# Seasonal Reproduction and Sexual Size Dimorphism of the African Helmeted Turtle, *Pelomedusa subrufa* (Family Pelomedusidae)

by

Aliki V. Strydom

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Supervisor: Professor J. H. van Wyk

Co-supervisor: Doctor A. J. Leslie

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*Declaration*: I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

*Signature*:

*Date*:

# - ABSTRACT -

*PELOMEDUSA SUBRUFA* is a freshwater turtle widely distributed throughout Africa and Madagascar, and is described as a Tropical to Sub-tropical species. I examined the female and male reproductive cycles of *P. subrufa*, over a 20-month period to determine whether they display a typical Tropical to Sub-tropical type reproductive cycle (pre-nuptial) or a typical Temperate Zone type reproductive cycle (post-nuptial). Blood and tissue samples were collected from wild specimens captured in the Western Cape, South Africa and these samples were supplemented by tissue samples obtained from museum specimens.

In female *P. subrufa* seasonal variation in related circulating reproductive hormones in the plasma (estradiol, progesterone, and testosterone) were analyzed using validated ELISA kits. Plasma vitellogenin (yolk precursor produced in liver) was measured using a newly developed universal vitellogenin ELISA for vertebrates (UNIVTG). Ovarian follicles were measured (± 0.1 mm) and female ovaries were staged macroscopically (non-active, pre-vitellogenic, vitellogenic, gravid), and results were confirmed via histological sectioning of ovaries and oviducts.

Females exhibited a cyclic reproductive pattern, with distinct phases of follicular enlargement (vitellogenesis), ovulation and a gravid period. Seasonal timing of the reproductive cycle coincided with those of other temperate zone freshwater turtles. Vitellogenic recrudescence began in summer (late December), and continued unabated through winter with ovulation occurring in the following spring (September-October). My data suggested that *P. subrufa* females mostly lay a single clutch of eggs during the late-spring summer period (September through January). Clutch size varied between 7 - 37 eggs, with the number of eggs being significantly correlated with maternal body size ($r = 0.82$, $P < 0.001$). Plasma estradiol and plasma vitellogenin concentrations peaked once during the ovarian cycle, typically

coinciding with the period of early- to mid-vitellogenesis in late summer. Plasma testosterone varied throughout the year, but significant increases were measured during the ovulation and mating period in spring. Plasma progesterone concentrations were significantly elevated during the gestation period prior to ovi-position in mid-summer (December).

In male *P. subrufa* spermatogenesis in mature specimens was distinctly seasonal and timing of the reproductive cycle coincided with those of other temperate zone freshwater turtles. Spermatogenic recrudescence began in summer, following emergence from a winter hibernation period (brumation) and spring mating. Peak testicular volume and maximum spermiogenic activity occurred in late summer and early autumn. Testicular regression commenced in autumn through winter. Spermatozoa were abundant in the ducti epididymi throughout the year. Plasma testosterone concentrations peaked once during the testicular cycle, typically coinciding with spermiogenesis in late summer, early autumn. Ducti epididymi diameter showed significant variation throughout the year, whereas the epithelial cell height showed no significant seasonal variation. Peak secretory activity coincided with spermiogenic activity and high circulating testosterone concentrations in late summer, early autumn. Testicular recrudescence was correlated with increasing ambient air temperatures, photoperiod and summer rainfall, whereas testicular regression, during late autumn, corresponded conversely with decreasing ambient air temperatures, photoperiod and rainfall. Female and male reproductive cycles were asynchronous in that the peak spermatogenic activity occurred in autumn at the time when most females were depositing yolk in growing ovarian follicles. Therefore, adult females displayed a typical post-nuptial vitellogemic cycle and adult males displayed a typical post-nuptial spermatogenic cycle.

Differences between sexes in body size are common in many animals, and the African helmeted turtle is no exception. Sexual size dimorphism (SSD) in *P. subrufa* was pronounced,

and using principal component analysis, it was clear that adult male *P. subrufa* was significantly larger than adult females. Using carapace length as the measure of body size (covariate), adult males, adult females, and juveniles differed significantly in absolute size of the carapace width, carapace depth, plastron length, plastron width, and head depth. However, there was no significant difference between adult males, adult females and juveniles in head width and head length. Therefore, adult males were larger than adult females in the seven traits measured, except in carapace depth where the females were significantly larger. In the occurrence of ontogenetic growth patterns, the adults grow at a slower rate than juveniles in plastron length. There was no significant difference between adults and juveniles in shell width, however in depth, the adults grow at a faster rate when compared to the juveniles. Adults significantly grow at a faster rate than juveniles in absolute head size as well. However, when these traits were used as a whole data set (eight traits measured), there was no difference in growth rate between adults of either sex. Similarly, there was no significant difference in adults compared to juveniles in shell size, however, adults grow at a faster rate than juveniles in absolute body size and head size. Differences in body size, and in the size of traits such as shell measurements and head measurements relative to absolute body size, were assessed to clarify SSD of *P. subrufa* in South Africa.

# - UITTREKSEL -

*PELOMEDUSA SUBRUFA* is 'n varswaterskilpad wat wyd verspreid oor Afrika en Madagascar voorkom en word beskryf as 'n Tropiese tot Sub-tropiese spesies. Die manlike en vroulike voortplantingspatroon van *P. subrufa* is oor 'n tydperk van 20 maande bestudeer om vas te stel of hul voortplanting ooreenstem met 'n tipiese tropiesie tot sub-tropiese voortplantingspatroon of 'n tipiese gematigde-sone voortplantingspatroon. Waterskilpaaie is uit damme in die Wes-Kaap, Suid-Afrika gevang en bloed- en weefselmonsters is versamel. Materiaal en data is aangevul deur weefselmonsters van waterskilpaaie wat in museumversamelings gehuisves word.

Ovarium follikels in *P. subrufa* wyfies is gemeet en die wyfies se ovariums is makroskopies gegradeer (onaktief, pre-vitellogenies, vitellogenies, dragtig) en resultate is deur histologiese snitte van die ovaria en ovidukte bevestig. Wyfies vertoon 'n sikliese voortplantingspatroon, met duidelike fases van follikulêre groei (vetllogenese), ovulasie en dragtigheid. Sirkulerende voortplantingshormone in die bloedplasma (estradiol, progesteroon en testosteroon) is ook geanaliseer met behulp van gevalideerde hormoonspesifieke ELISA bepalings. Plasma vitellogeen ('n dooiervoorloper wat in die lewer vervaardig word) konsentrasies is ook bepaal met 'n nuut ontwikkelde, universele (spesifiek vir werweldiere) vitellogeen ELISA (UNIVTG). Seisoenale tydsberekening van die voortplantingsiklus het ooreengestem met dié van ander varswaterskilpaaie vanuit die Gemagtigde-sone. Vitellogenese het in die somer begin en duur voort deur die grootste gedeelte van die somer, herfs en winter gevolg deur ovulasie in die daaropvolgende lente (September – Oktober). Die data ingewin stel voor dat *P. subrufa* wyfies meestal een broeisel eiers tydens laat lente-somer lê (September tot Januarie). Broeiselgrootte het gewissel tussen 7-37 eiers, met die hoeveelheid eiers wat

beduidend met moederlike liggaamsgrootte gekorreleer was ($r = 0.82$, $P < 0.001$). Plasma estradiol en vitellogeen konsentrasies het een keer tydens die ovariumsiklus gepiek, en gewoonlik saamgeval met vroeë tot middel vitellogenese in die laat somer. Plasma testosteroon het dwarsdeur die jaar gevarieër, maar beduidende toenames is gemeet tydens ovulasie en die paartydperk in die lente. Plasma progesteroon konsentrasies was beduidend hoër tydens dragtigheid kort voor eierlegging in die middel van die somer (Desember).

In volwasse *P. subrufa* mannetjies was spermatogenese sterk seisoenaal en het die voortplantingsiklus ooreengestem met dié van ander varswaterskilpadspesies wat in die gematigde streke voorkom. Na 'n oorwinteringsperiode (brumasie), volg die paringstydperk gedurende die lente. 'n Nuwe spermatogeniese siklus het in die somer begin. Maksimale spermatogeniese aktiwiteit en testis-volume word in die laat somer en vroeë herfs bereik. Testikulêre regressie neem in aanvang in die herfs en duur voort tot na paringstyd in die lente. Tydens testikulêre regressie word spermatosoë in die ducti epididymi gestoor. Plasma testosteroon konsentrasies het in die laat somer en vroeë herfs gedurende die testikulêre siklus, spermiogenese (sperm produksie fase), gepiek. Die grootte (omtrek) van die ducti epididymi het beduidende variasie dwarsdeur die jaar getoon, terwyl epiteel selhoogtes geen beduidende seisoenale variasie getoon het nie. Piek sekretoriese aktiwiteit het saamgeval met spermiogeniese aktiwiteit en hoë vlakke van sirkulerende testosteroon tydens laat somer en vroeë herfs. Testikulêre groei het goed gekorreleer met toenemende omgewingstemperatuur, fotoperiode en reënval, terwyl testikulêre regressie in herfs met 'n daling in omgewingstemperature, fotoperiode en reënval gekorrespondeer het. Die vroulike en manlike voortplantingspatrone was nie goed ge-sinkroniseerd nie, deurdat piek spermatogeniese aktiwiteit tydens herfs voorgekom het, gedurende die tyd waarin meeste wyfies besig was om

dooier in groeiende ovarium follikels neer te lê. Daarteenoor vertoon die mannetjies testikulêre regressie tydens die pre-ovulatoriese fase en ovulasie periode van die wyfies. Dus toon volwasse wyfies 'n tipiese gematigde sone vitellogeniese patron en volwasse mannetjies 'n tipiese gematigde sone spermatogeniese patroon.

'n Verskil in liggaamsgrootte tussen die geslagte is 'n algemene verskynsel by baie diere en *P. subrufa* is geen uitsondering nie. Daar was wesenlike geslagtelike grootteverskille (SSD) in *P. subrufa* en 'n hoofkomponent analise (PCA) het getoon dat daar beduidende morfometriese verskille tussen volwasse mannetjies en wyfies was. Deur karapakslengte as 'n maatstaf vir liggaamsgrootte te gebruik (mede-veranderlike), het volwasse mannetjies, volwasse wyfies en onvolwassenes beduidend verskil ten opsigte van absolute grootte van hul karapaksbreedte, karapaksdiepte, plastronlengte, plastronbreedte en kopdiepte. Geen beduidende verskil in kopbreedte en koplengte in volwasse mannetjies, volwasse wyfies en onvolwassenes is gevind nie. Derhalwe was volwasse mannetjies groter as volwasse wyfies in sewe van die liggaamseienskappe wat gemeet is, buiten vir karapaksdiepte waar die wyfies beduidend groter was. In terme van die voorkoms van ontogenetiese groeipatrone het volwassenes teen 'n stadiger tempo as onvolwassenes in plastronlengte toegeneem. Daar was geen beduidende verskil in die groeitempo van dopbreedte tussen volwassenes en onvolwassenes nie, alhoewel dopdiepte van volwassenes teen 'n vinniger tempo gegroei het as dié van onvolwassenes. Absolute kopgrootte van volwassenes het ook teen 'n vinniger tempo gegroei as in onvolwassenes. Wanneer hierdie eienskappe as 'n volledige datastel gebruik word (al agt gemete eienskappe), wil dit voorkom asof daar geen verskil in groeitempo van volwassenes van die onderskeidelike geslagte is nie. Daar was geen beduidende verskil tussen volwassenes en onvolwassenes, ten opsigte van dopgrootte nie, alhoewel volwassenes klaarblyklik teen 'n vinniger tempo in liggaamsgrootte en

kopgrootte toeneem. Verskille in liggaamsgrootte en grootte van ander veranderlikes, soos byvoorbeeld dop- en kop-eienskappe, relatief tot absolute liggaamsgrootte, word aangebied om geslagsdimorfisme in *P. subrufa* vir die eerste keer te beskryf.

*Dedication*: To my family, THE STRYDOMS, for their support, patience and perseverance.

*"God is the only comfort, He is also the supreme terror: the thing we most need, and the thing we most want to hide from."* *C. S. Lewis*

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# - PREFACE -

POPULATIONS of tortoises, terrapins and turtles are experiencing a variety of survival pressures worldwide. *Pelomedusa subrufa* is currently not considered a threatened species, but due to an increasing human population, destruction and/or habitat alterations, and road casualties, this species may be in danger of premature extinction. Apart form anecdotal notes on ecology and reproduction, no detailed physiological information is available for this species. In particular, data is lacking on the dynamics of gonadal cycles, as well as seasonal timing and endocrine control of reproductive activity. Conservation management of this key member in freshwater ecosystems is therefore impossible. Initiating and continuing basic biological and conservation research becomes vital, when formulating management plans needed for a wild population to thrive and survive.

In addition, the aquatic lifestyle of *P. subrufa* renders the species suitable as a bio-indicator in the monitoring of environmental health, specifically the effect of water quality on reproductive output. Several features of the reproductive physiology of freshwater turtles have the potential to be employed as biomarkers, for example, sexual dimorphism, hormonal concentrations and gonadal differentiation. Academically this study will contribute to the enrichment of our scientific knowledge of the only living species of the genus *Pelomedusa*.

Therefore, my thesis is structured as follows: A General Introduction provides life-history and distribution information on the family Pelomedusidae as well as a brief description of *P. subrufa*. Chapter 1 is an overview of the basic reproductive cycles found in reptiles in general and then specifically in freshwater turtles. This chapter also indicates aspects of the timing, control, and synchronization of these reproductive cycles. In chapters 2 and 3 I present extensive information about the female and male reproductive cycles of *P. subrufa* in their natural habitat,

in a Temperate Zone region. In chapter 4, baseline information is provided on sexual size dimorphism in *P. subrufa* occurring in South Africa.

# - GENERAL INTRODUCTION -

## Life History, Distribution, and General Description of *Pelomedusa subrufa*

TURTLES first appeared in the Late Triassic. The living forms belong to a pair of lineages that split in the early Cretaceous. The side-necked turtles (Suborder*: Pleurodira*) are one of the two major groups of living turtles (the other group, Suborder*: Cryptodira*) (Zug, 1993; Kardong, 1995). Pleurodires are defined by the lateral plane of retraction of the neck, with correlated muscular development and lateral processes on the cervical vertebrae (Pritchard, 1979). The pelvis is fused to both carapace and plastron (Pritchard, 1979; Hofstra, 1995), and the jaw closing muscles operate over a process of the pterygoid (Gaffney, 1975). Cryptodires have a vertical retraction movement (Kardong, 1995). Pleurodires live exclusively in the Southern Hemisphere, i.e. Australia, Africa, Madagascar and South America, and are confined to freshwater habitats (Iverson, 1992; Zug, 1993). Within the living forms, two families have historically been recognized, the Chelidae (10 genera and 35+ species) only found in Australia / New Guinea and South America, and the Pelomedusidae (5 genera and 25+ species) found only in Tropical and Subtropical South America, Africa and Madagascar (Zug, 1993; Kardong, 1995). Of the family Pelomedusidae there are two genera in South America (*Peltocephalus* and *Podocnemis*), two in Africa (*Pelomedusa* and *Pelusios*) and three in Madagascar (*Erymnochelys*, and the two genera of Africa) (Zug, 1993). However, due to recent molecular, morphological and paleontological studies (Shaffer *et. al.*, 1997) suborder Pleurodira has been divided into three families: (1) Pelomedusidae (2 genera and 17 species) occurring in subSaharan Africa, Madagascar and other Indian islands, (2) Chelidae (10 genera and 40 species) found in Australia

and South America and (3) Podocnemidae (2 genera and 7 species) found in tropical South America and there is one monotypic genus in Madagascar. Podocnemidae and Pelomedusidae were originally considered as subfamilies: Podocneminae and Pelomedusinae (Kuchling, 1999). The Pelomedusidae fossil record extends back to the Upper Cretaceous of North America and Europe, thus indicating that they probably evolved in the Northern Hemisphere and were formerly more widespread (Ernst and Barbour, 1989).

The African genus, *Pelomedusa* (Wagler, 1830), is composed of only one species, *Pelomedusa subrufa* (Lacépède, 1788) better known as the African helmeted or marsh turtle (Ernst and Barbour, 1989; Branch, 1998). This species is medium-sized (> 20 cm), with a poorly ossified shell, a large midplastral fontanelle that persists until late in life, as well as a small, widely separated mesoplastra, and a fully emarginate skull roof (Pritchard, 1979). The carapace is olive to brown, hard, flat and thin, with no nuchal scute (Zug, 1993; Hofstra, 1995). The plastron has no plastral hinge (Alderton, 1993), the hind feet have a webbed fringe, and both the fore- and hindfeet possesses five toenails (Ernst and Barbour, 1989; Hofstra, 1995; Branch, 1998). Adult males have concave plastra and long, thick tails, whereas females have flat plastra and short tails (Ernst and Barbour, 1989; Branch, 1998). Both male and female have two small tentacles (of unknown function) beneath the chin, and musk glands beneath the fourth to eighth marginals (Loveridge, 1941; Branch, 1998).

This turtle species was originally known as *Testudo subrufa* (Iverson, 1992). There are three subspecies: 1) *P. s. subrufa* - Lacépède (1788) (Common African Helmeted Turtle), which ranges from Somali and Sudan, west to Ghana and south to the Cape of Africa and on Madagascar;

2) *P. s. olivacea* – Schweigger (1812) (North African Helmeted Turtle), which ranges from Ethiopia, west to Senegal, Nigeria and Cameroon, to Saudi Arabia and Yemen; and

3) *P. s. nigra* – Gray (1863) (Black Helmeted Turtle), which ranges from Natal to the Free State and east of the Cape Province to the line joining Kuruman, Kimberley, Graaf Reinet, and Grahamstown, in South Africa (Loveridge, 1941; Pritchard, 1979; Bour, 1986; Ernst and Barbour, 1989).

*Pelomedusa subrufa* is described as a tropical to sub-tropical species (Branch, 1998), and occurs in a variety of freshwater habitats, e.g. seasonal ponds, shallow and temporary water bodies or in marshes, and rivers (Ernst and Barbour, 1989; Zug, 1993; Alderton, 1997; Branch, 1998). They aestivate, when these temporary ponds dry up (the exact time of year for this aestivation is uncertain), sometimes far from their original pond (Ernst and Barbour, 1989; Branch, 1998). South African species are known to hibernate during the winter months (Loveridge, 1941). *Pelomedusa subrufa* starts migrating to the nearest pond after good rains, and many are killed on roads (Branch, 1998). They are known to bask on warm days in Subtropical and warmer Temperate areas, but apparently the sun is too hot for them to bask in the more Tropical areas (Ernst and Barbour, 1989). They have been found up to an altitude of 6 000 feet (3 100 m) (Pritchard, 1979). They are poor swimmers and instead depend on bottom walking (Zug, 1993). They feed opportunistically on invertebrates and vertebrates and appear to be almost entirely carnivorous (Loveridge, 1941; Pritchard, 1979; Ernst and Barbour, 1989; Zug, 1993), however Branch (1998) refers to this species as being omnivorous.

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# - CHAPTER 1 -

## Turtle Reproductive Cycles: An Overview

### PATTERNS OF REPRODUCTION

KNOWLEDGE of reproductive patterns is basic to many studies concerning evolution and ecology (Moll, 1979). A wide variety of reproductive patterns and controls exist both within and among species of fish, amphibia and reptiles (Whittier and Crews, 1987). Reptiles display three fundamental reproductive patterns, based on the degree of variation in gonadal activity throughout the year: (1) continuous or acyclic reproduction (aseasonal) with roughly similar levels of gonadal activity in all months, (2) continuous or cyclic reproduction (aseasonal), but with gonadal activity fluctuating across months, and (3) discontinuous or seasonal reproduction, where periods of gonadal activity alternate with periods of gonadal quiescence (Licht, 1984; Callard and Kleis, 1987). Breeding cycles consists of a series of events – spermatogenesis in the male, maturing of follicles and ovulation in the female, the coming in contact of sperm and oocytes, fertilization, embryonic development and birth / hatching (Van Tienhoven, 1968). Tropical species have traditionally been considered to be reproductively acyclic (Whittier and Crews, 1987) however, according to Kuchling (1999) acyclic reproduction as described by Licht (1984) has not been demonstrated in any reptilian species. In most reptile species investigated, reproduction is seasonal and generally only one ovarian cycle is observed each year. There are however exceptions to the pattern of only one ovarian cycle per year (Van Tienhoven, 1968). Chelonians on the other hand have either continuous reproduction (aseasonal) or discontinuous reproduction (seasonal) (Kuchling, 1999). Seasonal reproductive cycles are not necessarily synonymous with annual reproductive cycles, i.e. an individual that breeds in spring every two to three years has a seasonal cycle, but not an annual one (Whittier and Crews, 1987), for example

*Erymnochelys madagascariensis* has a biennial ovarian cycle (Kuchling, 1993) and *Sphenondon punctatus* has an ovarian cycle of two to three years (Cree *et. al.*, 1992). In seasonal reproduction the reproductive processes are so timed that the young will be born when there are optimal conditions for their development. Thus, time of ovulation, time of mating, length of incubation, etc. is synchronized rather accurately but still allow sufficient leeway for special conditions.

Two basic spermatogenic cycles exist in adult male turtles exhibiting discontinuous (i.e. seasonal) reproduction: pre- and post-nuptial cycles. In pre-nuptial cycles the onset of spermatogenesis (recrudescence) immediately precedes the mating period, with spermiogenesis coinciding with ovulation in females. Alternatively, in post-nuptial cycles recrudescence ensues after the mating season – sperm is produced well in advance of ovulation in females and is stored in the ducti epididymi or vas deferens in males (Lofts, 1977; St. Girons, 1982; Bradshaw, 1986; Van Wyk, 1995; Kuchling, 1999), or in the isthmus of the oviduct in females (Palmer and Guillette, 1988), until utilized. Winter and early spring months are the time of germinal quiescence, while recrudescence is confined to the warm summer months, culminating in peak spermiogenesis during autumn (Kuchling, 1999). Therefore, males in the temperate zone can produce mature sperm as much as half a year before they will be used to fertilize eggs. Furthermore the spermatogenic cycle begins as the ovarian cycle is winding down and vice versa. This is made possible by the ability of both sexes (as mentioned above) to store mature sperm over long periods (Moll, 1979), eg. female *Malaclemys terrapin* (Barney, 1922), *Terrapene carolina* (Ewing, 1943) and *Chelydra serpentina* (Smith, 1956) can store sperm for several years, and eggs may be fertilized four years after insemination.

According to Moll (1979) the female reproductive cycle can be divided into four phases: (1) follicular enlargement (vitellogenesis), (2) ovulation and intrauterine period (gestation), (3) nesting period (oviposition) and (4) latent period (germinal quiescence). Differences between reproductive patterns are mainly caused by the length of time between successive clutches and the number of follicles that reach maturity during each clutch interval (Licht, 1984; Callard and Kleis, 1987). Therefore, females exhibiting a discontinuous (i.e. seasonal) reproductive pattern also have two basic vitellogenic cycles: pre- and post-nuptial cycles. In pre-nuptial cycles the onset of vitellogenesis is characterized by rapid follicular growth immediately before ovulation, with the ovaries remaining relatively quiescent during most of the year. This phenomenon is usually well synchronized with the period of spermiation in males (as mentioned above). Alternatively, in post-nuptial cycles the onset of vitellogenesis (recrudescence) commences shortly after the breeding season and is characterized by an extended period of follicular growth (Licht, 1984). Therefore, vitellogenesis begins in the late summer or autumn months and after a possible break during winter hibernation (brumation), continues until completion in spring (Kuchling, 1999). In contrast to the pre-nuptial cycle, in species exhibiting post-nuptial spermatogenesis / vitellogenesis, male and female cycles are usually asynchronous. Counter intuitively, most temperate zone turtles exhibit a post-nuptial cycle (Lofts, 1987; Jameson, 1988).

Patterns of reproduction in animals have evolved to maximize an individual's contribution of genetic information to the next generation. Thus, the pattern of reproduction may be influenced by generation time, age to reproduction, life expectancy and age-specific mortality as well as by the predictability of the environment, ecological niche, and body shape and size. All patterns of reproduction evolve in some manner as to maximize the "benefits" of reproduction while minimizing its "costs". Not all adult members of a population breed every

year and there is evidence that absence of reproduction in some individuals during particular years is related to the energetic cost of reproduction, eg. female *Cordylus giganteus* (Van Wyk, 1991). The phenomenon is most often observed in females, where the energetic cost of vitellogenesis is great. Assuming high year-to-year survivorship, an individual that does not reproduce in a given year can divert energy into growth and may possibly produce a larger number of offspring the following year. However, an increase in clutch size would be offset by the loss of a complete, albeit smaller, clutch of the previous year (Whittier and Crews, 1987).

