

**REPRODUCTION AND ITS SEASONAL VARIATION IN  
THE SOUPFIN SHARK, *GALEORHINUS GALEUS*.**

BY

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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 other university for a degree.

Signature.

Date.

**ABSTRACT**

The soupfin shark, *Galeorhinus galeus*, is a circum-global shark species of great economical importance. Their reproductive biology is reasonably well understood, but intraspecific differences between global populations necessitate the study of populations separately. For this study, 70 male and 74 female specimens were collected along the southwestern coast of South Africa between 34°8'S; 18°27'E and 34°24S; 21°25'E. Morphological changes were described and serum steroid hormone concentrations evaluated by enzyme-linked immunosorbent assay (ELISA) over an almost-complete reproductive cycle. Males display an annual spermatogenic cycle that starts with a significant peak in testosterone concentrations coinciding with the start of spermatogenesis around April-May. Testosterone levels decrease to reach a minimum in late-winter after which spermogenesis commences. The prevalence of spermigenic cysts in the testis increase to reach a peak between February and April, after which mating occurs until about July. Females (aplacentally viviparous) are suggested to have a triennial cycle, similar to the Brazilian & Australian populations, but different from the Californian & Mediterranean populations. During the first year after parturition there is not much reproductive development. The following year sees increased vitellogenic activity in ovarian follicles and development of the oviducal glands and uteri. Ovulation then occurs towards the end of that year. Mating is suggested to precede ovulation by two to three months necessitating sperm storage by the females in their oviducal glands. A 12-15 month gestation ensues and ends in parturition the following summer. Testosterone and estradiol levels fluctuate together and are involved in the preparation for ovulation and oviducal gland development. Progesterone levels show two distinct peaks during the year, in both immature and mature females, and could not yet be functionally linked to any reproductive activity or condition. This cycle is not very tightly synchronised among the South African females.

## OPSOMMING

Die vaalhaai, *Galeorhinus galeus*, kom wêreld-wyd voor en is van groot ekonomiese belang in baie lande. Die spesie se voortplantings biologie is reeds redelik deeglik ondersoek maar intraspesifieke verskille tussen verskillende wêreld populasies noodsaak die bestudering van populasies afsondelik. Daar is 70 mannetjies en 74 wyfies versamel vir hierdie studie langs die Suid Afrikaanse suid-wes kus tussen  $34^{\circ}8'S$ ;  $18^{\circ}27'E$  and  $34^{\circ}24S$ ;  $21^{\circ}25'E$ . Morfologiese veranderinge is beskryf en serum hormoon konsentrasies is ge-evalueer deur middel van die ELISA tegniek vir 'n amper-volleldige voortplanting siklus. Mannetjies vertoon 'n jaarlikse spermatogeniese siklus met 'n piek in testosteroon konsentrasies aan die begin van spermatogenese in April-Mei. Hierna daal testosteroon vlakke tot minimum vlakke in die laat-winter, waarna spermogenese begin. Die proporsie spermogeniese siste in die testis vermeerder tot maksimum waardes bereik word tussen Februarie en April. Paring vind hierna plaas tot omtrent Julie. Wyfies (aplasentaal vivipaar) het 'n voorgestelde driejaarlikse siklus, soortgelyk aan die Brasiliaanse & Australiese populasies maar anders as die Kaliforniese & Mediterreneense populasies. Tydens die eerste jaar na bevalling vind daar nie veel voortplantings ontwikkeling plaas nie. Die daaropvolgende jaar verhoog vitellogeniese aktiwiteit in die ovarium follikels en ontwikkeling van die dopkliere en uteri. Ovulasie vind nader aan die einde van hierdie jaar plaas. Paring vind heel moontlik twee tot drie maande voor ovulasie plaas, wat noodsaak dat die wyfies sperm moet stoor in hulle dopkliere. 'n 12-15 maande dratyd begin na ovulasie en eindig in geboorte teen die volgende somer. Testosteroon en estradiol konsentrasies fluktueer saam en speel 'n rol tydens voorbereidings vir ovulasie en dopklier ontwikkeling. Progesteron vlakke piek twee maal gedurende die jaar, in beide onvolwasse en volwasse wyfies, maar dit kon nog nie funksioneel geassosieer word met enige voortplantings aktiwiteit of toestand nie. Die siklus is nie baie nou gesinkroniseer onder die Suid Afrikaanse wyfies nie.

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I would like to dedicate this work to the man who inspired it and whose sudden, untimely death in August 2000 will forever remain a deep loss to all who had the privilege of knowing him, a man of the sea, Dion Sadie.

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## 1. General introduction

The soupfin shark, *Galeorhinus galeus*, (alias tope-, vitamin- or school shark) is an active, strong swimming, coastal-pelagic species of temperate continental and insular waters of the world's oceans (Compagno 1984). Being a member of the family Triakidae, *G. galeus* is a long-lived (up to 53 years old (Olsen 1984)) and slow growing species with low fecundity and is therefore highly susceptible to overexploitation (Freer 1992). In Southern Africa the soupfin shark occurs from southern Namibia all around the coast as far east as East London (Compagno 1984) and has constituted a principle component of the local commercial fisheries since World War II (Freer 1992). It is most abundant in catches at Gansbaai from early winter until about early summer (Mr. A. Nouwers, pers. comm.) but is caught almost all year round at Stilbay although in smaller numbers.

Like about half the genera of the family Triakidae, *G. galeus* is aplacentally yolk-sac viviparous (Hamlett & Koob 1999), generally meaning that the young are born alive but feed exclusively on the yolk within their eggs and receive no maternal nourishment during embryonic development. This mode of reproduction would previously have been described as a form of ovoviparity, but the classification of reproductive modes has in more recent years become more complex (Otake 1990). They aggregate in schools that segregate sexually once they reach maturity. The males and females meet annually on the continental shelf where mating supposedly takes place and after gestation, which could be anything from six months (Olsen 1954) to 12 months (Ripley 1946), females liberate their young in shallower waters near the coast (Ripley 1946; Olsen 1954; Freer 1992).

