in 1.2 ml assay buffer solution.

5. *Sample preparation*

Plasma of softshell turtle was extracted with diethyl ether prior to assay. The advantages of an extraction were to concentrate endogenous ligand from

other materials, to improve specificity and to avoid damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978, cited in Suchinda Malaivijitnond, 1994).

5.1 *Extraction of unknown plasma and quality control*

Two aliqouts of each sample (500 µl for female, and 10 µl for male) and 3 replicates of each quality control were transferred into cone tubes and reconstituted with 5 ml fresh diethyl ether. Five millilitres of fresh diethyl ether only was added to two tubes and labelled as ether blank. Every tubes was subjected to extraction under agitation machine for 3 minutes. The ether was allowed to settle. The tubes were dipped in 95 % ethanol containing chips of dry ice. An aqueous layer froze and the upper ether layer was decanted to an assay tube. The ether was evaporated to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution. The assay tubes were mixed under agitation machine for 1 minute, allowed to stand for 10 minutes then mixed again for 1 minute. This solution was then ready to be used for assay.

5.2 *Recovery of extraction*

Two aliquots of 500 µl and 10 µl of the male pooled plasma were added with 10 µl of recovery tracer in cone tubes, mixed and allowed to equilibrate with the tracer for 30-60 minutes at room temperature. The mixed aliquots were extracted with fresh diethyl ether and separated for the ether layer as well as the unknown plasma. The ether was evaporated to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution, and mixed like the unknown sample. The reconstitution was decanted to counting vial. Two recovery totals were prepared by adding 10 µl of recovery tracer and 500 µl of assay buffer solution into assay tubes, then transferred to counting vials. Five millilitres of scintillation fluid was added to counting vials. After equilibration, each vial was counted in a β-counter for 5 minutes. Recovery of extraction was calculated by:

$$\text{Recovery of extraction (\%)} = \frac{\text{Sample recovery counts} \times 100}{\text{Recovery total counts}}$$

Recovery of extraction in this study ranged from 83.51 to 92.10 %. This recovery factor was merely used for monitoring efficiency of extraction in each assay. It was not applied to calculation of testosterone concentration. Hence, an underestimation for testosterone level should be remarked.

Assay procedures:

Various reagents were added to yield a final volume of 900 µl in an assay tube. Three replicates of zero antigen or maximum binding (B0) tubes were prepared by adding 500 µl of assay buffer solution in assay tubes. A 100 µl amount of antiserum to testosterone was added to the B0, extracted unknown sample, quality controls, and serial dilutions of standard in 500 µl of assay buffer solution in assay tubes. Three non-specific binding (NSB) tubes were prepared by adding 600 µl of assay buffer solution in assay tubes. Three total count (TC) tubes were prepared by adding 800 µl of assay buffer solution in assay tubes. The latter two triplicates were prepared without added antiserum. A 100 µl amount of testosterone working tracer was added to each tube, then mixed and incubated at 4°C for 18-24 hours. Contents of testosterone assay tubes are summarized in table 3-2.

Table 3-2 Summary of contents of assay tubes in testosterone assay.

	Buffer	**Standard or Sample**	**Antiserum to testosterone**	**Testosterone tracer**	*Incubate*	**Charcoal suspension**
Total count	800 µl	-	-	100 µl	*at*	-
NSB	600 µl	-	-	100 µl	*4 ℃*	200 µl
B0	500 µl	-	100 µl	100 µl	*18-24*	200 µl
Standard or Sample	-	500 µl	100 µl	100 µl	*hours*	200 µl

Separation of antibody-bound hormone was performed in an ice bath by addition of 200 µl of cold charcoal suspension which was stirred continuously with magnetic stirrer except for the total count tubes. Each tube was mixed under agitation machine for 1 minute and allowed to stand for 30 minutes at 4°C, then centrifuged at 1,100 x g, 4°C for 15 minutes. The supernatant was decanted into a counting vial. Five millilitres of scintillation fluid was added to each vial, and allowed to equilibrate for 60 minutes. Each vial was counted in a β-counter for 5 minutes.

A dose response curve was plotted using $\log_{10}$ dose against standard bound-NSB (counts per minute), and regression analysis was performed. Result for each sample (fmol/tube) was read from the regression line of the standard curve. This result was transformed to concentration per millilitre (fmol/ml) according volume of the sample. It was then multiplied by molecular weight of testosterone

(288.43) and divided by 10^6 in order to express the concentration of testosterone as nanogram per millilitre (ng/ml).

Validation of assay:

1. *Parallelism*

The high levels of softshell turtle testosterone were paralleled against the standard curve of human testosterone as shown in figure 3-4. There was no significant difference between the slopes of these two regression lines.

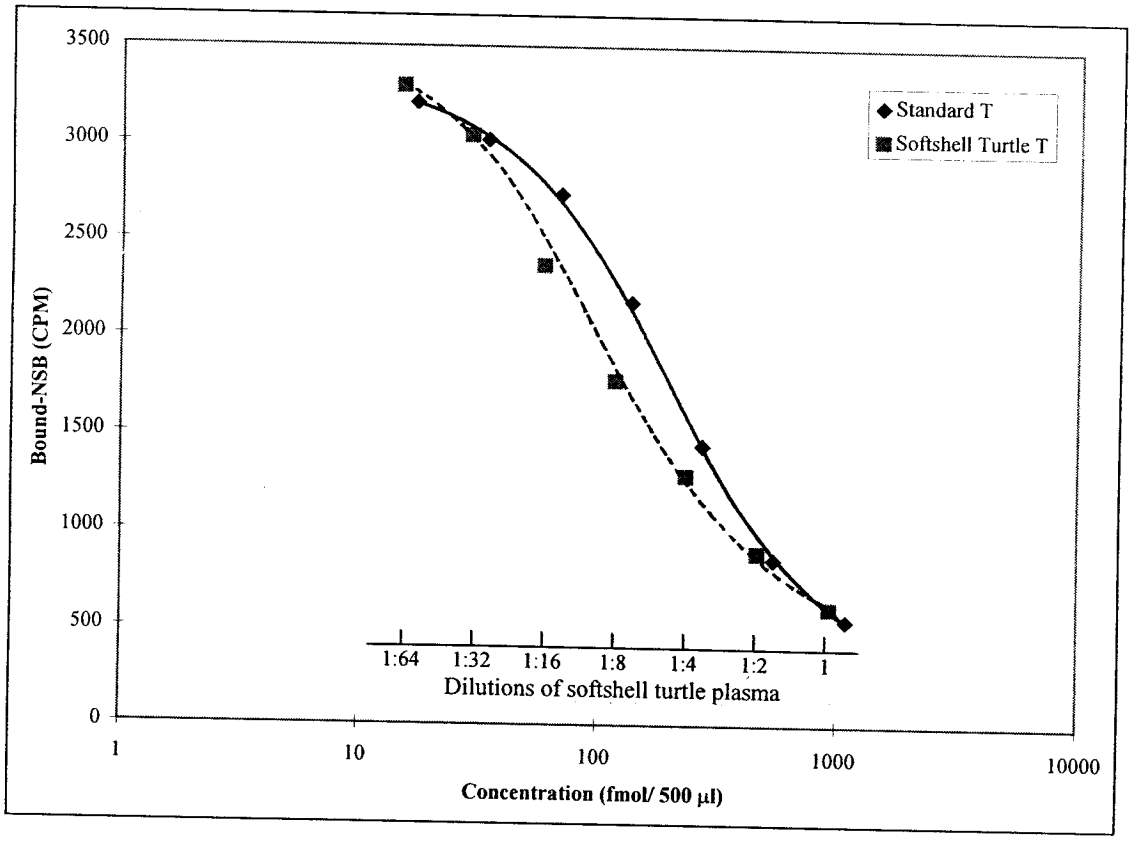

Figure 3-4 Parallelism of immunoreactivities of human testosterone standard and serial dilutions of softshell turtle plasma.

2. *Specificity*

The cross reactions of steroids likely to be present in plasma were tested by WHO matched reagent programme as listed below (Sufi, Donaldson, Jeffcoate, 1990).

- Testosterone %Cross reaction = 100.0
- 5α Dihydrotestosterone %Cross reaction = 1.3
- Δ 4-Androstenedione %Cross reaction = 3.5

- 5α-Androstanediol %Cross reaction = 1.3
- Androstane 3α diol %Cross reaction = 0.7
- Androstane 3β diol %Cross reaction = 1.8

3. *Sensitivity*

The sensitivity of testosterone assay was 3.31 pg/ml.

4. *Precision*

The intra-assay and inter-assay variation of testosterone assay are presented as coefficients of variation in table 3-3.

Table 3-3 Precision of testosterone assay.

Quality Control	Mean (fmol / 10 µl)	Coefficient of Variation	
		Intra-assay (n=10)	Inter-assay (n=30)
Males	659.08 ± 72.48	5.34 - 8.81	11.00
Females	478.32 ± 70.27	4.50 - 5.19	14.69

5. *Accuracy*

The Pearson's correlation coefficient between the determined and added values of testosterone was 0.999 ($p < 0.01$).

3.3.2 Estradiol-17β assay

To perform radioimmunoassay for estradiol of softshell turtles, estradiol of softshell turtles was allowed to compete with (2, 4, 6, 7-^{3}H) estradiol in binding to mouse antibody to estradiol-6CMO-BSA. Dextran-charcoal was utilized for separation of free hormone from antibody-bound hormone. The following method procedures were followed the WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Reagent preparation:

1. *Estradiol antiserum*

Estradiol antiserum was produced in mice by monoclonal antibody technique in response to estradiol-6CMO-BSA conjugate. It was provided in lyophilized form. The freeze dried antiserum is stable for several years if stored at 4°C. The content of one bottle of antiserum was reconstituted with 10 ml assay

buffer solution, allowed to stand for 5-10 minutes and mixed before use with a final dilution of 1: 210,000 in assay tubes.

2. *Estradiol tracer*

Solution of (2, 4, 6, 7-^{3}H) estradiol that was provided in the concentration of 9.25 MBq (250 μCi) was transferred from sealed ampule to a 25 ml volumetric flask. The ampule was rinsed with a solution of toluene: ethanol (9:1 by volume), and the washings was added to the contents of the flask. The volume of the stock solution was made up to 25 ml with the toluene: ethanol (9:1) solution. The stock solution with concentration of 370 kBq or 10 μCi/ml was stored in a dark bottle at -20°C.

Working tracer was prepared as follows: 400 μl of the stock solution was transferred into a bottle and the solvent was allowed to evaporate. The residue was redissolved with 12 ml assay buffer solution. The bottle was allowed to stand for 30 minutes in order for resolvation of the tracer.

3. *Estradiol standard*

This was provided in ethanolic solution at a concentration of 150 nmol/l. An ampule and pipettes to be use were cooled to 4 °C. After the ampule was opened, aliquots of 3 or 4 x 100 μl were transferred to the 15 ml bottles. These aliquots were stored at 4°C until needed. When required, 10 ml assay buffer solution was added to the bottle and heated to 40°C in a water bath for 30 minutes. The solution was allowed to cool to 4°C and mixed vigorously before use. This solution (solution B) contained 1.5 nmol/l or 1,500 fmol/ml estradiol and was stable for 2-3 weeks when stored at 4°C. Upon assay, the solution B was serially diluted to obtain the following triplicate standard dose levels: 750, 375, 188, 94, 47, 23 and 12 fmol/500 μl.

4. *Recovery tracer*

Recovery tracer was prepared to monitor the recovery of extraction. Four hundred microlitres of stock tracer was transferred to a vial, the solvent was allowed to evaporate and redissolved in 1.2 ml assay buffer solution.

5. *Sample preparation*

Plasma of softshell turtles was double extracted with diethyl ether prior to assay. The advantages of an extraction were to concentrate endogenous

ligand from other materials, to improve specificity and to avoid damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978, cited in Suchinda Malaivijitnond, 1994). The double extraction could concentrate and recover much more amount of hormone from sample (Suchinda Malaivijitnond, 1994)

5.1 *Extraction of unknown plasma and quality control*

Two aliqouts of each sample (200 µl for female, and 500 µl for male) and 3 replicates of each quality control were transferred into cone tubes and reconstituted with 4 ml fresh diethyl ether. Four millilitres of fresh diethyl ether only was added to two tubes and labelled as ether blank. Every tubes was subjected to extraction under agitation machine for 3 minutes. The ether was allowed to settle. The tubes were dipped in 95 % ethanol containing chips of dry ice. An aqueous layer froze and the upper ether layer was decanted to an assay tube. Every tubes were repeated extraction with 4 ml diethyl ether. The second ether layer was poured over the first one in the assay tube and allowed to evaporate to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution. The assay tubes were mixed under agitation machine for 1 minute, allowed to stand for 10 minutes then mixed again for 1 minute. This solution was then ready to be used for assay.

5.2 *Recovery of extraction*

Two aliquots of 500 µl and 200 µl of the female pooled plasma were added with 10 µl of recovery tracer in cone tubes, mixed and allowed to equilibrate with the tracer for 30-60 minutes at room temperature. The mixed aliquots were double extracted with fresh diethyl ether and separated for the ether layer as well as the unknown plasma. The ether was evaporated to dry in a heating block. Remaining residue was redissolved with 500 µl of assay buffer solution, and mixed like the unknown sample. The reconstitution was decanted to a counting vial. Two recovery totals were prepared by adding 10 µl of recovery tracer and 500 µl of assay buffer solution into assay tubes, then transferred to counting vials. Five millilitres of scintillation fluid was added to counting vials. After equilibration, each vial was counted in a β-counter for 5 minutes. Recovery of extraction was calculated by:

$$\text{Recovery of extraction (\%)} = \frac{\text{Sample recovery counts} \times 100}{\text{Recovery total counts}}$$

Recovery of extraction in this study ranged between 80.18 % and 80.95 %. This recovery factor was merely used for monitoring efficiency of extraction in each assay. It was not applied to calculation of estradiol concentration. Hence, an underestimation for estradiol level should be remarked.

Assay procedures:

Various reagents were added to yield a final volume of 900 µl in an assay tube. Three replicates of zero antigen or maximum binding (B0) tubes were prepared by adding 500 µl of assay buffer solution in assay tubes. One hundred microlitres of antiserum to estradiol was added to the B0, extracted unknown sample, quality controls, and serial dilutions of standard in 500 µl of assay buffer solution in assay tubes. Three non-specific binding (NSB) tubes were prepared by adding 600 µl of assay buffer solution in assay tubes. Three total count (TC) tubes were prepared by adding 800 µl of assay buffer solution in assay tubes. The latter two triplicates were prepared without added antiserum. A 100 µl amount of estradiol working tracer was added to each tubes, then mixed and incubated at 4°C for 18-24 hours. Contents of estradiol assay tubes are summarized in table 3-4.

Table 3–4 Summary of contents of assay tubes in estradiol assay.

	Buffer	**Standard or Sample**	**Antiserum to estradiol**	**Estradiol tracer**	*Incubate*	**Charcoal suspension**
Total count	800 µl	-	-	100 µl	*at*	-
NSB	600 µl	-	-	100 µl	*4 ℃*	200 µl
B0	500 µl	-	100 µl	100 µl	*18-24*	200 µl
Standard or Sample	-	500 µl	100 µl	100 µl	*hours*	200 µl

Except for the total count tubes, separation of antibody-bound hormone was performed in an ice bath by addition of 200 µl of cold charcoal suspension which was stirred continuously with magnetic stirrer. Each tube was mixed under agitation machine for 1 minute and allowed to stand for 30 minutes at 4°C, then centrifuged at 1,100 x g, 4°C for 15 minutes. The supernatant was decanted into a counting vial. Five millilitres of scintillation fluid was added to each vial, and allowed to equilibrate for 60 minutes. Each vial was counted in a β-counter for 5 minutes.

A dose response curve was plotted using $\log_{10}$ dose against standard bound-NSB (counts per minute), and regression analysis was performed. Result for each sample (fmol/tube) was read from the regression line of the standard curve. This result was transformed to concentration per millilitre (fmol/ml) according to volume of the sample. Then it was multiplied by molecular weight of estradiol (272.39) and 10^{-3} in order to express the concentration of estradiol as picogram per millilitre (pg/ml).

Validation of assay:

1. *Parallelism*

The high levels of softshell turtle estradiol were paralleled against the standard curve of human estradiol as shown in figure 3-5. There was no significant difference between the slopes of these two regression lines.

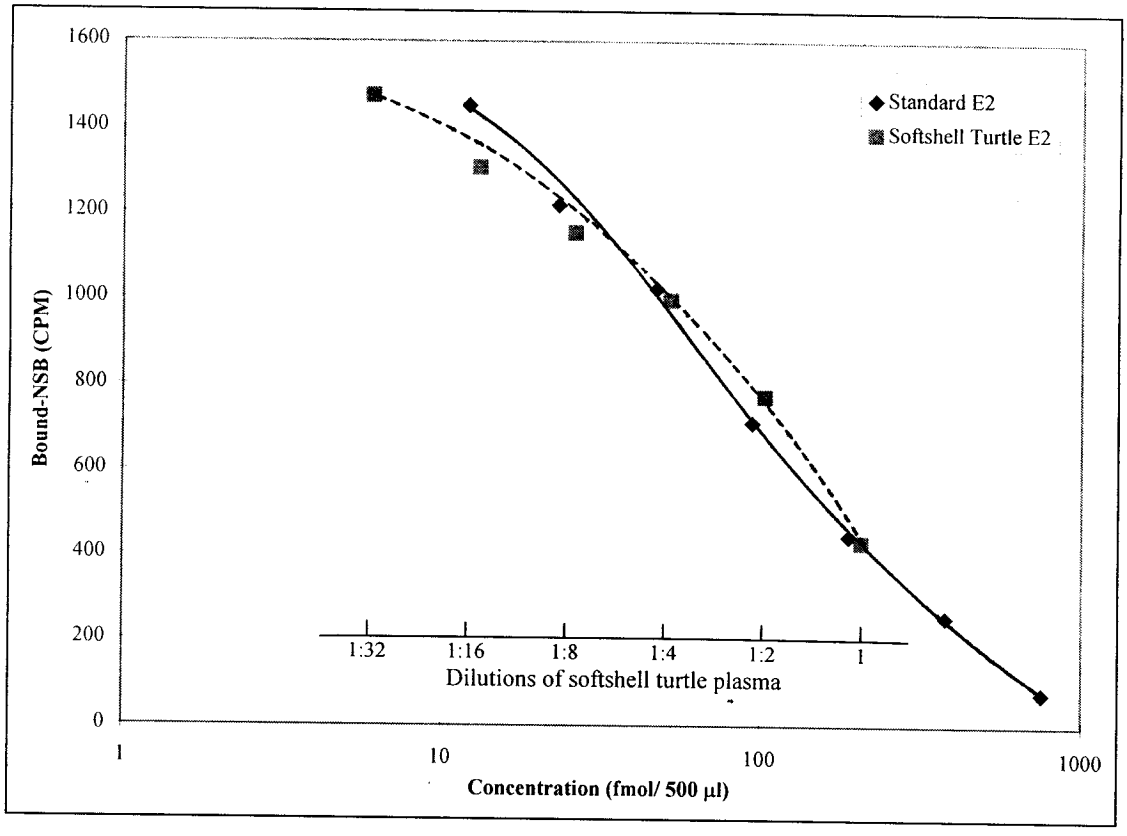

Figure 3-5 Parallelism of immunoreactivities of human estradiol standard and serial dilutions of softshell turtle plasma.

2. *Specificity*

The cross reactions of steroids likely to be present in plasma were tested by WHO matched reagent programme as listed below (Sufi, Donaldson, Jeffcoate, 1990).

- Estradiol %Cross reaction = 100.0
- Estriol %Cross reaction = 0.8
- Estrone %Cross reaction < 0.02
- Cortisol %Cross reaction < 0.02
- Progesterone %Cross reaction = 0.02
- Testosterone %Cross reaction < 0.02

3. *Sensitivity*

The sensitivity of estradiol assay was 1.57 pg/ml.

4. *Precision*

The intra-assay and inter-assay variation of estradiol assay are presented as coefficients of variation in table 3-5.

Table 3-5 Precision of estradiol assay.

Quality Control	Mean (fmol / 200 µl)	Coefficient of Variation	
		Intra-assay (n=10)	Inter-assay (n=30)
Males	22.41 ± 3.33	4.45 - 9.67	14.86
Females	124.05 ± 15.44	7.36 - 9.09	12.45

5. *Accuracy*

The Pearson's correlation coefficient between the determined and added values of estradiol was 0.969 ($p < 0.01$).

3.3.3 Progesterone assay

To perform radioimmunoassay for progesterone of softshell turtles, progesterone of the softshell turtles was allowed to compete with (1, 2, 6, 7-^{3}H) progesterone in binding to mouse antibody to progesterone-3CMO-BSA. Dextran-charcoal was utilized to separate free hormone from antibody-bound hormone. The following method procedures followed the WHO matched reagent programme (Sufi, Donaldson and Jeffcoate, 1990).

Reagent preparation:

1. *Progesterone antiserum*

Progesterone antiserum was produced in mice by monoclonal antibody technique in response to progesterone-3CMO-BSA conjugate. It was provided in lyophilized form. The freeze dried antiserum is stable for several years if stored at 4°C. The content of one bottle of antiserum was reconstituted with 10 ml assay buffer solution, allowed to stand for 5-10 minutes and mixed before use with a final dilution of 1: 210,000 in assay tubes.

2. *Progesterone tracer*

Solution of (1, 2, 6, 7-^{3}H) progesterone that was provided in the concentration of 3.7 MBq (100 μCi) was transferred from sealed ampule to a 10 ml volumetric flask. The ampule was rinsed with a solution of toluene: ethanol (9:1 by volume), and the washings was added to the contents of the flask. The volume of the stock solution was made up to 10 ml with the toluene: ethanol (9:1) solution. The stock solution with concentration of 370 kBq or 10 μCi/ml was stored in a dark bottle at -20°C.

Working tracer was prepared as follows: 400 μl of the stock solution was transferred into a bottle and the solvent was allowed to evaporate. The residue was redissolved with 12 ml assay buffer solution. The bottle was allowed to stand for 30 minutes in order for resolvation of the tracer.

3. *Progesterone standard*

This was provided in ethanolic solution at a concentration of 250 nmol/l. An ampule and pipettes to be use were cooled to 4 °C. After the ampule was opened, aliquots of 3 or 4 x 100 μl were transferred to the 15 ml bottles. These aliquots were stored at 4°C until needed. When required, 10 ml assay buffer solution was added to the bottleand heated to 40°C in a water bath for 30 minutes. The solution was mixed vigorously and allowed to cool to 4°C before use. This solution (solution B) contained 2.5 nmol/l or 2,500 fmol/ml progesterone and was stable for 2-3 weeks when stored at 4°C. Upon assay, the solution B was serially diluted to obtain the following triplicate standard dose levels: 1250, 625, 313, 156, 78, 39 and 20 fmol/500 μl.