Bruce (1975) and Murphy (1968) have offered alternative evolutionary arguments for the occurrence of irregular nonannual breeding among some females. Bruce (1975) suggested that, in addition to energetic savings, selection might favour spreading reproduction over a number of years under certain conditions. Murphy (1968) has provided a mathematical model of these ideas in which it is assumed that females that skip reproduction live longer (see Hirshfield, 1980), that there is a long time to maturity and that mortality of young may be high or intermittent. The model predicts that a female producing young every few years over a long period of time will contribute more sexually reproducing offspring than a female that reproduces every year (Whittier and Crews, 1987).

## TIMING OF REPRODUCTION

Timing of reproduction in a population is determined by (1) when the most offspring survive and (2) when parents, most often females, are capable of energetically supporting the production of viable young at the least cost to themselves (Guillette and Mendez de la Cruz, 1993). Further, timing of reproduction not only reflects current conditions but also is influenced by factors regulating the total lifetime production of offspring. All organisms have to balance

the allocation of resources to growth, maintenance and reproduction (Kuchling, 1999). Optimal resource allocation theories generally predict that it is optimal to grow early in life and at a particular age or size, to stop growing and start reproducing at the maximum rate until the end of life (Koztowski, 1992). All chelonians keep growing throughout much of their life, although growth slows once individuals reach sexual maturity (Andrews, 1982). Therefore, they allocate resources simultaneously to growth and reproduction during much of their adult life (Kuchling, 1999).

*Sexual Maturity*

Onset of maturity in most chelonians correlates more with attainment of some minimum size than with age (Cagle, 1950; Ernst, 1971; Gibbons, 1968; Legler, 1960). Two wide-ranging species are apparent exceptions that mature at various sizes but at the same age throughout its range: *Sternotherus odoratus* (Tinkle, 1961; Gibbons, 1970), and *Chrysemys picta* (Christiansen and Moll, 1973; Moll, 1973). Both mature at smaller sizes in lower latitudes (Moll, 1979). In *Pseudemys scripta*, another wide-ranging species, size at maturity varies little within the temperate zone (Cagle, 1950), but Tropical populations mature at larger sizes (Moll and Legler, 1971). Males of many species mature smaller and often earlier than females (e.g. *Graptemys barbouri, Malaclemys terrapin, Melanochelys trijuga, Terrapene ornate, Trionyx muticus*) – advantages: reduced intersexual competition, rapid maturation of males and increased reproductive capacity of females. In other species both sexes mature at similar sizes (e.g. *Clemmys guttata, Chelydra serpentina, Kinosternon flavescens, Macroclemys temmincki, Rhinoclemys funereal*). In a few species, females may mature at the smaller size (e.g. *Gopherus agassizi, Kinoternon leucostomum, Sternotherus minor*) – latter two types' advantages:

uncertain, but known that males are more competitive and aggressive for females and space, therefore large size is advantageous (Gibbons *et. al.*, 1982).

The large *Chelonia mydas* (Hendrickson, 1958) and the small *Sternotherus carinatus* (Tinkle, 1958) may mature in 4 to 6 years, whereas the medium-sized desert tortoise (*Gopherus agassizii*) may require 15 to 20 years (Woodbury and Hardy, 1948). When considering separate populations, factors such as heredity, habitat productivity, and length of growing season may be more important determinants of maturation time than mere size (Moll, 1979).

*Associated versus Dissociated*

In most species that have been studied with respect to the environmental cues and physiological mechanisms influencing reproduction, three events: gonadal growth, sex steroids, hormone secretion and sexual behavior are functionally associated (Whittier and Crews, 1987). In lizards and snakes exhibiting a pre-nuptial spermatogenic cycle, a single plasma testosterone peak measured at the time of maximum spermiogenesis in summer is generally the rule (Lofts, 1987). However, many species of snakes and freshwater turtles do not fit this pattern and have a dissociated reproductive pattern in which gonadal activity and sexual behaviour (hormone secretion) peak at different times of the year. In species known to exhibit a post-nuptial spermatogenic cycle a single plasma testosterone peak is also seen, however plasma testosterone concentrations peak in autumn during the time of maximum spermiogenesis (Lofts and Tsui, 1977; Johnson *et. al.*, 1982; Licht *et. al.*, 1985). This is especially well seen in male squamates exhibiting a post-nuptial spermatogenic cycle (Crews, 1984). Although previous studies showed a single testosterone peak associated with spermatogenesis rather than mating in a typical post-nuptial spermatogenic cycle, studies on cordylid lizards indicate a bimodal testosterone cycle (Flemming, 1993; Van Wyk, 1995). In the lizard *C. giganteus*, a brief testosterone peak

characterized the mating period associated with female ovulation in spring. During this period males exhibit testicular regression but abundant spermatozoa in storage in the ducti epididymi and vas deferens (Van Wyk, 1995). However, the suggested "associated versus dissociated" reproductive tactics has not been established in any females exhibiting a post-nuptial vitellogenic cycle.

## CONTROL OF REPRODUCTION

Mechanisms that control seasonal reproduction may be viewed as lying on a continuum between two extremes (Whittier and Crews, 1987), the relative importance of which varies among cycle phases and species (Kuchling, 1999). At one extreme, pre-programmed or closed control mechanisms (endogenous), seasonal reproduction is entirely determined by endogenous cycles and is not influenced by external cues. At the other extreme of this continuum lies labile control (exogenous) in which seasonal reproduction occurs as a result of responses to environmental cues. In predictable environments, species usually display seasonal reproductive cycles, whereas in unpredictable environments aseasonal reproductive cycles are usually found. Therefore, in predictable environments individuals of a population that rely on pre-programmed control mechanisms may have an advantage, because they are ready to reproduce and need not rely on external influences to initiate reproduction. In less predictable environments, pre-programmed control may be disadvantageous if reproductive responses occur under inappropriate conditions. Labile control mechanisms, although they may retard a reproductive response until conditions are favourable, result in fewer risks in unpredictable environments. Many organisms exhibit intermediate control mechanisms between these two extremes, i.e. preparatory events leading to reproduction, including gonadal growth, can be influenced by endogenous factors, but final stages of maturation as well as reproductive behaviour can be

controlled by integration of specific cues (Whittier and Crews, 1987). Another intermediate stage of control is represented by the "hour glass" mechanism, in which external events initiate but have no further influence on an endogenously controlled cycle (Gwinner, 1971). Therefore, sexes are either stimulated by different factors or react to the same factors in an antipodal manner (Moll and Legler, 1971).

*Endogenous control*

The generation of a complete annual cycle through endogenous timing mechanisms, a complete circannual rhythm requires that internal factors alone be capable of both inducing and terminating a gonadal cycle at approximately yearly intervals. Such an endogenous periodicity should be expressed without any external influence, under constant environmental conditions that are permissive to reproductive activity. A complete circannual rhythm has not been demonstrated yet for any chelonian species (Kuchling, 1999).

There is an internal rhythm of reproduction that is primitive and depends primarily upon the alternation of periods of rest and activity as shown in nearly all animals. In correlation with this rhythm, hormones are periodically elaborated by the gonads and act upon the accessory organs and secondary sexual characters where these exist. But in the higher animals the internal rhythm is brought into special relation with seasonal changes and other external environmental phenomena, these not merely conditioning the metabolic processes but in part at any rate, acting exteroceptively through the nervous system and probably through the hypothalamus upon the anterior pituitary and thence upon the testis and ovary (Amoroso and Marshall, 1965). Androgens, for example, have been implicated in the control of spermatogenesis and mating activity in many chelonian species (Norris, 1987). In most chelonians exhibiting post-nuptial

cycles, mating behaviour occur during testicular quiescence, i.e. at a time when circulating androgens are expected to be low (Whittier and Crews, 1987).

In *Testudo hermanni* for example, spermatogenesis is primarily under temperature control however, spermatocytogenesis also commenced spontaneously (although at a very slow rate) if hibernation (brumation) conditions were extended until June. The termination of spermatogenesis also required an endogenous readiness of the hypothalamus-pituitary-gonad system to react upon low temperature: from June to August a constant temperature of 5 °C and constant darkness did not induce regression and spermatocytogenesis continued strongly, whereas the same treatment during September and November caused immediate regression of the testes (Kuchling, 1982).

In any case an endogenous component seems to be involved in testicular regression of chelonians. In *Chrysemys picta* females (Ganzhorn and Licht, 1983), seasonal differences in responsiveness of the ovaries to environmental stimuli are also partly related to some endogenous cycle in their sensitivity to exogenous factors. All these experimental results with temperate zone chelonians suggest the possibility, but do not prove that an endogenous circannual rhythm or a circannual clock may underlie chelonian gonadal cycles. However, during the quiescence phase in *Lissemys punctata*, neither ovarian growth nor steroidogenesis can be stimulated by various photothermal treatments (Sarkar *et. al.*, 1996).

Age / size is another endogenous factor. Older males may begin spermatogenesis earlier and continue it longer in the year than younger / smaller males (Moll and Legler, 1971; White and Murphy, 1973). Females parallel this situation in that older / larger individuals nest earlier and in species laying multiple clutches, continue nesting later than younger / smaller members of the population (Moll, 1979).

*Exogenous control*

A variety of environmental factors such as temperature, photoperiod, rainfall, moisture, humidity and food supply, as well as sexual behaviour and social interactions may act as proximate cues to regulate reproduction in reptiles (Duvall *et. al.*, 1982). Environmental factors may directly trigger physiological processes, they may be permissive factors for physiological processes and for the expression of endogenous circannual rhythms, or they may be external *zeitgebers* to synchronize circannual rhythms with the time of the year (for review see Kuchling, 1999).

## SYNCHRONIZATION OF REPRODUCTION

Seasonal patterns of reproduction in a population of animals are a result of a synchronous response of individual's physiological mechanisms influencing reproduction. Synchrony in a population may result from endogenous circannual controls initiated at birth or puberty or perhaps more commonly, from the influence of exogenous factors on reproductive controlling mechanisms (Whittier and Crews, 1987). However, male and female gonadal cycles of chelonians exhibiting a post-nuptial reproductive cycle are generally not synchronized (as mentioned above). In temperate zone species exhibiting a post-nuptial reproductive cycle, spermatogenesis starts in spring and spermiogenesis and spermiation reach a peak in the summer or early autumn months before a period of germinal quiescence occurs from late autumn to early spring months. Temperate zone females on the other hand, typically show a more or less pronounced period of ovarian quiescence during summer, with vitellogenesis starting in late summer or autumn and progressing (with a break during brumation) until spring, when ovulation and oviposition occur (Kuchling, 1999).

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# - CHAPTER 2 -

## Female Reproductive Cycle of the African Helmeted Turtle,

## *Pelomedusa subrufa*

### INTRODUCTION

STUDIES on reproductive patterns and their hormonal control in animals is central to our understanding of life history patterns and necessary for structuring proper management strategies for our aquatic and terrestrial ecosystems. The lack of basic data or bias towards a small core of well studied species regarding reproduction, its endocrine regulation and seasonal variation thereof, will limit our understanding of life history variations and possible effects of environmental change (Janssen, *et. al.*, 1995; Norris, 1996). Willingham and Crews (2000) pointed out that one problem facing researchers in the field of endocrine disruption is finding model animals for *in vivo* studies of the effects of endocrine-disrupting contaminants (EDC's). They listed several reasons why freshwater turtles are suitable models for such studies. It is however clear, that before implementing southern African turtles as models in environmental assessment studies, the acquisition of a basic knowledge regarding the seasonal and annual variations of reproductive cycles of local populations is a prerequisite (Mitchell, 1985).

There is a wide variety of reproductive traits in the class Reptilia, ranging from oviparity and viviparity to parthenogenesis (Kuchling, 1999). Differences between reproductive patterns are primarily due to length of time between successive clutches and the number of follicles that reach maturity during each clutch interval. These patterns can be classified as seasonal (discontinuous) breeding or aseasonal (continuous) breeding. Seasonal breeding patterns are characterized by periods of reproductive (gonadal) activity alternating with periods of quiescence

(Licht, 1984; Callard and Kleis, 1987) and with aseasonal breeding there is continuous reproductive activity throughout the year (Callard and Kleis, 1987). Licht (1984) divides continuous breeding into two categories: (1) breeding throughout the year with reproductive activity being similar in all months (acyclic), and (2) breeding throughout the year, but with variable levels of reproductive activity (cyclic). Kuchling (1999) suggested that based on available data all chelonians fit either the continuous cyclic reproductive pattern, or the seasonal (discontinuous) reproductive pattern.

The mechanisms that control seasonal reproduction are described by Whittier and Crews (1987) as lying on a continuum between two extremes: pre-programmed or closed control (endogenous cycles) versus labile or open control (exogenous cues). The relative importance of each varies among cycle phases and species (Kuchling, 1999). Exogenous factors that affect the reproduction of turtles include photoperiod, temperature, rainfall, humidity, food availability, sexual behaviour and social interaction, while endogenous factors include the hypothalamus-pituitary-gonad axis, reproductive steroid hormones, age and body size (Moll, 1979; Duvall *et. al.*, 1982; Kuchling, 1999). Although most field studies attempt to correlate gonadal changes with climatic variables, these descriptive studies provide little insight into the physiological mechanisms that synchronize chelonian reproductive cycles (Kuchling, 1999). The effect of handling stress has an additional impact on chelonian reproduction. Observations on stressed captive *Chelydra serpentina* suggest that the response of reproductive processes to stress varies in intensity according to the state of the ovarian cycle and the condition of the gonads. It is easier to block gonadal growth at the start of a new gonadal cycle than to inhibit or interrupt vitellogenesis once it has begun (Mahmoud and Licht, 1997; Kuchling, 1999). Kuchling (1999) therefore suggests that once a new gonadal cycle has begun, stress effects on reproduction are

less pronounced in species with primarily pre-programmed control mechanisms than in species with labile control mechanisms.

The reproductive biology of female turtles has been well documented. Seasonal changes occurring in gonadal structures have been investigated in genera like *Chelydra* (Lewis *et. al.*, 1979; Congdon *et. al.*, 1987), *Chrysemys* (Powell, 1967; Gibbons, 1968; Ernst, 1971; Christiansen and Moll, 1973; Gist *et. al.*, 1990), *Clemmys* (Powell, 1967), *Kinosternon* (Christiansen and Dunham, 1972; Mahmoud and Klicka, 1972; Mendonça and Licht, 1986; Iverson, 1999), *Macroclemys* (Dobie, 1971), *Pseudemys* (Cagle, 1944; Moll and Legler, 1971), *Sternotherus* (Mahmoud and Klicka, 1972; Mendonça and Licht, 1986; Etchberger and Ehrhart, 1987), and *Terrapene* (Altland, 1951; Legler, 1960). However, the majority of these species are Northern hemisphere turtles, leaving the Southern hemisphere species mostly unstudied.

According to Moll (1979), the female reproductive cycle can be divided into four phases: (1) follicular enlargement (vitellogenesis), (2) ovulation and intra-uterine period, (3) the nesting period, and (4) a latent period (germinal quiescence). In reptiles exhibiting seasonal vitellogenesis, ovarian cycles conform to two basic types. (a) In pre-nuptial cycles, the onset of vitellogenesis is characterized by rapid follicular growth immediately before ovulation, with the ovaries remaining relatively quiescent during most of the year. This usually coincides with the period of spermiation in males. (b) Alternatively, in post-nuptial cycles the onset of vitellogenesis (recrudescence) commences shortly after the breeding season, and is characterized by an extended period of follicular growth (Licht, 1984). Therefore, vitellogenesis begins in summer or early autumn and after a possible break during brumation, continues until completion in spring. Depending on the geographic location, age and species, vitellogenesis may be completed either before or after brumation (Kuchling, 1999).

The role of reproductive steroid hormones during the female reproductive cycle varies among species. In *Chelydra serpentina*, plasma progesterone concentrations peak during the luteal period whereas plasma estrogen concentrations peak immediately before ovulation (Lewis *et al.*, 1979). In *Geochelone nigra* plasma estradiol concentrations are highest at the peak of the mating season, plasma progesterone concentrations peak at the beginning of the nesting season and plasma testosterone concentrations peak during the second half of the mating season (Schramm *et. al.*, 1999).

Despite all the studies that have been conducted on freshwater turtles, female reproductive cycles in members of the family Pelomedusidae remains poorly known when compared to that of other turtle families from the Northern hemisphere. In particular, data regarding the dynamics of gonadal cycles as well as endocrine control of reproductive activity are lacking. *Pelomedusa subrufa* is widespread throughout Africa and Madagascar where it inhabits inland freshwater bodies (Loveridge, 1941; Jaques, 1966; Pritchard, 1979; Ernst and Barbour, 1989; Iverson, 1992; Zug, 1993; Hofstra, 1995; Branch, 1998; Van Wyk, 1998). This species is indigenous to Africa and is described as a Tropical to Sub-tropical species (Branch, 1998), yet its range extends into the warm temperate zone at the most southern tip of Africa, where summer rainfall is replaced with winter rainfall. Although Kuchling (1999) suggests a general evolutionary conservatism regarding the phenomenon of post-nuptial vitellogenesis in turtles, more studies are needed to test this generalization, specifically studies from the temperate Southern hemisphere. Moreover, the correlation between steroid hormones and the reproductive cycle is unknown. The fact that *P. subrufa* inhabits not only freshwater bodies in pristine areas, but also in intense farming areas, industrial areas, sewage discharge areas and areas contaminated with organochlorine pesticides (e.g. DDT through attempting to combat the

malaria epidemic), makes it an ideal aquatic bio-indicator species to study the impact of EDC's in ecosystem degradation (Willingham and Crews, 2000).

The objective of this study was to study changes in histology and circulating hormones during the natural reproductive cycle of female *P. subrufa*. I address the general lack of knowledge regarding reproductive biology of Southern hemisphere freshwater turtles and also evaluate the conservatism of post-nuptial vitellogenesis in freshwater turtles (Kuchling, 1999). The question about synchronization between male and female steroid hormone cycles controlling mating behaviour in reptiles exhibiting post-nuptial reproductive cycles (Whittier and Crews, 1987) is also addressed.

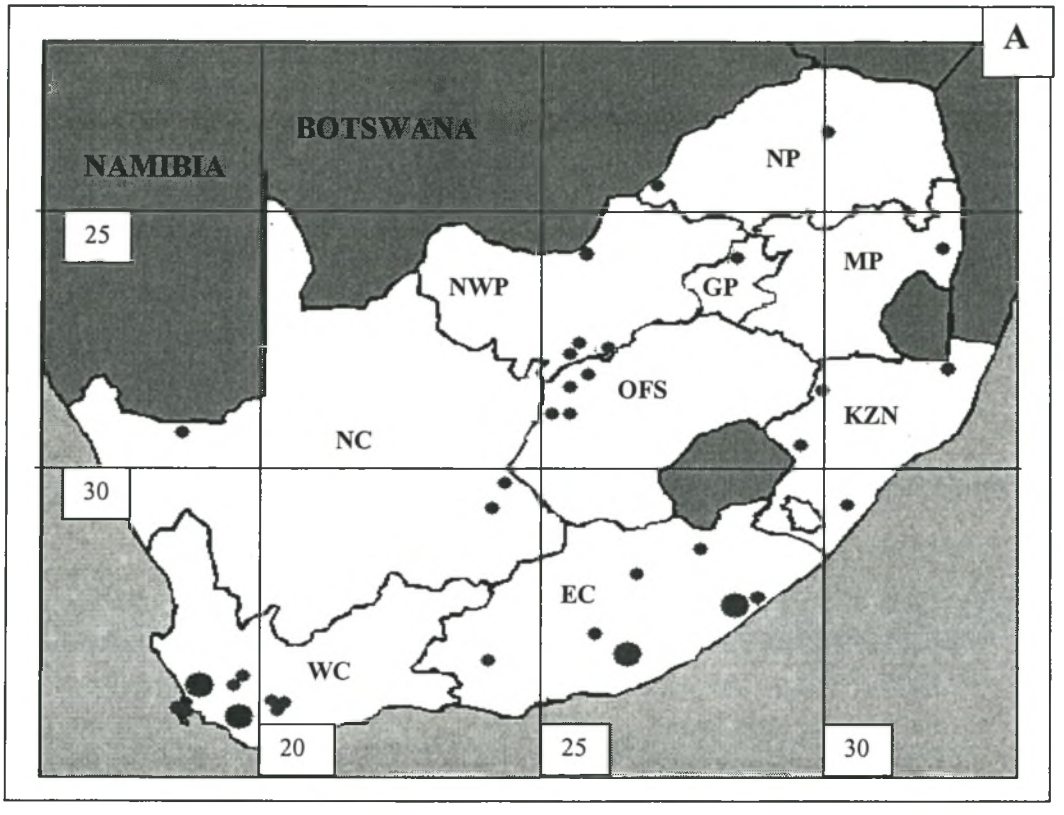
## MATERIALS AND METHODS

### *Study Areas and Climate*

Live turtles were trapped in farm dams in Klapmuts (33° 51' S; 18° 50' E; altitude 177 m) and Caledon (34° 12' S; 19° 21' E; altitude 128 m), Western Cape, South Africa. In addition, preserved specimens were obtained from the South African Museum in Cape Town (Western Cape), the National Museum in Bloemfontein (Orange Free State) and the Transvaal Museum in Pretoria (Gauteng), South Africa (Figure 1A) for subsequent dissection.

Meteorological data were obtained from weather stations at Elsenburg, 10 km from Klapmuts and at Boontjieskraal, 5 km from Caledon. The study areas are characterized by 600 mm and 400 mm of annual rainfall respectively, occurring mostly in winter months, with a distinct dry season during the summer months. Mean monthly maximum and minimum ambient air temperatures varied markedly between seasons. Photoperiod was calculated using a formula (Van Leeuwen, 1981, Figures 2A and 2B).

FIGURE 1. Geographic distribution of *P. subrufa* samples used for the categorizing of the reproductive stages in this study. (A) Female distribution of museum specimens (red circles, N = 37) and trapped specimens (blue circles, N = 14). (B) Male distribution of museum specimens (blue circles, N = 61) and trapped specimens (red circles, N = 20). Each small circle represents one sample, and larger circles represent two or more samples per collection site. In South Africa there are winter rainfall regions: Western Cape (WC), Eastern Cape (EC) and the southern part of the Northern Cape (NC), and summer rainfall regions: the northern part of the NC, Orange Free State (OFS), Kwa-Zulu Natal (KZN), Mapumalanga (MP), Gauteng (GP), North Western Province (NWP) and Northern Province (NP).