Their commercial importance globally has resulted in a reasonably thorough understanding of a number of aspects of their biology (Ripley 1946; Moulton *et al.* 1989; Stevens 1990; Peres & Vooren 1991; Moulton *et al.* 1992; Officer *et al.* 1996; Walker 1999) while Bass *et al.* (1975), Van der Elst (1981), Freer (1992) and McClusky (1988) have been the main contributors regarding the South African stock. What has become clear from the available data is that distinct differences occur in some of the reproductive aspects amongst geographically separate *G. galeus* populations (Walker 1999). Such differences between conspecific shark populations have also been reported for a number of other species (Breeder & Rosen 1966; Wass 1973; Gubanov 1978; Compagno 1984). These intraspecific differences necessitate the acquisition of reproductive data locally for each fishery and careful generalisation when comparing with data from other populations (Wourms 1977).

Our understanding of the reproductive cycles in sharks is largely limited to small, inshore species and larger, commercially harvested species. The most common problem here is obtaining a large enough sample that is representative of the entire reproductive season (Wourms 1977; Parsons & Grier 1992). The main difficulty in obtaining such a sample is caused by the seasonal migrations that many species undertake who are consequently only obtainable during certain times of the year. Among the Triakidae the most seasonal reproductive data exists for the genus *Mustelus*. The placental *Mustelus canis* (Hisaw & Abramowitz 1939; TeWinkel 1963) and aplacental *Mustelus lenticulatus* (Francis & Mace 1980) females both have an annual cycle and 10-11 month gestation period. An annual cycle and 11-12 month gestation period was described for the aplacental female *Mustelus antarcticus* (Lenanton *et al.* 1990). In a joint study on the placental *Mustelus griseus* and aplacental *Mustelus manazo*, Teshima (1981) described a 10-month gestation period for both species. Data for other triakid species indicate no seasonality in reproductive activity for *Iago omanensis* females (Fishelson & Baranes 1998) and an annual cycle for *Triakis semifasciata* (Kusher *et al.* 1992). A 10-month gestation period is also reported for *Galeorhinus japonicus* (Chen & Mizue 1973).

Ripley (1946) attempted to unravel the reproductive cycle for the female *G. galeus* from Southern California but had to base his conclusions on a rather small sample size. He suggested a 12-month gestation period, fertilisation between February and April and parturition by June (Ripley 1946). For the Australian population Olsen (1954) suggested a gestation period of six months but with only half of the female population being pregnant each year (Olsen 1954) and is interpreted as a biennial female cycle (Peres & Vooren 1991). The testes of the males from the same stock reach their maximum size between March and April and all males have discharged seminal vesicles by the end of July, indicating that mating had taken place (Olsen 1954). The Brazilian *G. galeus* population is the last global population that has been reasonably well investigated (Peres & Vooren 1991). Here a triennial female cycle is proposed with males being ready for mating over an extended five-month period (Peres & Vooren 1991).

In spite of some efforts to investigate the reproductive cycle of the South African *G. galeus* (McClusky 1988; Freer 1992), data is still fragmentary (Walker 1999). Freer (1992) reports that the gestation period cannot be less than six months, but incomplete sampling of females prevented any direct estimations to be made. In a study of mainly the male reproductive system, McClusky (1988) included data of six females, representing four months of the year, in view of the total absence of such data on South African *G. galeus* females at the time. He

also reported on spermatogenic activity for males over six months of the year and cautioned that it should be considered as preliminary. Again no firm conclusions could be drawn.

In the present study the focus is firstly on determining, for both sexes, whether the South African *G. galeus* reproduces seasonally and to describe such a seasonal cycle if it exists. This would be done on both a morphological (females), histological (males) and endocrinological (both sexes) basis. Secondly, the purpose is also to correlate morphological conditions with the hormone concentrations (testosterone, estradiol and progesterone) since very little such data exist for sharks. This study provides the first account of steroid hormone levels for the Triakidae family. The respective hormone concentrations were determined by enzyme-linked immunosorbent assay (ELISA). This technique is very seldom used for non-mammalian steroid hormones and has never been used before for quantifying shark hormones. Most studies quantify hormone levels by radio-immuno assay, but it requires relatively large sample volumes (~200 µl), long incubation times (up to 24 hours) and necessitates the use of radioactive materials. ELISA's need much smaller sample volumes (~25 µl), much shorter incubation times (one to two hours) and uses no biologically hazardous materials, making it a more ideal assay. This will hopefully pave the way for future studies of hormone levels on especially small, non-mammalian animals where small blood volume has always been a limiting factor.

Chapter one of this report serves as a general introduction to this study. Chapter two is a literature review of the morphology of the reproductive systems found among sharks, also covering reproductive endocrinology and seasonality. This chapter also summarises our current knowledge on the biology and, more specifically, the reproduction of the South African population of *G. galeus*. Chapter three looks at the reproductive cycle of the males on a histological and endocrinological basis while Chapter four focuses on the morphology and endocrinology of the female reproductive cycle. Chapter five ends this report with a discussion and conclusions on the synchronisation of the male and female reproductive cycles.

## 2. Reproduction in sharks: A focus on seasonal cycles

Living sharks (neoselachians) are classified into three groups, namely Galeomorphii, Squatinomorphii and Squalomorphii and together with the skates and rays (Batoidii) these four superorders constitute the subclass Elasmobranchii (Compagno 1973).

### 2.1 HISTORICAL AND CURRENT VIEWS

Shark reproduction has interested researchers for a long time, mainly because of their phylogenetic position as primitive vertebrates and the strategies employed by them that have usually been associated with higher vertebrates, for example internal fertilisation, highly specialised forms of viviparity and vertebrate endocrinology (Wourms 1977). Also, the fact that elasmobranchs reproduce in modes ranging from strict oviparity to placental viviparity, makes them an important and ideal group for the study of reproductive modes (Chieffi 1967).