4. *Recovery tracer*

Recovery tracer was prepared to monitor the recovery of extraction. Four hundred microlitres of stock tracer was transferred to a vial, the solvent was allowed to evaporate and redissolved in 1.2 ml assay buffer solution.

5. *Sample preparation*

Plasma of softshell turtle was extracted with diethyl ether prior to assay. The advantages of an extraction were to concentrate endogenous ligand from other materials, to improve specificity and to avoid damage to endogenous ligand or tracer with potentially damaging enzymes in the sample (Chard, 1978, cited in Suchinda Malaivijitnond, 1994).

5.1 *Extraction of unknown plasma and quality control*

Two aliqouts of each sample (50 µl for female and 500 µl for male) and 3 replicates of each quality control were transferred into cone tubes and reconstituted with 5 ml fresh diethyl ether. Five millilitres of fresh diethyl ether only was added to two tubes and labelled as ether blank. Every tubes was subjected to extraction under agitation machine for 3 minutes. The ether was allowed to settle. The tubes were dipped in 95 % ethanol containing chips of dry ice. An aqueous layer froze and the upper ether layer was decanted to an assay tube. The ether was evaporated to dry in a heating block. The remaining residue was redissolved with 500 µl of assay buffer solution. The assay tubes were mixed under agitation machine for 1 minute, allowed to stand for 10 minutes then mixed again for 1 minute. This solution was then ready to be used for assay.

5.2 *Recovery of extraction*

Two aliquots of 50 µl of the female pooled plasma were added with 10 µl of recovery tracer in cone tubes, mixed and allowed to equilibrate with the tracer for 30-60 minutes at room temperature. The mixed aliquots were extracted with fresh diethyl ether and separated for the ether layer as well as the unknown plasma. The ether was evaporated to dry in a heating block. Remaining residue was redissolved with 500 µl of assay buffer solution, and mixed like the unknown sample. The reconstitution was decanted to a counting vial. Two recovery totals were prepared by adding 10 µl of recovery tracer and 500 µl of assay buffer solution into assay tubes, then transferred to counting vials. Five millilitres of scintillation fluid was added to counting vials. After equilibration, each vial was counted in a β-counter for 5 minutes. Recovery of extraction was calculated by:

$$\text{Recovery of extraction (\%)} = \frac{\text{Sample recovery counts}}{\text{Recovery total counts}} \times 100$$

Recovery of extraction in this study ranged from 60.35 to 60.57 %. This recovery factor was merely used for monitoring efficiency of extraction in each assay. It was not applied to calculation of progesterone concentration.

According to Sufi, Donaldson and Jeffcoate (1990), it was essential to make sure that the extract is properly dissolved before analysis. In case of progesterone it might be necessary to heat the reconstituted extract at 50°C for 15-40 minutes to ensure that the progesterone was completely dissolved. But after many trials, it was found that heating assay tube which was not single use could dissolve uncontrol impurities resulted in unacceptable poor precision. Progesterone assay was thus conducted without heating the assay tube. Low recovery of extraction was compensated by better precision. By the way, an underestimation for progesterone assay should be remarked.

Assay procedures:

Various reagents were added to yield a final volume of 900 µl in an assay tube. Three replicates of zero antigen or maximum binding (B0) tubes were prepared by adding 500 µl of assay buffer solution in assay tubes. A 100 µl amount of antiserum to progesterone was added to the B0, extracted unknown sample, quality controls, and serial dilutions of standard in 500 µl of assay buffer solution in assay tubes. Three non-specific binding (NSB) tubes and three total count (TC) tubes were prepared by respectively adding 600 µl and 800 µl of assay buffer solution in assay tubes. The latter two triplicates were prepared without added antiserum. A 100 µl amount of progesterone working tracer was added to each tube, then mixed and incubated at 4°C for 18-24 hours. Contents of progesterone assay tubes are summarized in table 3-6.

Table 3-6 Summary of contents of assay tubes in progesterone assay.

	Buffer	**Standard or Sample**	**Antiserum to progesterone**	**Progesterone tracer**	*Incubate*	**Charcoal suspension**
Total count	800 µl	-	-	100 µl	*at*	-
NSB	600 µl	-	-	100 µl	*4 ℃*	200 µl
B0	500 µl	-	100 µl	100 µl	*18-24*	200 µl
Standard or Sample	-	500 µl	100 µl	100 µl	*hours*	200 µl

Separation of antibody-bound hormone was performed in an ice bath by addition of 200 µl of cold charcoal suspension which was stirred continuously with magnetic stirrer except for the total count tubes. Each tube was mixed under agitation machine for 1 minute and allowed to stand for 30 minutes at 4°C, then centrifuged at 1,100 x g, 4°C for 15 minutes. The supernatant was decanted into a counting vial. Five millilitres of scintillation fluid was added to each vial, and allowed to equilibrate for 60 minutes. Each vial was counted in a β-counter for 5 minutes.

A dose response curve was plotted using $\log_{10}$ dose against standard bound-NSB (counts per minute), and regression analysis was performed. Result for each sample (fmol/tube) was read from the regression line of the standard curve. This result was transformed to concentration per millilitre (fmol/ml) according to volume of the sample. It was then multiplied by molecular weight of progesterone (314.47) and 10^{-6} in order to express the concentration of progesterone as nanogram per millilitre (ng/ml).

Validation of assay:

1. *Parallelism*

Due to limited amount of softshell turtle plasma, only medium level of progesterone was available. Dilution curve of softshell turtle plasma containing medium level of progesterone was paralleled against the standard curve of human progesterone as shown in figure 3-6. There was no significant difference between the slopes of these two regression lines.

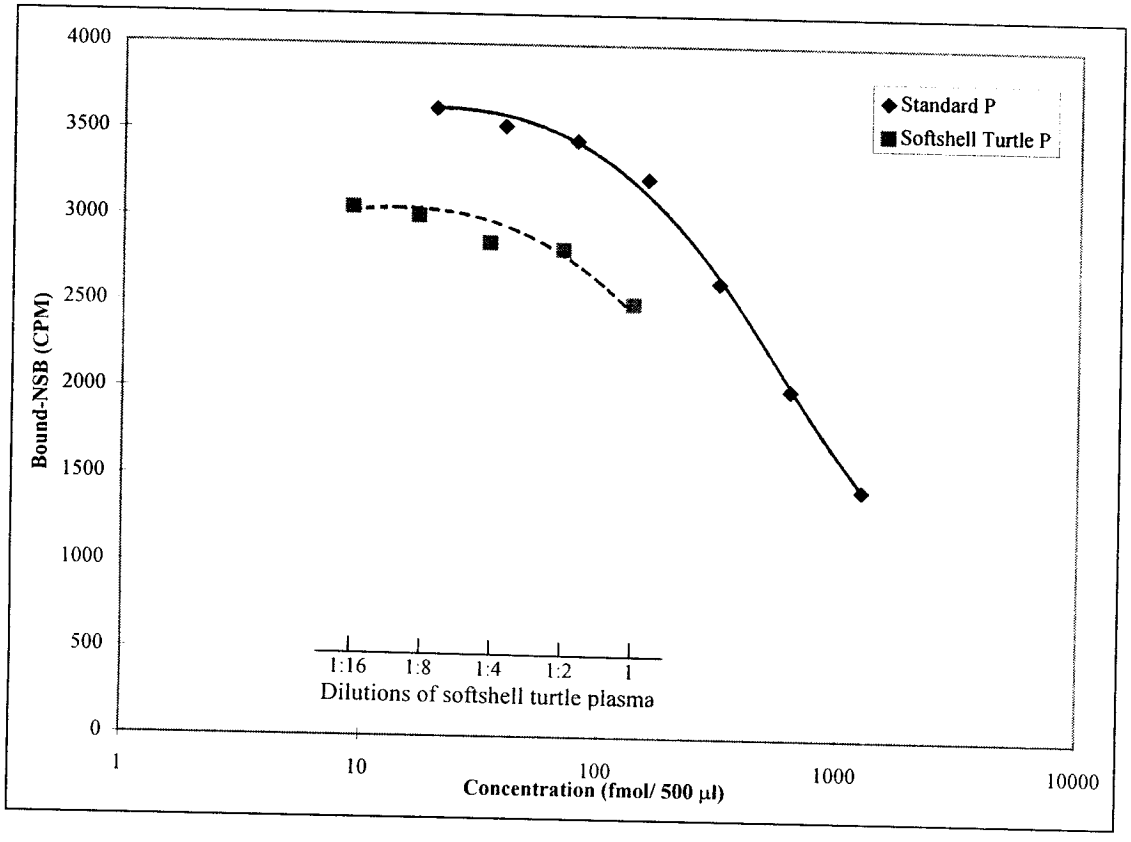

Figure 3-6 Parallelism of immunoreactivities of human progesterone standard and serial dilutions of softshell turtle plasma.

2. *Specificity*

The cross reactions of steroids likely to be present in plasma were tested by WHO matched reagent programme as follows (Sufi, Donaldson, Jeffcoate, 1990).

- Progesterone	%Cross reaction = 100.0
- Cortisol	%Cross reaction = 0.002
- Testosterone	%Cross reaction = 0.02
- 17α Hydroxyprogesterone	%Cross reaction = 1.6
- 20α Dihydroprogesterone	%Cross reaction = 0.04

3. *Sensitivity*

The sensitivity of progesterone assay was 7.52 pg/ml.

4. *Precision*

The intra-assay and inter-assay variation of progesterone assay are presented as coefficients of variation in table 3-7.

Table 3-7 Precision of progesterone assay.

Quality Control	*Mean* (fmol / 50 µl)	Coefficient of Variation	
		Intra-assay (n=10)	Inter-assay (n=30)
Males	348.22 ± 50.53	8.70 - 9.75	14.51
Females	373.96 ± 51.37	8.08 - 9.46	13.74

5. *Accuracy*

The Pearson's correlation coefficient between the determined and added values of progesterone was 0.921 ($p < 0.01$).

3.3.4 Analysis

Annual reproductive cycles of male and female softshell turtle were analyzed according to the following two categories.

1. Temporal changes in hormonal levels

Levels of steroids of each sex in each sampling, monthly levels as well as seasonal levels were calculated for mean and standard error of the mean (S.E.M.). The means were compared among samplings, months and season using analysis of variance (ANOVA), and Duncan's multiple range test was used to classify homogeneous subsets of the means. Probability of $p \leq 0.05$ was considered to be significantly different.

2. Correlation to climatic factors

Mean levels of each samplings, months and seasons were analyzed for correlation with climatic data of Bangkok area (Meteorological department, 1997) by bivariate correlation method. Significant correlation was considered from probability of $p \leq 0.05$.

General calculations were performed on computer by Microsoft Excel for Windows 95 version 7.0. Statistical analysis were performed on computer by SPSS for Windows release 7.0.

Chapter 4

Results and Discussion

4.1 Sexual dimorphism

In order to minimize size bias, every morphological characters of *Amyda cartilaginea* were transformed to various parameters in relative to carapace length (CL). It was found that the male softshell turtle exhibited significantly higher degree of plastron to cloaca (PC/CL), tailbase to cloaca (TC/CL), cloaca to tail tip (CT/CL), tail length (TL/CL), tail width (TW/CL), and weight (W/CL). The females showed significantly higher degree of bony disc length 1 (BDL1/CL), bony disc length 2 (BDL2/CL), plastron length 2 (PL2/CL), height (H/CL), head length without snout (HL/CL), and head length with snout (HLs/CL). While other parameters were not significantly different between sex. Table 4-1 presents mean and S.E.M. of the mentioned parameters of male and female softshell turtle.

In addition to the above parameters, tail length beyond carapacial margin was also compared between sexes. The length was transformed from subtraction of plastron to rear margin of carapace from addition of plastron to cloaca and cloaca to tail tip {(PC+CT)-PR}. The results were transformed into relative numbers to capapace length and compared between sexes. It was found that the males showed significant higher tail length beyond carapacial margin than females as displayed in table 4-1.

Body outline of softshell turtle was determined from subtraction of carapace length by plastron width (CW-PW) in relative to carapace length. It was found that there was no significant difference between the two sexes as shown in table 4-1.

Cloacal position was determined from division of tailbase to cloaca with tail length (TC/TL). It was found that cloacal position of the males was significantly more posterior than the females as shown in table 4-1.

Table 4-1 Mean and S.E.M. of parameters of morphological characters of *Amyda cartilaginea*.

Parameters	Mean ± S.E.M.	
	Males (n = 80)	Females (n = 54)
Bony Disc Length 1/CL	0.6548[a] ± 0.0035	0.6722[b] ± 0.0052
Bony Disc Length 2/CL	0.5570[a] ± 0.0042	0.5814[b] ± 0.0061
Carapace Width/CL	0.7222[a] ± 0.0044	0.7292[a] ± 0.0053
Plastron Length 1/CL	0.5155[a] ± 0.0029	0.5257[a] ± 0.0046
Plastron Length 2/CL	0.6645[a] ± 0.0034	0.6771[b] ± 0.0050
Plastron Width/CL	0.6818[a] ± 0.0055	0.6895[a] ± 0.0052
Height/CL	0.2638[a] ± 0.0023	0.2764[b] ± 0.0039
Plastron to Rear margin of carapace/CL	0.3526[a] ± 0.0021	0.3738[a] ± 0.0049
Plastron to Cloaca/CL	0.3015[a] ± 0.0026	0.2625[b] ± 0.0034
Tailbase to Cloaca/CL	0.0690[a] ± 0.0022	0.0301[b] ± 0.0016
Cloaca to Tail tip/CL	0.0712[a] ± 0.0014	0.0623[b] ± 0.0022
Tail Length/CL	0.1394[a] ± 0.0029	0.0923[b] ± 0.0027
Tail Width/CL	0.0963[a] ± 0.0017	0.0783[b] ± 0.0016
Head Length without snout/CL	0.2730[a] ± 0.0020	0.2795[b] ± 0.0026
Head Length with snout/CL	0.2961[a] ± 0.0025	0.3088[b] ± 0.0027
Head Width/CL	0.1748[a] ± 0.0022	0.1755[a] ± 0.0019
Head Height/CL	0.1399[a] ± 0.0009	0.1398[a] ± 0.0013
Weight/CL	0.3615[a] ± 0.0180	0.2447[b] ± 0.0136
Tail length beyond Carapace/CL	-0.010[a] ± 0.0037	-0.049[b] ± 0.0037
Carapace Width-Plastron Width/CL	0.0404[a] ± 0.0040	0.0394[a] ± 0.0021
Tailbase to Cloaca/Tail Length	0.4901[a] ± 0.0091	0.3255[b] ± 0.0143

Remark Significant differences ($p<0.05$) of the means between males and females are indicated by differences in superscript letters.

Every morphological characters of each sex were plotted against carapace length (CL). The regression analysis revealed significant linear regression line ($p<0.05$) of both sexes in every pair of morphological characters and CL. Slopes and elevation of regression line in every pairwise were compared between sexes. The results are presented in figure 4-1 to 4-20.

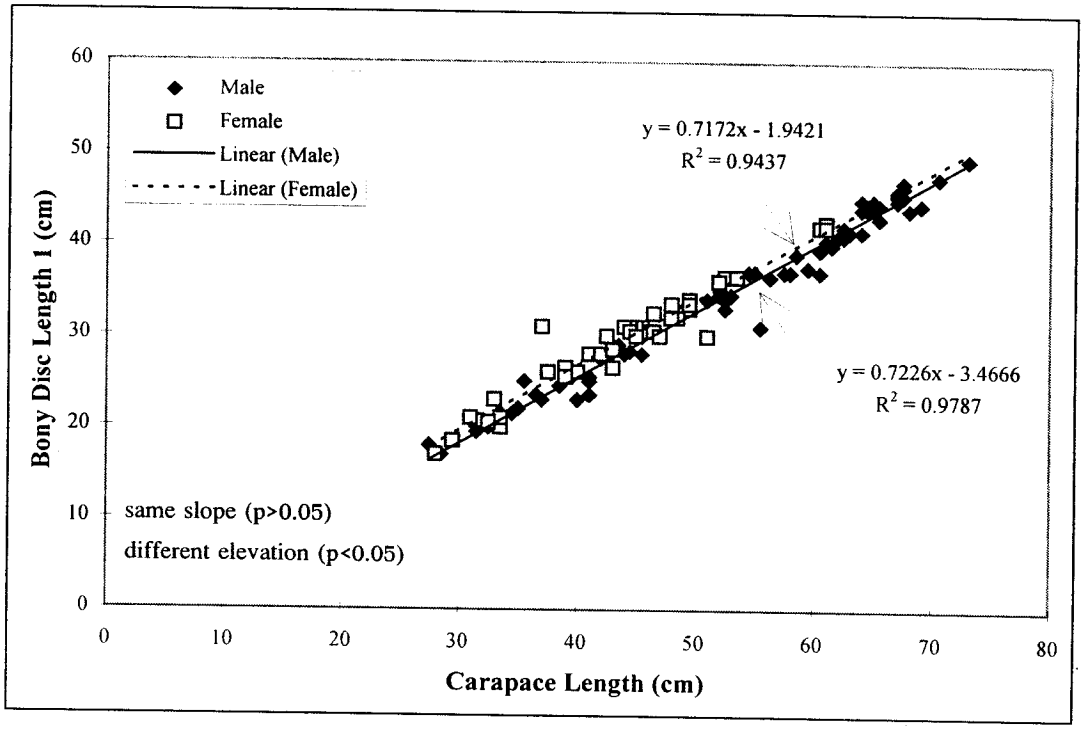

Figure 4–1 Linear regression lines relating bony disc length 1 to carapace length of *Amyda cartilaginea* ($p<0.05$).

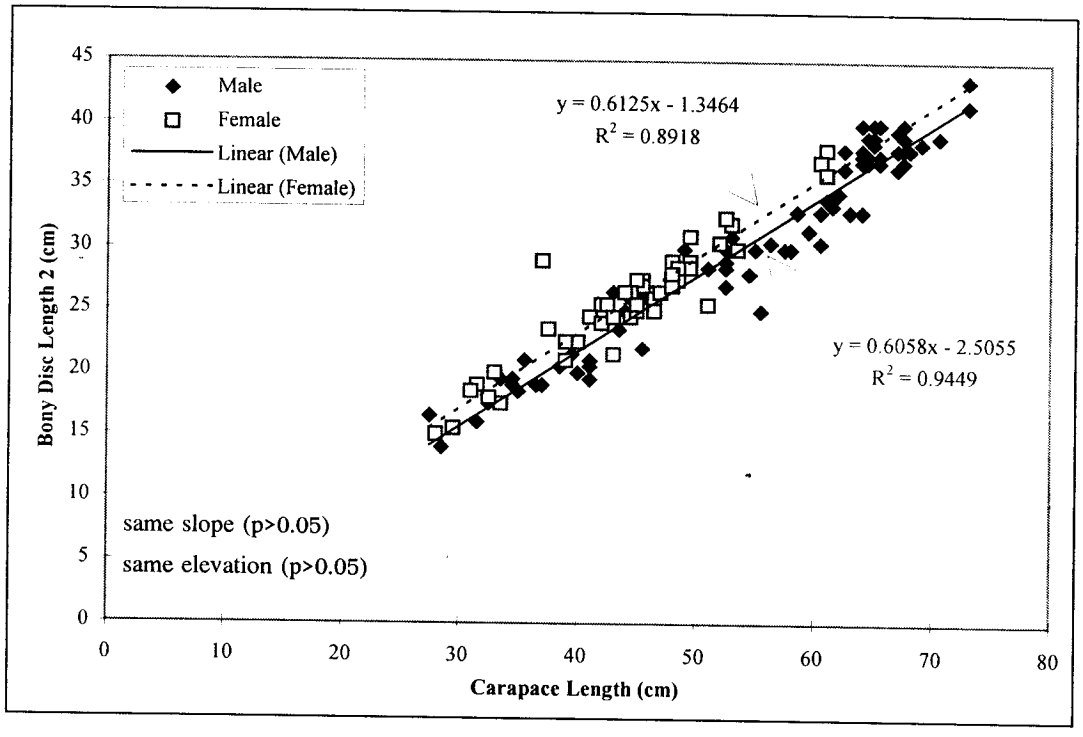

Figure 4–2 Linear regression lines relating bony disc length 2 to carapace length of *Amyda cartilaginea* ($p<0.05$).

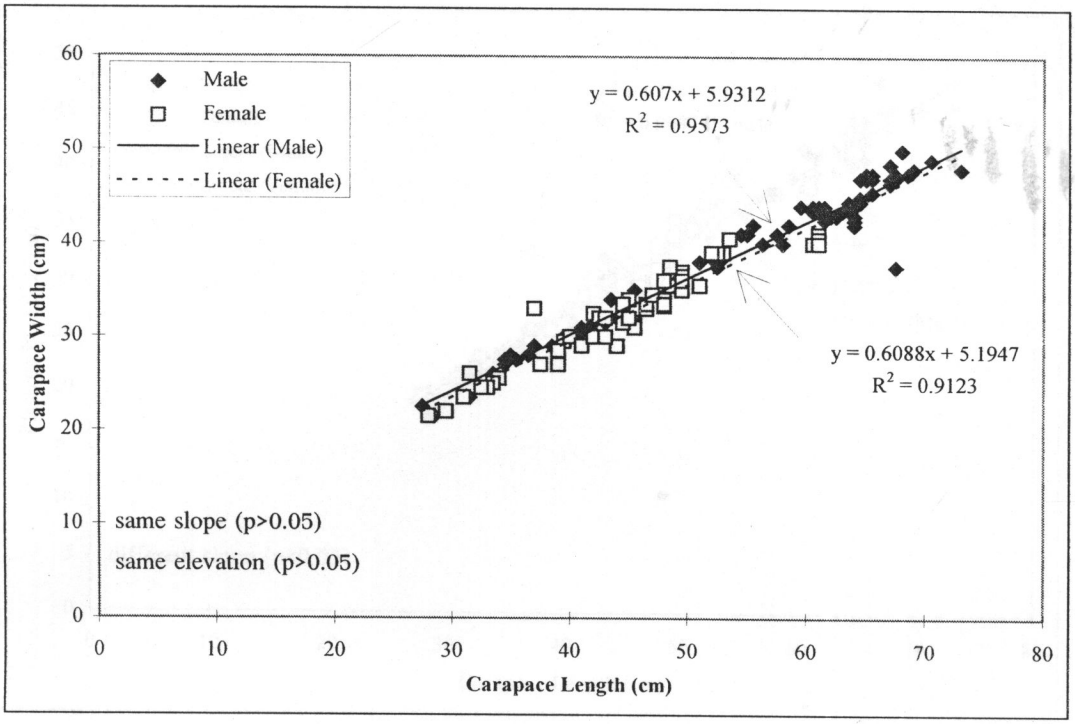

Figure 4-3 Linear regression lines relating carapace width to carapace length of *Amyda cartilaginea* ($p<0.05$).