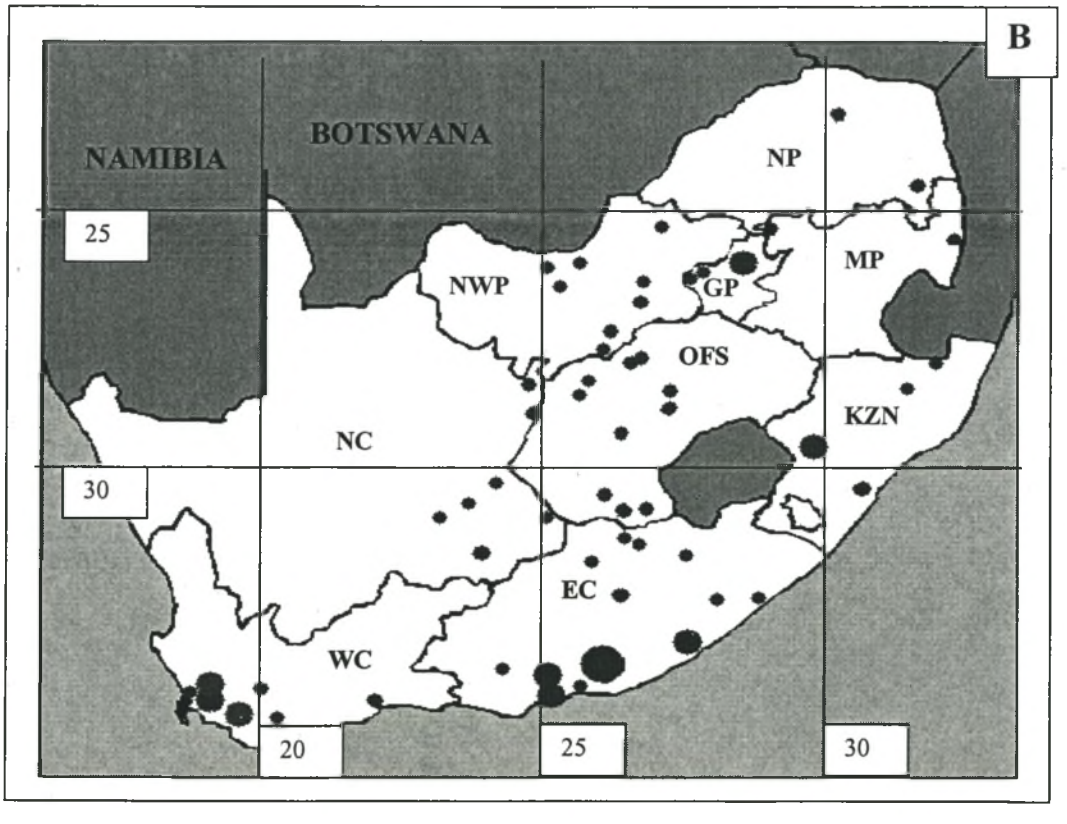
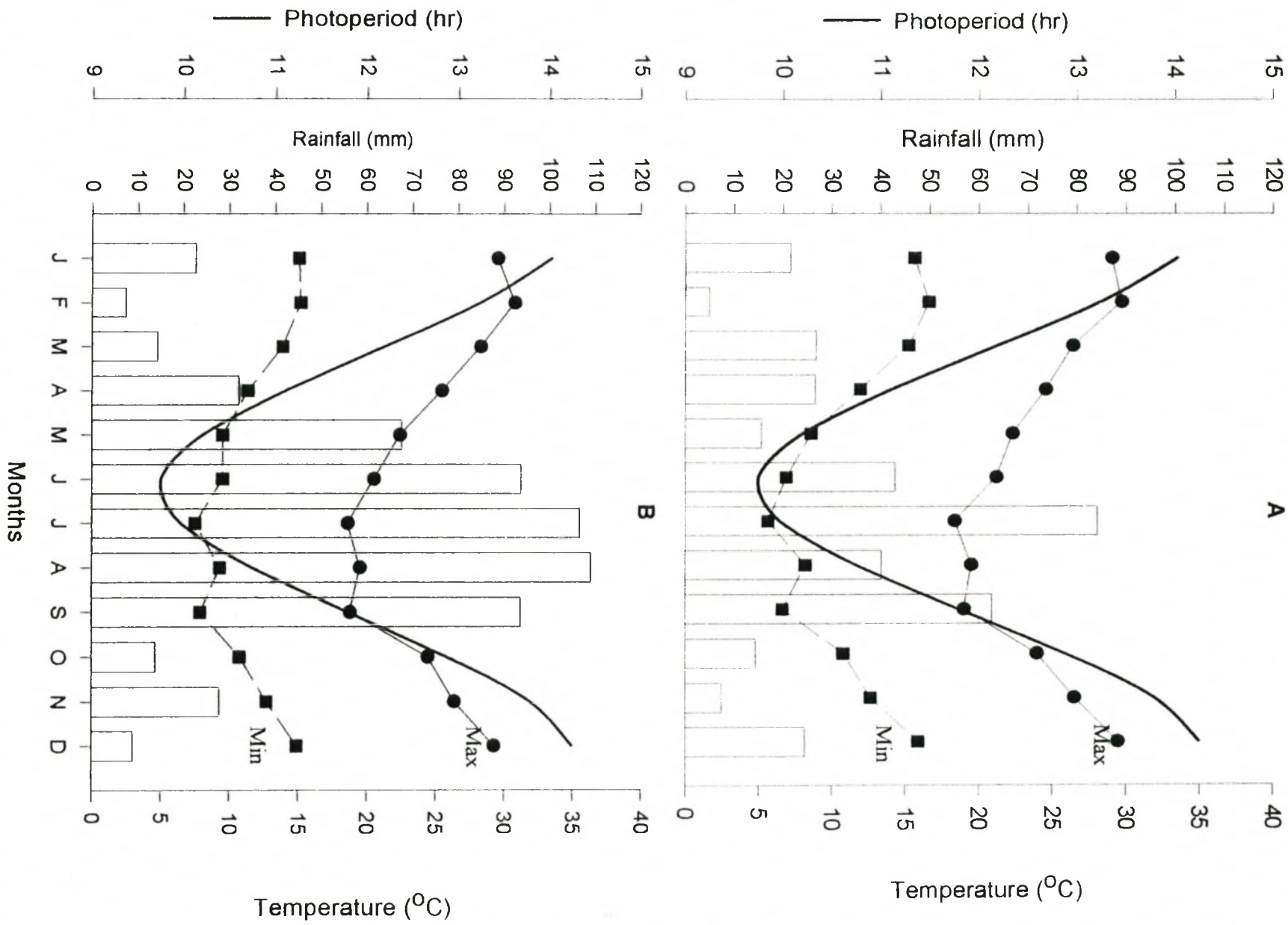



FIGURE 2. Total monthly rainfall (bar graph), maximum ambient air temperatures (closed circles), minimum ambient air temperatures (closed squares) and photoperiod (line), recorded at (A) Boontjieskraal (34° 12' S; 19° 21' E, altitude 127 m), 5 km from the Caledon study area and (B) at Elsenburg (33° 51' S; 18° 50' E, altitude 177 m), 10 km from the Klapmuts study area.



*Trapping and Blood Sampling Procedures*

Turtles were trapped from September 1999 through April 2001 at approximately bimonthly intervals. Turtle trapping was unsuccessful from April through August and I assumed that turtles were in brumation during this time period. Baited funnel hoop-traps were used, with bait ranging from pilchards, ox liver and pig heart, to fish guts (Legler, 1960; Iverson, 1979; Plummer, 1979; Kennett, 1992; Mansfield *et. al.*, 1998). Traps were checked twice weekly. Body mass was recorded using a portable Panasonic electronic balance (± 0.1 g). A small hole was drilled through the plastron (approximately in the area of the heart) using a cordless drill and 1.25 mm drill point. Blood samples were collected by heart puncture (Stephens and Creekmore, 1983; Jacobson and Schumacher, 1992) using a 2 ml heparinized syringe and a 1,5 inch, 23-gauge needle. The volume of blood drawn was dependent on body mass, i.e. 2 ml / kg (Stephens and Creekmore, 1983) and all blood samples were taken within a few minutes of initial handling. Blood samples were transported to the laboratory on ice, separated by centrifugation and stored at – 80 °C for later analysis of plasma estradiol (E), plasma progesterone (P), plasma testosterone (T) and plasma vitellogenin (VTG) concentrations. To investigate the possibility of post-capture effects on the aforementioned hormones (Licht *et. al.*, 1985; Lance and Elsey, 1986; Mahmoud *et. al.*, 1989) blood samples were again collected within a few days of capture in the laboratory from four turtles before they were sacrificed.

Carapace length of both museum specimens and captured specimens, were measured using calipers (± 0.1 mm). Only captured turtles were permanently marked by notching of the carapace, using the numbering system of Cagle (1939) and Honegger (1979). The hole drilled in the plastron for blood sampling was closed with pratley-putty before turtles were returned to the

site of capture. Two to three of the largest specimens were transported to the laboratory for autopsy.

*Plasma Hormone and Vitellogenin Analyses*

Plasma was mixed with diethyl ether after which the diethyl ether phase was collected, dried and extracted with 0.1% human serum albumin in phosphate buffered saline (pH = 7). Extraction efficiency was determined by adding known amounts of tritiated T to four random aliquots of plasma, extracting them together and determining the respective recoveries by scintillation counts against a 100% tritiated control. Extraction efficiency was 84%. Estradiol, P and T were then measured in duplicate using ELISA (enzyme-linked immunosorbant assay) kits (IBL, Hamburg, Germany), with reference to a standard curve based on standards prepared in human serum. Plasma vitellogenin was measured using a newly developed universal vitellogenin ELISA for vertebrates (UNIVTG) (Pool and Van Wyk, 2001).

In house validations of ELISA kits were conducted and adequate sensitivity, specificity and parallelism indicated that these specific hormone ELISA's were reliable for quantifying turtle steroid hormones. For intra- and inter-assay coefficients of variation for these assays see Table 1.

*Autopsy and Tissue Sampling*

Turtles were sacrificed by injecting lethal doses of sodium pentobarbitone (Eutha-naze, Ref. 83/91) directly into the heart. The reproductive organs (ovaries and oviducts) were excised and fixed in 70% ethanol (Humason, 1967). After dissection turtle carcasses were fixed in 4% buffered formalin for ten days, washed in running tap water and subsequently preserved in 70% ethanol.

TABLE 1. In house validation of ELISA's used for quantifying estradiol (E) in pg/ml, progesterone (P) in ng/ml and testosterone (T) in ng/ml, in *P. subrufa*. The validation showed that these assays were reliable for quantifying turtle steroid hormones.

| | N | Intra-assay % CV | Inter-assay % CV |
|---|---|---|---|
| Estradiol | 34 | 7.42 | 7.34 |
| Testosterone | 34 | 4.77 | 6.65 |
| Progesterone | 34 | 2.86 | 8.13 |
| Vitellogenin | 34 | < 10.00 | < 10.00 |

The ovaries were examined for reproductive condition (stages) and the ten largest follicles were measured (± 0.1 mm), using calipers and a dissecting microscope. Reproductive condition was determined using as criteria the mean diameter (mm) of the ten largest follicles of each ovary, visible presence of yolk, and the presence of eggs in the oviduct, therefore reproductive stages were based on the gross morphology of the ovary and the presence of *in utero* eggs. Reproductive categories were adapted from Moll (1979) and defined as follows:

Stage 1 – ovarian follicle diameters < 3.5 mm (non-active phase) without eggs in the oviduct;

Stage 2 – ovarian follicle diameters between 3.5 mm and 10 mm (pre-vitellogenic) without eggs in the oviduct;

Stage 3 – ovarian follicle diameters > 10 mm (vitellogenic) without eggs in the oviduct;

Stage 4 – ovarian follicle diameters < 3.5 mm with eggs in the oviduct (gravid with ovaries in a non-active phase);

Stage 5 – ovarian follicle diameters between 3.5 mm and 10 mm with eggs in the oviduct (gravid with pre-vitellogenic follicles).

Averages of follicle diameters for turtle individuals in each reproductive category (stage) were calculated. The length and width (± 0.01 mm) of each egg in the oviducts (left and right) were measured using calipers, as well as the length of each oviduct with and/or without eggs (± 1 mm).

*Histological Analysis*

Selected ovaries in each stage were prepared for histology using standard histological procedures (Humason, 1967; Bancroft and Stevens, 1977). Sections were also taken from the oviducts including the uterine tube and isthmus regions, which were used to determine the presence or absence of spermatozoa. Tissues were dehydrated in graded alcohols, cleared in

toluene, embedded in paraffin wax (Paraplast Plus: melting point: 56°C), sectioned at 8 μm with a Jung rotary microtome and stained with Harris' hematoxylin and eosin (Humason, 1967; Bancroft and Stevens, 1977).

*Data Analysis*

All data for adult females were combined to calculate means (x) and standard errors (SE) of the mean for the reproductive condition categories. Data were normalized by logarithmic or square root transformations and subsequently subjected to the Lillifors normality test and Levene's test for homogeneity of variances. It was possible to employ parametric statistics on all but two of the data sets. Non-parametric statistics was performed on the latter datasets. A one-way analysis of variance (ANOVA) and a Least significant difference (LSD) multiple comparisons procedure was used to detect significant variation between monthly samples and reproductive conditions. To test whether dimensions of the follicles were affected by variation in body size, linear regression analysis was performed with variables being regressed on carapace length. Body size had no significant effect on mean follicle size ($P > 0.05$) and logarithmic transformation values were used for ANOVA and Kruskal-Wallis analyses. Linear regression was also used to test whether the number of eggs, egg length and egg width was affected by body size. Pearson's product-moment correlation analyses were employed between reproductive categories (follicle size) and climatic variables. For this purpose data were partitioned into two subsets: (1) December through March, including those months during which vitellogenesis was initiated and progressively increased in activity; and (2) July through November, including those months during which vitellogenic activity peaked and ovulation occurred. Climatic data included total monthly rainfall, maximum and minimum ambient air temperatures and photoperiod.

Significance levels for all tests was set at $P < 0.05$ unless otherwise stated. Mean values are presented with ± standard error (SE). The STATISTICA (StatSoft, Inc. 1984-2000) software package was used for all statistical procedures and graphical presentations.

## RESULTS

### *Gross Reproductive Morphology*

Gross morphology of the ovaries and oviducts of the African helmeted turtle corresponded to that described for other turtle species (Ashley, 1962; Moll, 1979; Solomon and Baird, 1979; Owens, 1980; Kuchling, 1999). The bulk of the ovary is composed of numerous follicles. At the onset of breeding, the ovaries assume a grape-like appearance owing to the rapid increase of follicle size (Franchi, 1962, Figure 3A).

### *Size at Sexual Maturity*

Individual female sizes ranged from 40-260 mm carapace length. Female *P. subrufa* with yolked follicles, oviducal eggs or corpora lutea varied from 120-260 mm carapace length. Although the smallest gravid female measured 123 mm in carapace length, a few females with carapace lengths smaller than 170 mm were found to be non-gravid during the breeding season. It was therefore assumed that females attained reproductive maturity at approximately 130-160 mm carapace length. Females ranging between 160-210 mm carapace length were encountered most frequently during the trapping period.

### *Ovarian Cycle and Vitellogenesis*

Mean ovarian follicle diameter (Figure 4) of the largest follicles did not increase significantly with body size ($P > 0.05$) and was therefore not adjusted for carapace length.

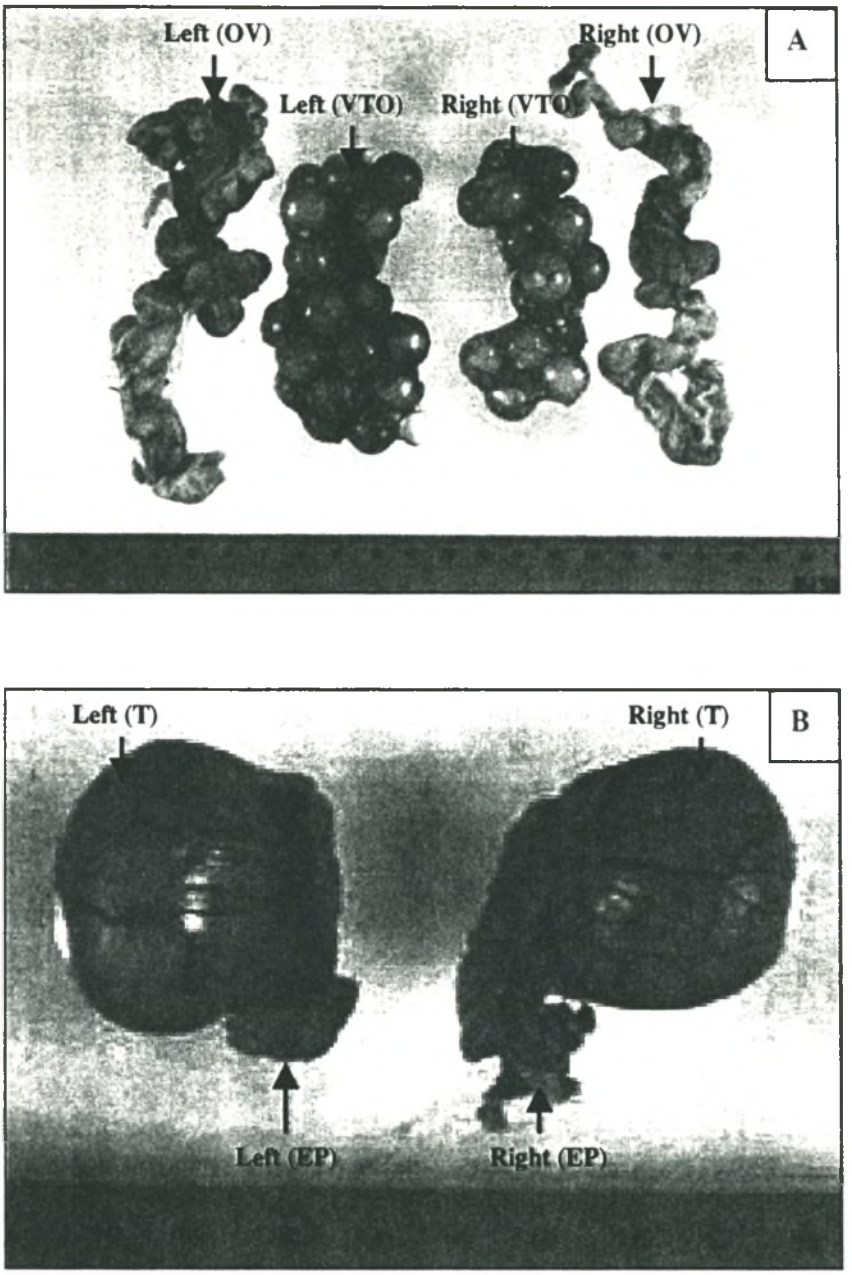



FIGURE 3. Photomicrograph of the left and right reproductive organs of *P. subrufa*. (A) Female vitellogenic ovaries (VTO) and oviducts (OV) of a specimen trapped in October 2000. (B) Male testes (T) and ducti epididymi (EP) of a specimen trapped in March 2000.

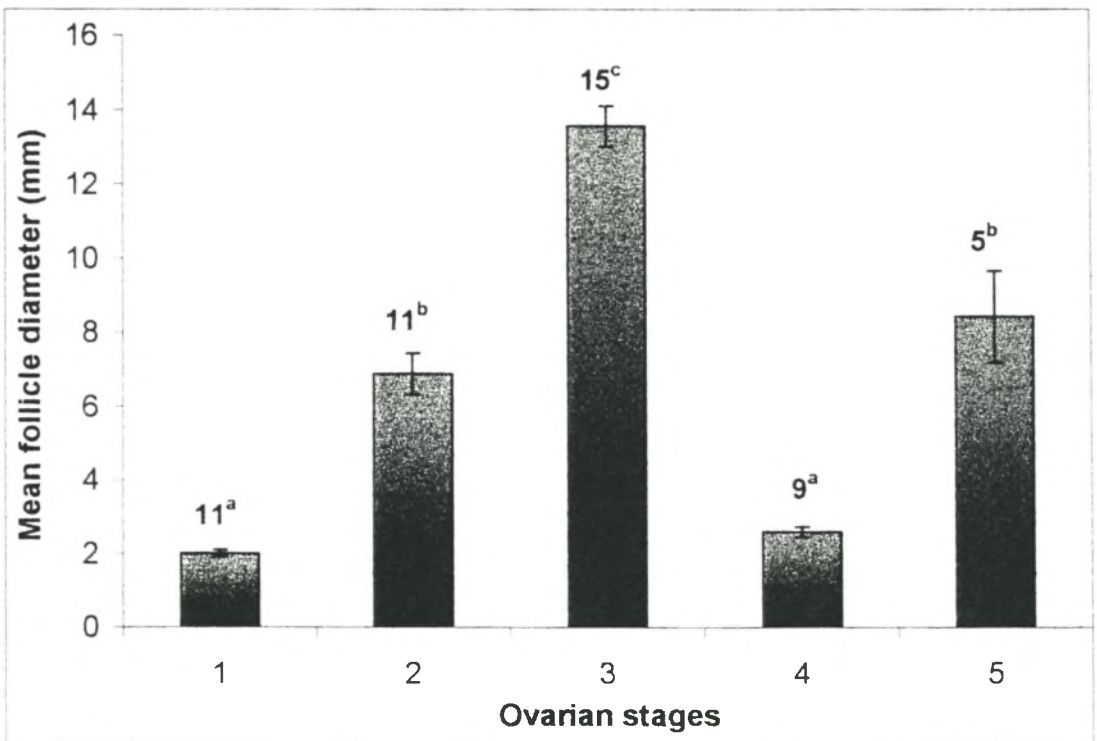



FIGURE 4. Variation among the female reproductive categories (stages) in *P. subrufa* follicle diameter (N = 51), measured in millimeters (mean ± SE). The number of turtles for each reproductive stage is at the top of each bar graph, and bars with different alphabetic superscripts are significantly different (ANOVA, LSD multiple comparisons procedure, $P < 0.05$ per comparison).

Significant seasonal variation in follicle diameter was apparent (Kruskal-Wallis: $H_{9,51} = 22.04$, $P < 0.05$). Follicles began to develop (late September to early October) and some were at pre-vitellogenic stage by early summer (November) and continued to enlarge well into winter (Table 2). Vitellogenesis for some females began in summer (late December) and the associated ovarian follicular increment continued through to next spring (September - October), when ovulation occurred (Figure 5). After brumation, most females had vitellogenic follicles ready for ovulation. Females with oviducal eggs were found from spring through summer (September – January). The eggs were ovoid and the shell was generally hard and white. Gravid females (N = 14) contained 9-38 eggs with the number of eggs correlating significantly with body size ($F_{1,13} = 24.33$, $P < 0.001$; Figure 6). It would be of little use to calculate a mean clutch size for this species, as the mean value would be strongly influenced by the size distribution of females examined. Therefore, it would be best to calculate mean clutch size by using a predictive regression.

Expected clutch size = 0.2841*carapace length – 36.34

($r = 0.82$, $r^2 = 0.67$, n = 14, length in mm)

Broadly speaking, seven eggs would be the minimum clutch size produced by females of about 150 mm in length, and the clutch size increases by one egg for each 5 mm increase in carapace length. The maximum clutch size would therefore be predicted at approximately 37 eggs and the mean clutch size for female *P. subrufa* would be ± 21 eggs. This generalization needs to be interpreted cautiously however, as growth rates are highly variable and appear to stabilize at markedly different maximum sizes (Georges, 1983). The means (± SE) and ranges for length and width (± 0.01 mm) of eggs still found in the oviduct were as follows: 35.66 ± 0.63 (38.51 – 30.67); 21.63 ± 0.36 (23.35 – 19.57).

TABLE 2. Seasonal changes in female ovarian activity of *P. subrufa*. Reproductive conditions were determined using as criteria the gross morphology of the ovary and the presence of eggs in the oviducts. Reproductive categories were adapted from Moll (1979). See text for full description of the reproductive categories (stages).

| | | Ovarian stages with number of turtles in each stage | | | | |
|---|---|---|---|---|---|---|
| | N | 1 | 2 | 3 | 4 | 5 |
| January | 5 | | 2 | 1 | | 2 |
| February | 8 | 1 | 4 | 3 | | |
| March | 6 | | 1 | 4 | | 1 |
| April | 2 | 1 | 1 | | | |
| May | 6 | 6 | | | | |
| June | 0 | | | | | |
| July | 1 | | | 3 | | |
| August | 0 | | | | | |
| September | 6 | 1 | | 4 | 1 | |
| October | 6 | 1 | | 2 | 3 | |
| November | 6 | 1 | 1 | | 2 | 2 |
| December | 6 | | 3 | 1 | 1 | 1 |

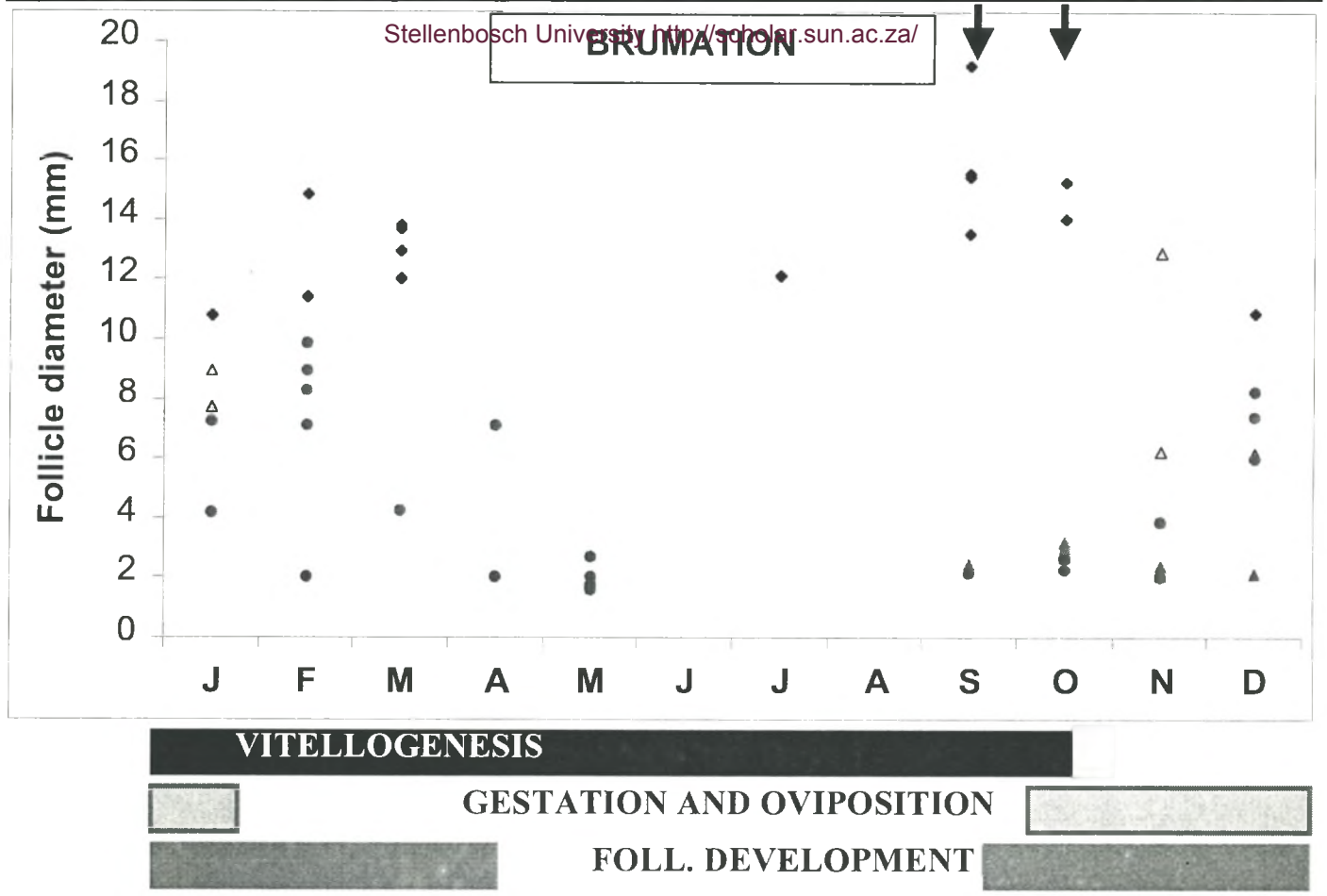



FIGURE 5. Relation of mean diameter of ten largest ovarian follicles (mm) in *P. subrufa* ovaries to time of year (N = 51). Brumation period is indicated at the top of the graph and the arrows indicate ovulation / mating period. Each symbol represents one turtle and five female reproductive groups are indicated: (1) non-active phase (pink circles), (2) pre-vitellogenic (orange circles), (3) vitellogenic (blue diamonds), (4) gravid with non-active phase ovaries (green tri-angles), (5) gravid with pre-vitellogenic ovaries (yellow tri-angles with black outline). See text for detailed description of reproductive categories.