Sharks are more similar to amphibians and amniotes than to teleosts with regards to many aspects of their reproduction (Chieffi 1967; Dodd 1983). When compared to teleosts, though, our knowledge of shark reproduction is at best fragmentary, selective and mostly descriptive (Dodd 1983). In 1997, a database was created for the United Nation's Food and Agriculture Organisation with the aim to help fisheries managers prevent over-exploitation of their shark stocks and to understand the limitations associated with commercial shark utilisation. This database was severely limited by the lack of biological and fisheries data available for sharks and emphasised the great need for shark research, especially studies concerning their reproduction.

The reproduction of both sexes has been thoroughly described in only a few selachian species. *Cetorhinus maximus* (Matthews 1950), *Scyliorhinus canicula* (Metten 1939) and *Squalus acanthias* (Simpson & Wardle 1967; Ketchen 1972) are the best examples. For recent, comprehensive reviews on elasmobranch reproduction, see Hoar (1969), Wourms (1977), Dodd (1983), Callard (1991), Hamlett (1999) and Hamlett & Koob (1999). Specific aspects of elasmobranch reproduction that have been reviewed, include viviparity (Amoroso 1960; Wourms 1981), reproductive cycles (Breeder & Rosen 1966; Dodd 1983; Callard 1991), development (Chieffi 1967), endocrinology (Dodd *et al.* 1960; Chieffi 1967; Dodd 1972; Donaldson 1973; Dodd 1975), gonad structure (Pratt 1988) and reproductive modes (Amoroso 1960; Breeder & Rosen 1966; Wourms 1981; Dodd 1983; Wourms *et al.* 1988; Otake 1990).

This review will focus on the seasonality in shark reproduction and other aspects that are linked to seasonality in reproduction (i.e. global intraspecific variation, classifying stages of development).

## 2.2 REPRODUCTIVE CYCLES IN GENERAL

### 2.2.1 Background

Wourms (1977) reviewed reproduction and development in chondrichthyan fishes and considers their diversity in patterns of reproduction and development. The scarcity of information on shark reproductive cycles is mainly due to the difficulty of getting a sufficient sample size that is representative of the complete reproductive cycle and preferably extends over more than one cycle. This problem is especially relevant in species that undertake seasonal migrations to deeper waters and are therefore not obtainable all year round, for example *C. maximus* (Matthews 1950) and *Galeorhinus galeus* (Freer 1992).

### 2.2.2 Environmental control

Annual environmental cycles such as displayed in temperature, photoperiods, physical oceanic changes (salinity, turbidity and glacier fluctuations) and food availability, amongst many other things, may have a significant effect on reproduction in fish and other marine vertebrates. Although the effect of environmental factors on the control of reproduction is widely acknowledged, it is still very ill understood. Yet we know that these govern reproduction to almost always be a cyclical phenomenon (Dodd 1972). The main environmental factor governing the reproductive cycle in *S. canicula* is temperature while photoperiod seems to have no effect (Dobson & Dodd 1977a). Temperature is also reported to act as queues for the migratory behaviour of *G. galeus* (Olsen 1954), juvenile *Carcharodon carcharias* (Casey & Pratt 1985) and *Lamna* spp. (Templeman 1963). The influences of other environmental factors on reproduction in viviparous species are still unknown, though probably quite important (Dodd 1983).

The diversity of reproduction in sharks, as displayed in their reproductive modes, embryonic development, morphology and endocrinology, has been the topic of many reviews in the past (Amoroso 1960; Breeder & Rosen 1966; Hoar 1969; Wourms 1981; Dodd 1983; Wourms *et al.* 1988; Otake 1990). It can thus be expected that the endocrine control of reproduction within this domain of diverse strategies may differ among the different elasmobranch taxa (Tricas *et al.* 2000).

### 2.2.3 Endocrine control of reproductive cycles

The gonads and other organs concerned with reproduction are closely linked with the environment by means of the endocrine system, as reviewed by Chieffi (1967), Hoar (1969) and Hamlett & Koob (1999). Environmental changes are relayed to specific centres in the brain via the sensory system.

These signals trigger neurosecretions that in turn regulate the activity of the pituitary gland. Gonadotropins are released from the ventral lobe of the pituitary, which in turn induces steroid synthesis in the gonads (see Figure 1). The presence of steroids in the testes of elasmobranchs was demonstrated by Chieffi & Lupo di Prisco (1961), but the actual site of steroid synthesis within the testis remains unclear (Hamlett 1999). Possible candidate sites from various studies cited in Hamlett (1999) include the interstitial cells, stromal cells and Sertoli cells. The latter shows activity for  $\Delta_{5,3}\beta$ -hydroxysteroid dehydrogenase in elasmobranchs, which plays an essential role in steroid biosynthesis and is normally present in Leydig cells in mammals (Simpson & Wardle 1967). It is also still uncertain whether the testis is the only source of plasma steroids in male sharks (Hamlett 1999). The ovary is the site of sex steroid synthesis and secretion in females, but, as with the males, there is some doubt about exactly where in the ovary this occurs (Hamlett & Koob 1999). It is currently believed that small ovarian follicles predominantly produce estradiol, large follicles mainly testosterone and that the corpora lutea in viviparous species produce mainly progesterone (Hamlett & Koob 1999).