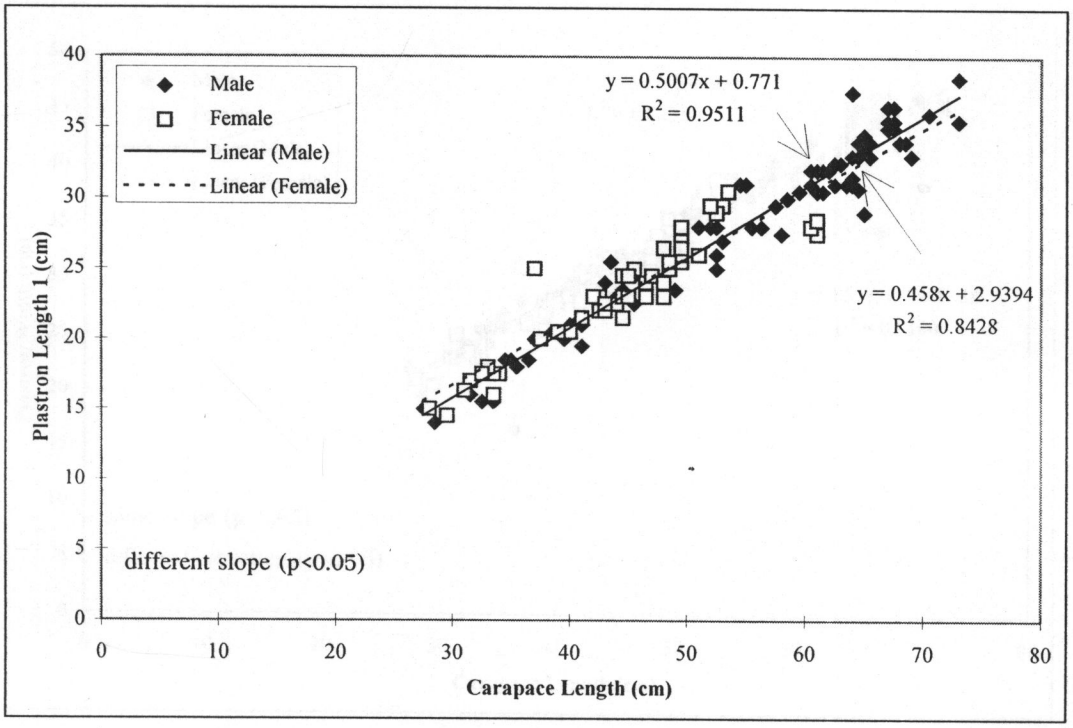

Figure 4-4 Linear regression lines relating plastron length 1 to carapace length of *Amyda cartilaginea* ($p<0.05$).

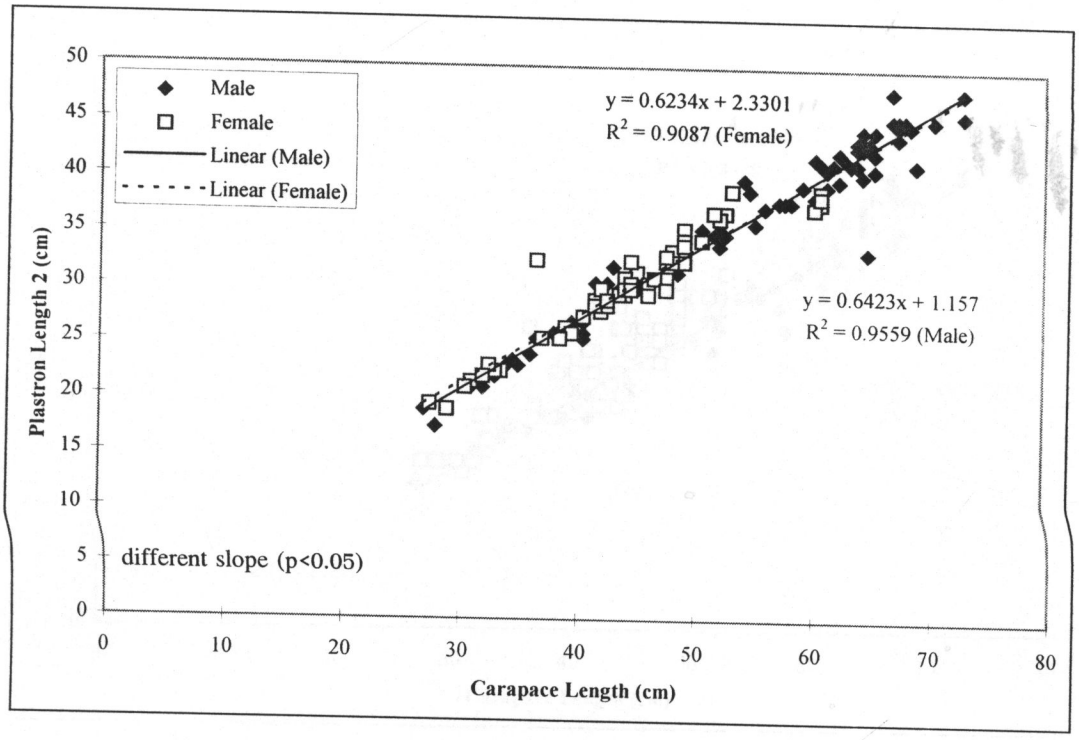

Figure 4-5 Linear regression lines relating plastron length 2 to carapace length of *Amyda cartilaginea* ($p<0.05$).

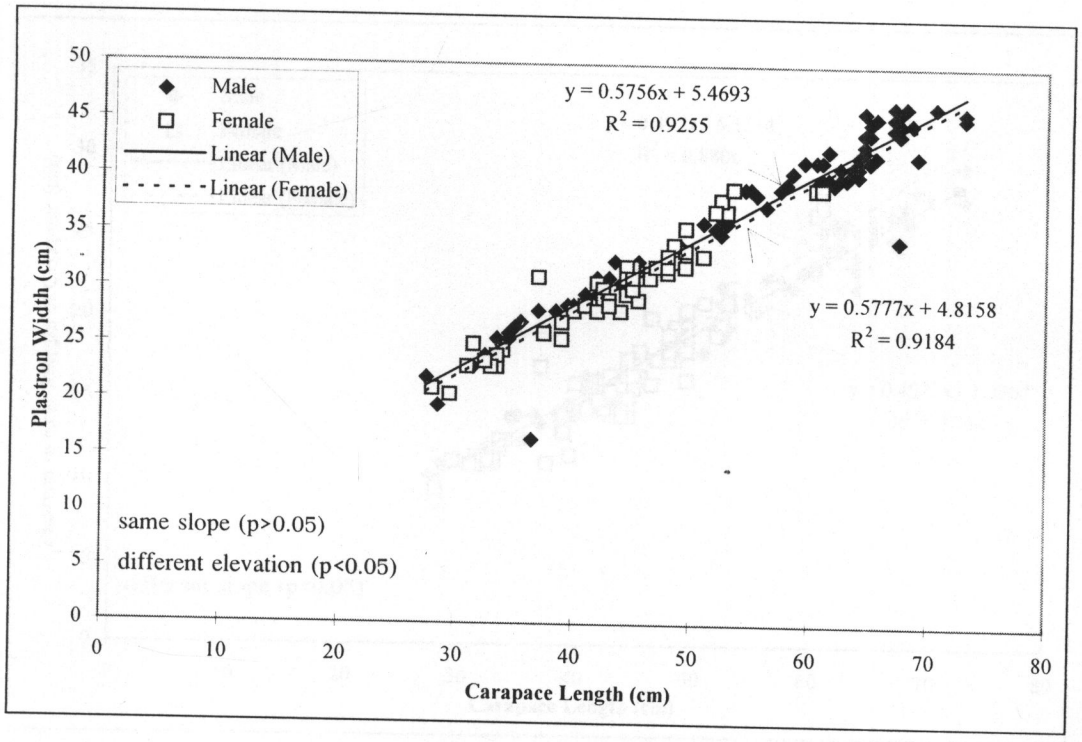

Figure 4-6 Linear regression lines relating plastron width to carapace length of *Amyda cartilaginea* ($p<0.05$).

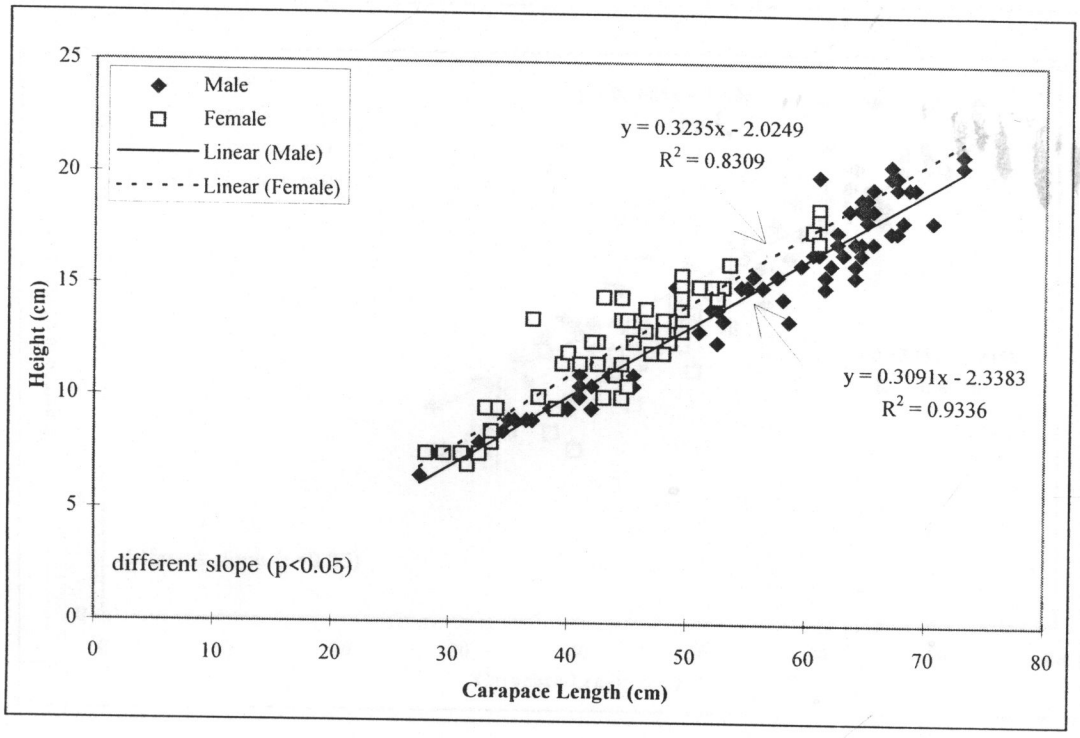

Figure 4-7 Linear regression lines relating height to carapace length of *Amyda cartilaginea* ($p<0.05$).

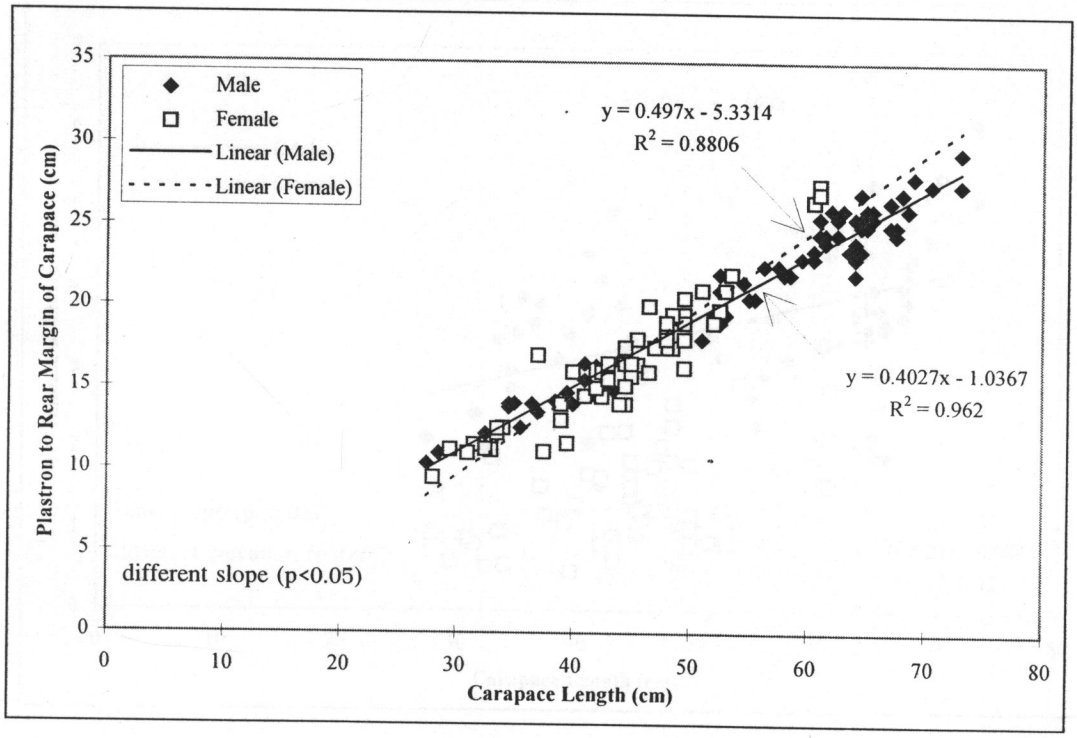

Figure 4-8 Linear regression lines relating plastron to rear margin of carapace to carapace length of *Amyda cartilaginea* ($p<0.05$).

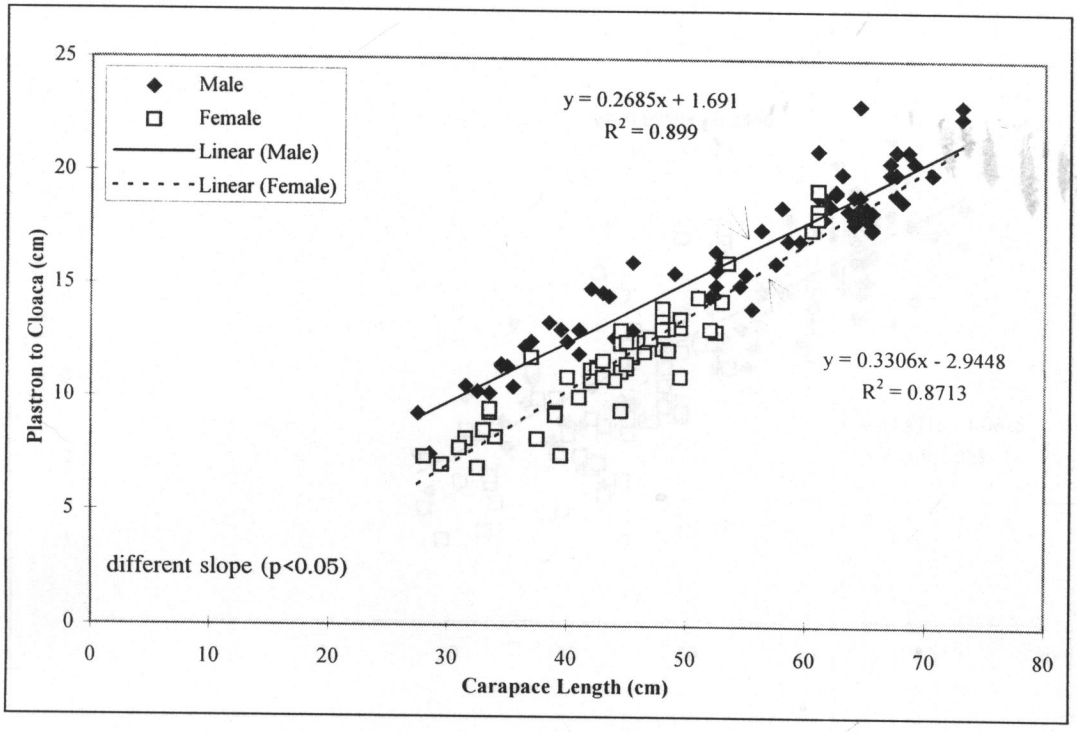

Figure 4-9 Linear regression lines relating plastron to cloaca to carapace length of *Amyda cartilaginea* ($p<0.05$).

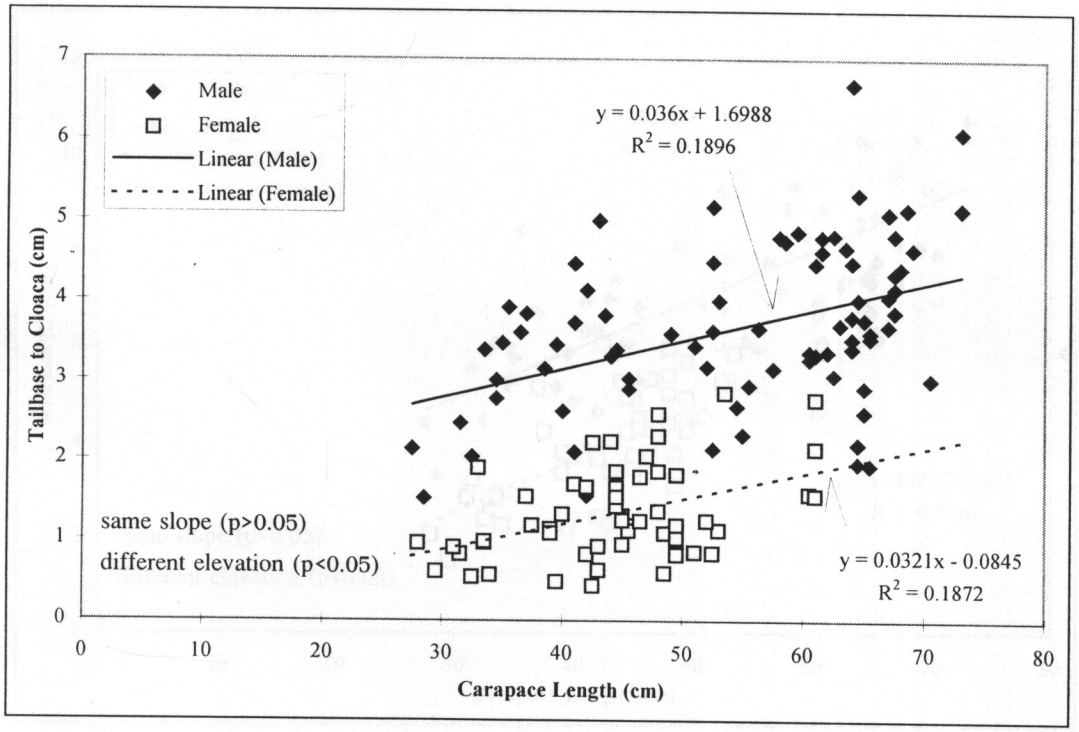

Figure 4-10 Linear regression lines relating tailbase to cloaca to carapace length of *Amyda cartilaginea* ($p<0.05$).

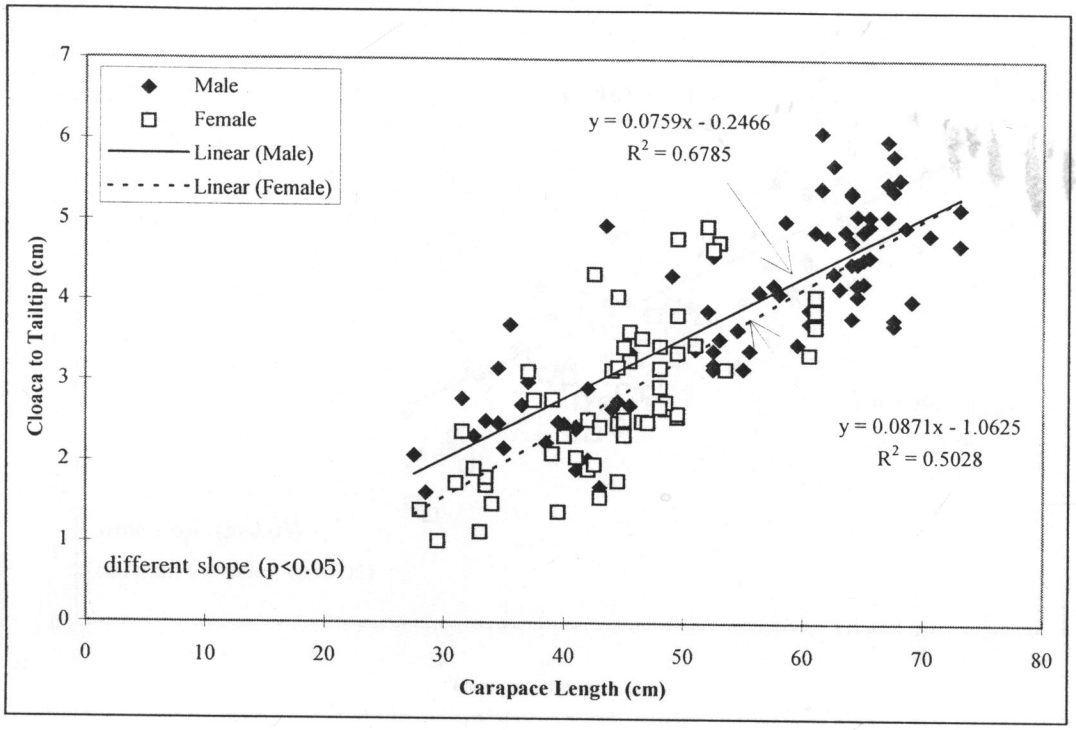

Figure 4-11 Linear regression lines relating cloaca to tail tip to carapace length of *Amyda cartilaginea* ($p<0.05$).