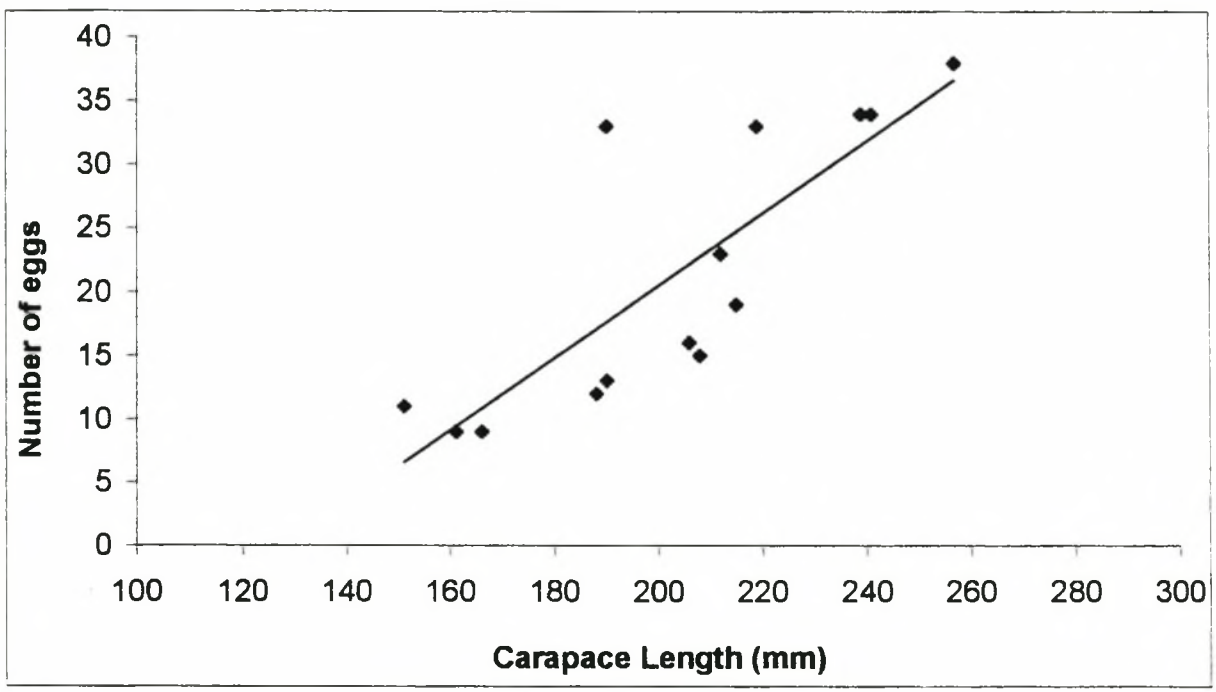



FIGURE 6. Standard curve (linear regression) of known number of eggs (N = 14) on the y-axis measured against the carapace length (mm) on the x-axis ($r = 0.82$, $y = 0.2841x - 36.339$, $P < 0.001$).

*Seasonal Variation in Circulating Plasma Steroid Hormone Concentrations*

Estradiol (E)

Mean plasma E concentrations varied significantly throughout the active season (late August through April) (Figure 7A; Kruskal-Wallis: $H_{4,33} = 11.10$, $P < 0.05$). Mean plasma E concentrations were basal at spring emergence (September) and increased during spring (October). A further increase occurred during early summer (November - December), with E peaking significantly ($P < 0.05$) in mid- to late summer (January - February). This peak occured after ovulation/mating and also after nesting, which coincided with the onset of vitellogenesis. Plasma E concentrations decreased significantly to basal concentrations at the beginning of autumn (March) and were still low after spring emergence. One female had a significantly elevated estradiol concentration in September (353.97 pg/ml), even higher than the peak in January - February, and was therefore excluded from the statistical analyses as an outlier.

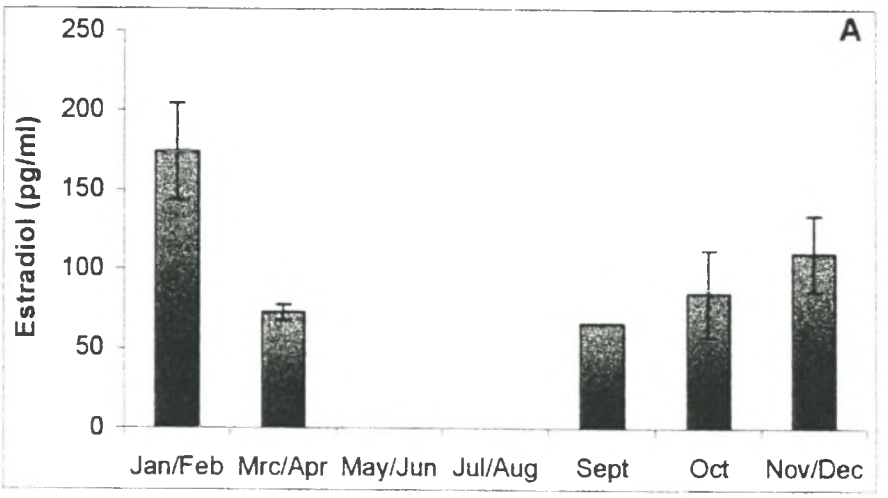
Progesterone (P)

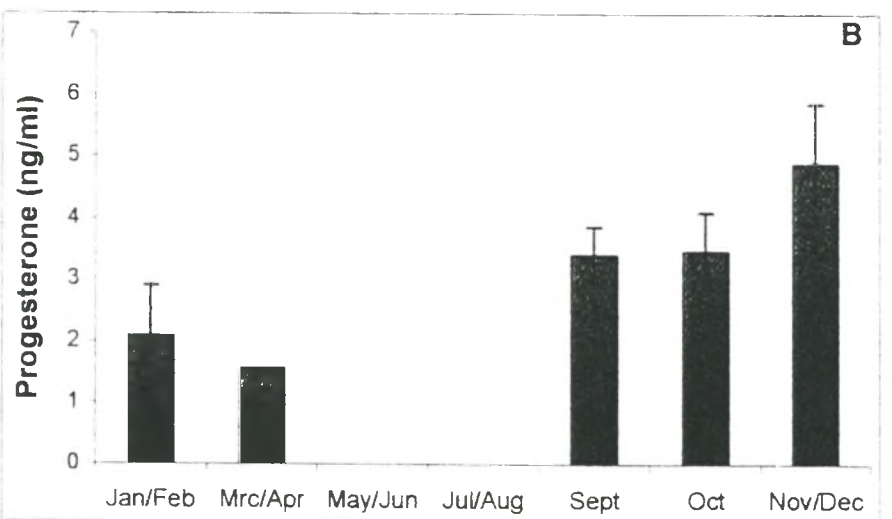
Mean plasma P concentrations did not vary significantly throughout the active season (Figure 7B; ANOVA: $F_{4,29} = 0.89$, $P > 0.05$). However, plasma P concentrations increased during spring corresponding to the late vitellogenic/ovulatory and mating period, and remained high peaking in summer (November - December), which corresponded with the gestation period. Plasma P concentrations had decreased by mid- to late summer (January - February), corresponding with the end of oviposition and eventually returning to basal concentrations by autumn (March - April).

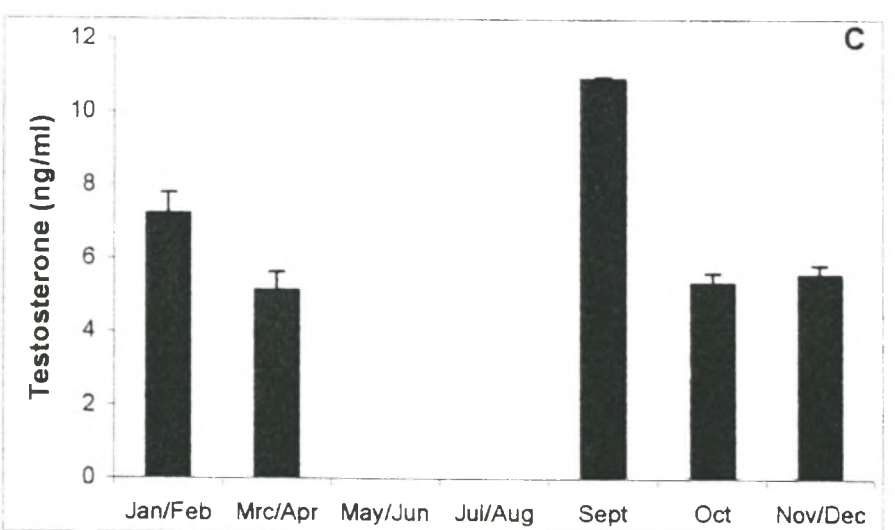
Testosterone (T)

Significant variations in mean plasma T concentrations were observed throughout the active season (late August through April) (Figure 7C; ANOVA: $F_{4,29} = 9.07$, $P < 0.001$). A peak

FIGURE 7. Monthly variation in the mean plasma (A) estradiol (N = 34), (B) progesterone (N = 34) and (C) testosterone (N = 34) concentrations, during the annual reproductive cycle of female *P. subrufa*. Values presented represent the mean ± SE.



characterized the cycle in early spring (September), during the mating/ovulation period. During late spring to summer (October - December), plasma T concentrations decreased significantly, corresponding to the start of the gestation period with a second peak in mid- to late summer (January - February). Plasma T concentrations returned to basal levels in autumn (March - April).

*Seasonal Variation in Circulating Plasma Vitellogenin (VTG) Concentrations*

Plasma VTG concentrations coincided with plasma E concentrations, therefore also varying significantly throughout the active season (ANOVA: $F_{4,29} = 4.86$, $P < 0.05$). Plasma VTG concentrations also coincided with the onset of vitellogenesis in January (Figure 8), but were significantly low at spring emergence (September), steadily increasing from October through December, with peak VTG concentrations in summer (January to February). At the beginning of autumn (March) plasma VTG concentrations decreased significantly and remaining at basal levels until after spring emergence.

*Post-Capture Effects*

Significant differences in mean plasma concentrations for estradiol (Student's paired t-test: $t = 0.87$, $P > 0.05$), progesterone ($t = 0.90$, $P > 0.05$), testosterone ($t = 1.01$, $P > 0.05$) and vitellogenin ($t = -0.81$, $P > 0.05$) were not evident for the four turtles trapped in October. The means and standard errors for the plasma hormone concentrations are tabulated in Table 3.

*Climatic Correlations*

Vitellogenesis in vitellogenic females (stage 3), correlated with decreasing ambient air temperature (December - March: $r = -0.98$, $P < 0.05$) and photoperiod ($r = -0.99$, $P < 0.001$), but with increasing rainfall ($r = 0.82$, $P < 0.05$). The increase in sexual activity after brumation, during the preparatory and ripening phases of the follicles were associated with increasing

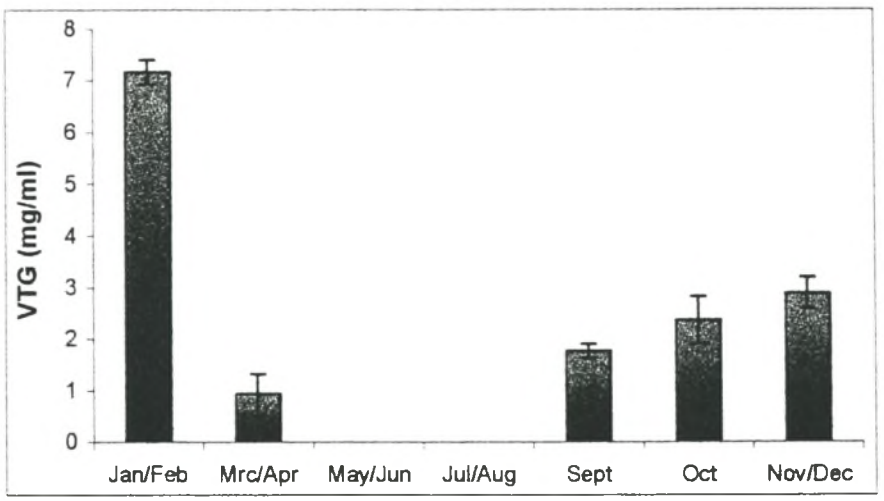



FIGURE 8. Monthly variation in the mean plasma vitellogenin (VTG) concentrations (N = 34), during the annual reproductive cycle of female *P. subrufa*. Values presented represent the mean ± SE.

TABLE 3. Means and standard errors for the reproductive hormone concentrations of estradiol, progesterone, testosterone and vitellogenin in the field and in the laboratory used in the post-capture effects analyses.

| | Field | Laboratory |
|---|---|---|
| Estradiol (pg/ml) | 92.18 ± 43.50 | 49.65 ± 13.43 |
| Progestrone (ng/ml) | 4.21± 2.51 | 2.78 ± 1.19 |
| Testosterone (ng/ml) | 6.36 ± 1.11 | 6.00 ± 0.81 |
| Vitellogenin (mg/ml) | 1.67 ± 0.64 | 2.71± 0.99 |

photoperiod ($r = 0.99$, $P < 0.001$) and temperature ($r = 0.95$, $P < 0.05$) but did not correlate with rainfall ($r = -0.79$, $P > 0.05$).

*Oviduct*

The oviduct of *P. subrufa* had an average length of ± 420 mm without eggs and ± 490 mm when eggs were present, which depended on the size of the turtle (as previously mentioned) but this was not statistically analyzed (Figure 4A). Eggs appeared to move in single file with a complement occupying ± $^2/_3$ of the oviduct's length. Eggs found in the oviduct from September - October were not in the oviduct for very long, indicated by their thin shells. The ovaries supported this observation as corpora lutea found were still large, whereas eggs found after October were in the lower part of the oviduct with a much harder shell. This phenomenon coincides with the size of the corpora lutea found, for the corpora lutea were small and could only be seen with a stereo-microscope. No albumen-covered eggs were found in the oviduct (Owens, 1980), which indicated that this phase of egg development happens rapidly (early-on) in this species. No spermatozoa were found in the oviducts examined. Histologically the oviduct of *P. subrufa* appeared similar to that described for *Chelonia mydas* (Solomon and Baird, 1979), *Chrysemys picta* (Motz and Callard, 1991) and *Gopherus polyphemus* (Palmer and Guillette, 1988).

*Female-Male Synchrony*

A summary of the timing of reproductive events in both female and male (Chapter 3) *P. subrufa* is presented in Figure 9. It is evident that male and female gonadal activities are asynchronous. Abundant spermatozoa observed in the ducti epididymi throughout the year ensures the availability of spermatozoa during the suspected mating period in spring at the time of ovulation.

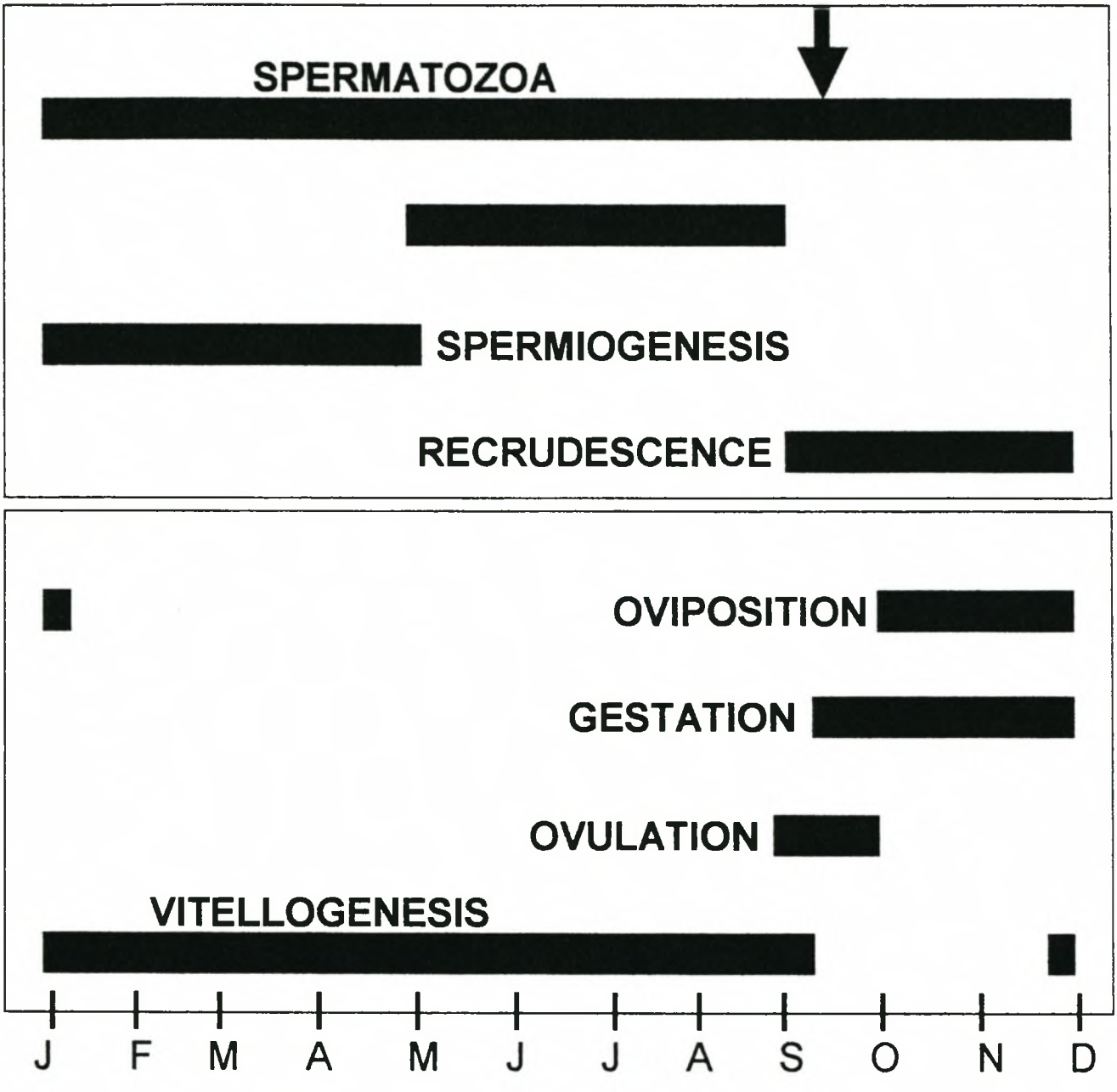



FIGURE 9. Summary diagram indicating the asynchrony between (A) male and (B) female *P. subrufa*, in timing of annual reproductive cycles. The arrow indicates the mating period.

## DISCUSSION

### *Reproductive Cycle*

Reproductive activity in temperate turtles is highly seasonal (Moll, 1979). The classical pattern of post-nuptial vitellogenesis as described by Moll (1979) and Kuchling (1999) and also known as an aestival cycle (St. Girons, 1982), is exhibited in most temperate zone turtles (single- and multi-clutched females). Females generally have an annual cycle, where vitellogenesis begins in late summer or autumn and after a break during brumation, continues until completion the following spring. Reproduction in chelonian species is known to vary between geographic location, populations, individuals and years (Boscott, 1962). Depending on these factors, together with age and species, the yolk accumulation may be completed either before or after brumation (Moll, 1979; Kuchling, 1999). In *Chrysemys picta* (Powell, 1967; Ernst, 1971) and *Terrapene carolina* (Altland, 1951) ovarian follicles become mature before brumation and when turtles emerge in spring they are ready to ovulate. In *Emydura krefftii* and other emydid turtles, yolk accumulation begins in late summer and continues unabated through winter (Mahmoud and Klicka, 1972; Georges, 1983).

The post-nuptial cycle is in strong contrast to the classic spring-summer cycle, also known as a pre-nuptial cycle, described for many temperate lizards and snakes (see review Fitch, 1970; Van Wyk, 1994), crocodilians (Lance, 1989; Thorbjarnarson, 1994), tortoises (Schramm, *et. al.*, 1999) and turtles (Singh, 1977; Etchberger and Ehrhart, 1987; Rostal *et. al.*, 1998). A pre-nuptial cycle is characterized by rapid follicular growth immediately before ovulation, with the ovaries remaining relatively quiescent during most of the year (Licht, 1984, Kuchling, 1999). According to Kuchling (1999), in a pre-nuptial cycle the male and female gonadal cycles of turtles are synchronized so that ovulation and spermiation occur at the time of mating. In

contrast to this, in a post-nuptial cycle, the male and female gonadal cycles are not synchronized and sperm storage takes place. In male *P. subrufa* peak testicular activity occurs in late summer / early autumn and declines towards the end of autumn / winter. Testicular activity ceases and remains regressed throughout winter (Chapter 3) with sperm storage in the ducti epididymi. Although no live turtles were examined from May through August, follicular growth in female *P. subrufa* commences in late August / early September and peaks during spring the following year (September – October) prior to ovulation (Figure 5). The exact period of ovulation in these turtles is still unknown. However, ovulation did occur as indicated by either oviducal eggs or large corpora lutea. For the timing of spring ovulation to coincide with spermiation in a post-nuptial species is presumably prevented by low ambient winter temperatures that inhibit spermatogenesis (Ganzhorn and Licht, 1983; Mendonça and Licht, 1986). In the temperate zone turtle, *Chrysemys picta*, warm temperatures inhibit follicular growth and cause regression of ovaries. These follicles grow at lower temperatures with ovulation only occurring when temperatures rise once again. This coincides with the female reproductive cycle of *P. subrufa*. This is in contrast to the males in which the onset of and peak spermatogenic activity is associated with increasing ambient air temperatures (Ganzhorn and Licht, 1983). Burger (1937) concluded that photoperiod in testicular recrudescence in *Pseudemys scripta* is more important than temperature, and field observations have led to the conclusion that photoperiod may control the rather precise timing of nesting in some species (Whillians and Crossman, 1977). Experimental evidence for squamate reptiles has demonstrated that temperature tends to be a dominant factor in seasonal reproductive cyclicity (Licht, 1983) and that increasing ambient air temperature is one of the major controlling factors involved in the onset of reproductive activity in temperate oviparous species (Licht, 1984; St. Girons, 1985). However, in *Lissemys punctata*

*granosa* it seems that successful completion of the breeding cycle is associated with temperature, photoperiod and moisture changes (Singh, 1977). In the warm temperate environment occupied by *P. subrufa* in South Africa, the wet seasons vary from winter to summer rainfall. Although rainfall is seasonal in the various habitat regions, temperature and photoperiod are more important for the successful completion of female *P. subrufa* vitellogenic cycle. If reproductive cycles are timed so that offspring are born during optimal conditions thereby enhancing their chances of survival (Moll, 1979) it would make sense to look at the probability of endogenous or self contained proximate factors, together with exogenous rhythms as suggested by Kuchling (1999). The fact that male and female cycles are asynchronous in *P. subrufa* suggests that sexes either respond to different environmental cues and endogenous rhythms, or differentially to the same cues. This asynchrony in timing of the onset of gonadal activity corresponds closely to asynchrony described for most temperate zone turtles (Altland, 1951; Christiansen and Dunham, 1972; Lofts and Tsui, 1977; Georges, 1983).