Therefore, the pituitary hormones directly regulate gametogenesis, development of gonadal endocrine tissues, metabolism and behaviour. Some of these effects are taken over by the gonadal hormones (androgens, estrogens and progestins), which also co-ordinate some aspects of gamete production, fertilisation and sexual behaviour. Confirmation of the role of pituitary gonadotropins in sperm production comes from the occurrence of a degenerating band of ampullae in the testis of *S. canicula* following hypophysectomy (Dodd *et al.* 1960). Testosterone, estradiol and progesterone are the main reproductive steroids and are by far the most widely investigated. The general pattern of regulation is similar for most vertebrates, but the details can vary greatly even among closely related species. To illustrate this point Hoar (1969) compares the spotted dogfish, *S. canicula*, with the spiny dogfish, *S. acanthias*. Female *S. canicula* have no obvious breeding season and spermatogenesis in the males is continuous throughout the year. In *S. acanthias* on the other hand, there is only a two-month breeding period in females which neatly coincides with maximum sperm accumulation in males, followed by a pause in spermiogenesis (Hoar 1969).

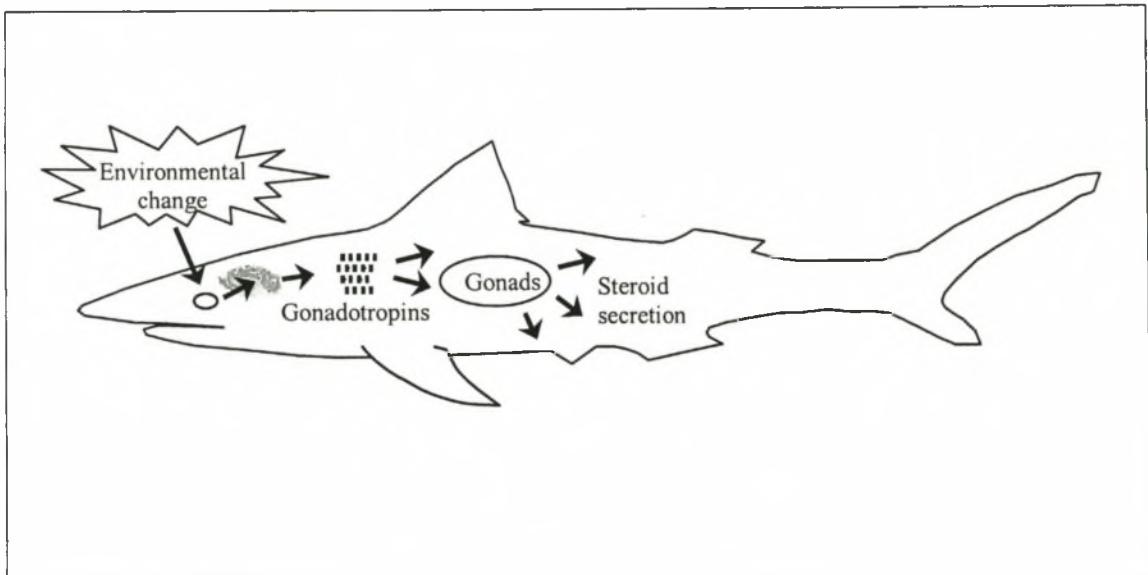


Figure 1. A simplified diagram describing the sequence of events that lead to an endocrine response. The main stimulus is environmental change, which is then detected by the sensory system and leads to production of gonadotropins. These in turn stimulate steroid secretion in the gonads.

## 2.2.4 Types of reproductive cycles

The most frequently used method for describing seasonal changes in the testes of male elasmobranchs has been the use of a gonado-somatic index and it was found that some species showed marked seasonal changes while others don't (Hamlett 1999). When mentioning the reproductive cycle of a species, it is important to remember that to say, for example, a species is reproductively active all year round, does not mean that it is the case for each individual of that species. It just means that regardless of when a population is sampled, you will find some individuals that are reproductively active and that the reproductive activity in such a case is not synchronised on species or population level. Sometimes the males and females of a species have different lengths of seasonal reproductive activity and it would lead to confusion if the sexes were not described separately.

There are three types of reproductive cycles found among male elasmobranchs. Firstly, we find species in which the males are actively producing sperm all year round with seemingly no seasonal variation in activity, for example *C. maximus* (Matthews 1950), *Raja erinacea* (cited in Wourms 1977), *P. glauca* (Pratt 1979) and *S. californica* (Natanson & Cailliet 1986). The next category belongs to those species whose males have annual testicular activity that attains maximum sperm accumulation shortly before mating and ovulation is to take place. This cycle could be referred to as pre-nuptial and is found in *Mustelus canis* (Hisaw & Abramowitz 1939), *S. acanthias* (Simpson & Wardle 1967), *Mustelus manazo* and *Mustelus griseus* (Teshima 1981) and *Rhizoprionodon terraenovae* (Parsons 1983). Males that fall into the third category of reproductive cycles also have an annual spermatogenic cycle but attain maximum sperm production some time before mating takes place. In these post-nuptial males a new spermatogenic cycle starts in the testes shortly after mating had occurred. At the end of this cycle sperm production ceases, the testes degenerate and the sperm are stored in the vesiculae seminales for extended periods of time until they mate. Examples of such reproductive cycles are seen in *Sphyraena mokarran* and *Eusphyra blochii* (Stevens & Lyle 1989) and *Mustelus antarcticus* (Lenanton *et al.* 1990). It seems, though, as if the division between these last two categories are arbitrary and dependent on the period of time that maximum sperm accumulation precedes mating for. This distinction should become better defined as more information becomes known in the future.

Three main types of reproductive cycles are described by Wourms (1977) that mainly pertain to female elasmobranchs. Firstly, there are those females that breed throughout the year like *S. canicula* (Metten 1939) and *Chlamydoselachus anguineus* (cited in Wourms 1977). These females lack any sign of a breeding season. A second type of female cycle involves

those with a partially defined annual cycle like that of *M. antarcticus* (Lenanton *et al.* 1990) and *R. erinacea* (cited in Wourms 1977). In this case the females are reproductively active throughout the year but seem to have some seasonal bouts of increased activity in terms of the percentage females that are, for example, gravid at that time. The third category of females described by Wourms (1977) have well-defined annual (*S. acanthias*, cited in Wourms 1977), *Carcharias taurus* (Gilmore 1993)) or biennial (*M. canis*, (Hisaw & Abramowitz 1939) reproductive cycles. A triennial cycle was reported for *G. galeus* from Brazil (Peres & Vooren 1991) and would belong to this category as well. Sharks that breed according to similar reproductive cycles seem to have ecological factors as common denominator rather than phylogenetic relatedness. The genus *Heterodontus* have species that would be classified into all three these above-mentioned categories (cited in Wourms 1977) while annual, biennial and triennial cycles have been reported for different *G. galeus* populations (Walker 1999). The relative constancy of the deep-sea habitat of *C. anguineus* was suggested to be the reason for its year-round reproductive activity (cited in Wourms 1977).