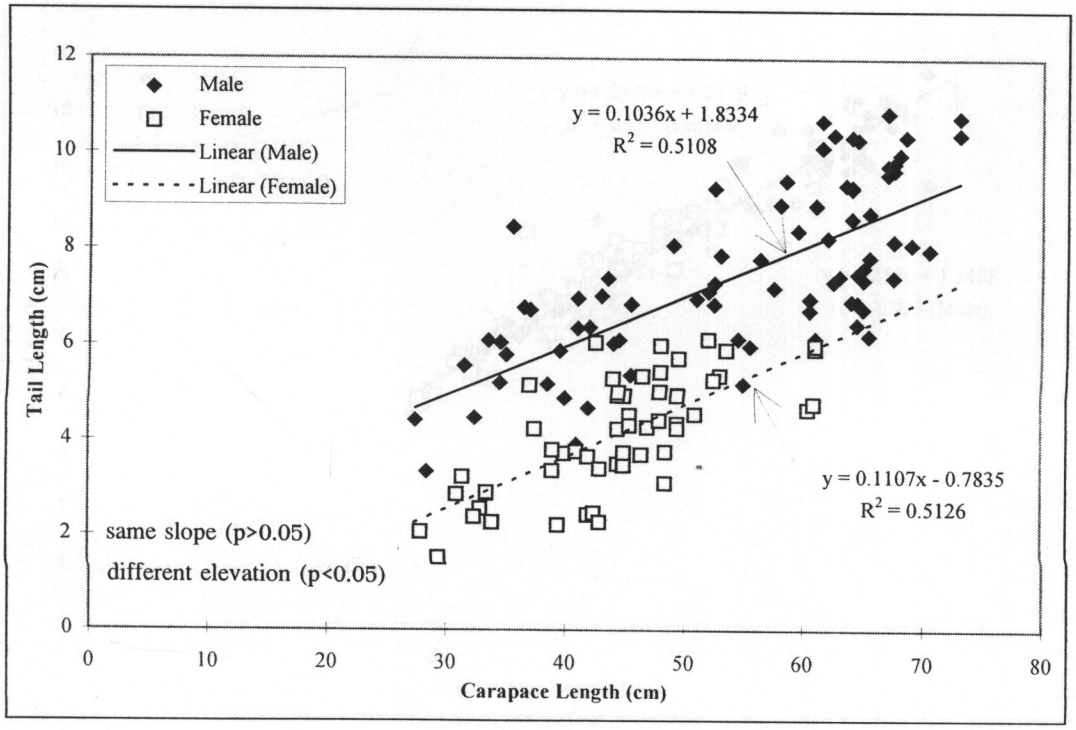

Figure 4-12 Linear regression lines relating tail length to carapace length of *Amyda cartilaginea* ($p<0.05$).

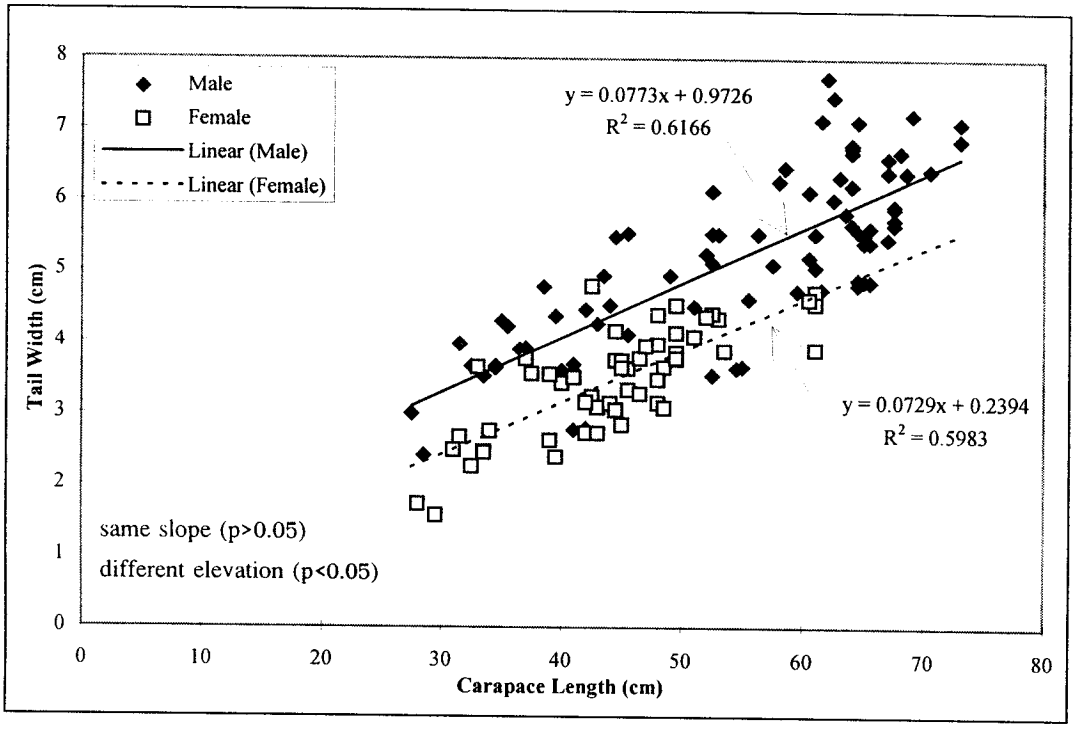

Figure 4-13 Linear regression lines relating tail width to carapace length of *Amyda cartilaginea* ($p<0.05$).

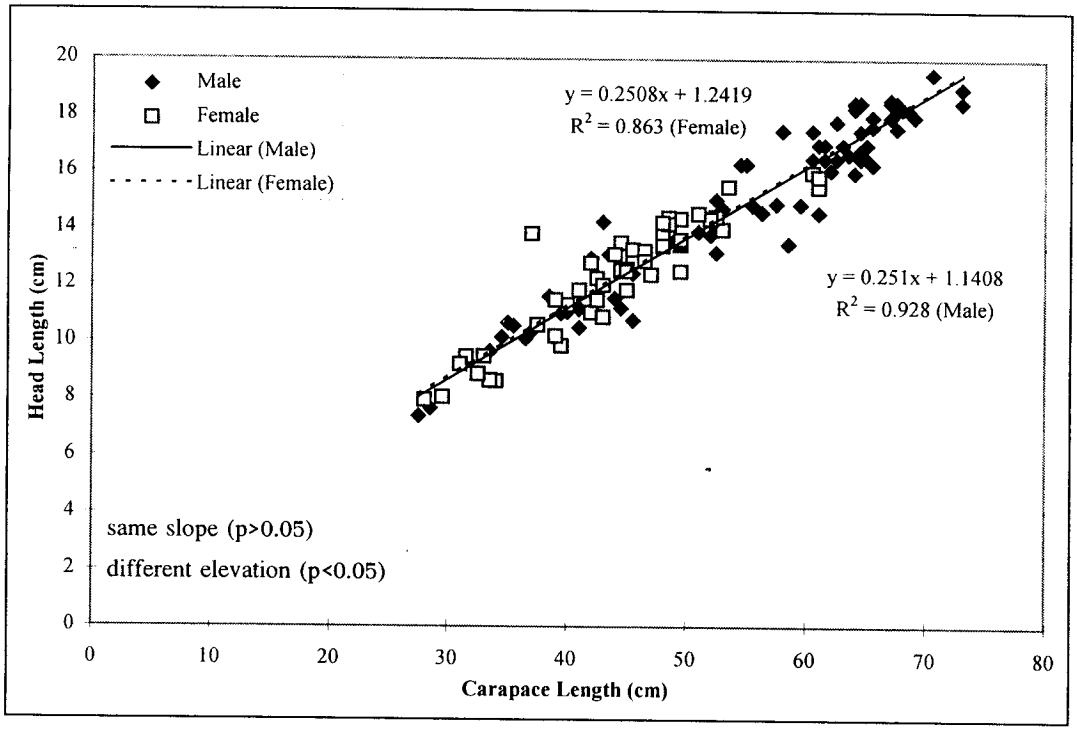

Figure 4-14 Linear regression lines relating head length without snout to carapace length of *Amyda cartilaginea* ($p<0.05$).

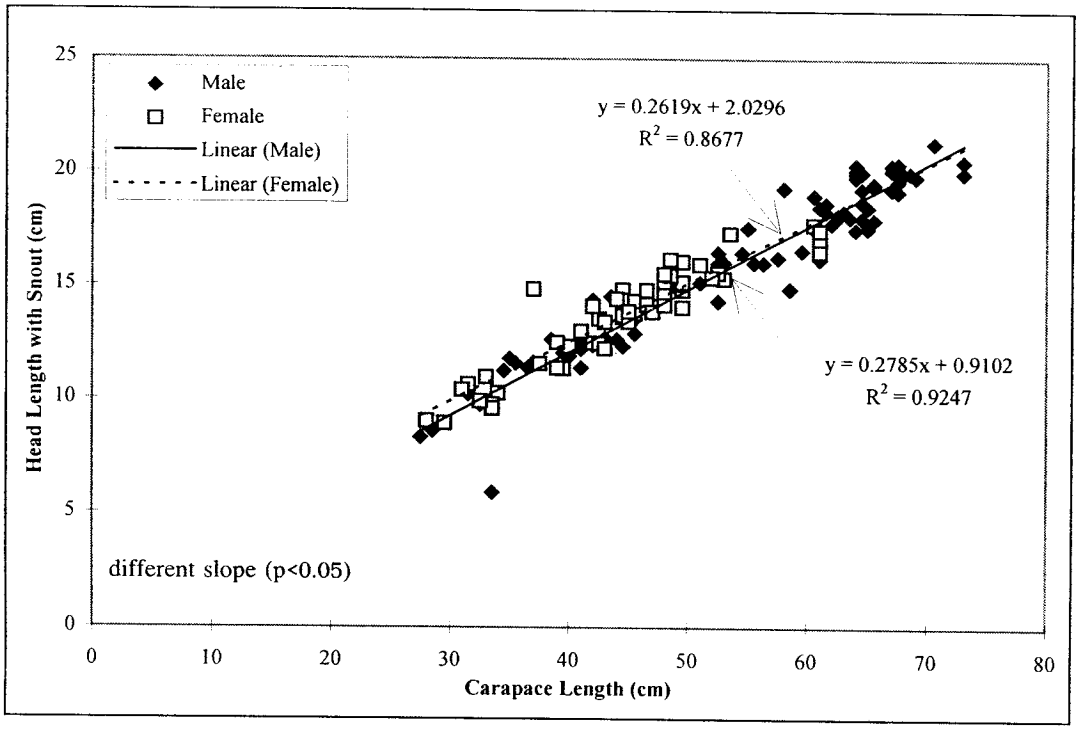

Figure 4-15 Linear regression lines relating head length with snout to carapace length of *Amyda cartilaginea* ($p<0.05$).

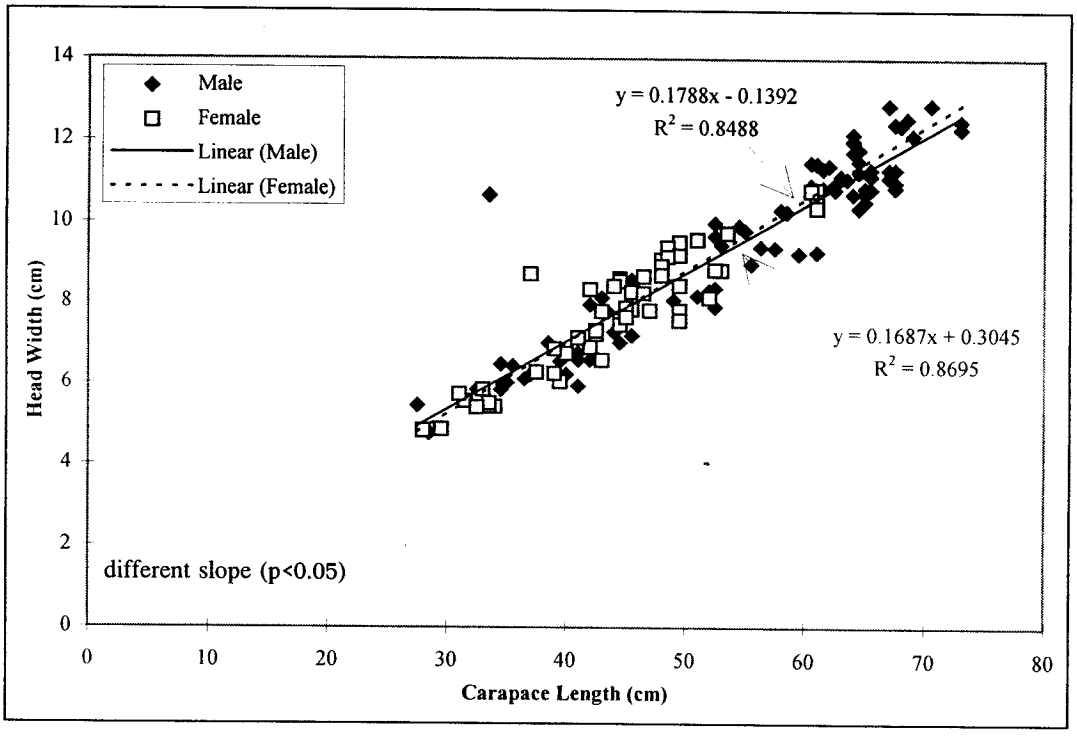

Figure 4-16 Linear regression lines relating head width to carapace length of *Amyda cartilaginea* ($p<0.05$).

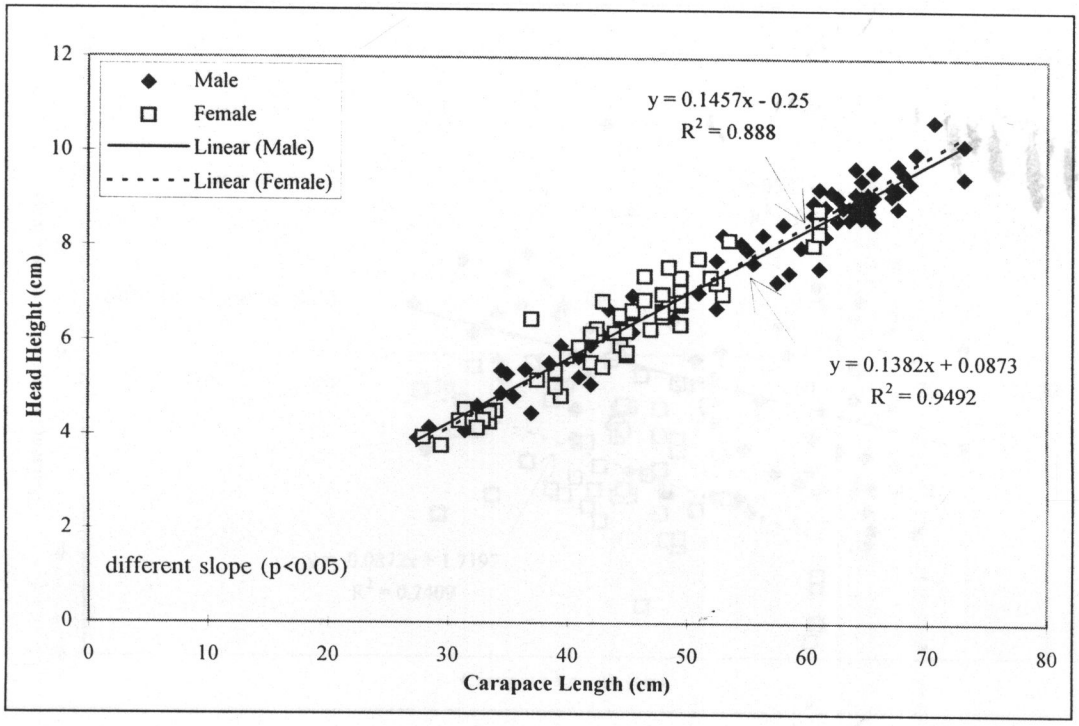

Figure 4-17 Linear regression lines relating head height to carapace length of *Amyda cartilaginea* ($p<0.05$).

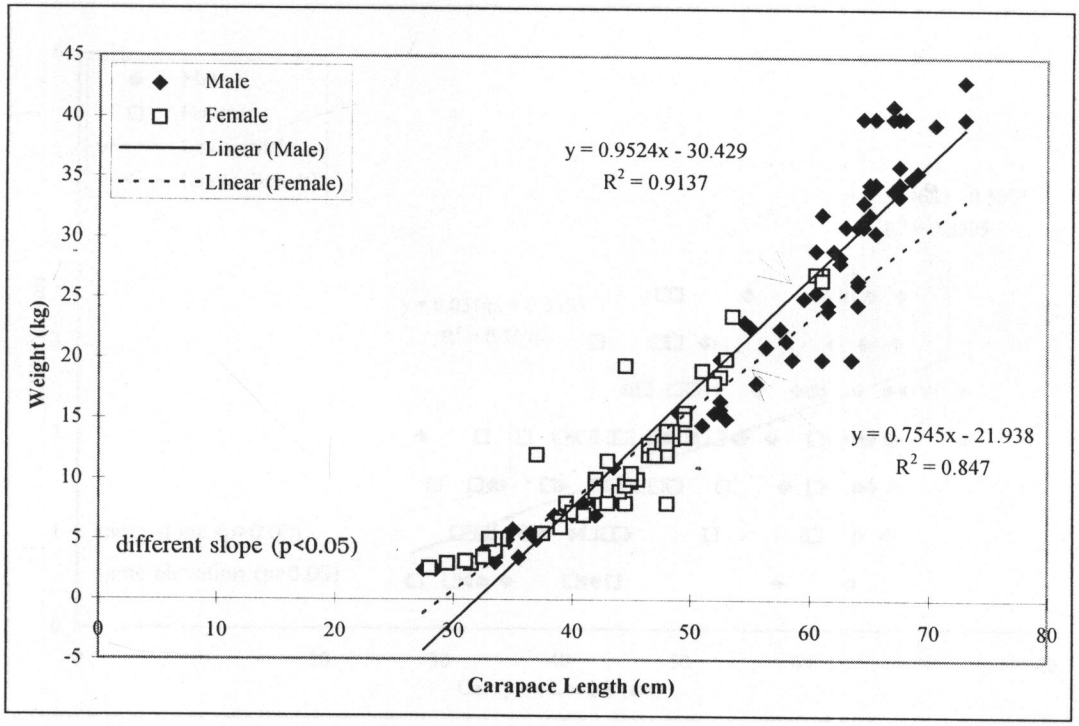

Figure 4-18 Linear regression lines relating weight to carapace length of *Amyda cartilaginea* ($p<0.05$).

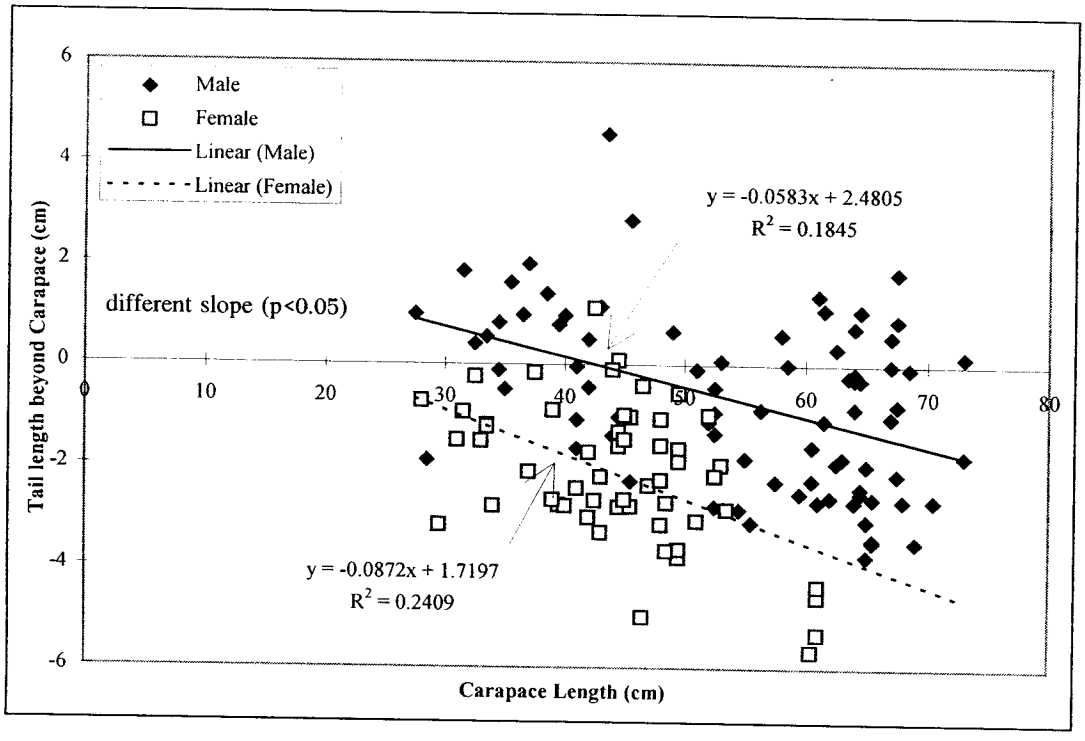

Figure 4–19 Linear regression lines relating tail length beyond carapace to carapace length of *Amyda cartilaginea* (p<0.05).

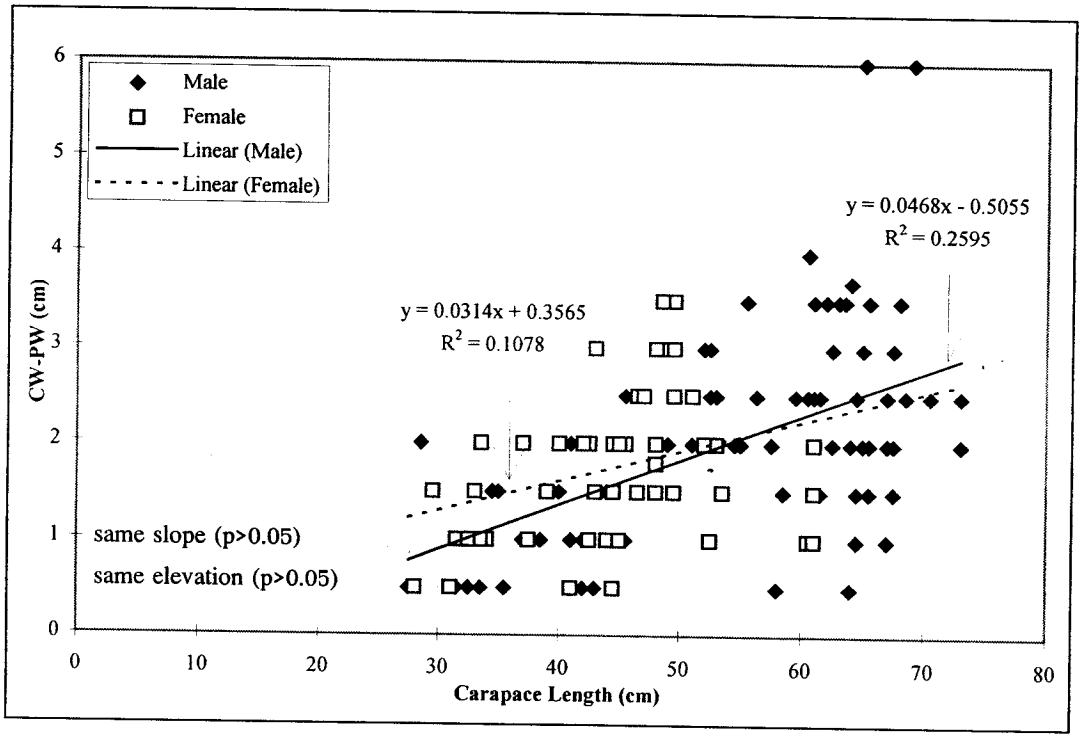

Figure 4–20 Linear regression lines relating CW-PW to carapace length of *Amyda cartilaginea* (p<0.05).