*Reproductive Endocrinology*

It is well known that plasma T plays a primary role as the precursor for E synthesis. Plasma E concentrations correlate with vitellogenesis and progesterone appears to have an important role in the ovulatory process (Chieffi and Pierantoni, 1987). The changes in mean plasma estradiol, progesterone, testosterone and vitellogenin concentrations (Figures 7A to 7C and 8) described for *P. subrufa* throughout the active season, are seasonal and typical of those observed in many other temperate zone reptiles. Plasma E concentrations reflect important physiological events in the reproductive cycle, rising during ovulation and peaking at the onset of vitellogenesis (Edwards and Jones, 2001). In vertebrates, after the initial endo- or exogenous stimulus and its transmission through the body, circulating estradiol concentrations build up

rapidly immediately prior to vitellogenesis. Vitellogenin is then synthesized in the liver and secreted into the blood stream to be taken up by the oocytes (Tata and Smith, 1979). Direct induction of VTG *in vitro* has already established that estradiol is the sole inducer (Follett and Redshaw, 1974) and its continuous presence is required during the lag period for the maintenance of vitellogenin synthesis (Ho, 1987; Tata and Smith, 1979). In reproductively active female *P. subrufa*, annual changes in mean plasma E concentrations and mean VTG concentrations reflect important physiological events in the reproductive cycle, rising during the onset of follicular development (October - December) and peaking during the onset of vitellogenesis in late December - January. Progesterone has been suggested to be the "antigonadal" hormone in lizards, however the specific inhibitory effect on VTG uptake and in gestation remains unclear (Yaron and Widzer, 1978; Edwards and Jones, 2001). Progesterone may slow the rate of ovarian development, delaying the next ovulation and gestation, so that the young are born when conditions are optimal for their survival (Callard *et. al.*, 1992). Progesterone may also influence the rate of development (Gemmell, 1995) or delay oviposition by reducing oviducal contractibility (Guillette *et. al.*, 1991). Elevated plasma P concentrations during gestation also inhibit E-stimulated follicular growth (Yaron and Widzer, 1978) so that vitellogenesis is not initiated during pregnancy (Callard *et. al.*, 1992). The elevated mean plasma P seen well before oviposition (September - October) and the peak seen during November - December in *P. subrufa*, is likely to be related to the antigonadal actions of the multihormone control of gestation and oviposition. A significant increase of plasma P concentrations during follicular maturation and ovulation has been reported for a number of turtles, eg. *Chrysemys picta* (Callard *et. al.*, 1978), *Sternotherus odoratus* (McPherson *et. al.*, 1982), *Chelydra serpentina* (Lewis *et. al.*, 1979), *Chelonia mydas* (Licht *et. al.*, 1979; Licht *et. al.*, 1980),

*Lepidochelys oliveaca* (Licht, 1982; Licht *et. al.*, 1982), *Lepidochelys kempi* (Rostal *et. al.*, 1998) and also in the snake *Naja naja* (Bona-Gallo *et. al.*, 1980). Although only determined in a few female oviparous reptile species, plasma T concentrations appear to be an important secretory product of the ovary (Arslan *et. al.*, 1978; Callard *et. al.*, 1978; Owens, 1997; Staub and DeBeer, 1997). In reproductively active female *P. subrufa* mean plasma T concentrations peak (10.91 ± 0.063 ng/ml) during late vitellogenesis and ovulation (September) and then decline rapidly in October. The magnitude of this T peak is relatively high for a female reptile. By comparison, plasma T in females peak between 0.3 – 0.4 ng/ml in the oviparous snake *Naja naja* (Bona-Gallo *et. al.*, 1980), and the turtles *Caretta caretta* (Wibbels, *et. al.*, 1990) and *Lepidochelys kempi* (Rostal *et. al.*, 1998). Peak plasma T concentrations are higher in female *Alligator mississippiensis* (1.12 ng/ml) (Guilette *et. al.*, 1997), *Chrysemys picta* (4.5 ng/ml) (Callard *et. al.*, 1978), and *Tiliqua nigrolutea* (6.3 ng/ml) (Edwards and Jones, 2001). Only the tuatara, *Sphenodon punctatus* (11.4 ng/ml) (Cree *et. al.*, 1992) is reported to have a higher peak in plasma T concentrations in females than in *P. subrufa* females. However, in *S. punctatus* the vitellogenic cycle lasts 18-24 months (Cree *et. al.*, 1992).

Recent studies suggest that stress caused by capture, confinement and repeated blood sampling in male and female amphibian and reptilian species, can alter the hormonal levels of sex steroids, gonadotropin and corticosteroids from the natural conditions. This has been determined in bullfrogs (Mendonça *et al.*, 1985), turtles (Mendonça and Licht, 1986; Mahmoud *et al.*, 1989), alligators (Lance and Elsey, 1986; Mahmoud *et al.*, 1996) and tuatara (Cree *et al.*, 1992). Licht *et. al.* (1985) found that plasma concentrations of reproductive hormones decreased within a few hours after capture, which they attributed to stress. Kuchling (1999) suggested that the change in hormone levels might be the animals normal response to disturbance and if the

disturbance does not persist, the hormonal levels will return to normal. Mahmoud and Licht (1997) suggested that the response to stress induced by captivity on *Chelydra serpentina* varies in intensity during the testicular and ovarian phases. Thus, the degree of stress depends on the hormonal levels and the condition of the gonads during the course of the cycle. In *P. subrufa* there was not sufficient evidence to support the idea that the plasma concentrations of sex steroids decrease after capture. It appears that the suggested intensity variation during the course of the cycle, depending on hormonal levels and condition of the gonads (Mahmoud and Licht, 1997), does to some extent explain the variation found in the hormonal levels of these females (Table 3).

*Conclusion*

Reproduction in female *P. subrufa* is clearly a seasonal phenomenon and as in most temperate zone turtles, male and female reproductive cycles was found to be asynchronized. The results of the present chapter indicate that the pattern of vitellogenesis in *P. subrufa* (tropical and sub-tropical species) does not differ from that of other temperate zone turtles and therefore, displays a post-nuptial vitellogenic cycle.

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# - CHAPTER 3 -

# Male Reproductive Cycle of the African Helmeted Turtle,

# *Pelomedusa subrufa*

## INTRODUCTION

REPTILES display three fundamental reproductive patterns, based on the degree of variation in gonadal activity throughout the year: (1) continuous or cyclic reproduction with roughly similar levels of gonadal activity in all months, (2) continuous reproduction, but with gonadal activity fluctuating across months and (3) discontinuous or seasonal reproduction, where periods of gonadal activity alternating with periods of gonadal quiescence (Licht, 1984). Based on available data, all chelonians fit into the latter two categories (Kuchling, 1999). The reproductive biology of freshwater turtles, in particular, is well documented. Gonadal cycles in freshwater turtles based on gravimetric and histological studies have been described by Mahmoud and Klicka (1972), Moll (1979), Georges (1983), Licht (1984), Mahmoud and Cyrus (1992) and Kennet (1999). Other studies have dealt with endocrine cycles related to seasonal changes in steroid levels (Callard *et. al.*, 1978; Ho *et. al.*, 1981; Licht, 1982; McPherson *et. al.*, 1982; Licht *et. al.*, 1985a; Mendonça and Licht, 1986). Although most studies have focused on reproduction in females and selection of fit offspring, the reproductive biology of male turtles is equally well known (Schrank and Ballinger, 1973; Trauth, 1979; Van Wyk, 1995). Two basic spermatogenic cycles exist in turtles exhibiting discontinuous (i.e. seasonal) reproduction: pre- and post-nuptial cycles. In pre-nuptial cycles, spermatogenesis (recrudescence) is completed immediately before the mating period, usually coinciding with ovulation in females. Alternatively, in post-nuptial cycles, recrudescence ensues after the mating season – sperm is

produced well in advance of ovulation in females and is stored in the ducti epididymi or vas deferens until utilized (Lofts, 1977; St. Girons, 1982; Bradshaw, 1986; Kuchling, 1999; Van Wyk, 1995). In contrast to the pre-nuptial spermatogenic cycle, in species exhibiting post-nuptial spermatogenesis, male and female cycles are asynchronous. Counter intuitively, most temperate zone turtles exhibit a post-nuptial cycle (Lofts, 1987; Jameson, 1988). Winter and early spring is the time of germinal quiescence, while recrudescence is confined to the warm summer months, culminating in peak spermiogenesis during autumn (Kuchling, 1999).

Androgens have been implicated in the control of spermatogenesis and mating activity in many chelonian species (Norris, 1987), although Crews (1984) pointed to the potential dissociation of mating behaviour from high androgen levels in squamates exhibiting post-nuptial cycles. In most chelonians exhibiting post-nuptial cycles, mating behaviour occur during testicular quiescence, i.e. at a time when circulating androgens are expected to be low (Whittier and Crews, 1987).

The majority of freshwater turtles studied to date are Northern hemisphere species, with a resultant lack of information on reproduction in Southern hemisphere species. The African helmeted or marsh turtle (*Pelomedusa subrufa*) is distributed widely throughout Africa and Madagascar, occurring inland in freshwater bodies (Loveridge, 1941; Jaques, 1966; Pritchard, 1979; Ernst and Barbour, 1989; Iverson, 1992; Zug, 1993; Hofstra, 1995; Branch, 1998; Van Wyk, 1998). Nevertheless, the reproductive biology of this turtle has received scant attention, with a complete lack of data on its gonadal cycles and associated endocrine control. *Pelomedusa subrufa* is described as a Tropical to Sub-tropical species (Branch, 1998), yet its range extends into the warm temperate zone at the southern most tip of Africa, where summer rainfall is replaced with winter rainfall. This renders the species ideal in testing Kuchling's suggested

evolutionary conservatism phenomenon of post-nuptial spermatogenesis in turtles and is not expressed in response to environmental cues. Furthermore, *P. subrufa* inhabits not only pristine freshwater bodies but also occur in industrial and agricultural areas, where it is exposed to sewage discharges and organochlorine pesticides such as DDT. This makes it an ideal aquatic bio-indicator species for the study of environmental change and ecosystem degradation (Willingham and Crews, 2000). The main objective of this chapter was to describe the gonadal histology and measure changes in circulating reproductive hormones during the natural reproductive cycle of male *P. subrufa*. Through this study I address the conservatism of post-nuptial spermatogenesis in freshwater turtles (Kuchling, 1999) as well as look at the suggested asynchrony between male and female hormone cycles controlling mating behaviour in a post-nuptial reproductive cycle (Whittier and Crews, 1987).

## MATERIALS AND METHODS

### *Study Areas and Climate*

Live turtles were captured in farm dams in Klapmuts (33° 51' S; 18° 50' E; altitude 177 m) and Caledon (34° 12' S; 19° 21' E; altitude 128 m), Western Cape, South Africa. In addition, preserved specimens were obtained from the South African Museum in Cape Town, the National Museum in Bloemfontein and the Transvaal Museum in Pretoria, South Africa (Figure 1B), for subsequent dissection.

Meteorological data were obtained from weather stations at Elsenburg, 10 km from Klapmuts and at Boontjieskraal, 5 km from Caledon. The study areas are characterized by 600 mm and 400 mm of annual rainfall respectively, occurring mostly in winter months, with a distinct dry season during the summer months. Mean monthly maximum and minimum ambient

air temperatures varied markedly between seasons. Photoperiod was calculated using a formula (Van Leeuwen, 1981, Figures 2A and 2B).

*Trapping and Blood Sampling Procedures*

Turtles were trapped from September 1999 through April 2001 at approximately bimonthly intervals. Turtle trapping was unsuccessful from late April through August and I assumed that turtles were in brumation during this time period. Baited funnel hoop-traps were used with bait ranging from pilchards, liver and pig heart, to fish guts (Legler, 1960; Iverson, 1979; Plummer, 1979; Kennett, 1992; Mansfield *et. al.*, 1998). Traps were checked twice weekly. Body mass was recorded using a portable Panasonic electronic balance (± 0.1 g). Blood samples were collected by heart puncture (Stephens and Creekmore, 1983; Jacobson and Schumacher, 1992), using a 2 ml heparinized syringe and a 1,5 inch 23-gauge needle. The volume of blood drawn was dependent on body mass, i.e. 2 ml / kg (Stephens and Creekmore, 1983) and all blood samples were taken within a few minutes of initial handling. Blood samples were transported to the laboratory on ice, separated by centrifugation and stored at – 80 °C for later analysis of plasma testosterone (T) concentrations. To investigate the possibility of post-capture effects on plasma T concentrations (Licht *et. al.*, 1985a; Lance and Elsey, 1986; Mahmoud *et. al.*, 1989) blood samples were again collected within a few days of capture in the laboratory from six turtles (three in April and three in October) before they were sacrificed. Similarly, blood samples were also collected from four turtles that were recaptured in the field in December, within the same week of first capture.

Carapace length of both museum specimens and captured specimens were measured using calipers (± 0.1 mm). Only captured turtles were permanently marked by notching of the

carapace, using the numbering system of Cagle (1939) and Honegger (1979). Two to three of the largest specimens were transported to the laboratory for autopsy.

*Plasma Testosterone*

Plasma was mixed with diethyl ether after which the diethyl ether phase was collected, dried and extracted with 0.1% human serum albumin in phosphate buffered saline (pH = 7). Extraction efficiency was determined by adding known amounts of tritiated T to four random aliquots of plasma, extracting them together and determining the respective recoveries by scintillation counts against a 100 % tritiated control. Extraction efficiency was 84 %. Testosterone was then measured in duplicate using ELISA (enzyme-linked immunosorbant assay) kits (IBL, Hamburg, Germany), with reference to a standard curve based on standards prepared in human serum.

In house validations of the testosterone ELISA kit were conducted and adequate sensitivity, specificity and parallelism indicated that this specific testosterone ELISA was reliable for quantifying turtle steroid hormones. Intra- and interassay coefficients of variation for this assay were 4.79 % and 6.65 % respectively.

*Autopsy and Tissue Sampling*

Turtles were sacrificed by injecting lethal doses of sodium pentobarbitone (Eutha-naze, Ref. 83/91) directly into the heart. Testes and ducti epididymi (Wolffian ducts) were excised and fixed in Bouin's solution (Humason, 1967; Bancroft and Stevens, 1977; Lofts and Tsui, 1977; Kuchling *et. al.*, 1981; Van Wyk, 1995). Differences in volume of the right and left testis were not consistent, therefore the mean testis volume of each individual was calculated. Testes were separated from the epididymi and the longest and shortest axes of the testes were measured to determine the volume, using the formula of an ellipsoid: $V = {}^{4}/_{3}\pi a^{2}b$, where 'a' is ½ the shortest

diameter and 'b' is ½ the longest diameter (Guillette and Bearce 1986). After dissection, turtle carcasses were fixed in 4 % buffered formalin for ten days, washed in running tap water and subsequently preserved in 70 % ethanol.

*Histological Analysis*

The central portion of the right testes and upper section of the ducti epididymi were prepared for analysis using standard histological techniques (Bancroft and Stevens, 1977). Tissues were dehydrated in graded alcohols, cleared in toluene, embedded in paraffin wax (Paraplast Plus: melting point: 56 °C), sectioned at 8 μm with a Jung rotary microtome and stained with Harris' hematoxylin and eosin (Humason, 1967).

Stages of the spermatogenic cycle are reflected by variation in seminiferous tubules histology (Lofts and Tsui 1977, Van Wyk 1995). A total of ten seminiferous tubule diameters and ten ductus epididymis tubule diameters (of approximate circular proportions) per turtle sample were measured (± 0.1 μm) using a Nikon microprojector. Spermatogenic activity was assessed qualitatively by adapting the classification scheme from Licht (1967), Mayhew (1971), Christiansen and Moll (1973), Lofts and Tsui (1977), and Dubois *et.al.* (1988). Accordingly, male turtles were classified firstly by collecting month and then by spermatogenic stages. Five spermatogenic stages were recognized:

Stage 1: few spermatozoa are present and spermatogonia start forming at the germinal epithelium.

Stage 2: seminiferous tubules are involuted containing only Sertoli cells and spermatogonia.

Stage 3: numerous dividing spermatogonia and spermatocytes are present at the luminal margin.

Stage 4: primary and secondary spermatocytes are visible, with numerous early- and late-stage spermatids at the luminal margin.

Stage 5: the germinal epithelium is composed mainly of a few spermatids and numerous spermatozoa (spermiation).

Seasonal changes in the amount of sperm in the lumen of the accessory ducts were evaluated qualitatively as full, half-full, quarter-full or empty. At least ten measurements of epithelial cell heights in the ductus epididymis and seminiferous tubules for each turtle sample were measured (± 0.1 μm), using a Nikon mircoprojector. The epithelial thickness of each seminiferous tubule and ductus epididymis was determined by measuring the distance from spermatogenic cells touching the basement membrane to the luminal margin. Average values were calculated for each measured variable to obtain a mean for individual turtles.

*Data Analysis*

All data for adult males were combined to calculate means (x) and standard errors (SE) of the mean for the monthly samples or reproductive condition. Data were normalized by logarithmic or square root transformations and subsequently subjected to the Lillifors normality test and Levene's test for homogeneity of variances. It was possible to employ parametric statistics on all data sets except two data sets, on which non-parametric statistics were performed. A one-way analysis of variance (ANOVA) and a Least significant difference (LSD) multiple comparisons procedure was used to detect significant variation between monthly samples. To test whether dimensions of the testes and ducti epididymi were affected by variation in body size, linear regression analysis was performed with variables being regressed on carapace length. Where body size affected variation, data was adjusted using the residuals. The residuals of the regression line between the different data sets and the carapace length were used as an index (e.g. carapace length-adjusted testicular volume). Residuals explain the variance not explained by the data sets. The residuals are therefore referred to as "adjusted" (Van Wyk and Mouton, 1996).

Adjusted values were used in ANOVA and Kruskal-Wallis analyses. Student's paired t-test was used to determine significant variation of plasma T concentrations in the field and in the laboratory of individual turtles. Pearson's product-moment correlation analysis was employed between reproductive conditions and climatic variables. For this purpose data were partitioned into two subsets: (1) the recrudescence set, including those months during which spermatogenesis was initiated and progressively increased in activity (September through May), and (2) the testicular regression set, including those months during which spermatogenic regression occurred (May through September).

Significance levels for all tests were set at $P < 0.05$ unless otherwise stated. Mean values are presented with ± standard error (SE). The STATISTICA (StatSoft, Inc. 1984-2000) software package was used for all statistical procedures and graphical presentations.

## RESULTS

### *Gross Testes Morphology*

Gross morphology of the African helmeted turtle testes corresponded to that described for other turtle species (Moll, 1979; Lofts, 1987; Dubois *et. al.*, 1988; Kuchling, 1999). The testes were creamy yellow, with an elliptical body of flaccid consistency (Figure 3B) and were located adjacent to the kidneys (as described by Risley, 1938; Ashley, 1955; Fox, 1977; Solomon and Baird, 1979; Owens, 1980; Wilson *et. al.*, 1981; Lance, 1989). All turtles were regarded as sexually mature since spermatozoa were abundant in the ducti epididymi.

### *Size at Sexual Maturity*

Individual male sizes ranged from 40-290 mm carapace length. The smallest male to exhibit testicular enlargement and active spermiogenesis (free spermatozoa present) was 110 mm

carapace length. Abundant spermatozoa were present in the ducti epididymi. Larger males (110-140 mm carapace length) were found without the necessary gonadal activity and there were no spermatozoa present in the ducti epididymi. It was therefore assumed that males attained sexual maturity between 110-140 mm carapace length.

*Testicular Cycle and Spermatogenesis*

Mean adjusted testis volumes varied significantly throughout the year (Table 4, Figure 10A; ANOVA: $F_{8,65} = 6.73$, $P < 0.001$). Corresponding with testis volume, mean diameter (Table 4, Figure 10B; ANOVA: $F_{8,65} = 5.54$, $P < 0.001$) and mean epithelium cell height of the seminiferous tubules (Table 4, Figure 10C; Kruskal-Wallis: $H_{8,74} = 42.11$, $P < 0.001$) also varied significantly throughout the year.

Males start brumation during late autumn (April to May). During this time, the onset of testicular regression (stage 1, Figure 11A) was marked by decreases in both testis volume (Figure 10A) and seminiferous tubule diameter (Figure 10B). The spermatogenic epithelium of the seminiferous tubules (Figure 10C) also exhibited spermatogenic regression (Table 5). This was characterized by small numbers of spermatogonia, scattered around the periphery of the tubule and adjacent to the basement membrane, as well as by Sertoli cells nuclei outnumbering spermatogonial nuclei. Spermatogonia were scattered around the periphery of the tubule adjacent to the basement membrane. Few spermatozoa, spermatids and spermatocytes were visible in the enlarged lumina. Testicular regression (stage 1, Figure 11A) progressed through winter, until eventually only spermatogonia and Sertoli cells were recognizable in the seminiferous epithelia (Figure 10A); late May and from August to September.

Testicular recrudescence was apparent in early spring (August to mid-October), following the winter brumation period (Figure 11B). Spermatogenic epithelia consisted of three

TABLE 4. Variation in the mean testicular volume ($mm^3$), mean diameter and mean epithelial cell height of seminiferous tubules (µm) and the mean diameter and mean epithelial cell height of ducti epididymi (µm), by spermatogenic stage in *P. subrufa*. Similar superscripts indicate significant difference ($P < 0.05$) among means (ANOVA, LSD multiple comparisons test) ± SE.

| Stage | N | Testis Volume ($mm^3$) | Seminiferous tubule diameter (µm) | Seminiferous tubule epithelium cell height (µm) | Ductus epididymidis diameter (µm) | Ductus epididymidis epithelium cell height (µm) |
|---|---|---|---|---|---|---|
| 1 | 12 | $110.51 \pm 9.93^{a}$ | $248.00 \pm 18.05^{a}$ | $29.79 \pm 4.16^{a}$ | $474.23 \pm 49.26^{a}$ | $7.01 \pm 0.87$ |
| 2 | 15(14) | $76.05 \pm 8.41^{b}$ | $247.78 \pm 19.59^{b}$ | $34.18 \pm 3.51^{bc}$ | $(626.15 \pm 80.90)^{b}$ | $(7.28 \pm 0.73)$ |
| 3 | 12(11) | $110.20 \pm 21.87^{c}$ | $232.37 \pm 25.94^{c}$ | $25.59 \pm 1.55^{c}$ | $(414.17 \pm 66.36)$ | $(7.76 \pm 1.64)$ |
| 4 | 15(14) | $118.54 \pm 17.76^{b}$ | $281.32 \pm 30.68^{bc}$ | $35.61 \pm 5.69^{ab}$ | $(636.84 \pm 84.34)^{abc}$ | $(8.22 \pm 0.46)$ |
| 5 | 30 | $135.81 \pm 14.28^{abc}$ | $207.73 \pm 9.90^{abc}$ | $19.42 \pm 1.27^{ab}$ | $627.50 \pm 36.13^{c}$ | $7.86 \pm 0.34$ |

FIGURE 10. Monthly variation in the (A) adjusted testicular volume, (B) adjusted seminiferous tubule diameter and (C) adjusted seminiferous tubule epithelium cell height, during the annual reproductive cycle of male *P. subrufa*. Values presented, represent the mean transformed values ± SE (N = 84). Brumation period is indicated at the top of graph A. Spermatogenic stages are indicated at the bottom of graph C.