Another interesting phenomenon of female shark reproduction is that in some species the ova of the following generation undergo vitellogenesis while the previous generation's embryos are still developing in the uteri. Examples include *Mustelus lenticulatus* (Francis & Mace 1980), *R. terraenovae* (Parsons 1983), *M. antarcticus* (Lenanton *et al.* 1990) and some viviparous species cited in Lenanton *et al.* (1990). More information is needed to better understand its significance.

## 2.3 MALE REPRODUCTIVE SYSTEM AND CYCLICITY

### 2.3.1 Gross reproductive morphology

Shark species whose male reproductive morphology has been well studied include *S. canicula* (Metten 1939), *C. maximus* (Matthews 1950), *S. acanthias* (Jones & Geen 1977), *P. glauca* (Pratt 1979), *M. griseus* (Teshima 1981), *M. manazo* (Teshima 1981) and *G. galeus* (McClusky 1988).

Wourms' (1977) review offers a thorough description of the reproductive system of male sharks, but the more concise and up-to-date description of Hamlett (1999) will be briefly summarised here for the sake of terminology. The male reproductive system, as illustrated in Figure 2, consists of testes, accessory glands, genital ducts and secondary sex organs. The latter include the claspers (used for copulation) and the siphon sac, which is replaced by a clasper gland in batoids (skates and rays).

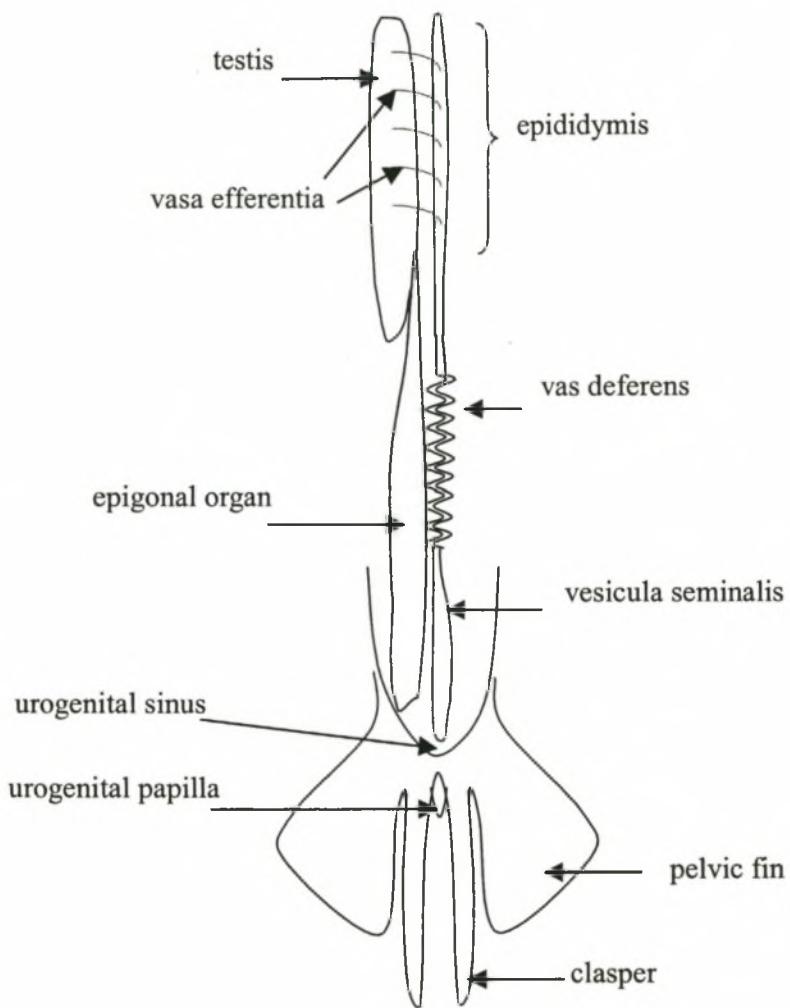


Figure 2. Schematic representation of the reproductive system of a male shark as viewed from the ventral side. Only the internal organs and ducts on the right hand side of the reproductive system are shown for ease of illustration.

### **2.3.2 The testis: macroscopically and histologically**

The testis is paired in elasmobranchs and is suspended from a mesorchium. It is responsible for the production of both germ cells and steroid hormones. The epigonal organ, which consists of lymphoid tissue, is closely associated with the testes only in elasmobranchs and not in holocephalans. It is a large organ in most sharks, but in the carcharhiniform *Gollum attenuatus*, the epigonal organ cannot be observed macroscopically (Yano 1993).

Pratt (1988) reviewed Elasmobranch gonad structure, giving most attention to selachian gonad types and only a preliminary description of batoid gonads was given. Three types of elasmobranch testes were described, namely diametric, radial and compound, and were found to be characteristic of different groups of sharks (see Figure 3). 1) Diametric testes, as found among carcharhinids and sphyraenids, refer to the lateral development of follicles across the diameter of the cylindrical testes. The germinal zone in this case is situated disto-laterally on the testes and development spreads medially. In cross section, this type of testis can easily be divided into zones corresponding to the different stages of spermatogenesis. 2) Radial testes are also cylindrical and were ascribed to lamnids and alopiids. Here the testis is divided into a highly variable number of lobes, each with a central germinal zone. Follicular development then ensues radially from the center outward. 3) The third type of testis, namely compound, is found in rajids and constitutes a combination of the radial and diametric modes of follicular development. This type of testis is less cylindrical, being laterally compressed, and appears lobular on the surface although it is not. It consists of islands of germinal centra, all situated on the disto-lateral surface. Cystic development then spreads from the center of each island along the surface until their borders meet, from where it submerges medially away from the surface (Pratt 1988).