To investigate position of cloacal opening, tailbase to cloaca (TC) were also plotted against Tail length (TL). The regression analysis revealed a significant linear regression line ($p<0.05$) of this relationship. Comparison of slope and elevation of the regression lines indicated that the slopes were significantly different between sexes as shown in figure 4-21.

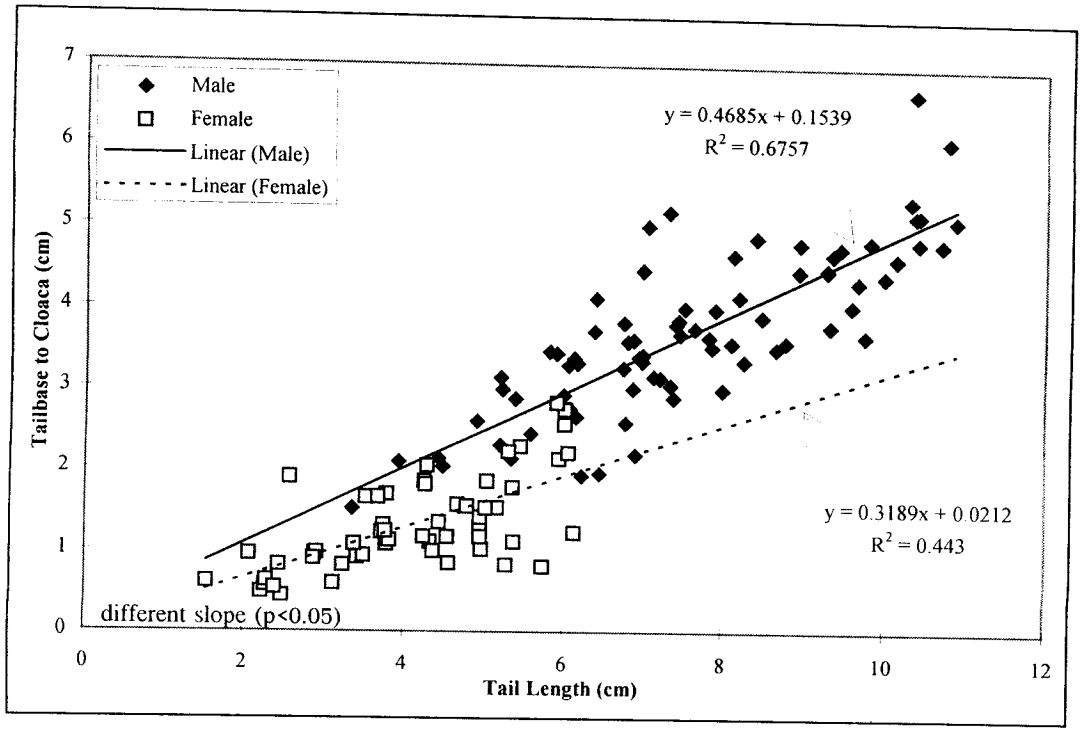

Figure 4-21 Linear regression lines relating tailbase to cloaca to tail length of *Amyda cartilaginea* ($p<0.05$).

Results from regression analysis and comparison of slope and elevation are summarized in table 4-2.

Table 4-2 Regression analysis of *Amyda cartilaginea* morphological characters and comparison of slope and elevation between sexes.

Sex	Linear regression equations	p	Significant difference in	
			Slope	Elevation
Male	BDL1 = 0.7226 CL - 3.4666	0.000	× ($p>0.05$)	✓ ($p<0.05$)
Female	BDL1 = 0.7172 CL - 1.9421	0.000		
Male	BDL2 = 0.6058 CL - 2.5055	0.000	× ($p>0.05$)	× ($p>0.05$)
Female	BDL2 = 0.6125 CL - 1.3464	0.000		
Male	CW = 0.607 CL + 5.9312	0.000	× ($p>0.05$)	× ($p>0.05$)
Female	CW = 0.6088 CL + 5.1947	0.000		
Male	PL1 = 0.5007 CL + 0.771	0.000	✓ ($p<0.05$)	-
Female	PL1 = 0.458 CL + 2.9394	0.000		

Table 4-2 (cont.) Regression analysis of *Amyda cartilaginea* morphological characters and comparison of slope and elevation between sexes.

Sex	Linear regression equations	p	Significant difference in	
			Slope	Elevation
Male	PL2 = 0.6423 CL + 1.157	0.000	✓ (p<0.05)	-
Female	PL2 = 0.6234 CL + 2.3301	0.000		
Male	PW = 0.5756 CL + 5.4693	0.000	× (p>0.05)	✓ (p<0.05)
Female	PW = 0.5777 CL + 4.8158	0.000		
Male	H = 0.3091 CL - 2.3383	0.000	✓ (p<0.05)	-
Female	H = 0.3235 CL - 2.0249	0.000		
Male	PR = 0.4027 CL - 1.0367	0.000	✓ (p<0.05)	-
Female	PR = 0.497 CL - 5.3314	0.000		
Male	PC = 0.2685 CL + 1.691	0.000	✓ (p<0.05)	-
Female	PC = 0.3306 CL - 2.9448	0.000		
Male	TC = 0.036 CL + 1.6988	0.000	× (p>0.05)	✓ (p<0.05)
Female	TC = 0.0321 CL - 0.0845	0.001		
Male	CT = 0.0759 CL - 0.2466	0.000	✓ (p<0.05)	-
Female	CT = 0.0871 CL - 1.0625	0.000		
Male	TL = 0.1036 CL + 1.8334	0.000	× (p>0.05)	✓ (p<0.05)
Female	TL = 0.1107 CL - 0.7835	0.000		
Male	TW = 0.0773 CL + 0.9726	0.000	× (p>0.05)	✓ (p<0.05)
Female	TW = 0.0729 CL + 0.2394	0.000		
Male	HL = 0.251 CL + 1.1408	0.000	× (p>0.05)	✓ (p<0.05)
Female	HL = 0.2508 CL + 1.2419	0.000		
Male	HLs = 0.2785 CL + 0.9102	0.000	✓ (p<0.05)	-
Female	HLs = 0.2619 CL + 2.0296	0.000		
Male	HW = 0.1687 CL + 0.3045	0.000	✓ (p<0.05)	-
Female	HW = 0.1788 CL - 0.1392	0.000		
Male	HH = 0.1382 CL + 0.0873	0.000	✓ (p<0.05)	-
Female	HH = 0.1457 CL - 0.25	0.000		
Male	W = 0.9524 CL - 30.429	0.000	✓ (p<0.05)	-
Female	W = 0.7545 CL - 21.938	0.000		
Male	TBC = -0.0583 CL + 2.4805	0.000	✓ (p<0.05)	-
Female	TBC = -0.0872 CL + 1.7197	0.001		
Male	(CW-PW) = 0.0468CL-0.5055	0.024	× (p>0.05)	× (p>0.05)
Female	(CW-PW) = 0.0314CL+0.3565	0.015		
Male	TC = 0.4685 TL + 0.1539	0.000	✓ (p<0.05)	-
Female	TC = 0.3189 TL + 0.0212	0.000		

Sexual dimorphism of Amyda cartilaginea was determined from both mean comparison of morphological characters and regression analysis of morphological characters relationships. Combination of the two analysis revealed significant difference between sexes in mean, or slope and elevation of regression line, for almost every morphological characters except carapace width (CW) and its transformed parameter (CW-PW). Thus it can be concluded that *Amyda cartilaginea* is sexually dimorphic.

The sexually dimorphic traits of softshell turtle could be divided into 3 groups according to degree of difference between sexes including male larger traits, female larger traits, and indistinctly different traits.

Male softshell turtles exhibited higher degree of plastron to cloaca (PC), tailbase to cloaca (TC), cloaca to tail tip (CT), tail length (TL), tail width (TW), tail length beyond carapace (TBC), cloaca position and weight (W). Except the weight, all parameters are related to tail size and position of cloacal opening which mainly involves in reproductive performance of softshell turtle. This is consistent with previous reports in many species of turtle including softshell turtle (Graham, 1979; Wirot Nutaphand, 1979; Gibbon and Lovich, 1990; van Dijk, 1992; Meylan, Moll and van Dijk, 1995). The longer, thicker tail and relative posterior position of cloacal opening might be benefitial to the males in order to extend the penis from cloacal opening to inseminate the females efficiently. Furthermore the longer and thicker tail could be useful to locate the female cloaca and assist in balancing during copulation.

Although the males showed higher degree of tail length beyond carapacial margin (TBC), plots of TBC against carapace length (CL) revealed that the tail length beyond carapacial margin is less obvious in the large male softshell turtle. Therefore the previous sexing technique indicating male from extension of tail beyond carapace (Anon Sirisuriyakamolchai, 1993; Sujin Nukwan, Panu Tavarutmaneegul and Anusin Inkuan, 1995) could lead to missidentify in large specimen. Hence other sexually dimorphic characters should be considered in addition to this criteria.

According to plots of weight (W) against carapace length (CL), the large softshell turtle seemed to exhibit much higher weight than linear proportion. Another regression models were tested and found that power and exponential

regression lines also showed significant relationships ($p<0.05$). This might indicated the plentiful of food in this turtle pond. Therefore the higher weight of males could be related to higher number of large males instead of the sexual difference.

Female softshell turtles showed higher degree of bony disc length 1 (BDL1), bony disc length 2 (BDL2), plastron length 2 (PL2), height (H), head length without snout (HL), and head length with snout (HLs). These parameters are less conspicuously related to reproductive behavior like the males. Larger in bony disc length, plastron length and height, which are related to larger female size, might be accounted for benefits in increasing female fecundity such as capable of producing more eggs (Berry and Shine, 1980).

The larger in female head length which is related to head size of softshell turtles might be related to difference in microhabitat utilization in order to decrease intersexual competition for resource. The finding offer support for the competition avoidance hypothesis (Darwin, 1889; Slatkins, 1984; Shine, 1989, 1990).

The indistinctly different traits included plastron length 1 (PL1), plastron width (PW), plastron to rear margin of carapace (PR), head width (HW), and head height (HH). Regression analysis of these parameters suggested a sexual dimorphism of softshell turtles but the patterns of difference remained invivid.

Plastron length, plastron width and plastron to rear margin of carapace which are account for size of softshell turtles might be related either to female fecundity or male combat. Thus the study for sexual size dimorphism of softshell turtles should be investigated in order to assimilate the dimorphism.

Plots of head width and head height against carapace length revealed the significant difference in slope of the regression lines between sexes. This restated the difference in head size which might be related to difference in microhabitat usage to decrease intersexual competition for resource, and offer support for the competition avoidance hypothesis (Darwin, 1889; Slatkins, 1984; Shine, 1989, 1990).

4.2 Annual reproductive cycle

4.2.1 Male reproductive cycle

Mean steroids levels in each sample of male softshell turtles are shown in table 4-3 and figure 4-22.

Table 4-3 Mean steroids levels of male *Amyda cartilaginea* in each sample.

Sample	n	Mean ± S.E.M.		
		Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
1/2 October 1996	3	$22.4842^{de} \pm 2.3495$	$40.7296^{cd} \pm 16.3395$	$0.3942^{c} \pm 0.0906$
2/2 Octobet 1996	-	-	-	-
1/2 November 1996	4	$25.0340^{e} \pm 2.9028$	$53.1612^{d} \pm 3.6206$	$0.4888^{c} \pm 0.1376$
2/2 November 1996	6	$16.9881^{bcde} \pm 4.3699$	$32.2805^{bc} \pm 3.1655$	$0.3499^{abc} \pm 0.0939$
1/2 December 1996	7	$11.5782^{abc} \pm 4.5941$	$36.1937^{cd} \pm 9.5368$	$0.3263^{abc} \pm 0.1122$
2/2 December 1996	5	$20.9805^{cde} \pm 3.5287$	$40.5056^{cd} \pm 6.6185$	$0.3446^{abc} \pm 0.0480$
January 1997	5	$9.2848^{ab} \pm 3.2832$	$38.9026^{cd} \pm 13.0646$	$0.2286^{ab} \pm 0.0732$
1/2 February 1997	7	$14.0072^{abcd} \pm 3.0406$	$24.8409^{abc} \pm 4.6282$	$0.2506^{ab} \pm 0.0275$
2/2 February 1997	7	$9.5862^{ab} \pm 3.4424$	$19.6181^{abc} \pm 5.4018$	$0.1788^{ab} \pm 0.0665$
March 1997	6	$7.6590^{ab} \pm 2.0728$	$10.7294^{a} \pm 2.4199$	$0.1771^{ab} \pm 0.0269$
April 1997	5	$8.2008^{ab} \pm 3.2975$	$24.8698^{abc} \pm 5.7844$	$0.1671^{a} \pm 0.0342$
May 1997	5	$9.7507^{ab} \pm 3.0756$	$17.1941^{abc} \pm 5.9587$	$0.1561^{a} \pm 0.0199$
June 1997	8	$5.2029^{a} \pm 1.1999$	$11.7681^{ab} \pm 2.2272$	$0.1400^{a} \pm 0.0323$
July 1997	4	$9.7254^{ab} \pm 3.0688$	$14.5271^{ab} \pm 2.2683$	$0.1829^{ab} \pm 0.0619$
August 1997	7	$3.7492^{a} \pm 0.9758$	$11.1681^{ab} \pm 1.2685$	$0.1478^{a} \pm 0.0240$
September 1997	1	7.0349	16.3404	0.0818

Remark * Significant differences ($p<0.05$) among each sample are indicated by differences in superscript letter.

** There was no male in the second sample (2/2 October 1996) and only one male in the 16th sample (September 1997).

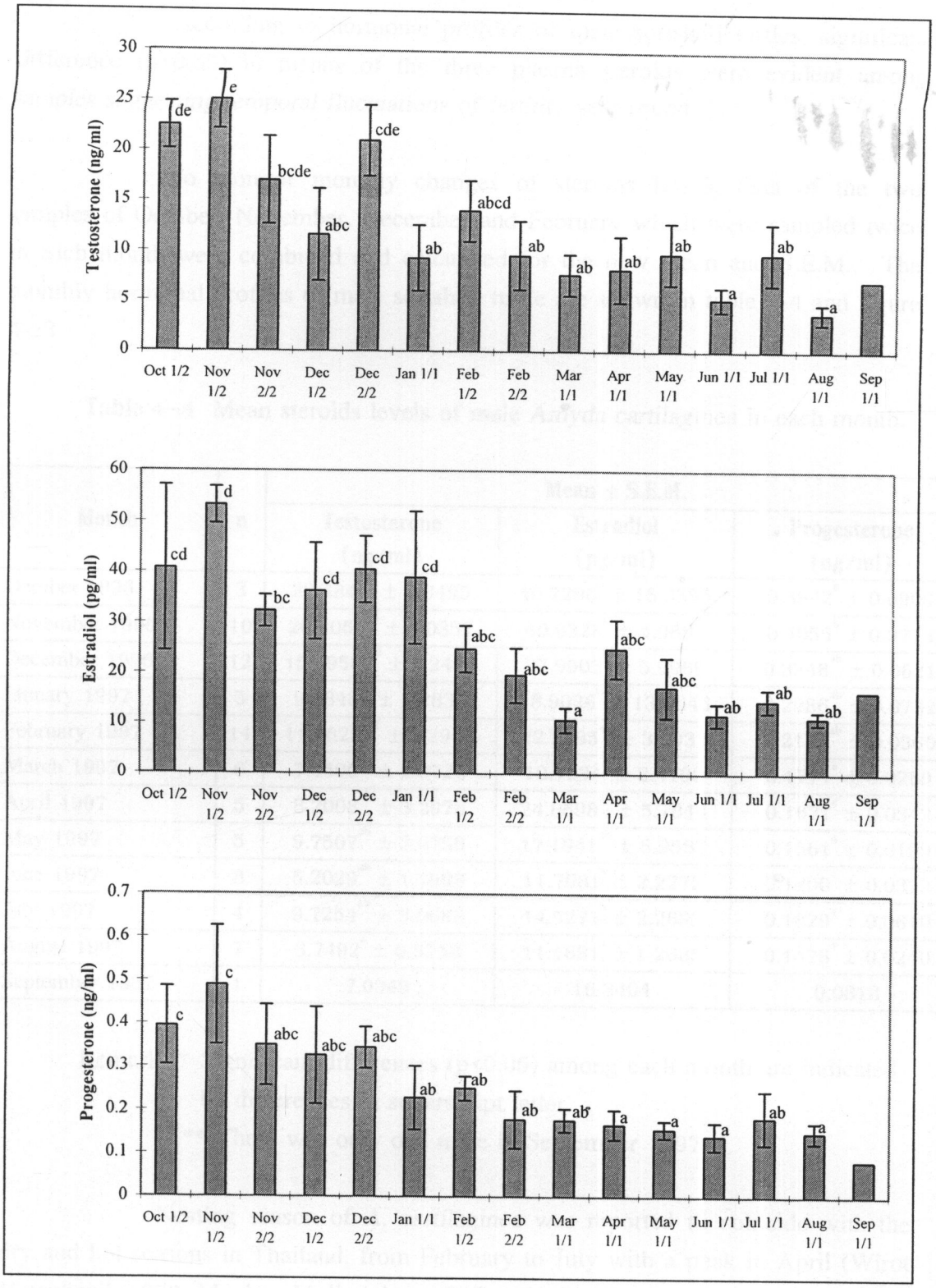

Figure 4-22 Plasma steroids profile of male *Amyda cartilaginea* in each sample. Error bar indicate standard error of the mean (S.E.M.). Difference in the above letter indicate significant difference ($p<0.05$) among samples.

According to hormonal profiles of male softshell turtles, significant difference ($p<0.05$) in means of the three plasma steroids were evident among samples suggesting temporal fluctuations of fertility year-round.

To monitor monthly changes of steroids levels, data of the two samples of October, November, December and February which were sampled twice in each month were combined and calculated for the new mean and S.E.M.. The monthly hormonal profiles of male softshell turtle are shown in table 4-4 and figure 4-23.

Table 4-4 Mean steroids levels of male *Amyda cartilaginea* in each month.

Month	n	Mean ± S.E.M.		
		Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
October 1996	3	$22.4842^{d} \pm 2.3495$	$40.7296^{b} \pm 16.3395$	$0.3942^{b} \pm 0.0906$
November 1996	10	$20.2065^{cd} \pm 3.0357$	$40.6328^{b} \pm 4.0884$	$0.4055^{b} \pm 0.0773$
December 1996	12	$15.4958^{bcd} \pm 3.2489$	$37.9903^{b} \pm 5.9989$	$0.3346^{ab} \pm 0.0621$
January 1997	5	$9.2848^{ab} \pm 3.2832$	$38.9026^{b} \pm 13.0646$	$0.2286^{ab} \pm 0.0732$
February 1997	14	$11.6628^{abc} \pm 2.2900$	$22.2295^{ab} \pm 3.4931$	$0.2147^{ab} \pm 0.0360$
March 1997	6	$7.6590^{ab} \pm 2.0728$	$10.7294^{a} \pm 2.4199$	$0.1771^{a} \pm 0.0269$
April 1997	5	$8.2008^{ab} \pm 3.2975$	$24.8698^{ab} \pm 5.7844$	$0.1671^{a} \pm 0.0342$
May 1997	5	$9.7507^{ab} \pm 3.0756$	$17.1941^{ab} \pm 5.9587$	$0.1561^{a} \pm 0.0199$
June 1997	8	$5.2029^{ab} \pm 1.1999$	$11.7681^{a} \pm 2.2272$	$0.1400^{a} \pm 0.0323$
July 1997	4	$9.7254^{ab} \pm 3.0688$	$14.5271^{a} \pm 2.2683$	$0.1829^{a} \pm 0.0619$
August 1997	7	$3.7492^{a} \pm 0.9758$	$11.1681^{a} \pm 1.2685$	$0.1478^{a} \pm 0.0240$
September 1997	1	7.0349	16.3404	0.0818

Remark * Significant differences ($p<0.05$) among each month are indicated by differences in superscript letter. .

** There was only one male in September 1997.

Nesting season of *A. cartilaginea* was reported to coincide with the dry and hot seasons in Thailand, from February to July with a peak in April (Wirot Nutaphand, 1979; Meylan, Moll and van Dijk, 1995). The nesting of softshell turtle population at Prayurawongsawas temple was found during March and May (Wichase Khonsue, 1993; Wachira Kitimasak, 1996). This nesting period was overlayed to the monthly steroids profile as shown in figure 4-23.

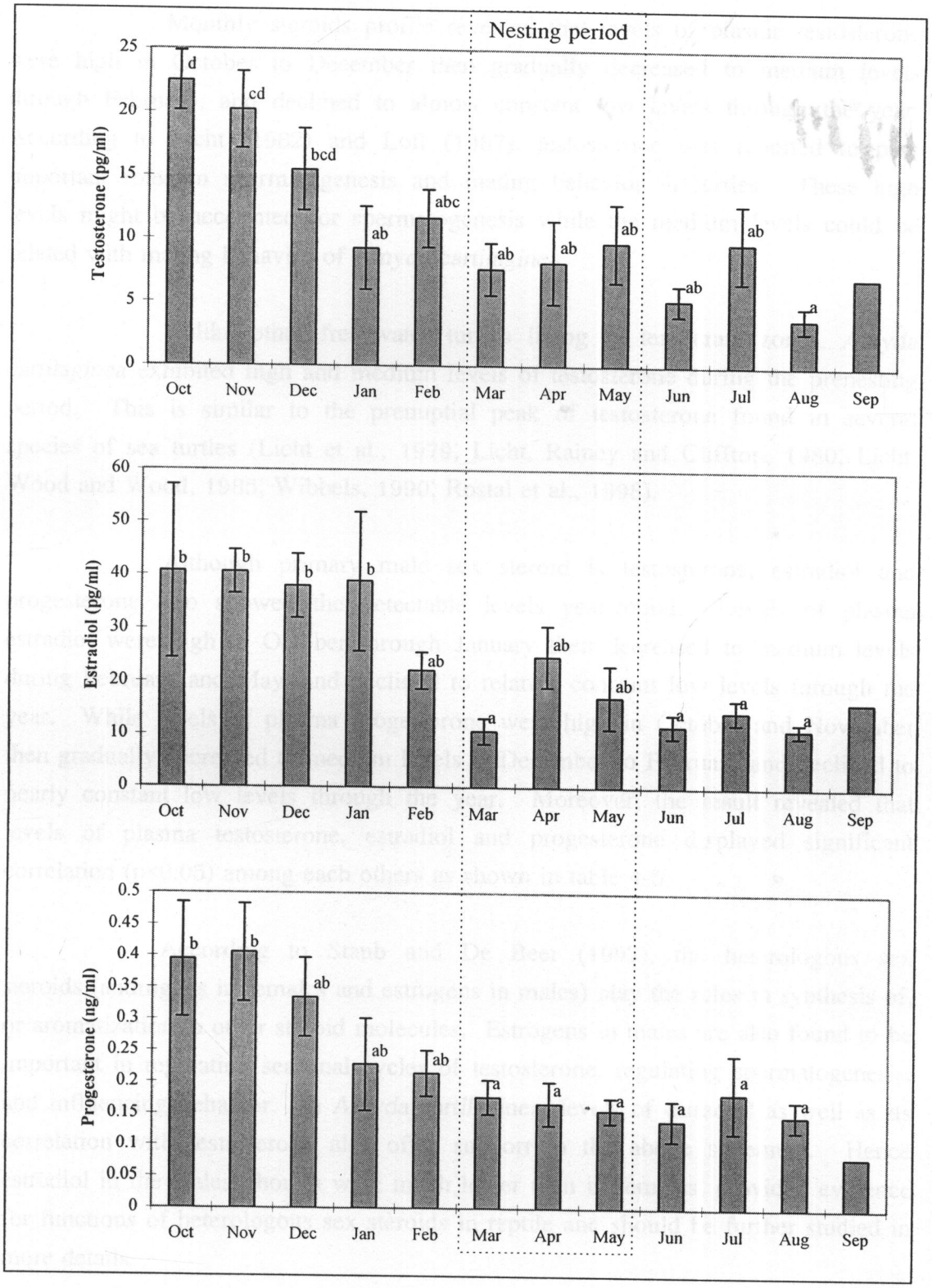

Figure 4-23 Monthly plasma steroids profile of male *Amyda cartilaginea*. Error bar indicate standard error of the mean (S.E.M.). Difference in the above letter indicate significant difference ($p<0.05$) among months.