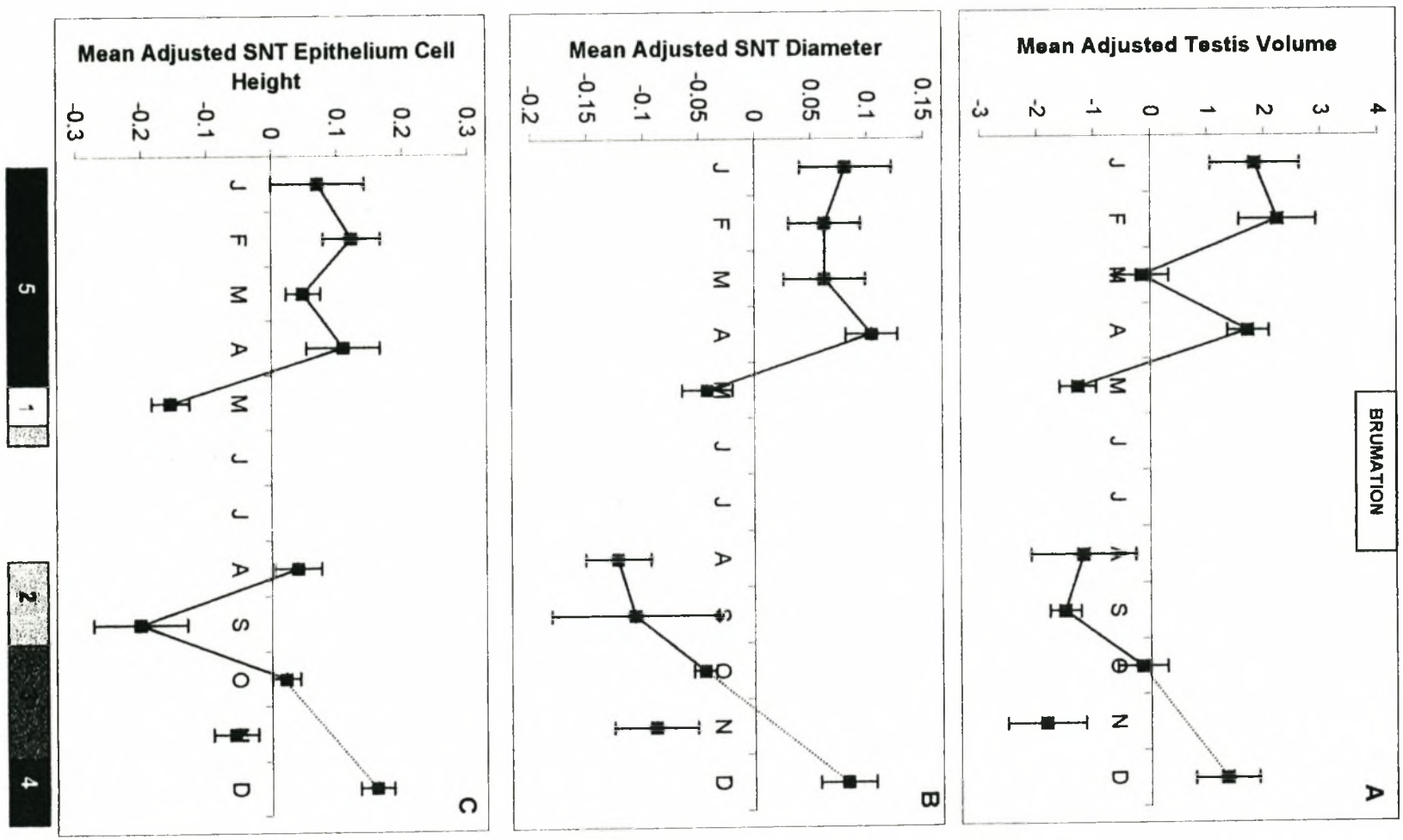

FIGURE 11. Photomicrographs of seminiferous tubules showing seasonal changes in spermatogenic activity in male *P. subrufa*. Spermatogenic categories: (A) Stage 1 x 250, (B) Stage 2 x 1000, (C) Stage 3 x 400, (D) Stage 4 x 400 and (E) Stage 5 x 250 (Table 4). SG = spermatogonia, SC = Sertoli cells, IC = interstitial cells, SS = spermatocytes, US = undifferentiated spermatids, MS = metamorphosing spermatids, SZ = spermatozoa, LU = lumen. See text for description of spermatogenic stages.

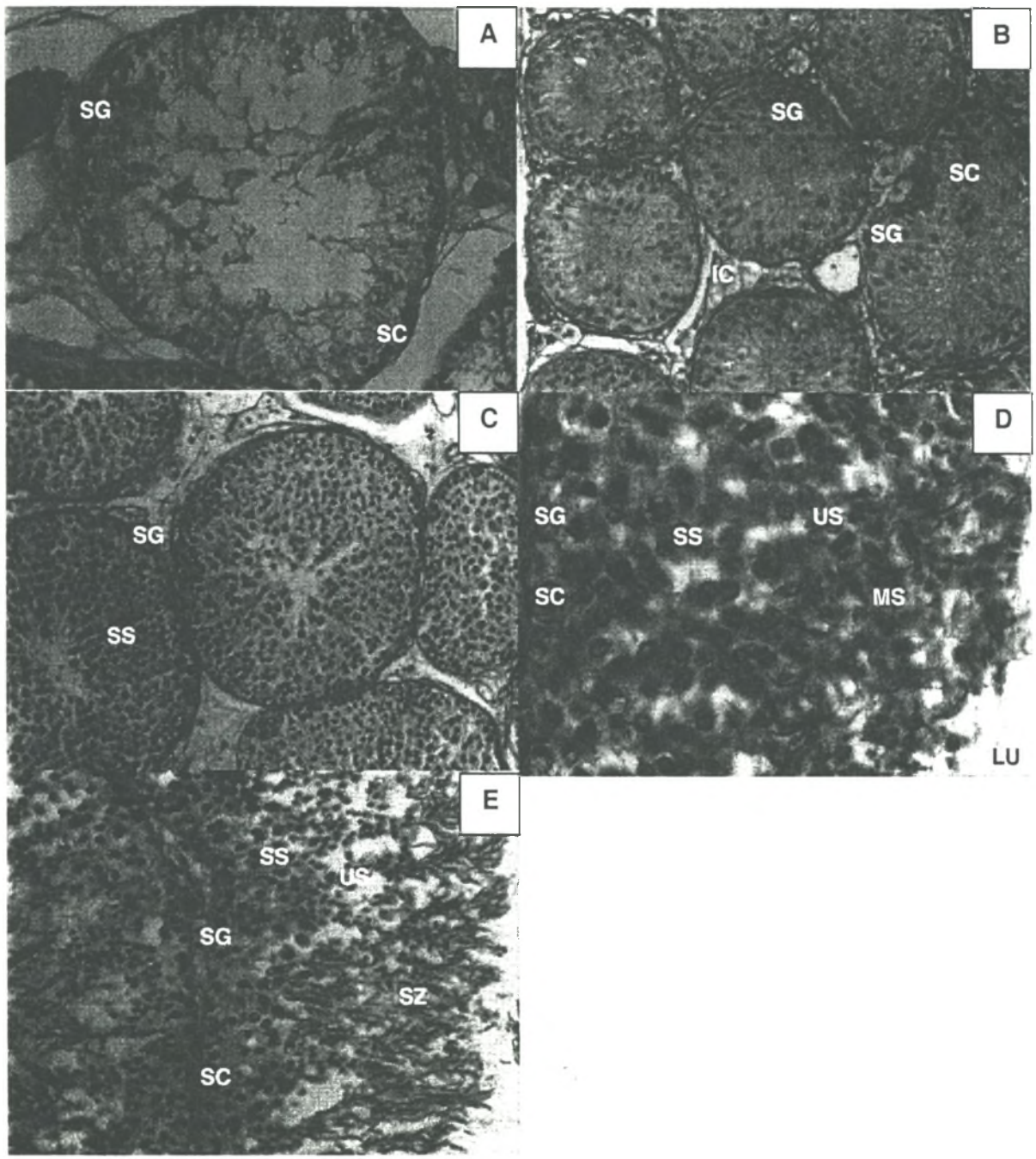

TABLE 5. Seasonal changes in male spermatogenic activity of *P. subrufa*. Classification of spermatogenic stages used was adapted mainly form Licht (1967), Mayhew (1971), Christiansen and Moll (1973), Lofts and Tsui (1977) and Dubois *et. al.* (1988). See text for description of the five spermatogenic stages used in this study.

| | | Spermatogenic stages with number of turtles in each stage | | | | |
|---|---|---|---|---|---|---|
| Month | N | 1 | 2 | 3 | 4 | 5 |
| January | 8 | | | | 2 | 6 |
| February | 8 | | | | | 8 |
| March | 8 | | | | | 8 |
| April | 5 | | | | | 5 |
| May | 19 | 11 | 5 | | | 3 |
| June | 0 | | | | | |
| July | 0 | | | | | |
| August | 2 | | 2 | | | |
| September | 3 | | 3 | | | |
| October | 13 | 1 | 5 | 7 | | |
| November | 10 | | | 5 | 5 | |
| December | 8 | | | | 8 | |

to four cell layers (stage 2), displaying resting spermatogonia with granular, rounded nuclei. The Sertoli cells, typified by larger vesicular nuclei, exhibited highly reduced nuclei during both stages 1 and 2 (Table 5). Lumina were occluded by a Sertoli syncitium in which residual spermatocytes, spermatids and spermatozoa were usually entrapped. Although the numbers of the Leydig cells were not determined, intertubular tissue was characterized by proliferation of Leydig cells. Testis volume and mean seminiferous tubule diameter remained unchanged when compared to stage 1.

Towards the end of spring and early summer (October-November), testicular volume (Figure 10A) and mean diameter of the seminiferous tubules (Figure 10B) increased further. Spermatogenic stage 3 (Figure 11C) was evident in most males (Table 5). Spermatogonia were abundant, showing mitotic activity and the appearance of primary as well as secondary spermatocytes, which outnumbered spermatogonia. Lumina were now well formed with Sertoli nuclei and spermatogonia located adjacent to the basement membrane and the other germinal cells arranged centrally in order of their development.

The presence of transforming spermatids (stage 4, Figure 11D) marked the onset of spermiation during early summer (November-December, Table 5). Acceleration of the spermiogenesis process over cell division during mid-summer (December) led to the abundance of metamorphosing spermatids and a moderate amount of mature spermatozoa at the luminal side of the seminiferous tubule.

Maximum testis volume (Figure 10A) and seminiferous tubule diameter (Figure 10B) were recorded in late summer (January to March). This coincided with peak spermiogenesis, as typified by spermatogenic stage 5 (Figure 11E). The seminiferous epithelia (Figure 10C), reduced in height and showing only rare divisions in spermatocytes, were dominated by

advanced metamorphosing spermatids. Mature and free spermatozoa were abundant and filled the lumina of the seminiferous tubules. Although the March sample was characterized by a significant decrease in testis volume (Figure 10A; March versus January, February and April, $P < 0.001$), testes exhibited increased seminiferous tubule diameters (Figure 10B; March versus May, August, September and October, $P < 0.001$) and spermatogenic epithelium heights (Figure 10C; March versus May, August and September, $P < 0.001$) combined with spermiogenic activity (stage 5, Table 5). The November sample was characterized by a significant decrease in testis volume (Figure 10A, $P < 0.001$), seminiferous tubule diameter (Figure 10B, $P < 0.05$) and seminiferous tubule epithelium cell height (Figure 10C, $P < 0.001$), however these turtle samples were all museum specimens and was therefore considered as an outlier.

Similarly, the mean adjusted testis volume (ANOVA: $F_{4,79} = 7.41$, $P < 0.001$) mean adjusted seminiferous tubule diameter (ANOVA: $F_{4,79} = 9.02$, $P < 0.001$) and epithelium cell height (Kruskal-Wallis: $H_{4,79} = 29.76$, $P < 0.05$) also varied significantly among the spermatogenic stages (Table 4).

*Plasma Testosterone*

Significant variation in plasma T concentrations occurred among months (ANOVA: $F_{5,42} = 6.11$, $P < 0.001$). Plasma T concentrations reached a prominent peak in March (ranging from 6.67 to 10.60 ng/ml), coinciding with peak spermiation (stage 5, Figure 12). Thereafter, plasma T concentrations decreased rapidly, ranging from 7.11 to 3.71 ng/ml during April (stage 5). There was no significant difference in plasma T concentrations between this point and that following emergence from brumation in August (stage 2). Plasma T concentrations continued to decrease during early spring (September), coinciding with stage 2 and 3 of spermatogenesis, early recrudescence. This period during late September (mid-spring) also coincided with the

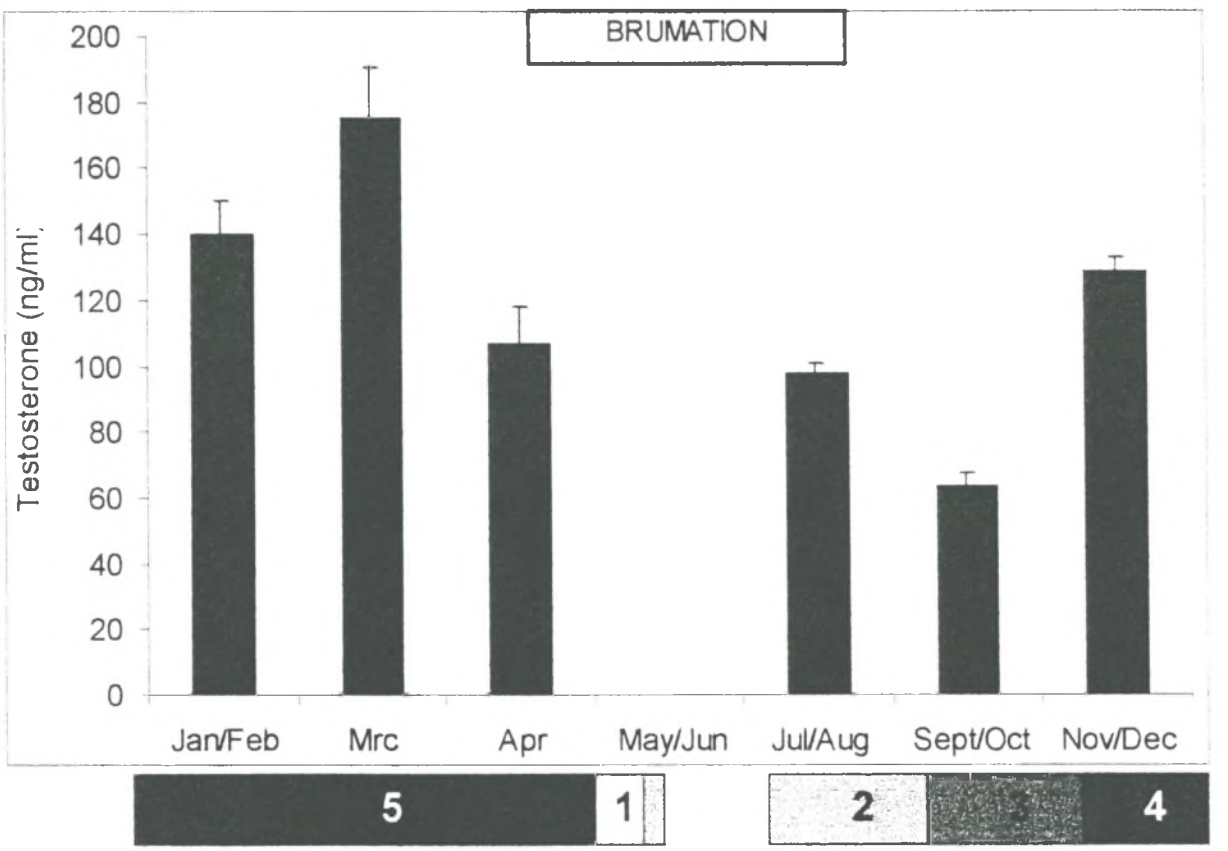


FIGURE 12. Monthly variation in the mean plasma testosterone (T) concentrations (ng/ml), during the annual reproductive cycle of male *P. subrufa* (N = 49). Values presented, represent the mean ± SE. Brumation period is indicated at the top of the graph. Spermatogenic stages are indicated at the bottom of the graph.

time when the mean testis volume, mean seminiferous tubule diameters and mean seminiferous tubule epithelium were at a seasonal low. Following the seasonal low documented for most testicular variables, plasma T concentrations similarly increased during mid-summer, November through February.

*Post-Capture Effects*

Plasma T concentrations showed no significant difference between the blood samples taken in the field and blood samples taken in the laboratory of the April sample (Student's paired t-test: $t = 1.58$, $P > 0.05$) or of the October sample ($t = 1.76$, $P > 0.05$). There was also no significant difference of the blood samples taken at first capture and blood samples taken within the same week in the field of the December sample ($t = 1.52$, $P > 0.05$). The means and standard errors for the plasma T concentrations are tabulated in Table 6.

*Ductus Epididymis*

Mean adjusted ducti epididymi diameter (ANOVA: $F_{8,74} = 2.51$; $P < 0.05$) revealed significant variation throughout the year (Figure 13), whereas mean adjusted epididymi epithelium cell height (Kruskal-Wallis: $H_{8,74} = 3.40$; $P > 0.05$) did not change significantly among months (Table 5). Abundant spermatozoa, along with eosinophilic secretions in the lumina of the ducti epididymi were present for all months, but the greatest concentration of spermatozoa were observed in autumn and spring (stage 5).

*Climatic Correlations*

Spermatogenic recrudescence in late summer / early autumn (February through April) was correlated with decreasing ambient air temperatures ($r = -0.95$, $P < 0.05$) and decreasing photoperiod ($r = -0.99$, $P < 0.05$), but increasing monthly rainfall ($r = 0.83$, $P < 0.05$). Conversely, spermatogenic regression in early spring through summer (September through

TABLE 6. Means and standard errors of the plasma T concentrations (ng/ml) of the blood samples taken in the field (field 1) and in the laboratory (laboratory 1) for turtles captured in April (N = 3) and October (N = 3), as well as the blood samples taken at first capture (field 1) and blood samples taken within the same week in the field (field 2), in December (N = 4).

| Month | Field 1 | Laboratory 1 | Field 2 |
|---|---|---|---|
| April | 4.75 ± 0.55 | 3.94 ± 0.45 | - |
| October | 3.51 ± 0.75 | 1.80 ± 0.49 | - |
| December | 6.48 ± 0.77 | - | 7.33 ± 0.79 |

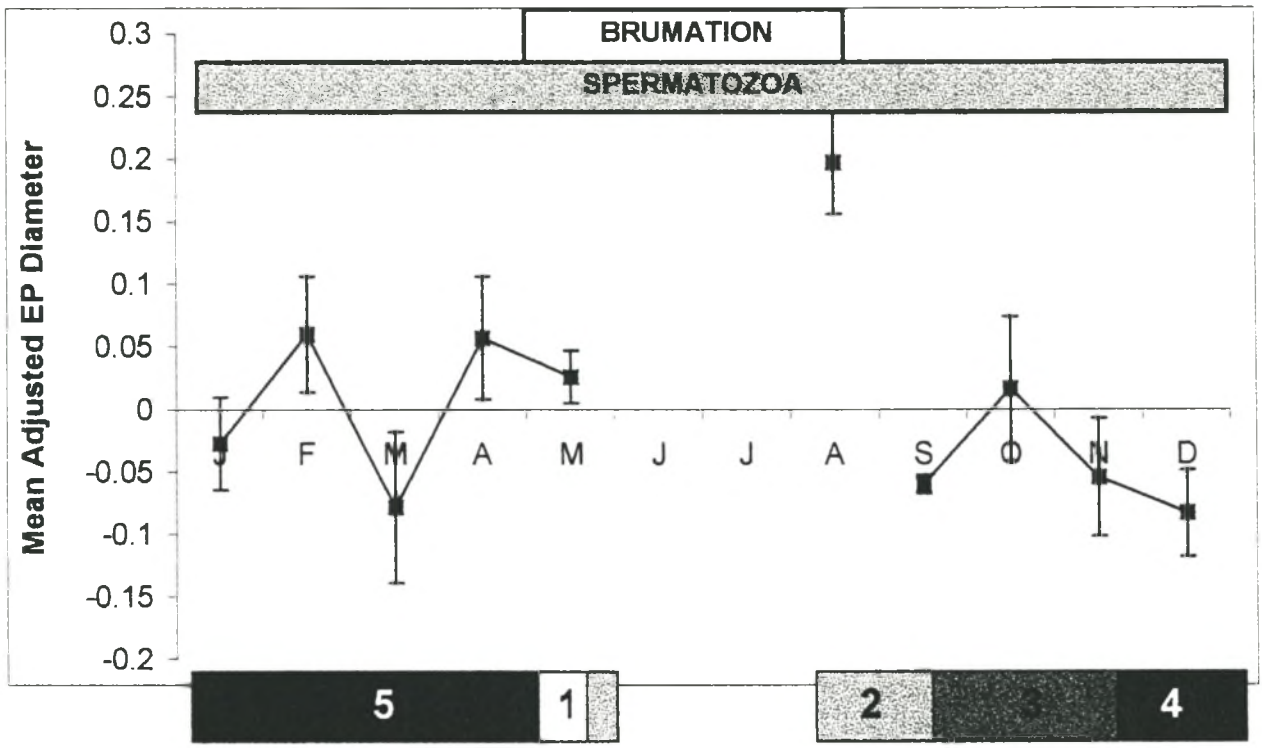


FIGURE 13. Monthly variation in the mean adjusted ducti epididymi diameters (N = 82), during the annual reproductive cycle of male *P. subrufa*. Values presented, represent the mean ± SE. Brumation period and mature spermatozoa found in the ducti epididymi (sperm storage) period is indicated at the top of the graph.

December) was correlated with increasing ambient air temperatures ($r = 0.94$, $P < 0.05$) and increasing photoperiod ($r = 0.99$, $P < 0.05$), but decreasing monthly rainfall ($r = -0.83$, $P < 0.05$).

*Male-Female Synchronization*

A summary of the timing of reproductive events in both male and female *P. subrufa* is presented in Figure 9 (Chapter 2). It was evident that male and female gonadal activities were asynchronous. Abundant spermatozoa in the ducti epididymi throughout the year however, ensure mating success in spring at the time of ovulation.

## DISCUSSION

The classical pattern of post-nuptial spermatogenesis as described by Lofts (1977), Moll (1979) and Kuchling (1999), or also known as an aestival cycle (St. Girons, 1982), is exhibited in most temperate zone turtles. Testicular activity peaks in late summer and is usually complete by the end of autumn, followed by testicular regression, sperm storage through winter (usually in the ducti epididymi) and mating activity in spring. Spermatogenesis is presumably inhibited by low ambient winter temperatures (Ganzhorn and Licht, 1983; Mendonça and Licht, 1986). The testes remain quiescent during the mating season in spring and early summer. This pattern was first established for temperate zone turtles by Risley (1933) in *Sternotherus odoratus* and then later confirmed for other temperate turtle species, e.g. *Clemmys caspica* (Lofts and Boswell, 1961), *Chelydra serpentina* (White and Murphy, 1973), *Chrysemys picta* (Ernst, 1971; Christiansen and Dunham, 1972), *Kinosternid turtles* (Mahmoud and Klicka, 1972), *Macroclemys temmincki* (Dobie, 1971), *Pseudemys scripta* (Moll and Legler, 1971), *Sternotherus minor* (Iverson, 1978), and *Trionyx sinensis* (Lofts and Tsui, 1977). The post-nuptial cycle is in strong contrast to the classic spring-summer or pre-nuptial cycle described for

many temperate lizards and snakes (for review see Fitch, 1970), alligators (Lance, 1989) and turtles (Singh, 1977). In this cycle the onset and peak of spermatogenic activity is associated with increasing ambient air temperatures (Ganzhorn and Licht, 1983).

In the warm temperate environment occupied by *P. subrufa* in South Africa, the wet seasons vary from winter rainfall in the southwestern regions to summer rainfall further north (Figure 1B). It is apparent from this study that temperature, rather than rainfall, acts as the environmental cue regulating the spermatogenic cycle of *P. subrufa*. As expected, peak testicular activity occurred in late summer / early autumn and declined towards the end of autumn / early winter. Testicular activity ceased and remained regressed throughout winter. However, in contrast to the typical post-nuptial cycle described earlier, spermatogenesis begins in spring. This corresponds with the finding of Fox (1952), with spermatogenesis beginning earlier in Southern hemisphere turtles. *Chelodina rugosa* and *Elseya dentate*, two Australian tropical turtles, possess spermatogenic cycles that mirror those of temperate zone turtles (Kennett, 1999). They are nevertheless classified as pre-nuptial because spermatogenesis begins before the breeding season (late winter). Spermiation also coincides with the start of ovulation (Kennett, 1999), which is not the case in *P. subrufa*. Mating was not observed in *P. subrufa*, but presumably commences in late spring / early summer (Ernst and Barbour, 1989; Branch, 1998). Jacques (1966) however, observed that mating takes place during the second half of summer, but he may have witnessed either late mating because those turtles were in captivity or witnessed pseudo-mating. Defining testicular cycles on the basis of their temporal relationship with the ovarian cycle may be too simplistic. A comparison of the reproduction of *Pseudemys scripta* in Panama and North America is a case in point. In both Panama and North America, male *P. scripta* follow a typical temperate zone type testicular cycle, but in North America females

ovulate from late April to July, while in Panama ovulation begins in late December and continues to May (Moll and Legler, 1971). This shift in the female cycle without any change in the male cycle effectively alters the temporal relationship between spermiation and ovulation, such that what was classified as post-nuptial breeding in North America becomes pre-nuptial breeding in Panama (Kennett, 1999).