### **2.3.3 The efferent duct system: sperm transport and storage**

Sharks have between two and six vasa efferentia while skates and rays have only one (Wourms 1977). The vasa efferentia joins the epididymis which is normally a heavily coiled structure. The epididymis leads to the vas deferens (ductus deferens) which, in some elasmobranchs, have sperm sacs attached to its posterior end as diverticula. The vas deferens is a straight, thin tube in juveniles, but becomes coiled and largely expanded when maturity is reached. There is considerable variation in size of the vas deferens among elasmobranchs and it is often responsible for storage of sperm or the site of spermatophore formation. Leydig's gland, which is the anterior part of the kidney that has lost its excretory function and is responsible for secreting most of the seminal fluid, empties into the vas deferens.

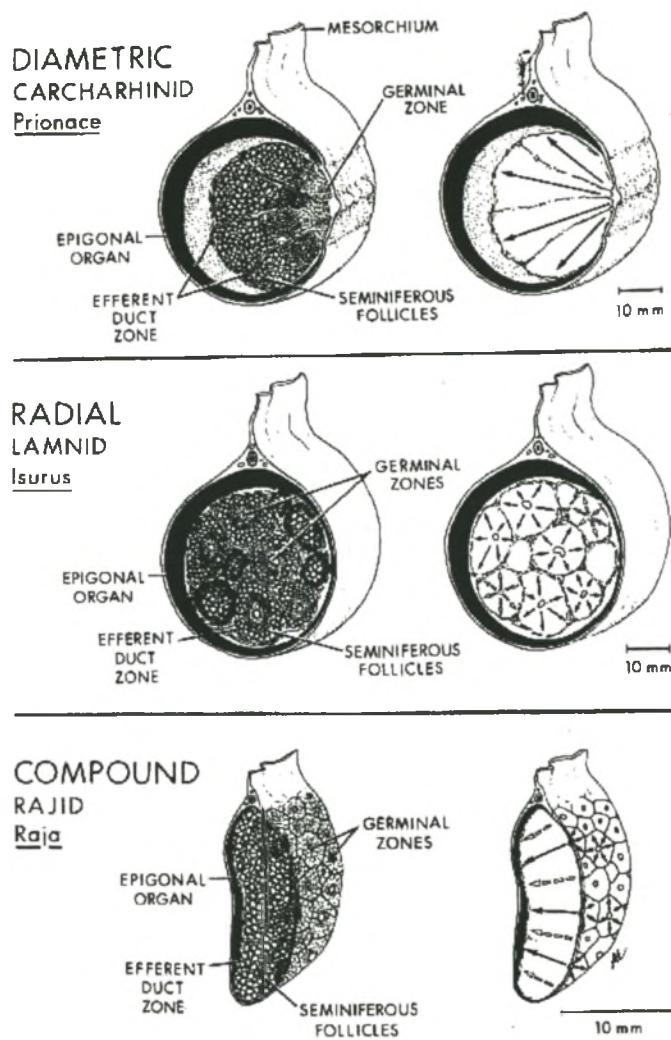


Figure 3. Diagram of the three types of mature elasmobranch testes in cross section, viewed from anterior. The illustrations on the left are exaggerated to show the development of the follicles. The accompanying illustrations on the right are schematic and the arrows indicate the direction of cyst development. Copied from Pratt (1988).

Sperm moves from the vas deferens into the vesicula seminalis, which in turn empties into the urogenital sinus. This sinus is formed by the joining of the two seminal vesicles and leads to the cloaca via the urogenital papilla (Hamlett 1999).

#### 2.3.4 Secondary sexual organs

Male elasmobranchs have two secondary sex organs, namely the claspers and siphon sacs (= clasper glands in batoids). Male sharks can easily be distinguished externally from females by the presence of claspers. These are paired modifications of the pelvic fins that are used as copulatory organs during mating to achieve internal fertilisation. Claspers have a rather complex internal structure and variations have been described in a great many species (cited in Hamlett (1999)). In sharks, sperm moves from the urogenital papilla into the clasper tube during copulation. The siphon sacs, consisting of blind ending pockets that open into the base of the claspers, then wash the sperm through the clasper tube. In *S. acanthias* the siphon sacs of mature animals contain a secretion that is rich in serotonin (Mann 1960). During copulation, muscle action fills the siphon sacs with seawater and then contracts it so that sperm is washed through the clasper groove into the female's oviduct. In batoids the clasper gland expels a secretion that pushes the sperm through the clasper tube. The secretion is also believed to have a function of sealing the clasper groove to form a solid tube since it coagulates on contact with seawater (Mann 1960).

#### 2.3.5 Classifying stages of maturity

In order to make sensible comparisons among species and between different studies on the same species, it is helpful if the different stages of maturity are described on the same basis. Some parameters, for example the presence of sperm, cannot serve as a conclusive indicator of reproductive activity by itself. Ideally, a combination of parameters should be used in order to prevent false and hasty conclusions. For example, in the absence of sperm the animal could be classified as immature, while it might actually only be between spermatogenic cycles at the time. The opposite may also be true since sperm are often present in males whose claspers are not yet rigid enough to be capable of mating (Bass *et al.* 1975). In most studies a combination of parameters are used (Pratt 1979). These include size and degree of calcification of the claspers, relative growth of the claspers, the degree of folding of the deferent ducts, testis size, siphon-sac development, presence of sperm in seminal vesicles and sperm sacs and vascular congestion around the cloaca (Templeman 1944; Kauffman 1950; Matthews 1950; Olsen 1954; Springer 1960; Clark & Von Schmidt 1965; Wass 1973; Bass *et*

*al.* 1975; Parsons 1983; Castro *et al.* 1988; Chen *et al.* 1988; Stevens & Lyle 1989; Capapè *et al.* 1990; Moreno & Moron 1992; Yano 1993).