Monthly steroids profile revealed that levels of plasma testosterone were high in October to December then gradually decreased to medium levels through February, and declined to almost constant low levels through the year. According to Licht (1982) and Loft (1987), testosterone was reported to play important roles in spermatogenesis and mating behavior in turtles. These high levels might be accounted for spermatogenesis while the medium levels could be related with mating behavior of *Amyda cartilaginea*.

Unlike other freshwater turtles living in temperate zones, *Amyda cartilaginea* exhibited high and medium levels of testosterone during the prenesting period. This is similar to the prenuptial peak of testosterone found in several species of sea turtles (Licht et al., 1979; Licht, Rainey and Cliffton, 1980; Licht, Wood and Wood, 1985; Wibbels, 1990; Rostal et al., 1998).

Although primary male sex steroid is testosterone, estradiol and progesterone also showed the detectable levels year-round. Levels of plasma estradiol were high in October through January then decreased to medium levels during February and May, and declined to relative constant low levels through the year. While levels of plasma progesterone were high in October and November then gradually decreased to medium levels in December to February and declined to nearly constant low levels through the year. Moreover, the result revealed that levels of plasma testosterone, estradiol and progesterone displayed significant correlation ($p<0.05$) among each others as shown in table 4-5.

According to Staub and De Beer (1997), the heterologous sex steroids (androgens in females and estrogens in males) play the roles in synthesis of, or aromatization to other steroid molecules. Estrogens in males are also found to be important in regulating seasonal cycles of testosterone, regulating spermatogenesis, and influencing behavior. In *Amyda cartilaginea*, levels of estradiol as well as its correlation with testosterone also offer support to the above statement. Hence estradiol in the males, though were much lower than in females, provided evidence for functions of heterologous sex steroids in reptile and should be further studied in more details.

Levels of progesterone, existed in less than 4 percent of testosterone, might be resulting from release of intermediate into the blood during the $\Delta 4$ pathway of testicular steroidogenesis (Johnson and Everitt, 1988).

Correlation of various climatic factors of Bangkok Metropolis area and levels of plasma sex steroids were analyzed and shown in table 4-5.

Table 4-5 Pearson's correlation coefficients relating climatic data of Bangkok Metropolis area and plasma sex steroid levels of male *Amyda cartilaginea*. Shaded cells indicate significant correlation ($p<0.05$).

	Max. temp.	Mean temp.	Min. temp.	Max. RH	Mean RH	Min. RH	Daily sunshine	Daily rainfall	T	E_2	P
Max. temp.	1.000										
Mean temp.	0.827	1.000									
Min. temp.	0.559	0.892	1.000								
Max. RH	-0.063	0.344	0.452	1.000							
Mean RH	-0.111	0.218	0.479	0.605	1.000						
Min. RH	0.033	0.311	0.501	0.380	0.749	1.000					
Daily sunshine	0.450	0.119	-0.176	-0.013	-0.408	-0.639	1.000				
Daily rainfall	-0.074	0.098	0.288	0.574	0.837	0.628	-0.367	1.000			
Testosterone	-0.552	-0.448	-0.372	-0.070	0.131	-0.113	-0.278	0.061	1.000		
Estradiol	-0.708	-0.732	-0.725	-0.127	-0.122	-0.297	-0.075	-0.062	0.820	1.000	
Progesterone	-0.621	-0.554	-0.514	-0.205	-0.059	-0.160	-0.310	-0.168	0.936	0.855	1.000

Estradiol profile showed significant correlation with minimum, mean and maximum temperature of Bangkok area ($p<0.05$), while plasma progesterone showed significant correlation with maximum temperature of Bangkok ($p<0.05$). The results are consistent with Whittier and Crews (1987) that photoperiod and temperature both serve as proximate cues for gonadal maturation.

Due to data availability, sunshine duration was used instead of photoperiod and showed no significant correlation with steroids level. The above result as well as the insignificant correlation of testosterone and climatic factors might suggest priority of endogenous over exogenous factors in controlling the reproductive cycle of the softshell turtle. This might be related to different control patterns of tropical organisms which are exposed to relative constant environment year-round.

4.2.2 Female reproductive cycle

Mean steroids levels in each sample of female softshell turtles are shown in table 4-6 and figure 4-24.

Table 4-6 Mean steroids levels of female *Amyda cartilaginea* in each sample.

Sample	n	Mean ± S.E.M.		
		Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
1/2 October 1996	5	$0.2828^{a} \pm 0.2373$	$207.957^{a} \pm 56.107$	$2.6649^{e} \pm 0.3622$
2/2 Octobet 1996	5	$0.1070^{a} \pm 0.1153$	$127.090^{a} \pm 7.0697$	$1.7587^{abcde} \pm 0.6057$
1/2 November 1996	2	$0.0652^{a} \pm 0.0269$	$215.513^{a} \pm 67.468$	$2.5648^{de} \pm 0.8863$
2/2 November 1996	3	$0.0661^{a} \pm 0.0117$	$101.397^{a} \pm 41.557$	$2.1737^{bcde} \pm 0.5822$
1/2 December 1996	4	$0.0743^{a} \pm 0.0100$	$240.930^{a} \pm 70.341$	$2.4463^{cde} \pm 0.4046$
2/2 December 1996	4	$0.0822^{a} \pm 0.0200$	$187.088^{a} \pm 56.075$	$2.2121^{bcde} \pm 0.3060$
January 1997	4	$0.1250^{a} \pm 0.0131$	$135.010^{a} \pm 58.024$	$1.3112^{abc} \pm 0.1506$
1/2 February 1997	3	$0.1551^{a} \pm 0.0441$	$164.968^{a} \pm 48.392$	$1.2569^{ab} \pm 0.1038$
2/2 February 1997	2	$0.1699^{a} \pm 0.0525$	$239.547^{a} \pm 25.033$	$1.7154^{abcde} \pm 0.3474$
March 1997	3	$0.2047^{a} \pm 0.0733$	$112.685^{a} \pm 16.218$	$1.1295^{ab} \pm 0.0618$
April 1997	4	$0.0486^{a} \pm 0.0170$	$90.075^{a} \pm 32.762$	$1.4631^{abcd} \pm 0.2639$
May 1997	3	$0.0411^{a} \pm 0.0038$	$79.671^{a} \pm 22.424$	$1.4834^{abcd} \pm 0.3885$
June 1997	2	$0.1551^{a} \pm 0.0420$	$70.817^{a} \pm 34.298$	$0.9582^{a} \pm 0.1431$
July 1997	-	-	-	-
August 1997	3	$0.1234^{a} \pm 0.0578$	$77.587^{a} \pm 17.346$	$0.8164^{a} \pm 0.0999$
September 1997	7	$0.0646^{a} \pm 0.0160$	$136.264^{a} \pm 22.267$	$1.0686^{ab} \pm 0.2139$

Remark * Significant differences ($p<0.05$) among each sample are indicated by differences in superscript letter.

** There was no female in the 14th sample (July 1997).

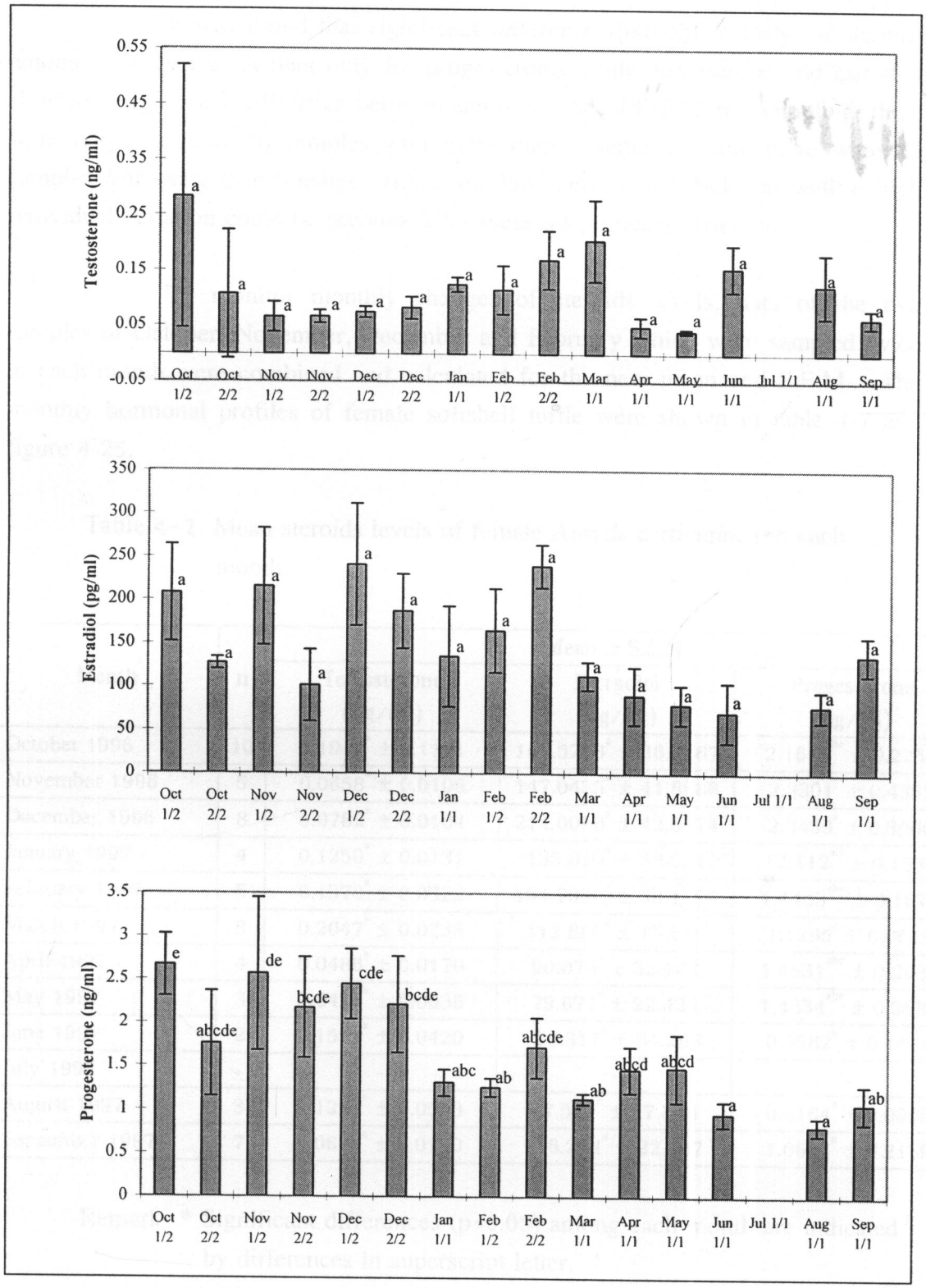

Figure 4-24 Plasma steroids profile of female *Amyda cartilaginea* in each sample. Error bar indicate standard error of the mean (S.E.M.). Difference in the above letter indicate significant difference ($p<0.05$) among samples.

It was found that significant difference ($p<0.05$) in mean of steroids among samples was evident only for progesterone, while testosterone and estradiol showed insignificant difference between samples. According to the sampling, there were only 7 out of 16 samples with more than 3 females while there were 13 samples with more than 3 males. Hence the low number of females as well as high individual variation could be accounted for these insignificant differences.

To monitor monthly changes of steroids levels, data of the two samples of October, November, December and February which were sampled twice in each month were combined and calculated for the new mean and S.E.M.. The monthly hormonal profiles of female softshell turtle were shown in table 4-7 and figure 4-25.

Table 4-7 Mean steroids levels of female *Amyda cartilaginea* in each month.

Month	n	Mean ± S.E.M.		
		Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
Octobet 1996	10	$0.1949^{a} \pm 0.1171$	$167.5233^{a} \pm 36.9667$	$2.1615^{bc} \pm 0.2256$
November 1996	5	$0.0658^{a} \pm 0.0106$	$147.0435^{a} \pm 41.8886$	$2.3301^{c} \pm 0.4352$
December 1996	8	$0.0782^{a} \pm 0.0104$	$214.0090^{a} \pm 42.8674$	$2.3459^{c} \pm 0.3060$
January 1997	4	$0.1250^{a} \pm 0.0131$	$135.010^{a} \pm 58.024$	$1.3112^{ab} \pm 0.1506$
February 1997	5	$0.1370^{a} \pm 0.0322$	$194.7999^{a} \pm 33.1499$	$1.4403^{abc} \pm 0.1671$
March 1997	3	$0.2047^{a} \pm 0.0733$	$112.685^{a} \pm 16.218$	$1.1295^{a} \pm 0.0618$
April 1997	4	$0.0486^{a} \pm 0.0170$	$90.075^{a} \pm 32.762$	$1.4631^{abc} \pm 0.2639$
May 1997	3	$0.0411^{a} \pm 0.0038$	$79.671^{a} \pm 22.424$	$1.4834^{abc} \pm 0.3885$
June 1997	2	$0.1551^{a} \pm 0.0420$	$70.817^{a} \pm 34.298$	$0.9582^{a} \pm 0.1431$
July 1997	-	-	-	-
August 1997	3	$0.1234^{a} \pm 0.0578$	$77.587^{a} \pm 17.346$	$0.8164^{a} \pm 0.0999$
September 1997	7	$0.0646^{a} \pm 0.0160$	$136.264^{a} \pm 22.267$	$1.0686^{a} \pm 0.2139$

Remark * Significant differences ($p<0.05$) among each month are indicated by differences in superscript letter.

** There was no female in July 1997.

The nesting period of softshell turtle population at Prayurawongsawas temple was overlayed to the monthly steroids profile as shown in figure 4-25.

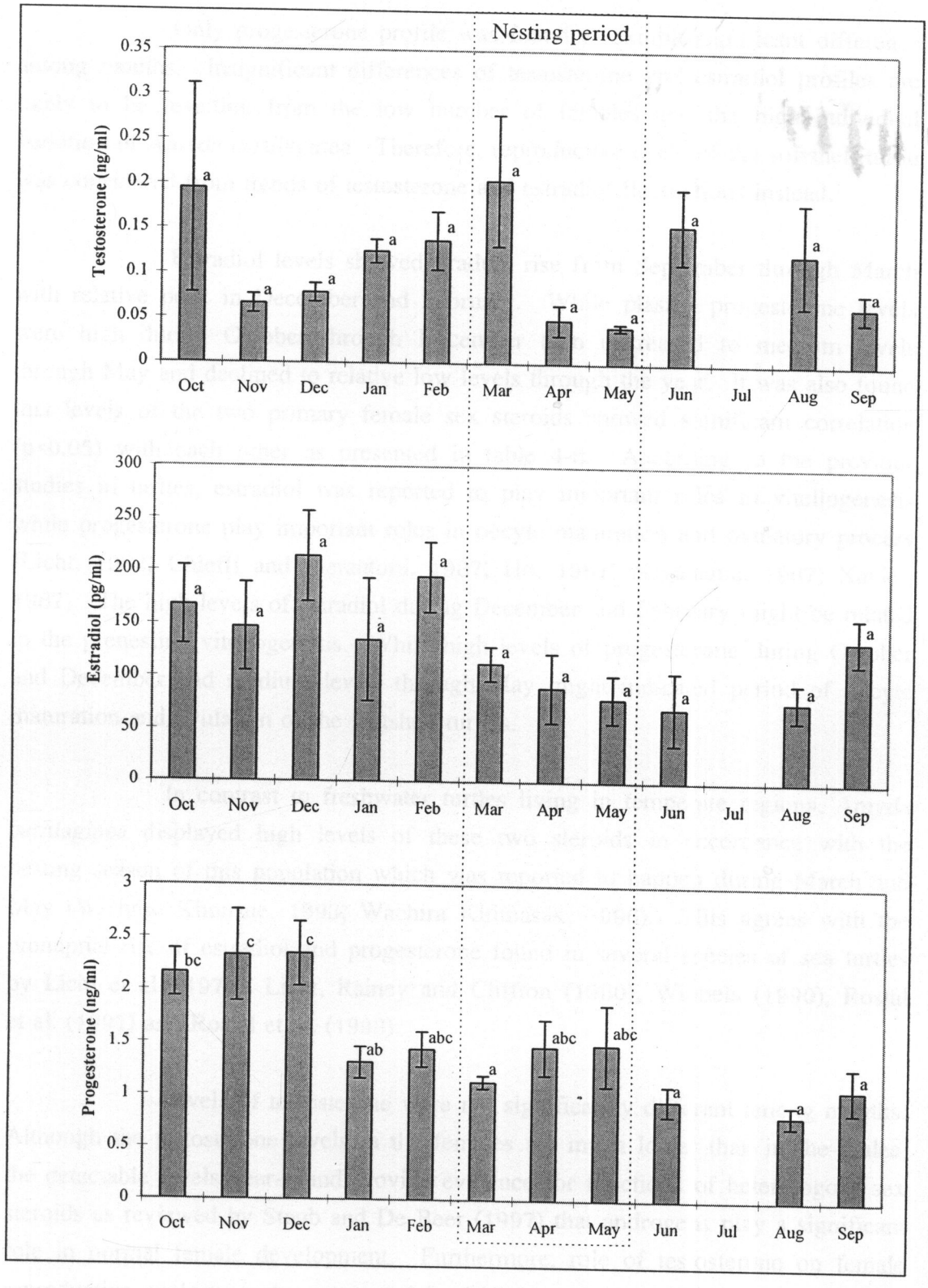

Figure 4–25 Monthly plasma steroids profile of female *Amyda cartilaginea*. Error bar indicate standard error of the mean (S.E.M.). Difference in the above letter indicate significant difference ($p<0.05$) among months.

Only progesterone profile was found to exhibit significant difference among months. Insignificant differences of testosterone and estradiol profiles are likely to be resulting from the low number of females and the high individual variation of *Amyda cartilaginea*. Therefore, reproductive cycle of the softshell turtle was considered from trends of testosterone and estradiol fluctuations instead.

Estradiol levels showed gradual rise from September through March with relative peak in December and February. While plasma progesterone levels were high during October through December then decreased to medium levels through May and declined to relative low levels through the year. It was also found that levels of the two primary female sex steroids showed significant correlation ($p<0.05$) with each other as presented in table 4-8. According to the previous studies in turtles, estradiol was reported to play important roles in vitellogenesis while progesterone play important roles in oocyte maturation and ovulatory process (Licht, 1982; Chieffi and Pierantoni, 1987; Ho, 1987; Nagahama, 1987; Xavier, 1987). The high levels of estradiol during December and February might be related to the prenesting vitellogenesis. While high levels of progesterone during October and December and medium levels through May might indicated period of oocyte maturation and ovulation of the softshell turtles.

In contrast to freshwater turtles living in temperate regions, *Amyda cartilaginea* displayed high levels of these two steroids in accordance with the nesting season of this population which was reported to happen during March and May (Wichase Khonsue, 1993; Wachira Kitimasak, 1996). This agrees with the prenuptial rise of estradiol and progesterone found in several species of sea turtles by Licht et al. (1979), Licht, Rainey and Cliffton (1980), Wibbels (1990), Rostal et al. (1997) and Rostal et al. (1998).

Levels of testosterone were not significantly different among months. Although the testosterone levels in the females are much lower than in the males, the detectable levels year-round provide evidence for functions of heterologous sex steroids as reviewed by Staub and De Beer (1997) that androgens play a significant role in normal female development. Furthermore, role of testosterone on female reproductive cycle was also suggested by Licht (1982) and Chieffi and Pierantoni (1987). This suggested that the functions of heterologous sex steroids in reptiles should be further studied in more details.

Correlation of climatic factors of Bangkok Matropolis area and levels of plasma sex steroids were analyzed and displayed in table 4-8.

Table 4-8 Pearson's correlation coefficients relating climatic data of Bangkok Metropolis area and plasma sex steroid levels of female *Amyda cartilaginea*. Shaded cells indicate significant correlation ($p<0.05$).

	Max. temp.	Mean temp.	Min. temp.	Max. RH	Mean RH	Min. RH	Daily sunshine	Daily rainfall	T	E_2	P
Max. temp.	1.000										
Mean temp.	0.827	1.000									
Min. temp.	0.559	0.892	1.000								
Max. RH	-0.063	0.344	0.452	1.000							
Mean RH	-0.111	0.218	0.479	0.605	1.000						
Min. RH	0.033	0.311	0.501	0.380	0.749	1.000					
Daily sunshine	0.450	0.119	-0.176	-0.013	-0.408	-0.639	1.000				
Daily rainfall	-0.074	0.098	0.288	0.574	0.837	0.628	-0.367	1.000			
Testosterone	-0.074	0.011	-0.038	0.112	0.111	-0.079	-0.003	-0.198	1.000		
Estradiol	-0.660	-0.775	-0.657	-0.357	-0.150	-0.488	-0.165	-0.053	0.080	1.000	
Progesterone	-0.471	-0.517	-0.508	-0.376	-0.115	-0.213	-0.351	-0.100	-0.185	0.676	1.000

Only estradiol profile showed significant correlation with minimum, mean and maximum temperature of Bangkok area ($p<0.05$). The finding that temperature serve as succeeding cues for gonadal maturation and reproductive cycle is consistent with the previous review by Whittier and Crews (1987).