Studies regarding the control of seasonality in reproduction have focused on a variety of exogenous factors including photoperiod, moisture, temperature, nutrition and social interaction (see Duvall *et. al.*, 1982; Marion, 1982; Licht, 1984 for reviews). Reproductive cycles of reptiles are mainly timed so that offspring are born under favourable conditions, thus enhancing their chances of survival (Moll, 1979). Reproductive cycles are not only controlled by exogenous factors, but also by endogenous rhythms. An example of the latter is refractoriness, where the gonads become insensitive to the stimulatory effects of exogenous cues (Kuchling, 1999). The refractory period is therefore useful in preventing reproduction when unfavourable conditions prevail and it may be the key factor in synchronization of reproductive cycles (Callard and Kleis, 1987). Most experimental work (Licht, 1984; St. Girons, 1985) suggests that increasing ambient air temperature is one of the major controlling factors involved in the onset of reproductive activity in temperate oviparous species. In accordance with this, spermatogenic recrudescence in male *P. **subrufa*** is confined to the relatively warm conditions of summer and autumn. The fact that male and female cycles are asynchronous in *P. **subrufa*** however, suggests that sexes respond to different environmental cues or differently to the same cues. It is clear that it is variation in the onset of spermatogenesis that results in asynchrony between male and female cycles in certain species.

The asynchrony in timing of the onset of gonadal activity between male and female *P. subrufa* corresponds closely to the asynchrony described for most temperate zone turtles (Lofts and Tsui, 1977; Georges, 1983). The degree of asynchrony in peak gametogenesis at the time of mating determines the extent of sperm storage by either males or females (Van Wyk, 1995). Although the mating period could not be ascertained precisely for *P. subrufa*, the magnitude of sperm storage in the ducti epididymidi decreased slightly after males emerged from brumation in August-September, which coincides with *Emydura krefftii* (Georges, 1983), *Emydura macquarii* (Parmenter, 1976) and *Chelodina longicoliis* (Chessman, 1978). Spring ovulation in females (Chapter 2) and the appearance of hatchlings in late summer may further support a spring mating period (Ernst and Barbour, 1989; Hofstra, 1995).

In lizards and snakes exhibiting pre-nuptial spermatogenic cycles, a single plasma androgen peak measured at the time of maximum spermiogenesis in summer is generally the rule (Lofts, 1987). This is also true for species known to exhibit a post-nuptial reproductive cycle, but plasma T concentrations peak in autumn during the time of maximum spermiogenesis (Lofts and Tsui, 1977; Johnson *et. al.*, 1982; Licht *et. al.*, 1985b; Van Wyk, 1995). Although previous studies showed a single androgen peak associated with spermatogenesis rather than mating in a typical post-nuptial spermatogenic cycle, studies in cordylid lizards indicated a bimodal androgen cycle (Flemming, 1993; Van Wyk, 1995). In the lizard, *Cordylus giganteus*, exhibiting classical post-nuptial spermatogenesis, the mating period associated with female ovulation in spring was characterized by a brief androgen peak (Van Wyk, 1995). During this period males exhibit testicular regression but abundant spermatozoa in storage in the ducti epididymi and vas deferens. Seasonal variation in plasma T concentrations of *P. subrufa* indicates a clear elevation coinciding with spermiogenesis in late summer and autumn (Figure 12). The rise in *P. subrufa*

plasma T concentrations occurs at a time when mean testicular volume is at a seasonal maximum, but the origin of this T remains obscure. Possibilities include intertubular synthesis by Leydig cells, intertubular synthesis by Sertoli cells, or an extratesticular origin (diZerega and Sherins, 1981). Few studies however, report on associated plasma androgen cycles and the question whether this bimodal androgen cycle could be regarded as a general phenomenon associated with post-nuptial spermatogenesis and asynchrony between male and female reproductive cycles, needs more study.

Licht *et. al.* (1985b) found that plasma concentrations of reproductive hormones decrease within a few hours after capture, which they attributed to stress. Kuchling (1999) suggested that the change in hormone levels might be a normal response to disturbance - if the disturbance does not persist, concentrations will return to normal. In *P. subrufa* there was not sufficient evidence to support the idea that plasma T concentrations decrease after capture.

The results of the present study established that the pattern of spermatogenesis of *P. subrufa*, although classified as a tropical to sub-tropical species, does not differ significantly from that of temperate zone turtles of the Northern hemisphere. Therefore it can be concluded that *P. subrufa* males have a post-nuptial spermatogenic cycle.

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# - CHAPTER 4 -

# Sexual Size Dimorphism in the African Helmeted Turtle, *Pelomedusa subrufa*

## INTRODUCTION

IN many species males and females attain different body sizes upon reaching sexual maturity, a phenomenon described as sexual size dimorphism (SSD). Sexual size dimorphism is assumed to have evolved as a result of three major causal factors: underlying sex-biased natural selection, intra-sexual selection and female fecundity (Stamps, 1995). These factors are not mutually exclusive and may contribute to various expressions of SSD (Huang, 1996). Ecologists, behaviouralists, and evolutionary biologists alike have attempted to understand the causal factors responsible for SSD since the 1970's (Clutton-Brock *et. al.*, 1977; Ralls, 1977; Shine, 1979). Such size differences were traditionally regarded as products of sexual selection (Darwin, 1874; Trivers, 1972), although several studies on birds and mammals suggest that ecological and physiological constraints may play an important role in the evolution of sexually dimorphic traits (Selander, 1972; Ralls, 1976; Clutton-Brock *et. al.*, 1977; Kolata, 1977; Myers, 1978). Others have suggested that SSD is a result of exogenous factors or natural selection, due to differential interactions of each sex with the environment (Schoener, 1966; Selander, 1966; Earhart and Johnson, 1970; Feduccia and Slaughter, 1974; Slatkin, 1984). Shine (1978, 1979) analysed sexual dimorphism of several reptilian and amphibian taxa and observed patterns consistent with predictions from sexual selection theory.

Generally two approaches have been used to address SSD: ultimate and proximate. Ultimate approaches examine the evolutionary history and functional significance of the

phenomenon, whereas proximate approaches explore the underlying ontogenetic, physiological and behavioural mechanisms (Hailman, 1982). Pronounced SSD is very common among turtles, with carapace length used as the standard parameter denoting body size (Berry and Shine, 1980; Fitch, 1981). Differences in body measurements and/or body proportions are commonly used to assess the proximate mechanism of sexually dimorphic traits (Fitch, 1981; Shine, 1989) and several different patterns of allometric growth in adults have been suggested to produce SSD (Hews, 1996). Documented examples range from species in which females far exceed males in size, such as *Graptemys barbouri*, *G. pulchra*, *Trionyx piniferus hartwegi* (Conant, 1975), *T. spiniferus* (Breckenridge, 1955), *Chrysemys picta* (Cagle, 1954), and *Deirochelys reticularia* (Gibbons and Lovich, 1990), to species in which males are larger, such as *Gopherus agassizi* (Woodbury and Hardy, 1948), *G. berlandieri* (Auffenberg and Weaver, 1969) and *Geochelone gigantea* (Gaymer, 1968). There are however, some species in which there is no difference between male and female size, e.g., *Sternotherus odoratus* (Tinkle, 1961; Gibbons and Lovich, 1990), *S. minor* (Iverson, 1977), and *Kinosternon flavescens* (Mahmoud, 1967).

Sexual selection pressures (i.e. male combat, female choice, male dispersal, male sequestration, etc.) suggest that the direction and degree of SSD should depend on the mating strategy of the male. Factors affecting optimal size in females (usually fecundity, but possibly predation at the nesting site) may be quite different than those affecting males. It is expected that these factors will be similar between related species so that most interspecific variability in SSD should be due to selective pressures acting on males. In species with male combat and/or forcible insemination, it is expected that males are as large or larger than females. Conversely, in groups where female choice is more important, selection is likely to favor small males as an adaptation to increase mobility (i.e. male dispersal). The specific strategy adopted by males of a

particular species should be strongly dependent on both habitat and species characteristics (e.g. forced insemination may not be a feasible strategy where females can easily elude males) (Berry and Shine, 1980). Food availability can also be one of the limiting resources for which the sexes compete (Schoener, 1967). When members of the larger sex eat larger prey than those of the smaller sex, the resulting SSD reduces intersexual food competition (Stamps, 1977; Perez-Mellado and De la Riva, 1993). However, the intersexual differences in foraging behaviour and diet are not always consistent with the ecological explanation of SSD (Perry, 1996).

The African helmeted or marsh turtle (*Pelomedusa subrufa*) is a widely distributed turtle, occurring in inland freshwater bodies of Africa and Madagascar (Loveridge, 1941; Jaques, 1966; Pritchard, 1979; Ernst and Barbour, 1989; Iverson, 1992; Zug, 1993; Hofstra, 1995; Branch, 1998; Van Wyk, 1998). *Pelomedusa subrufa* is described as a tropical to sub-tropical species (Branch, 1998), but at the most southern point of Africa the climate changes to warm temperate with specific seasonal rainfall. Data on sexual dimorphism traits are sparse and often equivocal for this species. Loveridge (1941) states that males are recognizable by their longer tails, and that their shells are narrower than females. Ernst and Barbour (1989) state that adult males have concave plastra and long, thick tails and that females have somewhat broader carapaces, flat plastra, and short tails. Branch (1998) agrees with Pritchard (1979) and Ernst and Barbour (1989) in that the males have longer tails, and narrower and flatter shells than females. The species is assumed to be an adult at an average carapace length of about 150-170 mm (Pritchard, 1979; Hofstra, 1995; Ernst and Barbour, 1989) and Branch (1998) estimates a total length of about 200-300 mm, with males growing to a larger size than females. Pritchard (1979) reported a male from Kingwilliamstown district, South Africa, with a shell length of approximately 325 mm. Differences in body size and in the size of traits such as shell measurements and head

measurements relative to overall body size are assessed to clarify SSD in *P. subrufa*. Possible explanations for the origin and maintenance of the observed SSD in shell size and head size are also discussed. Therefore the aim of this chapter was to provide baseline information on SSD in *P. subrufa*, in South Africa.

## MATERIALS AND METHODS

### *Study Areas*

Live turtles were captured from farm dams in Klapmuts (33° 51' S; 18° 50' E; altitude 177 m) and Caledon (34° 12' S; 19° 21' E; altitude 128 m), Western Cape, South Africa. In addition, preserved specimens were obtained from the South African Museum in Cape Town, the National Museum in Bloemfontein and the Transvaal Museum in Pretoria, South Africa (Figure 14).

### *Trapping and Measuring Procedures*

Turtles were trapped from September 1999 through April 2001 at approximately bimonthly intervals. Baited funnel hoop-traps were used with bait ranging from pilchards, ox liver and pig heart, to fish guts (Legler, 1960; Iverson, 1979; Plummer, 1979; Kennett, 1992; Mansfield *et. al.*, 1998). Traps were checked twice weekly. Body mass was recorded using a portable Panasonic electronic balance (± 0.1 g). Turtles were permanently marked by notching of the carapace, using the numbering system of Cagle (1939) and Honegger (1979).

Eight traits were measured on all captured turtles and museum samples: carapace length (CL), carapace width (CW), carapace depth (CD), plastron length (PL), plastron width (PW),

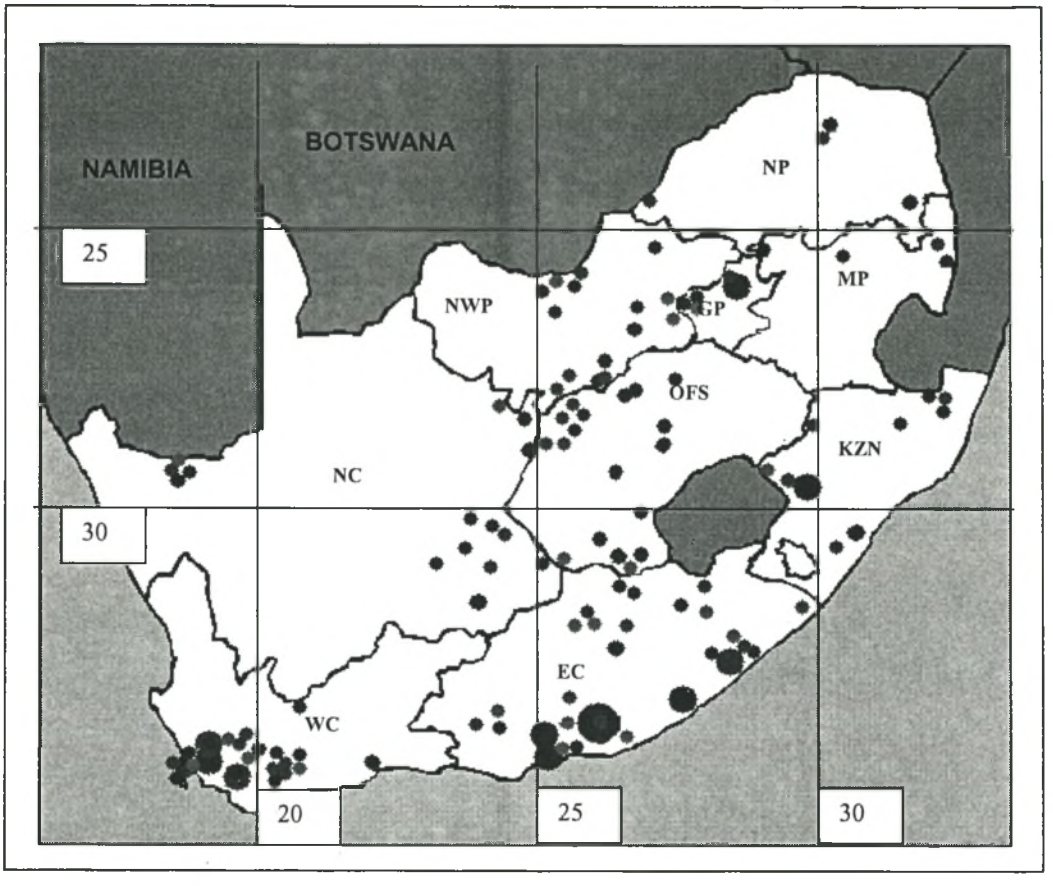



FIGURE 14. Geographic distribution of *P. subrufa* samples used in the sexual dimorphism study, South Africa. Adult males (blue circles, N = 116), adult females (red circles, N = 73) and juveniles (pink circles, N = 47). Museum samples and trapped samples were pooled. Each small circle represents one specimen, and larger circles represent two or more specimens per collection site. Winter rainfall regions include: Western Cape (WC), Eastern Cape (EC) and the southern part of the Northern Cape (NC). Summer rainfall regions include: the northern part of the NC, Orange Free State (OFS), Kwa-Zulu Natal (KZN), Mapumalanga (MP), Gauteng (GP), North Western Province (NWP) and Northern Province (NP).

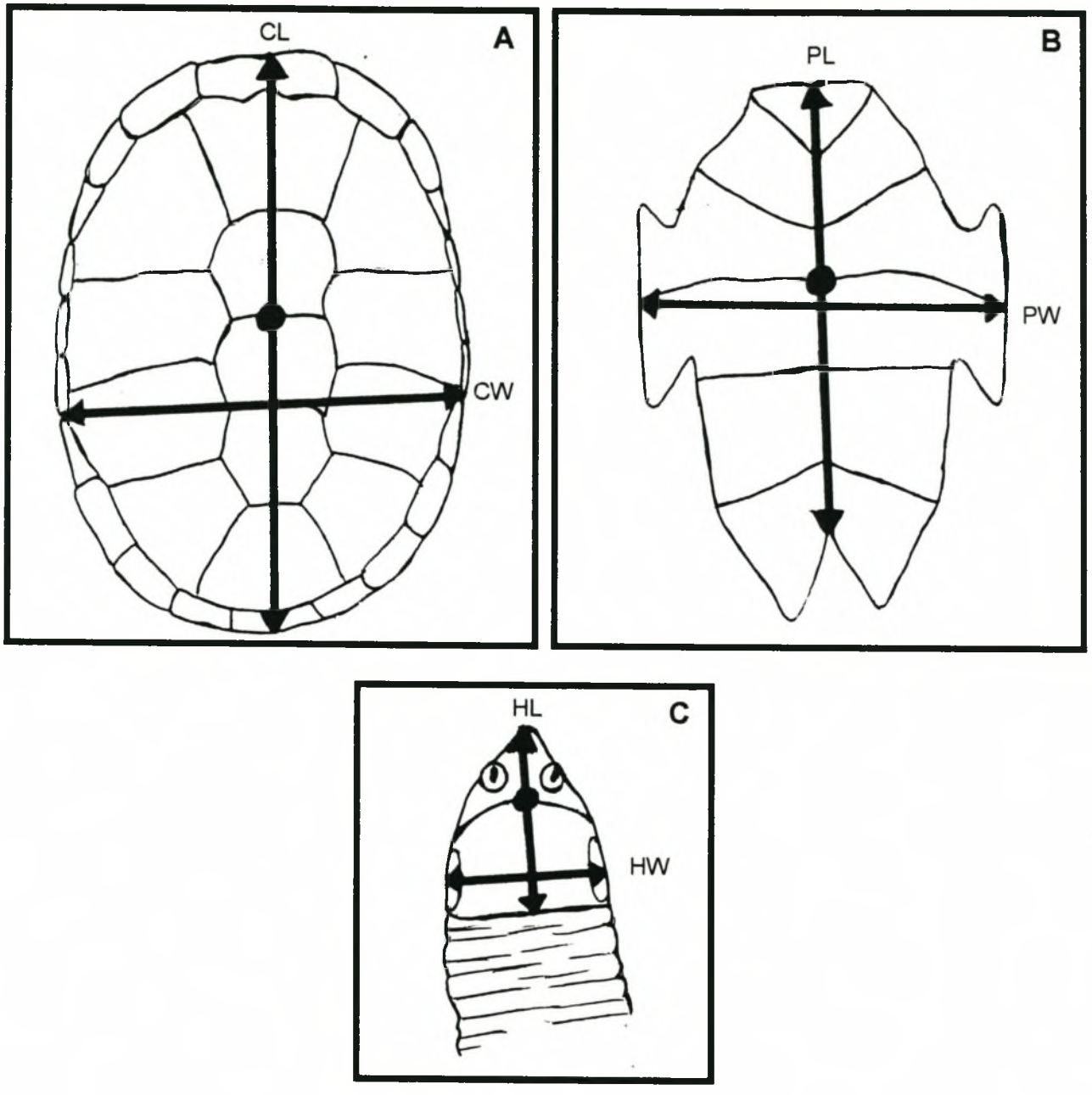
head width (HW), head length (HL), and head depth (HD) (Figure 15). All body measurements were taken to the nearest millimeter and all head measurements were taken to the nearest 0.1 mm. All measurements were log transformed prior to statistical analysis.

Sex was determined by the presence of known secondary sexual characteristics, eg. tail length (short tail in females and longer tails in males) and concave (males) or flat (females) plastron (Branch, 1998). Minimum size at sexual maturity was estimated using signs of vitellogenesis and active spermiogenesis.

*Data Analysis*

Principal component analysis (PCA) is used descriptively as a convenient way to summarize the relationships in a large set of observed variables, therefore assumptions regarding the distributions of variables are not in force (Tabachnick and Fidell, 1989). This statistical analysis attempts to maximize the variance within the sample, assuming all observations are from the same group (Ward and Smith, 1998). It also attempts to describe complex growth patterns in terms of a minimum number of basic trends and is therefore, best suited to disclose the nature and magnitude of size and shape variation (Jolicoeur and Mosimann, 1960). Principal component analysis was performed including all age / size groups (adult males, adult females, and juveniles). Three PCA's were performed to see if there was any significant differences between the adult males, adult females and juveniles, which might have been disguised by the overall body size (seven traits measured: CW, CD, PL, PW, HL, HW and HD). The second PCA included the data set limited to the shell size traits (four traits measured: CW, CD, PL and PW) and the third PCA only included traits related to head size (three traits measured: HL, HW and HD).

FIGURE 15. Schematic diagram of measurements taken for the (A) carapace, (B) plastron and (C) head of *P. subrufa*. Vertical arrows indicate the straight mid-line length, horizontal arrows indicate the straight line width, and the closed black circles indicate where the depth measurements were taken.



It is important to select an appropriate measure of body size for studies of SSD (Gibbons and Lovich, 1990) and as illustrated by Lovich *et. al.* (1990), linear measurements of CL is a better measure of body size than PL. Carapace length explained 99 % of the variance in the data, therefore CL was used as the covariate to factor out the effect of body size. An analysis of variance (ANOVA) was performed on CL to determine whether there was a significant difference among groups. An analysis of covariance (ANCOVA) was performed on the factor scores of the three categories: overall body size, shell size and head size (as indicated above), ANCOVA's were then performed on the remaining seven traits measured: CW, CD, PL, PW, HL, HW and HD. A *post hoc* comparisons test (Scheffe's test) was performed to determine differences among groups.

Significance levels for all tests were set at $P < 0.05$ unless otherwise stated. Mean values are presented with ± standard error (SE). The STATISTICA (StatSoft, Inc. 1984-2000) statistical software package was used for all statistical procedures and graphical presentations.

## RESULTS

Females reached sexual maturity between 120-160 mm CL approximately (Chapter 2), and males reached sexual maturity between 110-140 mm CL approximately (Chapter 3). For the purpose of this chapter, male and female juveniles were placed into one category because of low sample sizes (Figure 16).

### *Variation in Morphometric Traits*

All traits (overall body size)

The PCA of overall body size among the groups, the first principal component (PC1) explained 93.14 % of the variance in the data (Eigenvalue = 6.520). Component loadings for

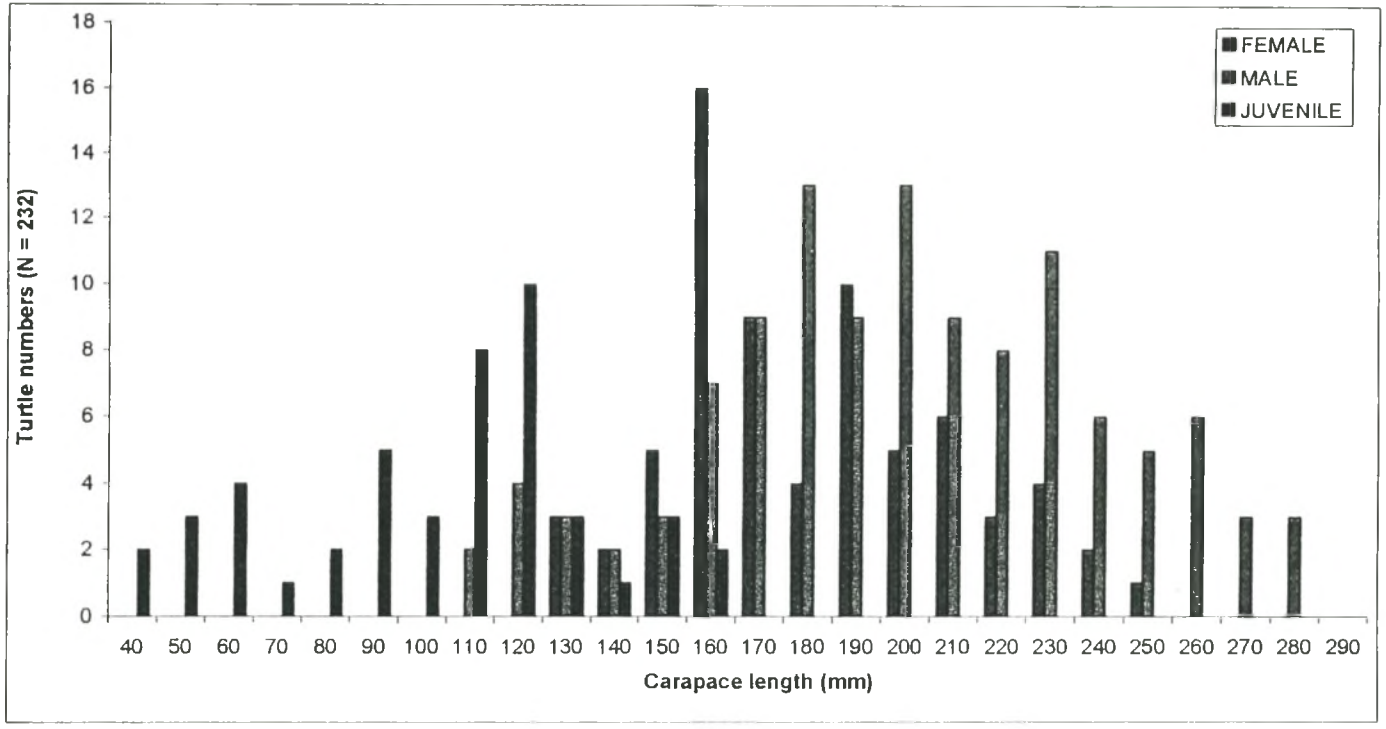



FIGURE 16. Distribution of body sizes (carapace length in mm) for adult male, adult female and juveniles of *P. subrufa* studied.