### 2.3.6 Spermatogenesis and its cyclic activity

Spermatogenesis in anamniotes takes place in the mature testis in units called spermatocysts (Callard 1991), previously known as ampullae. In contrast to this, spermatogenesis in amniotes occurs in seminiferous tubules. Spermatoblasts, which consist of a single Sertoli cell and its associated germ cells, join together to form the spermatocysts. Within the spermatocyst, the germ cells develop synchronously until the mature sperm are expelled through the efferent ducts. Asynchronous development within a spermatocyst, which is supposedly rare, has been recorded in *G. galeus* (McClusky 1988).

A brief summary of spermatogenesis in elasmobranchs from Wourms (1977) follows. Each spermatogonium divides and develops to form 16 primary spermatocytes that undergo meiosis to form 32 secondary spermatocytes. These then develop into spermatids that grow to become mature sperm during spermiogenesis. The latter process happens in much the same fashion in sharks as in other vertebrates. Mature sperm bundles are released from the follicle and flow into the collecting ductules, the vasa efferentia. The mature sperm are relatively large, exceeding 100 µm. The head (30–40 µm) is spirally twisted like a corkscrew and is 10 to 20 times larger than those of teleosts.

Spermatogenesis has been described as a seasonal phenomenon in the testes of only a handful of species, namely *S. acanthias*, *S. canicula*, *M. griseus*, *M. manazo*, *Sphyrna tiburo*, *C. limbatus* (cited in Parsons & Grier 1992) and *D. sabina* (Maruska *et al.* 1996). On the other hand, *C. maximus* (Matthews 1950), *S. californica* (Natanson & Cailliet 1986) and *P. glauca* (Pratt 1979) seem to maintain reproductive potential throughout the year in the absence of any obvious seasonal fluctuation in sperm production.

The diametric testis type of carcharhinids and sphyrmids, as described by Pratt (1988), reveals the different stages of spermatocyst development clearly when viewed in cross section. Distinct zones are evident as the spermatocyst development progresses diametrically across the width of the testis. Hamlett (1999) summarises seven spermatocyst stages that are described and used by the majority of authors:

Stage 1: <4 spermatogonia on the periphery of the spermatocyst

Stage 2: 4 - 8 spermatogonia with Sertoli cell nuclei migrating to the edge of the spermatocyst

Stage 3 - primary spermatocytes with large nuclei

Stage 4 - secondary spermatocytes with round, small nuclei

Stage 5 - appearance of spermatids with elliptical nuclei and emerging flagella

Stage 6 - loose aggregations of spermatozoa around the periphery of the spermatocyst

Stage 7 - sperm released into the collecting efferent ducts

The sizes of the spermatocysts increase up to stage four where they reach a maximum diameter (400 µm in *S. tiburo*, 350 µm in *S. canicula*). Some workers have described up to 18 different stages of spermatocyst development (cited in Parsons & Grier 1992), but such detail is normally deemed unnecessary. All of these stages of spermatocyst development are present throughout the year in the testes of most species (Parsons & Grier 1992), but the composition thereof differs throughout the year in seasonal males. These fluctuations in proportionate representation of the different spermatocyst stages are a useful tool in describing reproductive cycles, as was first used in *S. acanthias* (Simpson & Wardle 1967).

Seasonal changes in shark testes are most commonly expressed in terms of a gonadosomatic index (GSI) which is a dimension of the testis (weight or length) expressed as a percentage of the same dimension of the whole animal (Parsons & Grier 1992). The annual changes in GSI that occur are quite substantial for some species and are correlated with a defined mating season in most cases. In *D. sabina* the phases of changing GSI was closely correlated with changes in the testes structure (Maruska *et al.* 1996), which in turn correlated with changes in hormone levels (Tricas *et al.* 2000). A low GSI coincided with low spermatogenic activity in this case. Despite the value of the GSI to implicate readiness for mating, it should be remembered that peak testicular activity and mating does not always coincide and that mating season should not be determined by GSI alone (Parsons & Grier 1992). Peak mating activity can be indicated by the presence of enlarged testes, substantial amounts of semen in the seminal vesicles and swollen, bleeding claspers in adult males (Bass *et al.* 1975).

### 2.3.7 Male reproductive endocrine cycles

Information on circulating steroid hormone levels in male sharks is still rather limited (Hamlett 1999). Fortunately, there has been a recent surge of studies focussing on the cycles of steroid production in selachians. One example is the annually reproducing Atlantic stingray, *D. sabina* (Tricas *et al.* 2000). Androgen levels are low between reproductive seasons, show a primary increase at the onset of spermatocyst development, decrease after maximum testis growth and spermatocyst development and finally show a secondary increase when sperm maturation is at its peak. Estradiol and progesterone levels increase concurrently with spermatocyst formation, maximum testis weight and the primary androgen surge (Tricas *et al.* 2000).

In *S. canicula*, spermatogenesis is associated with high testosterone production levels (Tricas *et al.* 2000). The maximum testosterone levels occurred in February and then slumped to its minimum from May to July (Garnier *et al.* 1989). A second, milder peak in testosterone concentration appears in September, coinciding with sperm maturation (Garnier *et al.* 1989). The progesterone levels showed a weak cycle and plasma estradiol was constant throughout the year (Garnier *et al.* 1989).