An insignificant correlation between sunshine duration and steroids levels as well as the insignificant correlations of testosterone and progesterone, and climatic factors might suggest priority of endogenous over exogenous mechanisms in controlling the reproductive cycle of the softshell turtle. This might be related to different control patterns of tropical organisms which are exposed to relative constant environment through the year.

Changes in fertility year-round in terms of plasma sex steroids profiles of the males and the females indicate a seasonal reproductive cycle of softshell turtle. Unlike freshwater living in the temperate environment, the results suggest that *Amyda cartilaginea* might exhibit a prenuptial reproductive cycle

resulted in rising of plasma sex steroids prior to mating season and stable low levels in other period of the year as found in sea turtles of Family Cheloniidae and Family Dermochelyidae living in the tropical environments (Licht, Wood and Wood, 1985; Rostal et al., 1996; Rostal et al., 1998). Furthermore, the data provides evidence in agreement with Licht (1982) that an associated reproductive pattern also appears in turtles as well as a dissociated reproductive pattern which is mainly found in freshwater turtles living in the temperate zones.

According to the present study, the high levels of plasma sex steroids, indicating high gonadal activity, were found during winter while mating and nesting periods were evident in summer. This provided an appropriate environment for the softshell turtle offspring that after a period for incubation, the hatchling would born during rainy season which is the most fertile period of the tropical region. This offer support to Whittier and Crews (1987) that timing of reproduction in a population is determined by 1) when the most offspring survive and 2) when parents are capable of energetically supporting the production of viable young at the least cost to themselves.

In order to certify the findings, annual reproductive cycle of softshell turtles should be conducted by other means of reproductive indicators such as changes in level of vitellogenin, plasma gonadotropins, and gonadal development including spermatogenesis and follicular development.

The high individual variation in plasma sex steroids levels which was evident in the softshell turtle could be beneficial for an artificial selection of breeder males and females which is important for economic animal development programs. Moreover, the finding that endogenous factors might dominate over exogenous factors in controlling the reproductive cycle suggest a possibility to enhance fertility of the softshell turtle by mean of hormonal stimulation. This could be usefully applied to both economic animal development programs as well as endangered turtles recovery programs.

Chapter 5

Conclusions and Recommendations

5.1 Conclusions

5.1.1 Sexual dimorphism

Amyda cartilaginea is sexually dimorphic showing significantly different in various parameters of morphological characters between sexes. The sexually dimorphic traits of the softshell turtle which are related to reproductive performance included higher degree of tail length, tail width and relative posterior position of cloacal opening in the males, and higher degree of female size in relative to carapace length.

Other sexually dimorphic traits which are related to difference in head size might play important roles in decreasing intersexual competition for resource. This offer support to the competition avoidance hypothesis.

5.1.2 Annual reproductive cycle

Temporal changes in fertility in terms of plasma steroids profile of males and females indicated a seasonal reproductive cycle of the softshell turtle. The males exhibited high levels of testosterone in the prenesting period. While the females showed prenesting peak of estradiol and high levels of progesterone during the prenesting and perinesting period.

The results suggest that *Amyda cartilaginea* might exhibit prenuptial reproductive cycle resulted in rising of plasma sex steroids prior to mating season and stable low levels in other period of the year as have been found in sea turtles of family Cheloniidae and family Dermochelyidae. The findings suggest that associated reproductive pattern is also evident in softshell turtles.

Detectable levels of heterologous sex steroids i.e. androgens in females and estrogens in males were evident in both sexes. Male estradiol and progesterone showed well correlation with testosterone levels. While female

testosterone displayed insignificant difference among months. The results indicate evidence for functions of heterologous sex steroids in reptiles.

Estradiol levels of both sexes, and progesterone levels of the males showed significant correlation with temperature of Bangkok area. This confirms that temperature serve as proximate cues for gonadal maturation and reproductive cycles. The insignificant correlation of sunshine duration and steroids levels suggest priority of endogenous over exogenous factors which might contribute to different control mechanisms of tropical organisms that are exposed to relative constant environment year-round.

5.2 Recommendations

1. To properly identify sex, various sexually dimorphic traits should be accounted for consideration. Multivariate analysis methods could provide powerful tools for the identification and should be further investigated.

2. In order to assure prenuptial reproductive cycle of the softshell turtle, it is suggested to detect annual changes in other reproductive parameters such as levels of vitellogenin, plasma gonadotropins as well as gonadal development including spermatogenesis and follicular development.

3. Levels of heterologous sex steroids were evident in both male and female softshell turtle. The roles of heterologous sex steroids in both sexes of softshell turtle should be studied in more details.

4. According to climatic data of this study, sunshine duration was used in stead of photoperiod and resulted in invivid influence on reproductive cycles. To ascertain the roles of photoperiod as well as other climatic factors, weather station should be set up to record the exact weather conditions of the study area.

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Appendix I

Chemical reagents and instruments

Chemical reagents

- Bacto gelatin : Difco Laboratories, U.S.A.
- Buffer solution ready for use pH 4.0 (citrate-hydrochloric acid) : Merck, Germany
- Buffer solution ready for use pH 7.0 (phosphate) : Merck, Germany
- Charcoal Batch No. K220520 : WHO matched reagent programme
- Dextran Batch No. K0801/89 : WHO matched reagent programme
- Diethyl ether : Merck, Germany
- 1, 4-Dioxan : Merck, Germany
- Estradiol-17β standard Batch No. H02050701 : WHO matched reagent programme
- Antiserum to estradiol-17β Batch No. K158330 : WHO matched reagent programme
- (2, 4, 6, 7-^{3}H) estradiol-17β TRK 322 Batch 172 : Amersham International plc, U.K.
- Ethanol absolute : Merck, Germany
- Heparin : Leo Pharmaceutical Products, Denmark
- Hydrochloric acid : Merck, Germany
- Ketamine hydrochloride (Ketalar®) 50 mg/ml : Parke-Davis, Australia
- Ketamine hydrochloride (Ketamil®)100 mg/ml : Troy Laboratories PTY, Australia
- Methanol : Merck, Germany
- POPOP [2, 2'-p-Phenylene-bis (5-phenyloxazole)] : Merck, Germany
- PPO [2, 5-Diphenyloxazole] : Merck, Germany
- Progesterone standard Batch No. K079410 : WHO matched reagent programme
- Antiserum to progesterone Batch No. K873610 : WHO matched reagent programme
- (1, 2, 6, 7-^{3}H) progesterone TRK 413 Batch 69 : Amersham International plc, U.K.
- Sodium chloride : Merck, Germany

- Sodium dihyhrogen phosphate 1 hydrate : Merck, Germany
- di-Sodium hydrogen phosphate 2 hydrate : Merck, Germany
- Sodium hydroxide : BDH Chemical, U.K.
- Testosterone standard : WHO matched reagent programme
  Batch No. K079810
- Antiserum to testosterone : WHO matched reagent programme
  Batch No. K200710
- (1, 2, 6, 7-^{3}H) testosterone : Amersham International plc, U.K.
  TRK 402 Batch 85
- Thimerosal : Fluka Chemika, Switzerland
  (Sodium ethylmercurithiosalicylate)
- Toluene : Merck, Germany

Instruments

- Agitation machine : Direct Mix TS-100
  Thermal Kagaku Sangyo, Japan
- Balance : Right-A-Weigh
  W. M. Ainsworth & Sons, U.S.A
- Bottle top dispenser : Labmax dispenser 10 ml
  Witeg Wertheim, Germany
- Disposable syringe filter 0.22 μ : Cameo 25AS
  Micro Separations Inc, U.S.A.
- Freezer : Sharp FC-27
  Thai City Electrics, Thailand
- Heating block : Dri-Block DB-3
  Tecam, U.S.A.
- β-liquid scintillation counter : 1218 Rackbeta
  LKB wallac, Finland
- Magnetic stirer : Pyro-Magnestir king size
  Lab-Line Instruments, U.S.A.
- Mixer : Vortex-Genie 2
  Scientific Indistries,
- Needle and syringe : Terumo Corporation, Japan
- Pipettors : Pipetman P20, P200, P1000
  Gilson, France

- Pipettor tips : Plastibrand 200 µl, 1000 µl
  Brand, Germany
- pH meter : Corning pH meter 240, U.K.
- Refrigerated centrifuge : Coolspin 2 MSE
  International Equipment, U.S.A.
- Refrigerater : Sharp Nice 320
  Thai City Electrics, Thailand
- Repeating pipette : Handystep
  Brand, Germany
- Ultracentrifuge : Centrikon T-1160
  Kontron Instruments, Switzerland
- Ultrasonic cleaner : D-7700
  Elma, Germany
- Vernier caliper : 0.05 mm graduation
  Mitutoyo, Japan
- Waterbath : Lab line/Dubnoff Incu-shaker 3575-1
  Lab-Line Instruments, U.S.A

Appendix II

Reagents preparation

Preparation of steroid assay reagents

These reagents were prepared according to Sufi, Donaldson and Jeffcoate (1990).

1. Assay buffer solution

Sodium dihydrogen phosphate (anhydrous) NaH_2PO_4	2.35 g *
di-Sodium hydrogen phosphate (anhydrous) Na_2HPO_4	11.6 g *
Sodium chloride	8.8 g
Thimerosal	0.1 g
Gelatin	1.0 g

All constituents were dissolved in 750 ml distilled water except gelatin which was dissolved in a small volume of warm water before being added to the others. The pH of this buffer was checked with a pH meter and adjusted to be between 7.2 and 7.4 with 1N NaOH or 1N HCl. The volume was then made up to 1000 ml.

The buffer could be stable for up to one month when stored at 4°C. It was used as the diluent for all reagents in steroids assay.

Remark * If the hydrate form of these reagents was used, then the amounts taken must be increased in proportion to the degree of hydration.

2. Charcoal suspension

Charcoal	0.625 g
Dextran	0.0625 g
Assay buffer solution	100 ml

Dextran was dissolved in 100 ml assay buffer solution in a stoppered container. Charcoal was added, and the container was shaked vigorously for 30 seconds.

The charcoal suspension could be stable for up to one month when stored at 4°C. It was stirred vigorously on ice before use.

3. Scintillation fluid

PPO [2, 5-Diphenyloxazole]	12.5	g
POPOP [2, 2'-p-Phenylene-bis (5-phenyloxazole)]	0.75	g
Toluene	2.50	l
1, 4-Dioxan	500	ml

All constituents were mixed together homogenously and stored in a dark bottle. The solution could be stable at room temperature.

Preparation of hormone free plasma

Hormone free plasma was used as diluent for serial dilution of softshell turtle plasma containing a high level of steroid in parallelism check. It was prepared according to DePaolo et al. (1979).

Pooled softshell turtle plasma was added with steroid tracer (in case of this study ^{3}H-testosterone was used) to yield an approximately 2,000 CPM/ml. The plasma was added with dry activated charcoal at 10 % vol/vol ratio. The mixture was stirred at 4°C for 24 hours with a magnetic stirer. Subsequently, the mixture was cool centriguge for 30 minutes at 1,100 x g, followed by second centrifugation at approximately 160,000 x g for 1 hour at 4°C. The supernatant was filtered through a millipore 0.22 μm cellulose filter and frozen at -20°C in small aliquots of 1 ml until use.

The hormone free plasma was checked for remaining steroid by counting with a β-counter. It was found that this procedure could remove up to 95 % of steroid from softshell turtle plasma.

Appendix III

Sexual dimorphism data

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP005-10-96	Male	63.5			44.5	31	41.5	41	18.5	23.5	18.38	4.67	4.88	9.34	5.83	16.66	18.04	11.07	8.61	20
BP006-10-96	Female	44.5			32.5	23	30	31	13.5	14	9.43	1.66	1.76	3.52	3.74	13.53	14.81	8.61	6.505	8
BP007-10-96	Female	39.5			29.5	20.5	26.5		11.5	11.6	7.45	0.49	1.375	2.22	2.395	9.865	11.33	6.04	4.82	8
BP008-10-96	Female	34			25.5	17.5	22.5	24.5	9.5	12.54	8.27	0.575	1.47	2.27	2.76	8.615	10.25	5.415	4.51	5
BP009-10-96	Female	48			34	23	31	32	13	18.5	12.19	2.3	3.16	5.445	3.97	13.58	14.73	9.075	6.52	8
BP010-10-96	Female	44.5			31.5	21.5	29.5	29.5	14.5	16.5	12.41	1.86	2.48	4.25	3.745	13.04	14.38	8.54	6.55	8
BP011-10-96	Male	61			44	32	41.5	41.5	16.5	24.5	21	4.465	4.87	8.92	5.06	14.58	16.17	9.275	7.56	20
BP012-10-96	Male	68.5			47.5	34	45	45	19.5	26	21	5.145	4.935	10.36	6.4	18.23	20	12.55	9.375	35
BP013-10-96	Female	33.5	20	17.5	25	17.5	22.5	23	8	12.23	9.33	0.97	1.7	2.88	2.47	8.645	9.75	5.425	4.275	5
BP014-10-96	Female	45.5	31	27.5	31	25	31.5	29	12.5	16.45	11.8	1.185	3.62	4.54	3.33	12.87	13.78	8.27	6.6	10
BP015-10-96	Female	28	17	15	21.5	15	19.5	21	7.5	9.5	7.38	0.96	1.39	2.07	1.725	7.935	9.035	4.82	3.945	2.6
BP016-10-96	Female	42	28	25.5	32.5	23	29	30.5	12.5	16.15	11.23	0.83	1.9	2.435	2.74	11.03	12.48	6.9	5.535	10
BP017-10-96	Female	45.5	30.5	27	33	25	31.5	31	13.5	18	11.95	1.12	3.25	4.325	3.63	13.28	14.33	7.83	6.65	10
BP018-11-96	Male	44.5	28.3	26	33	23.5	30	31		16.5	12.72	3.385	2.75	6.11	5.49	11.18	12.32	7.015	6.285	8
BP019-11-96	Female	42.5	28	25.5	32	23	30	30	12.5	16	11.35	0.44	1.965	2.475	3.235	11.47	12.45	7.22	6.14	8
BP020-11-96	Female	53	36.5	32	39	29.5	37	37	15	21	14.3	1.13	4.73	5.37	4.34	14	15.3	8.815	7	20
BP021-11-96	Male	52.5	34	30	38	26	35	35	14.5	19.5	15.66	5.175	3.385	7.29	5.54	15.04	16.45	9.99	7.72	20
BP022-11-96	Male	64.5	44.5	39	45	30.7	40.5	44	16.5	27	23	5.325	5.075	10.3	7.12	16.81	18.72	11.5	8.645	40
BP023-11-96	Male	67	45.5	39.5	47	36.5	45.5	44.5	20.5	26.5	20.5	5.09	6	10.86	6.41	17.98	19.3	11.3	9.265	40
BP025-11-96	Male	65.5	43	37.5	45.5	34	42.5	42	19.5	25.5	18.31	1.94	4.56	6.215	4.86	17.65	19.42	11.3	9.62	40

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP026-11-96	Male	65.5	44.5	40	47.5	33	41	45.5	18.5	26	17.58	3.535	4.945	7.84	5.625	16.3	17.93	10.82	8.56	34.5
BP027-11-96	Male	62.5	42	38	43	31	40	41	17.5	24.5	19.13	4.82	5.7	10.4	6.03	17.82	18.14	10.82	9.08	28
BP028-11-96	Female	61	42	38	41	28	38	39	18.5	27	18.35	2.145	4.06	5.93	4.715	15.7	17	10.81	8.78	27
BP029-11-96	Male	53	34.5	31	38.5	27	35	36	13.5	19.5	16.04	4	3.53	7.88	5.53	14.74	16	9.45	8.25	15
BP030-11-96	Female	49.5	33	29	36	25.5	33.5	32.5	14	19.5	13.14	1.01	2.56	4.365	3.765	14.37	16.05	9.515	7.345	
BP031-11-96	Male	43	29	26.5	31.5	24	30.5	31	10	15.2	14.66	5	1.685	7.025	4.26	14.26	12.7	8.125	6.13	
BP032-11-96	Female	31.5	20.7	19	26	17	21.5	25	7	11.5	8.18	0.83	2.37	3.235	2.675	9.465	10.63	5.555	4.535	3
BP033-11-96	Male	64	45	40	43	31.5	43.5	41	16	24	17.78	3.52	5.365	8.645	6.75	18.45	20.13	11.98	8.785	
BP034-12-96	Male	33.5	21.5	19.5	26	15.5	22	25.5	8	12.13	10.17	3.38	2.5	6.1	3.52	9.66	5.885	10.66	4.34	3.1
BP035-12-96	Female	49.5	34	31	37	28	35.5	35.5	13	16.28	10.94	0.825	4.775	5.73	4.135	13.45	14.81	8.43	6.355	13.5
BP036-12-96	Female	37	31	29	33	25	32.5	31	13.5	17	11.77	1.545	3.11	5.16	3.765	13.83	14.82	8.715	6.46	12
BP037-12-96	Female	45	31	27.5	34	24.5	32.5	32	10.5	15.73	11.31	1.31	3.425	4.945	3.74	12.58	13.77	7.745	5.7	9.8
BP038-12-96	Female	52.5	36.5	32.5	39	29	36.5	38	14.5	19.78	12.94	0.845	4.645	5.275	4.41	14.44	15.55	8.825	7.23	18.5
BP039-12-96	Male	27.5	18	16.5	22.5	15	19	22	6.5	10.36	9.27	2.14	2.075	4.43	3	7.365	8.34	5.45	3.93	2.5
BP040-12-96	Male	49	33	30	36	23.5	31.5	34	15	19.19	15.53	3.585	4.315	8.085	4.945	13.63	14.92	8.07	6.48	15.5
BP041-12-96	Male	67.5	45.5	39	47.5	35.5	45.5	46	17.5	25	21	4.825	5.82	9.8	5.74	18.23	19.63	12.43	9.215	34.5
BP042-12-96	Male	44	28	25	32	23	30	30.5	11	16.73	12.67	3.3	2.66	6.04	4.525	11.54	12.65	7.275	6.16	8
BP043-12-96	Male	42	28	25.5	31.5	23	30.5	31	9.5	16.35	14.83	4.125	2.025	6.375	4.465	12.97	14.38	7.95	5.91	7
BP044-12-96	Male	62.5	41	36.5	43	32.5	42.5	40	17	25.5	19.22	3.065	4.355	7.325	7.46	16.5	18.05	10.94	8.58	28.5
BP045-12-96	Female	52	36	30.5	39	29.5	37	37	15	19	13.08	1.245	4.93	6.125	4.365	14.37	15.35	8.125	7.35	18

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP046-12-96	Male	64	45	37.5	42.5	31	43	42	16	23	18.25	4.48	4.475	9.27	5.67	18.3	20	12.01	9.685	26.5
BP047-12-96	Female	51	30	25.5	35.5	26	34.5	33	15	21	14.47	0.86	3.455	4.56	4.085	14.55	15.93	9.575	7.75	19
BP048-12-96	Female	33.5	21	17.5	25	16	22.5	24	8.5	12.5	9.465	0.985	1.8	2.905	2.455	8.63	9.58	5.5	4.465	4
BP049-12-96	Female	61	42.5	36	40.5	27.5	39	39	18	27.5	19.25	2.76	3.875	6	4.55	15.5	16.5	10.55	8.51	27
BP050-12-96	Male	65	45	39	47	29	33.5	44	18.5	26	18.05	3.765	4.88	7.625	5.415	16.5	17.55	10.72	9.15	34
BP051-12-96	Male	68	44	38	50	34	45.5	46.5	18	27	18.8	4.41	5.525	9.98	6.685	18.28	19.98	12.38	9.565	40
BP052-12-96	Male	32.5	20	17.5	24.5	15.5	21	24	8	12.18	10.27	2.04	2.315	4.48	3.66	8.875	9.75	5.84	4.61	4
BP053-12-96	Male	67.5	46.5	40	37.5	35	45	34.5	19.5	25	19.06	4.15	3.78	8.175	5.9	17.6	19.2	11.3	9.225	40
BP054-01-97	Female	42.5	30	25.5	30	22	28	29	11.5	14.47	11.25	2.22	4.33	6.05	4.79	12.24	13.55	7.31	6.265	8
BP055-01-97	Male	31.5	19.5	16	23.5	16	21	23	7.5	11.44	10.5	2.46	2.775	5.575	3.975	9.36	10.2	5.61	4.095	3.2
BP056-01-97	Female	48.5	32.5	27.5	35.5	25	33.5	32.5	12.5	17.5	12.12	1.09	2.66	3.775	3.65	14.4	16.15	9.39	7.565	13.4
BP057-01-97	Male	35.5	25	21	27.5	18	23	27	9	12.55	10.47	3.91	3.7	8.46	4.22	10.55	11.56	6.44	4.815	3.5
BP058-01-97	Female	49.5	33.5	28.5	36.5	27	34.5	33.5	15.5	19	13.53	1.03	3.825	4.965	4.525	13.52	15.07	7.8	6.775	15.5
BP059-01-97	Female	44.5	31	26.5	33.5	24.5	31	32	10	15.13	11.16	1.425	4.05	4.95	4.16	12.5	13.63	7.565	6.225	19.4
BP061-01-97	Male	67	46	38	48.5	35	48	46.5	20	26.5	20	3.68	5.475	9.745	6.6	18.6	20.13	12.88	9.27	41
BP062-01-97	Male	62	41	34.5	43	32	41.5	39.5	16	26	18.58	3.36	4.81	8.245	7.735	16.1	17.76	11.39	9.18	29
BP063-01-97	Male	64	45	38	43	31	43.5	41	15.5	23.5	18	3.8	5.34	9.31	6.22	18.32	19.81	12.17	9.15	26.2
BP064-02-97	Male	58.5	39	33	42	30	38	40.5	13.5	22	17	4.75	5.004	9.43	6.465	13.49	14.87	10.27	7.46	20
BP065-02-97	Male	56.3	36.5	30.5	40	28	37.5	37.5	15	22.5	17.5	3.655	4.12	7.8	5.535	14.61	16	9.401	8.265	21
BP066-02-97	Male	64	44	37	42	37.5	43	41.5	15.5	22	18	6.685	4.745	10.36	6.795	18.5	20.36	11.73	8.92	24.5