PC1 axis were: 0.990 PL + 0.987 HW + 0.986 CW + 0.983 PW + 0.967 CD + 0.965 HL + 0.872 HD. Although all component loading were high, PL and HW showed the highest loadings.

Shell size

In the analysis of the shell size, PC1 explained 97.95 % of the variance in the data (Eigenvalue = 3.914). Component loadings for PC1 axis were: 0.994 PL + 0.992 PW + 0.990 CW + 0.980 CD. Plastron length showed the highest component loading.

Head size

The principal component analysis of head size, PC1 explained 91.84 % of the variance in the data (Eigenvalue = 2.755). Component loadings for PC1 axis were: 0.980 HW + 0.967 HL + 0.927 HD. Head width showed the highest component loading.

*General Size Dimorphism*

Mean trait values

Adult males, adult females and juveniles differed significantly in absolute size of the CL, CW, CD, PL, PW and HD (ANOVA for CL, ANCOVA for rest and Scheffe's *post hoc* comparisons test). Head width and HL showed no significant difference between adult males, adult females and juveniles in absolute size. Adult males were larger than adult females in the seven traits measured, except CD where the females were larger (Table 7).

PCA Factor Scores

Analysis of covariance (ANCOVA) on PC1 (all traits) was performed between the different groups and there was a significant difference among the groups ($F_{2,232} = 8.57$; $P < 0.001$). Scheffe's *post hoc* comparisons test showed that each group was significantly different

TABLE 7. Comparison of means (± SE), and ranges between carapace length (CL), carapace width (CW), carapace depth (CD), plastron length (PL), plastron width (PW), head width (HW), head length (HL) and head depth (HD) measurements (all in mm) for adult female, adult male and juvenile *P. subrufa*. Adult male, adult female and juvenile (*Adults vs Juvenile* and *Male vs Female*) differed significantly in mean log carapace length (ANOVA, Scheffe's *post hoc* comparison test: $P < 0.001$). Comparisons of each trait were log transformed and analyzed using ANCOVA and Scheffe's *post hoc* comparisons test, using carapace length as the covariate. Superscripts indicate no significant difference ($P > 0.05$), whereas all other comparisons differed significantly ($P < 0.05$).

| | N | CL | CW | CD | PL | PW | HW | HL | HD |
|---|---|---|---|---|---|---|---|---|---|
| **Adult female** | 73 | 183.82 ± 3.45 | 139.92 ± 2.35 | 63.52 ± 1.49[a] | 162.22 ± 2.82 | 119.78 ± 2.17 | 37.45 ± 0.74[b] | 43.89 ± 0.86[c] | 21.16 ± 0.54 |
| **-range** | | 123 - 257 | 96 - 188 | 41 - 100 | 109 - 220 | 80 - 164 | 24.4 - 50.5 | 29.6 - 62.75 | 12.15 - 30.6 |
| **Adult male** | 116 | 204.52 ± 3.75 | 148.36 ± 2.37 | 62.13 ± 1.25[a] | 170.91 ± 2.91 | 124.23 ± 2.03 | 42.12 ± 0.82[b] | 48.83 ± 0.99[c] | 24.80 ± 0.57 |
| **-range** | | 110 - 284 | 79 - 205 | 34 - 97 | 92 - 232 | 65 - 178 | 24.05 - 62.1 | 15.5 - 72.5 | 13.2 - 41.75 |
| **Juvenile** | 47 | 107.45 ± 4.63 | 87.25 ± 3.45 | 34.91 ± 1.45 | 95.64 ± 4.28 | 72.53 ± 2.90 | 22.56 ± 0.86[b] | 26.38 ± 1.04[c] | 14.86 ± 0.55 |
| **-range** | | 41 - 165 | 36 - 129 | 13 - 53 | 33 - 150 | 28 - 109 | 10.7 - 35.0 | 11.7 - 40.2 | 8.4 - 25.7 |
| ***Adults vs Juvenile*** | | | | | | | | | |
| **F-value** | | 146.16 | 8.79 | 52.35 | 37.86 | 22.24 | 2.71 | 0.23 | 5.63 |
| ***P*** | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | > 0.05 | > 0.05 | < 0.05 |
| ***Male vs Female*** | | | | | | | | | |
| **F-value** | | 11.78 | 19 | 114.41 | 60.72 | 34.78 | 1.68 | 0.004 | 7.24 |
| ***P*** | | < 0.001 | < 0.001 | < 0.001 | < 0.001 | < 0.001 | 0.196 | 0.949 | < 0.05 |

from each other ($P < 0.001$) and that adult males attained the largest body size compared to adult females and juveniles (Figure 17A).

ANCOVA on PC1 scores from the Shell size PCA revealed a significant difference among groups ($F_{2,232} = 72.27$, $P < 0.001$). Scheffe's *post hoc* comparisons test revealed the same result as for the overall body size analysis ($P < 0.001$) (Figure 17B).

ANCOVA on PC1 scores from the Head size PCA revealed a significant difference among groups ($F_{2,232} = 4.62$, $P < 0.001$) and Scheffe's test showed that all groups differed significantly from each other group ($P < 0.05$) (Figure 17C). Adult males attained the largest head size. From the results of this analysis it was clear that sexually mature males were larger than females of the same age class and that PL and HW described the most variance in the body size of *P. subrufa*.

*Rate of Increase (Regression)*

Regression slopes showed that sexually mature males were not significantly different from females of similar CL in all seven traits measured (Table 8 and 9, $P > 0.05$). In shell size and head size, PL (99.4 %) and HW (98.0 %) explained the most variance in the data respectively. Therefore only these two traits were plotted to aid in visualizing the occurrence of ontogenetic growth patterns (Figures 18A and 18B respectively). Regression slopes showed a highly significant decrease in PL between adults and juveniles (Figure 18A, $P < 0.001$). There was no significant difference in shell width (CW and PW) between adults and juveniles however, there was a significant increase in depth (CD) in adults when comparing adults and juveniles ($P < 0.05$). Regression slopes for head size (HL, HW, and HD) showed a significant increase in adults compared to juveniles (Figure 18B, $P$s $< 0.05$). However, in the occurrence of ontogenetic growth patterns (Table 8), regression slopes showed no significant difference

FIGURE 17. Scatter plots of principal component analysis' factor scores (PC1) for (A) overall body size, (B) shell size and (C) head size relative to log carapace length in *P. subrufa*. Open tri-angles correspond to juveniles, whereas open circles correspond to adults of each sex, as indicated at the top of graph A. The slope of each regression is given next to the arrows. Text in brackets next to each set of arrows indicates the significance of comparisons of pairs of slopes (ANCOVA), diagonal arrows between juveniles and adults of each sex, and horizontal arrows between males and females: (NS) = $P > 0.05$, (•) = $P < 0.05$, and (••) = $P < 0.001$.

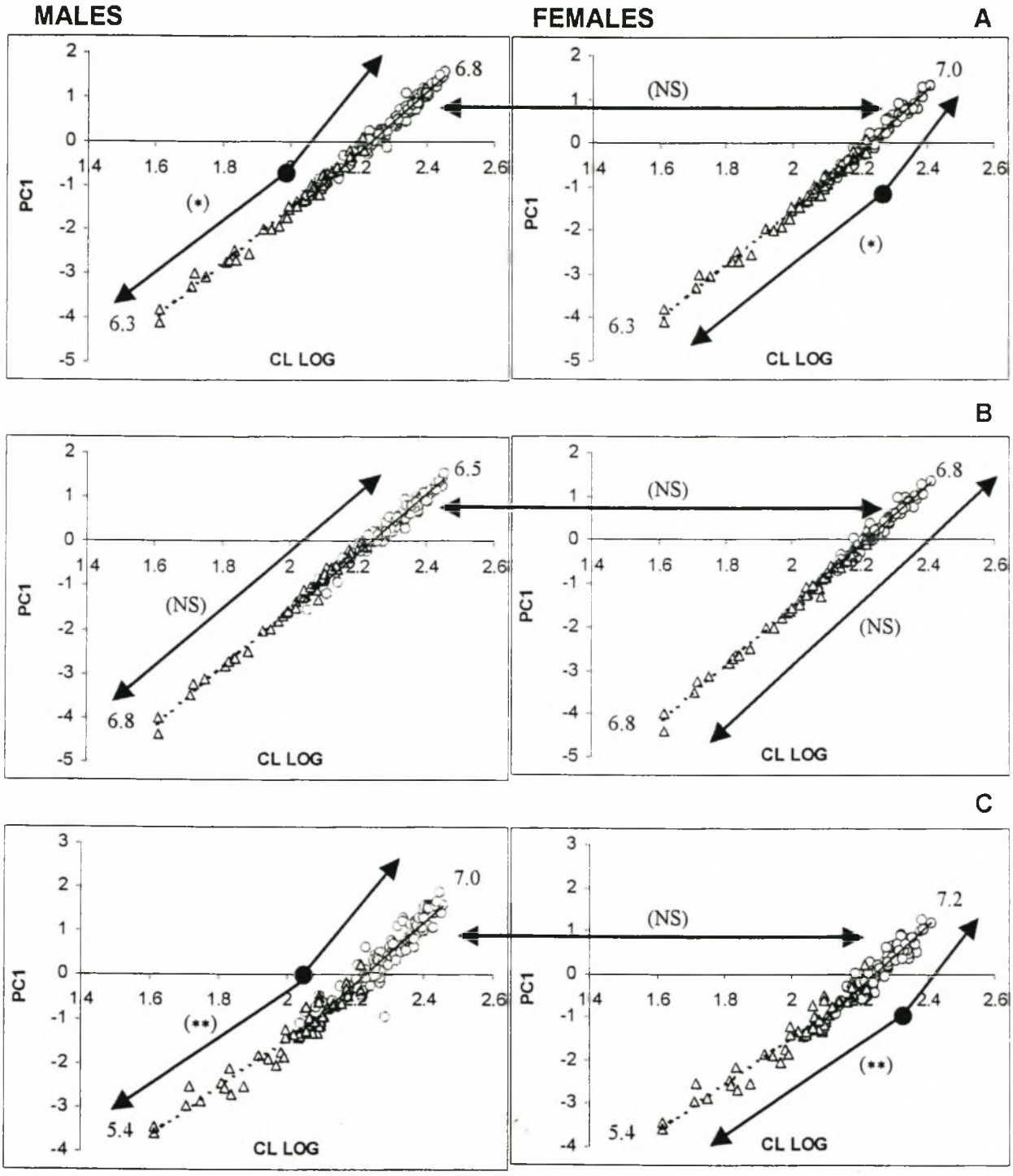


TABLE 8. Regression statistics comparing the relationships between the principal components analysis' (PCA) factor scores (overall body size, shell size and head size) and the log carapace length of *P. subrufa* by age / sex.

| | | Regression Model | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Trait** | **Age/Sex** | **$r^2$** | **F** | **d.f.** | ***P*** | **Slope** | **Intercept** | **SE** |
| Overall Body | Male | 0.967 | 3338.77 | 1,116 | < 0.001 | 6.831 | -15.28 | 0.115 |
| Size | Female | 0.955 | 1510.32 | 1,72 | < 0.001 | 7.046 | -15.68 | 0.107 |
| | Juvenile | 0.984 | 2781.47 | 1,46 | < 0.001 | 6.344 | -14.20 | 0.124 |
| Shell Size | Male | 0.966 | 3219.59 | 1,116 | < 0.001 | 6.578 | -14.75 | 0.113 |
| | Female | 0.948 | 1296.67 | 1,72 | < 0.001 | 6.838 | -15.14 | 0.112 |
| | Juvenile | 0.991 | 4988.60 | 1,46 | < 0.001 | 6.805 | -15.13 | 0.099 |
| Head Size | Male | 0.865 | 728.87 | 1,116 | < 0.001 | 7.008 | -15.63 | 0.253 |
| | Female | 0.854 | 415.81 | 1,72 | < 0.001 | 7.169 | -16.08 | 0.208 |
| | Juvenile | 0.932 | 620.38 | 1,46 | < 0.001 | 5.494 | -12.46 | 0.227 |

TABLE 9. Regression statistics comparing the relationships between the log of each trait (carapace width, carapace depth, plastron length, plastron width, head length, head width and head depth) and the log carapace length of *P. subrufa* by age / sex.

| | | Regression Model | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Trait** | **Age/Sex** | **$r^2$** | **F** | **d.f.** | ***P*** | **Slope** | **Intercept** | **SE** |
| Carapace Width | Male | 0.935 | 1641.33 | 1,116 | < 0.001 | 0.836 | 0.24 | 0.020 |
| | Female | 0.919 | 806.67 | 1,72 | < 0.001 | 0.850 | 0.20 | 0.018 |
| | Juvenile | 0.956 | 975.42 | 1,46 | < 0.001 | 0.883 | 0.15 | 0.029 |
| Carapace Depth | Male | 0.884 | 870.99 | 1,116 | < 0.001 | 1.000 | -0.52 | 0.033 |
| | Female | 0.832 | 353.00 | 1,72 | < 0.001 | 1.105 | -0.70 | 0.035 |
| | Juvenile | 0.945 | 768.95 | 1,46 | < 0.001 | 0.891 | -0.27 | 0.033 |
| Plastron Length | Male | 0.954 | 2369.52 | 1,116 | < 0.001 | 0.916 | 0.12 | 0.018 |
| | Female | 0.962 | 1821.57 | 1,72 | < 0.001 | 0.917 | 0.13 | 0.013 |
| | Juvenile | 0.995 | 8752.65 | 1,46 | < 0.001 | 1.043 | -0.14 | 0.011 |
| Plastron Width | Male | 0.924 | 1378.04 | 1,116 | < 0.001 | 0.863 | 0.10 | 0.023 |
| | Female | 0.81 | 303.20 | 1,72 | < 0.001 | 0.880 | 0.09 | 0.030 |
| | Juvenile | 0.991 | 4746.44 | 1,46 | < 0.001 | 0.913 | 0.01 | 0.014 |
| Head Length | Male | 0.737 | 319.43 | 1,116 | < 0.001 | 0.971 | -0.56 | 0.053 |
| | Female | 0.861 | 439.54 | 1,72 | < 0.001 | 0.956 | -0.52 | 0.027 |
| | Juvenile | 0.936 | 663.47 | 1,46 | < 0.001 | 0.836 | -0.28 | 0.033 |
| Head Width | Male | 0.942 | 1859.10 | 1,116 | < 0.001 | 0.999 | -0.69 | 0.023 |
| | Female | 0.875 | 497.19 | 1,72 | < 0.001 | 0.974 | -0.63 | 0.026 |
| | Juvenile | 0.968 | 1353.62 | 1,46 | < 0.001 | 0.811 | -0.29 | 0.023 |
| Head Depth | Male | 0.546 | 137.16 | 1,116 | < 0.001 | 0.850 | -0.57 | 0.071 |
| | Female | 0.499 | 70.77 | 1,72 | < 0.001 | 0.952 | -0.84 | 0.067 |
| | Juvenile | 0.634 | 77.91 | 1,46 | < 0.001 | 0.567 | 0.02 | 0.066 |

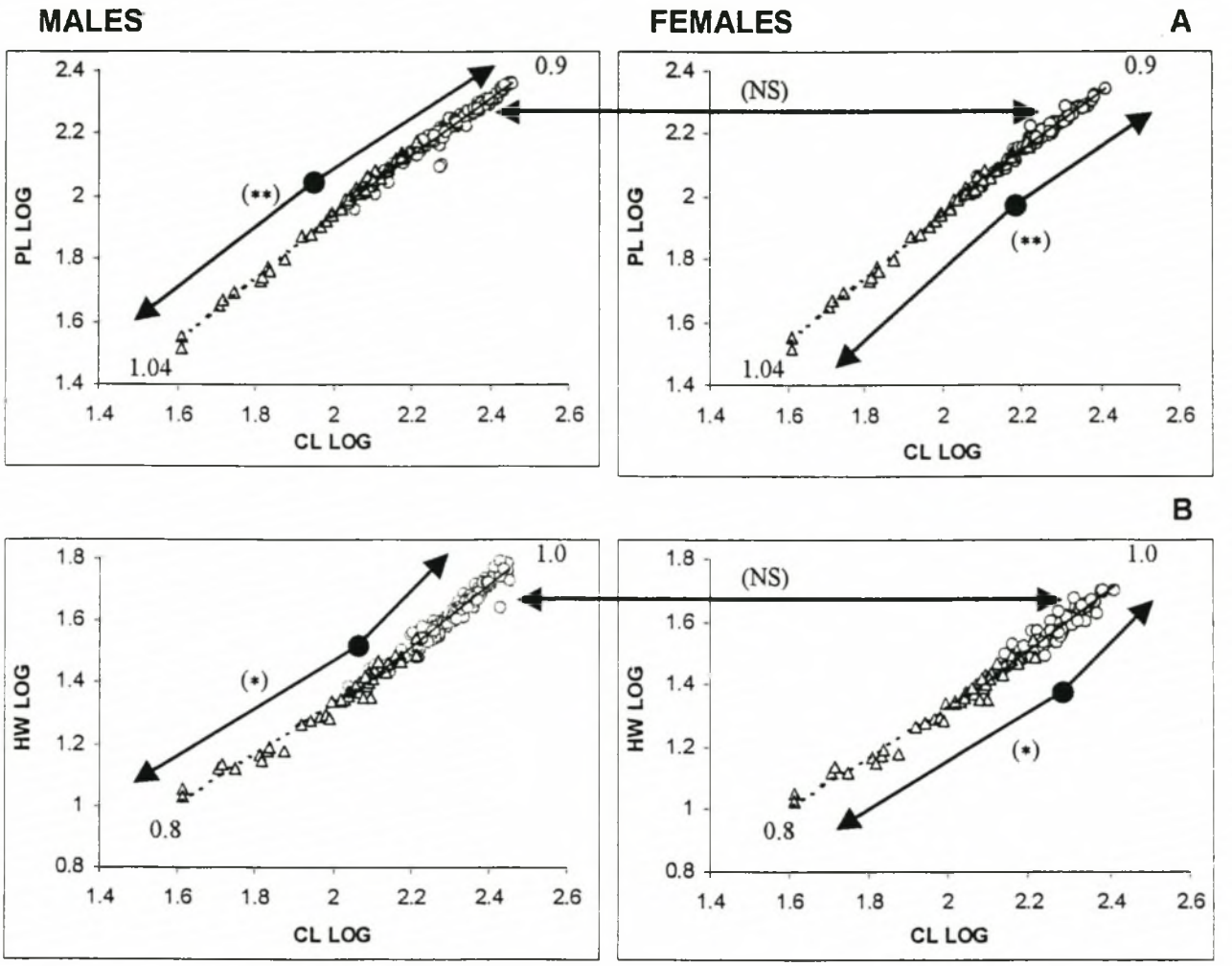



FIGURE 18. Scatter plots of log (A) plastron length (mm), and (B) head width (mm) relative to log carapace length in *P. subrufa*. Open triangles correspond to juveniles, whereas open circles correspond to adults of each sex, as indicated at the top of graph A. The slope of each regression is given next to the arrow. Text in brackets next to each set of arrows indicates the significance of comparisons of pairs of slopes (ANCOVA), diagonal arrow between juveniles and adult of each sex, and horizontal arrows between males and females: (NS) = $P > 0.05$, (*) = $P < 0.05$, and (**) = $P < 0.001$.

between males and females in overall body size (Figure 17A), shell size (Figure 17B), and head size (Figure 17C). In overall body size and head size, regression slopes showed a significant increase in adult males and adult females compared to juveniles (Figures 17A and 17C). However, in shell size there was no significant difference in the regression slopes between adults and juveniles (Figure 17B).

**DISCUSSION**

Adult *P. subrufa* exhibits strong SSD in all traits measured, except HL and HW. The growth patterns are similar to those observed in other aquatic "bottom-walking" turtle species in which the males are larger than females, eg. *Chelydra serpentina* (Ernst and Barbour, 1972), *Kinosternon hirtipes*, *K. integrum*, *Staurotypus triporcatus* (Berry and Shine, 1980), and *K. flavescens* (Lardy, 1975; Berry and Shine, 1980). Growth patterns are also similar to those observed in some semi-aquatic species, eg. *Clemmys muhlenbergii* (Ernst, 1977) and *Terrapene coahuila* (Berry and Shine, 1980), as well as in some terrestrial species, eg. *Geochelone carbonaria*, *G. denticulate* (Auffenberg, 1965), *G. berlandieri* (Auffenberg and Weaver, 1969) and *Malacochersus tornieri* (Loveridge and Williams, 1957). Berry and Shine (1980) found that patterns of SSD correlate with habitat type and male mating strategy. (1) In most terrestrial species, males engage in combat with each other. Males typically grow larger than females. (2) In semi-aquatic and "bottom-walking" aquatic species, male combat is less common, but males often forcibly inseminate females. Males are usually larger or equal in size to 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 turtle species with male combat or forcible insemination, the degree of male

size superiority increases with mean species body size. In this study no mating was observed in the field or in the laboratory, therefore it would be unwise to speculate whether males attain a larger body size than females because of either male-male combat or forcible insemination. However, turtles kept in a terrarium at the laboratory showed male aggression towards each other. Larger males would generally attempt to bite their smaller male counterparts when they approached any of the females in the terrarium, or when by chance they swam past the larger male. Although no conclusions can be made from the specific observation, if males are territorial and interact aggressively with each other and if male success is determined by body and/or head size relative to that of other males, selection should favour larger body and head size in males. It would therefore be expected that male body size would be equivalent to or larger than female body size and male head size would be larger than female head size (Cooper and Vitt, 1989).

Cooper and Vitt (1989) suggested that in adult *Sceloporus undulates*, head size dimorphism results from females increasing body size at a greater rate than head size. Another explanation for the difference in head size, could possible be explained by competitive displacement (Brown and Wilson. 1956). In the displacement model, the resources used by a given sex are determined to some extent by a particular trait. For example, larger individuals or individuals with a larger feeding apparatus may be able to consume larger food items than their smaller counterparts. Often differences in the distribution of such a trait lessen competition between the sexes for the limiting resource (Schoener, 1975; Gibbons and Lovich, 1990). Head size dimorphism in *Anolis* has been suggested to evolve from intersexual food competition (Preest, 1994). In *Tripidurus melanopleurus*, males have larger heads and feed on a wider range of food items than females (Perez-Mellado and De la Riva, 1993). The rationale is that females

allocate more energy to growth of reproductively significant characteristics in situations of limited resources (Mouton and Van Wyk, 1993). This coincides with the fact that these turtles' primary habitat, eg. freshwater bodies such as dams have a limited resource availability.

In South Africa, *P. subrufa* attains a larger overall body size compared to turtles elsewhere (Pritchard, 1979; Ernst and Barbour, 1989; Hofstra, 1995). Although the adult male turtle found by Pritchard (1979) near Kingwilliamstown, South Africa was approximately 40 mm longer in carapace length than any of the turtles examined in this study, it does correlate with the size of turtles found in South Africa (Branch, 1998). It would therefore seem that *P. subrufa* in South Africa attains a larger body size than their counterparts in Tropical to Sub-tropical regions. This correlates with Bergmann's rule, which states that geographic races of homeothermic species are usually larger in cooler than in warmer climates (Bergmann, 1847). Studies have been carried out to establish if this is also the case with poikilotherms. Ray (1960), Atkinson (1994), and Atkinson and Sibly (1997) found that poikilotherms grow to larger sizes when reared in cooler conditions. It has been hypothesized that if maintenance of the internal body temperature above ambient temperature, even for short periods of time, has a selective advantage for poikilotherms, then large individuals would be favoured at low temperatures because of their low surface to volume ratio (Lindsey, 1966). It has also been suggested that larger turtles are better able to withstand long periods of inactivity during the colder winter months, owing to a potential for accumulating greater energy stores (Galbraith *et. al.*, 1989).

The samples examined in this study were from several localities over its range in South Africa. Although any single specimen is not representative of its local population, the data are not specific to one population at one time, but instead apply to a large set of places within its range. Thus, this data presents a picture of the general SSD for *P. subrufa* across South Africa.

We can conclude that SSD of adults in the African helmeted turtle is significant, with adult males attaining a larger body and head size than adult females. The differences between the sexes can however vary according to sample size (Lovich *et. al.*, 1990) and/or because differences can vary temporally within a population according to sample size. For example, data on sizes of turtles captured at Georgian Bay between 1993 and 1995 were analyzed per year and they found that males had significantly larger mean CLs than females in 1993 and 1995, but there was no difference in 1994 (Litzgus and Brooks, 1998).

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