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP067-02-97	Male	61.5	40	34	42.5	30.5	39.5	40	15.5	24	19	4.8	6.1	10.69	7.14	17	18.65	11.32	8.87	24
BP068-02-97	Female	60.5	42	37	40	28	37.5	39	17.5	26.5	17.5	1.58	3.335	4.67	4.61	16	17.71	10.79	8.05	27
BP069-02-97	Male	37	23	19	29	20	25.5	28	9	13.5	12.5	3.83	2.98	6.73	3.915	10.36	11.61	6.225	4.465	5
BP070-02-97	Male	60.5	37	30.5	43.5	31	38.5	39.5	17.5	23.5	17.5	3.27	3.73	6.73	6.135	16.5	17.57	11.46	8.7	29
BP071-02-97	Male	67.5	45.5	38	47	35.5	44	45	17.5	24.5	20	4.34	5.385	9.65	5.67	18.5	20.39	10.99	9.745	33.5
BP072-02-97	Female	33	23	20	24.5	18	23	23	9.5	11.2	8.56	1.9	1.125	2.565	3.645	9.48	10.95	5.84	4.32	5
BP073-02-97	Female	49.5	33	28.5	35	26	32.5	32	15	20.5	13.5	1.19	3.355	4.95	3.86	12.5	14.05	9.185	7.05	15.5
BP074-02-97	Male	55	37	30	41	31	39	39	15	20.5	15.5	2.315	3.16	5.195	3.655	16.3	17.55	9.81	7.945	22.6
BP075-02-97	Male	52.5	33	27	38.5	25	34	36.5	12.5	22	16.5	4.49	4.565	9.27	6.14	14.5	15.98	9.67	7.255	15.5
BP076-02-97	Male	36.5	23.5	19	28	18.5	24	16.5	9	14	12.27	3.595	2.7	6.78	3.9	10.11	11.38	6.11	5.38	5.5
BP077-02-97	Female	43	26.5	21.5	32	22	28.5	29	14.5	16.5	11.64	0.635	1.555	2.28	3.09	12	13.41	7.785	6.84	11.5
BP078-02-97	Male	42	27.5	24	30.5	23	29	29.5	10.5	14.5	11.15	1.555	2.91	4.69	2.8	11.06	12.37	6.59	5.07	8.1
BP079-02-97	Male	64.5	44	37	45	33	44.5	42.5	19	25.5	18.5	2.2	4.475	6.895	4.89	18.5	20.03	11.79	9.085	33
BP080-02-97	Male	28.5	17	14	21.5	14	17.5	19.5	7.5	11	7.5	1.525	1.61	3.35	2.415	7.64	8.615	4.765	4.155	2.5
BP081-02-97	Male	58	37	30	40	27.5	38	39.5	14.5	22	18.5	4.8	4.1	8.93	6.28	17.5	19.32	10.31	8.49	21.5
BP082-02-97	Female	44	31	26.5	29	22.5	29.5	28	11	14	10.77	2.23	3.13	5.3	3.14	13.1	14.42	8.415	6.175	9
BP083-02-97	Female	29.5	18.5	15.5	22	14.5	19	20.5	7.5	11.22	7.03	0.615	1.009	1.53	1.57	8.04	8.94	4.85	3.77	3
BP084-03-97	Female	40	26	22.5	30	20.5	26	28	12	16	10.89	1.325	2.32	3.74	3.425	11.3	12.33	6.745	5.67	7.25
BP085-03-97	Male	67.5	47	37	47	36.5	45.5	44	20	24.5	20	3.86	3.71	7.42	5.94	18.5	19.95	10.86	8.85	36
BP086-03-97	Male	52.5	34.5	28.5	39	28	36	36.5	14	21	15	3.62	3.21	6.855	5.12	14.11	15.55	8.365	7.26	16.5

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP087-03-97	Female	46.5	32.5	26	33	24	30.5	31.5	13	16	12.05	1.79	3.535	5.355	3.775	13.23	14.8	8.655	7.375	12
BP088-03-97	Male	54.5	37	28	41	31	40	39	15	21.5	15	2.67	3.65	6.14	3.635	16.3	16.44	9.92	8.08	23
BP089-03-97	Male	45.5	28	22	35	24.5	31	32.5	11	18	13	2.89	2.7	5.38	4.11	10.76	12.9	7.2	6.2	10
BP090-03-97	Male	67	45	36.5	46.5	35.5	45.5	45.5	17.5	25	20.5	4.05	5.07	9.575	5.47	18.5	20.33	11.11	9.11	34
BP091-03-97	Male	65.5	43	37	47	34	44.5	45.5	17	26	17.5	3.6	5.06	8.76	5.415	18	19.52	11.15	9.09	30.5
BP092-03-97	Female	44.5	30.5	24.5	31.5	23	30	31	11.5	17.5	13	1.54	3.17	5.02	3.05	12.54	13.7	7.46	5.9	9.5
BP093-04-97	Male	69	44.5	38.5	48	33	41.5	42	19.5	28	20.5	4.65	4.01	8.11	7.215	18	19.83	12.14	9.99	35.5
BP094-04-97	Female	41	28	24.5	29	21.5	27.5	28.5	11.5	14.5	10	1.7	2.055	3.775	3.5	11.82	13	7.135	5.87	7
BP095-04-97	Female	53.5	36.5	30	40.5	30.5	39	39	16	22	16	2.84	3.15	5.9	3.88	15.5	17.3	9.74	8.14	23.5
BP096-04-97	Male	34.5	21.5	19.5	27	18.5	23	25.5	8.5	14	11.4	3	2.475	5.215	3.635	10.16	11.24	5.84	5.365	5.4
BP097-04-97	Female	42	28	24	30	23	28.5	28	12.5	15	10.76	1.665	2.52	3.67	3.16	12.79	14.1	8.335	6.15	9
BP098-04-97	Male	52	34.5	30.5	38.5	28	35	35.5	14	19.5	14.5	3.17	3.88	7.115	5.25	13.8	15.36	8.31	7.32	15.5
BP099-04-97	Male	41	25	21	29	19.5	25.5	28.5	11	15.5	11.95	2.1	1.9	3.925	2.78	10.5	11.38	5.935	5.23	7.1
BP100-04-97	Female	46.5	30.5	25	33.5	23	29.5	31	14	20	12.5	1.24	2.5	3.715	3.275	12.86	14.2	8.24	6.875	12.8
BP101-04-97	Male	65	44.5	38.5	47	33.5	43.5	45	19	26	18	2.6	4.235	6.765	5.52	17	18.5	10.85	8.9	34.5
BP102-05-97	Male	34.5	21.5	19	27.5	18.5	23	26	8.5	13.81	11.48	2.77	3.15	6.06	3.66			6.48	4.88	5
BP103-05-97	Female	39	26.5	22.5	27	20.5	25.5	25.5	9.5	13	9.33	1.14	2.765	3.81	3.545	11.47	12.5	6.86	5.34	6.5
BP104-05-97	Male	61.5	40	33.5	44	32	41	42.5	15	24.5	18	4.62	5.415	10.13	4.75	16.5	18.3	10.85	8.24	24.5
BP105-05-97	Male	59.5	37.5	31.5	44	30.5	39.5	41.5	16	23	17	4.865	3.47	8.39	4.73	14.88	16.54	9.235	8	25
BP106-05-97	Female	48	32.5	29	33.3	26.5	32.5	31.5	12	17.5	13.5	1.865	2.93	5.035	3.15	13.81	15.17	8.735	6.645	12

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP107-05-97	Male	60.5	39.5	33	44	32	42	41.5	16.5	23	17.5	3.355	3.9	6.965	5.205	17.5	19	10.91	8.955	25.5
BP108-05-97	Male	57.5	37	30	41	29.5	38	39	15.5	22.5	16	3.15	4.2	7.195	5.1	14.9	16.23	9.38	7.27	22.5
BP109-05-97	Female	49.5	33.5	28.5	36	26.5	34	33.5	14.5	18	13.5	1.82	2.6	4.265	3.78	13.62	15.15	7.565	6.83	15
BP110-06-97	Male	65	44	40	47.5	34.5	43	41.5	18	25	18.5	2.91	4.525	7.365	4.87	17	18.5	10.52	8.755	32
BP111-06-97	Male	43.5	29	23.5	34	25.5	32	32.5	11	14.87	14.5	3.81	4.94	7.385	4.94	13.1	14.53	7.78	6.675	11
BP112-06-97	Male	35	22	18.5	28	18.5	23.5	26.5	9	14.04	11.38	3.465	2.17	5.8	4.3	10.66	11.78	6.015	5.285	5.8
BP113-06-97	Female	43	28.5	24.5	30	22.5	29	28.5	10	15.55	10.9	0.925	2.43	3.41	2.735	10.87	12.22	6.58	5.445	8
BP114-06-97	Male	38.5	24.5	20.5	29	20.5	26	28	9.5	14.18	13.33	3.145	2.24	5.19	4.78	11.59	12.63	7.01	5.525	7
BP115-06-97	Male	39.5	25.5	21.5	30	20	26	28.5	9.5	14.73	13.02	3.445	2.49	5.885	4.37	10.99	12.04	6.555	5.9	7.5
BP116-06-97	Female	48.5	32	28.5	37.5	25.5	32.5	34	13.5	19.5	13.09	0.6	2.74	3.115	3.085	14.19	15.45	9.145	6.805	14
BP117-06-97	Male	51	34	28.5	38	28	35.5	36	13	18	14.5	3.43	3.4	6.97	4.51	13.92	15.14	8.175	7.035	14.5
BP118-06-97	Male	64.5	44	37.5	44.5	33	43.5	43	18.5	25	18.5	1.965	4.07	6.44	4.82	17.5	19.3	11.24	9.44	31.5
BP119-06-97	Male	73	49.5	43.5	48	38.5	48	45.5	21	29.5	23	6.095	4.71	10.78	7.1	19	20.5	12.3	10.18	43
BP120-07-97	Male	64.5	44.5	37	47	34	43	46	17	23.5	19	4.015	4.205	7.495	5.575	16.5	18	10.37	8.85	31
BP121-07-97	Male	70.5	47.5	39	49	36	45.5	46.5	18	27.5	20	3.015	4.83	7.985	6.435	19.5	21.3	12.88	10.67	39.5
BP122-07-97	Male	73	49.5	41.5	48	35.5	46	46	20.5	27.5	22.5	5.15	5.16	10.41	6.86	18.5	20	12.49	9.485	40
BP123-07-97	Male	64	41.5	33	44	33	41.5	40.3	17	25.5	19	3.405	3.8	6.92	6.685	16	17.5	10.7	8.825	31
BP124-08-97	Male	63	41.5	33	43.5	32.5	42	40	16.5	26	20	3.695	4.17	7.44	6.34	17	18.3	11.14	8.855	31
BP125-08-97	Male	61	40.5	34	43.5	30.5	38	40	20	25.5	19	3.325	3.8	6.15	5.535	17	18.5	11.44	9.25	32
BP126-08-97	Female	45	30	25	32	24.5	30.5	30	13.5	16.5	11.5	0.95	2.335	3.49	2.85	11.82	13.42	7.875	6.21	10

NUMBER	SEX	CL	BDL1	BDL2	CW	PL1	PL2	PW	H	PR	PC	TC	CT	TL	TW	HL	HLs	HW	HH	Weight
BP127-08-97	Male	40	23	20	30	21	27	28.5	9.5	14	12.5	2.61	2.465	4.9	3.6	11.05	11.88	6.23	5.615	7
BP128-08-97	Male	52.5	34	29	37.5	28	35.5	35	14.5	19	14.5	2.14	3.155	5.335	3.535	13.17	14.33	7.9	6.71	15.8
BP129-08-97	Male	55.5	31	25	42	28	36	38.5	15.5	20.5	14	2.935	3.39	5.985	4.615	14.86	16.01	8.975	7.665	18
BP130-08-97	Female	32.5	20.5	18	24.5	17.5	22	23.5	7.5	11.25	6.865	0.545	1.905	2.385	2.26	8.855	9.9	5.4	4.15	3.5
BP131-08-97	Male	41	23.5	19.5	30.5	21	26	29.5	10	16.5	13	4.465	2.41	6.965	3.6	11.2	12.2	6.585	5.65	7
BP132-08-97	Male	45.5	30	26	32	22.5	30	31	10.5	16.5	16	3.02	3.37	6.855	5.54	12.42	13.55	8.58	6.96	10
BP133-08-97	Female	45	30	25.5	32	23	30	31	10.5	16.5	12.5	1.25	2.525	3.76	3.64	12.5	13.87	7.64	5.76	10.5
BP134-09-97	Female	37.5	26	23.5	27	20	25.5	26	10	11.1	8.175	1.185	2.76	4.24	3.555	10.59	11.54	6.28	5.16	5.5
BP135-09-97	Female	47	30	26.5	34.5	24.5	31	32	12	17.5	12.63	2.05	2.49	4.285	3.95	12.37	13.85	7.81	6.255	12
BP136-09-97	Female	48	32	27	33.5	24	30	32	13.5	18	13.06	1.365	2.675	4.435	3.475	13.41	14.15	8.92	6.995	13
BP137-09-97	Female	48	33.5	28	36	26.5	33	33	13	19	13.98	2.575	3.435	6	4.39	14.24	15.5	8.685	6.615	14
BP138-09-97	Female	39	25.5	21	28.5	20.5	25.5	27	9.5	14	9.23	1.09	2.1	3.375	2.63	10.2	11.36	6.25	5.01	6
BP139-09-97	Female	61	42	36	40	28.5	38.5	39	17	27	18	1.56	3.68	4.785	3.9	15.86	17.44	10.37	8.295	26.5
BP140-09-97	Female	31	21	18.5	23.5	16.3	21	23	7.5	11	7.77	0.91	1.73	2.87	2.49	9.2	10.38	5.73	4.3	3.2
BP141-09-97	Male	41	25.5	20.5	31	21	27	29	10.5	15.5	13.02	3.72	2.43	6.355	3.69	11.14	12.56	6.76	5.79	8

Appendix IV

Plasma steroids data

	SEX	Sampling	Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
BP005-10-96	M	1	25.5428	26.7196	0.4028
BP006-10-96	F	1	0.0178	217.5749	1.7628
BP007-10-96	F	1	0.0855	213.1619	2.3976
BP008-10-96	F	1	1.2305	19.8089	
BP009-10-96	F	1	0.0204	215.8144	3.3017
BP010-10-96	F	1	0.0598	373.4230	3.1977
BP011-10-96	M	1	24.0442	22.1665	0.2331
BP012-10-96	M	1	17.8655	73.3027	0.5467
BP013-10-96	F	2	0.2651	10.5858	1.5057
BP014-10-96	F	2	0.0773	163.8100	1.5186
BP015-10-96	F	2	0.0517	25.7346	2.1276
BP016-10-96	F	2	0.0665	249.9049	1.8342
BP017-10-96	F	2	0.0743	185.4144	1.8073
BP018-11-96	M	3	19.1075	52.4481	0.2119
BP019-11-96	F	3	0.0921	282.9816	1.6784
BP020-11-96	F	3	0.0383	148.0453	3.4511
BP021-11-96	M	3	27.5319	60.4440	0.3720
BP022-11-96	M	3	21.5582	43.4664	0.5132
BP023-11-96	M	3	31.9385	56.2864	0.8581
BP025-11-96	M	4	24.9451	45.8207	0.6208
BP026-11-96	M	4	28.6029	35.9048	0.2994
BP027-11-96	M	4	25.0764	26.2307	0.2085
BP028-11-96	F	4	0.0870	60.5679	1.6892
BP029-11-96	M	4	9.3888	27.7179	0.1402
BP030-11-96	F	4	0.0647	184.5070	3.3330
BP031-11-96	M	4	1.9429	25.4502	0.1747
BP032-11-96	F	4	0.0467	59.1155	1.4990
BP033-11-96	M	4	11.9726	32.5590	0.6561
BP034-12-96	M	5	1.7570	45.1077	
BP035-12-96	F	5	0.0477	67.1874	3.4483
BP036-12-96	F	5	0.0726	411.6976	2.6842
BP037-12-96	F	5	0.0815	239.0443	1.5664
BP038-12-96	F	5	0.0955	245.7896	2.0864
BP039-12-96	M	5	0.1131	2.7211	0.1289
BP040-12-96	M	5	29.1893	46.0783	0.2629
BP041-12-96	M	5	21.4766	72.3438	0.8715
BP042-12-96	M	5	21.8684	30.4135	0.2784

	SEX	Sampling	Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
BP043-12-96	M	5	1.2988	5.3353	0.1485
BP044-12-96	M	5	5.3441	51.3564	0.2673
BP045-12-96	F	6	0.0474	200.0480	1.5227
BP046-12-96	M	6	24.1430	61.1684	0.2848
BP047-12-96	F	6	0.1395	240.9873	1.7947
BP048-12-96	F	6	0.0666	26.3305	
BP049-12-96	F	6	0.0753	280.9873	3.3189
BP050-12-96	M	6	30.9213	34.3466	0.3369
BP051-12-96	M	6	18.9102	33.3417	0.4392
BP052-12-96	M	6	9.3376	23.9333	0.2022
BP053-12-96	M	6	21.5906	49.7381	0.4598
BP054-01-97	F	7	0.1122	44.6648	1.1675
BP055-01-97	M	7	7.0056	81.3683	
BP056-01-97	F	7	0.1459	234.7298	1.4742
BP057-01-97	M	7	0.9774	1.6094	0.1137
BP058-01-97	F	7	0.1475	235.7999	0.9663
BP059-01-97	F	7	0.0943	24.8464	1.6370
BP061-01-97	M	7	20.1129	46.8340	0.1280
BP062-01-97	M	7	5.6919	26.4007	0.4305
BP063-01-97	M	7	12.6360	38.3006	0.2424
BP064-02-97	M	8	14.8446	20.4608	0.2500
BP065-02-97	M	8	27.9485	32.2529	0.3360
BP066-02-97	M	8	13.6792	29.7223	0.2049
BP067-02-97	M	8	16.9542	13.2622	0.2175
BP068-02-97	F	8	0.0957	94.6093	1.1325
BP069-02-97	M	8	0.9516	5.3353	0.1292
BP070-02-97	M	8	10.3230	33.3675	0.3092
BP071-02-97	M	8	13.3490	39.4855	0.3074
BP072-02-97	F	8	0.0502	142.5933	1.1752
BP073-02-97	F	8	0.1994	257.7028	1.4631
BP074-02-97	M	9	0.0515	1.6721	0.0576
BP075-02-97	M	9	9.7679	13.8030	0.2333
BP076-02-97	M	9	15.8864	26.9436	0.1092
BP077-02-97	F	9	0.1174	214.5143	1.3680
BP078-02-97	M	9	1.5046	36.4631	0.0328
BP079-02-97	M	9	19.0781	21.2036	0.5414
BP080-02-97	M	9	0.1527	2.3767	0.0783

	SEX	Sampling	Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
BP081-02-97	M	9	20.6626	34.8648	0.1988
BP082-02-97	F	9	0.2224	264.5801	2.0629
BP084-03-97	F	10	0.1937	145.1160	1.1649
BP085-03-97	M	10	9.7878	12.1969	0.1395
BP086-03-97	M	10	11.5834	7.5545	0.1092
BP087-03-97	F	10	0.0837	95.9591	1.2145
BP088-03-97	M	10	0.1244	2.7641	0.1530
BP089-03-97	M	10	13.9974	7.6474	0.1652
BP090-03-97	M	10	5.5667	14.9671	0.1975
BP091-03-97	M	10	4.8940	19.2468	0.2980
BP092-03-97	F	10	0.3367	96.9790	1.0092
BP093-04-97	M	11	8.6874	19.7939	0.2378
BP094-04-97	F	11	0.0798	72.8708	1.6815
BP095-04-97	F	11	0.0220	4.5823	1.0567
BP096-04-97	M	11	3.4735	20.7842	0.1120
BP097-04-97	F	11	0.0165	142.3359	1.0068
BP098-04-97	M	11	7.5486	16.6858	0.0713
BP099-04-97	M	11	1.0953	19.2369	0.1682
BP100-04-97	F	11	0.0762	140.5101	2.1073
BP101-04-97	M	11	20.1995	47.8485	0.2460
BP102-05-97	M	12	1.6714	0.9379	0.0780
BP103-05-97	F	12	0.0445	50.2022	0.7287
BP104-05-97	M	12	15.3739	9.5477	0.1869
BP105-05-97	M	12	10.9454	17.3146	0.1786
BP106-05-97	F	12	0.0336	123.6834	1.7006
BP107-05-97	M	12	3.6707	21.8540	0.1753
BP108-05-97	M	12	17.0921	36.3166	0.1618
BP109-05-97	F	12	0.0453	65.1270	2.0210
BP110-06-97	M	13	3.3005	3.8540	0.0829
BP111-06-97	M	13	10.4053	20.7527	0.0951
BP112-06-97	M	13	0.9851	7.8615	0.0903
BP113-06-97	F	13	0.1132	36.5185	0.8151
BP114-06-97	M	13	4.5031	9.7627	0.1097
BP115-06-97	M	13	2.5361	4.4968	0.1476
BP116-06-97	F	13	0.1971	105.1150	1.1013
BP117-06-97	M	13	9.8253	16.8634	0.1118
BP118-06-97	M	13	6.2832	12.8612	0.3604

	SEX	Sampling	Testosterone (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
BP119-06-97	M	13	3.7846	17.6930	0.1224
BP120-07-97	M	14	14.5938	18.6703	0.0820
BP121-07-97	M	14	9.4553	13.3835	0.0844
BP122-07-97	M	14	13.6976	17.4300	0.2268
BP123-07-97	M	14	1.1548	8.6245	0.3383
BP124-08-97	M	15	6.2001	11.4465	0.2259
BP125-08-97	M	15	4.8246	7.1070	0.1622
BP126-08-97	F	15	0.0203	78.0615	0.9281
BP127-08-97	M	15	7.2654	8.3765	0.0820
BP128-08-97	M	15	4.2668	15.8715	0.1157
BP129-08-97	M	15	1.1180	15.3978	0.2321
BP130-08-97	F	15	0.2201	47.3080	0.6170
BP131-08-97	M	15	2.0350	10.4499	0.0741
BP132-08-97	M	15	0.5347	9.5276	0.1427
BP133-08-97	F	15	0.1299	107.3920	0.9044
BP134-09-97	F	16	0.0234	76.3622	0.5416
BP135-09-97	F	16	0.0106	187.1239	0.8142
BP136-09-97	F	16	0.1107	74.2290	1.1547
BP137-09-97	F	16	0.0746	183.7000	0.5669
BP138-09-97	F	16	0.1094	181.1085	0.9780
BP139-09-97	F	16	0.0311	181.8583	1.2213
BP140-09-97	F	16	0.0927	69.4697	2.2038
BP141-09-97	M	16	7.0349	16.3404	0.0818

Biography

Mr. Noppadon Kitana was born on the 22nd of May 1972 in Chanthaburi province. He graduated his bachelor's degree of science in zoology in 1993 from the Department of Biology, Faculty of Science, Chulalongkorn University. He continued his graduated study for a master's degree of science in zoology at the same institute in 1994. He was awarded a two-year scholarship by the University Development Committee(UDC), Ministry of University Affairs in 1996. After his graduation, he works as a full-time lecturer at the Department of Biology, Faculty of Science, Chulalongkorn University.