In *S. tiburo* the association of testosterone, dihydrotestosterone and progesterone with the spermatogenic cycle is similar to that found in *S. acanthias* (Manire & Rasmussen 1997). The levels of these three steroids all increased concurrently along with testicular development and declined once spermatogenesis had peaked. The concentration of testosterone reached a maximum of 303 ng/ml, which is very high when compared to most vertebrates. At the time of mating, testosterone and dihydrotestosterone levels were declining together with the GSI. Progesterone covaries with testosterone and dihydrotestosterone in *S. tiburo*, but varies independently of testosterone and dihydrotestosterone in *S. canicula* and *D. sabina* (Manire & Rasmussen 1997). Although yet an unresolved matter, progesterone is considered to be a precursor for the other steroids in male elasmobranchs (Manire & Rasmussen 1997). Furthermore, the significant relation of progesterone to stress in *S. tiburo* suggests a dual function of progesterone in this species (Manire & Rasmussen 1997). The way that the level of estradiol in male *S. tiburo* varied, was very different from that of testosterone, progesterone and dihydrotestosterone and was the only one of these hormones that was present in significantly lower concentrations in males than in females (Manire & Rasmussen 1997). The role of estradiol seems to be limited to the initiation of testicular recrudescence in *S. tiburo* and further work is needed to investigate other possible regulatory roles (Manire & Rasmussen 1997).

In a study involving 11 species from the family Carcharhinidae, Rasmussen & Gruber (1990) presented the first data on serum levels of testosterone, estradiol, progesterone, dihydrotestosterone and corticosterone for this family of sharks. Unfortunately sampling occurred only during spring and autumn and consequently only limited seasonal correlations could be made. Estradiol and testosterone were both found to be important in the sexual development of immature carcharhinoid sharks and especially testosterone had a reasonably wide range in *Carcharhinus perezi* (Rasmussen & Gruber 1990). A mature *Carcharhinus leucas* male topped the testosterone concentrations with 358 ng/ml, which is one of the highest yet recorded in any vertebrate (Rasmussen & Gruber 1990).

Earlier work on *Raja radiata* and *Raja ocellata* indicates testosterone levels that are comparatively high in males. During the reproductive cycle of *R. radiata*, testosterone displays an almost 10-fold range (Idler & Truscott 1966). Some of the recorded minimum and maximum levels of testosterone, estradiol and progesterone are summarised in Table 1.

## 2.4 FEMALE REPRODUCTIVE SYSTEM AND CYCLICITY

### 2.4.1 Gross reproductive morphology

Shark species whose female reproductive morphology has been well studied include *S. canicula* (Metten 1939), *C. maximus* (Matthews 1950), *Galeorhinus japonicus* (Chen & Mizue 1973), *P. glauca* (Pratt 1979), *M. griseus* (Teshima 1981), *M. manazo* (Teshima 1981) and *C. taurus* (Gilmore *et al.* 1983). Wourms (1977), Hoar (1969) and Hamlett & Koob (1999) provide extensive reviews on the reproductive morphology of female sharks. The female reproductive system consists of ovaries, an ostium and paired anterior oviducts, oviducal glands, isthmi and uteri as illustrated in Figure 4.

### 2.4.2 The ovaries

In his review of elasmobranch gonad structure, Pratt (1988) identified two major types of elasmobranch ovary, namely external and internal. External ovaries occur in carcharhinids and sphyraenids on the distal surface of the epigonal organ. Typically only the right ovary is present and it consists of follicles that are contained under a single layer of generative tissue through which the ova are discharged during ovulation. In various species several stages of follicular development occur simultaneously. Batoids also have external ovaries, but they differ from carcharhinids and sphyraenids in that some variation exists within this group. Marked asymmetry is reported in *Squatina argentina* (cited in Capapè *et al.* 1990), the majority of cases in *S. californica* (Natanson & Cailliet 1986) and in *Squatina dumeril* (Merriman & Olsen 1949; Backus 1957), where the right ovary is either atretic or non-functional. Both ovaries are active and functional in *Squatina squatina* and *Squatina oculata* (Capapè *et al.* 1990). *S. squatina* does, however, display functional asymmetry in the ovary by having more maturing oocytes in the right ovary than in the left, while *S. oculata* is fully symmetrical in this respect (Capapè *et al.* 1990).

Table 1. Summary of the minimum and maximum recorded levels (in ng/ml) of testosterone, estradiol and progesterone in mature male elasmobranchs. The ranges for mature and immature *Galeorhinus galeus* recorded in this study are included in **bold**.

Species	Testosterone min	max	Estradiol min	max	Progesterone min	Max
<b>Mature</b>						
<i>Dasyatis sabina</i> <sup>a</sup>	0.01 <sup>g</sup>	17.3 <sup>h</sup>	0.005	0.27	0.01	0.77
<i>Scyliorhinus canicula</i> <sup>b</sup>	5	21 <sup>i</sup>	<0.1	<0.1	0.1	0.9
<i>Sphyrna tiburo</i> <sup>c</sup>		303		0.08		11.6
<i>Carcharhinus perezi</i> <sup>d</sup>	0.85	3.1	0	0.1	0.03	0.21
<i>Carcharhinus leucas</i> <sup>d</sup>		358				
<i>Raja radiata</i> <sup>e</sup>	28	102				
<i>Raja ocellata</i> <sup>e</sup>	22	208				
<i>Galeorhinus galeus</i> <sup>f</sup>	<b>54.3</b>	<b>1498</b>				
<b>Immature</b>						
<i>Galeorhinus galeus</i> <sup>f</sup>	<b>0.4</b>	<b>12.1</b>				

<sup>a</sup> Tricas *et al.* (2000)

<sup>b</sup> Garnier *et al.* (1989)

<sup>c</sup> Manire & Rasmussen (1997)

<sup>d</sup> Rasmussen & Gruber (1990)

<sup>e</sup> Idler & Truscott (1966)

**This study**

<sup>g</sup> between reproductive seasons

<sup>h</sup> sperm maturation

<sup>i</sup> maximum sperm in vesicula seminalis

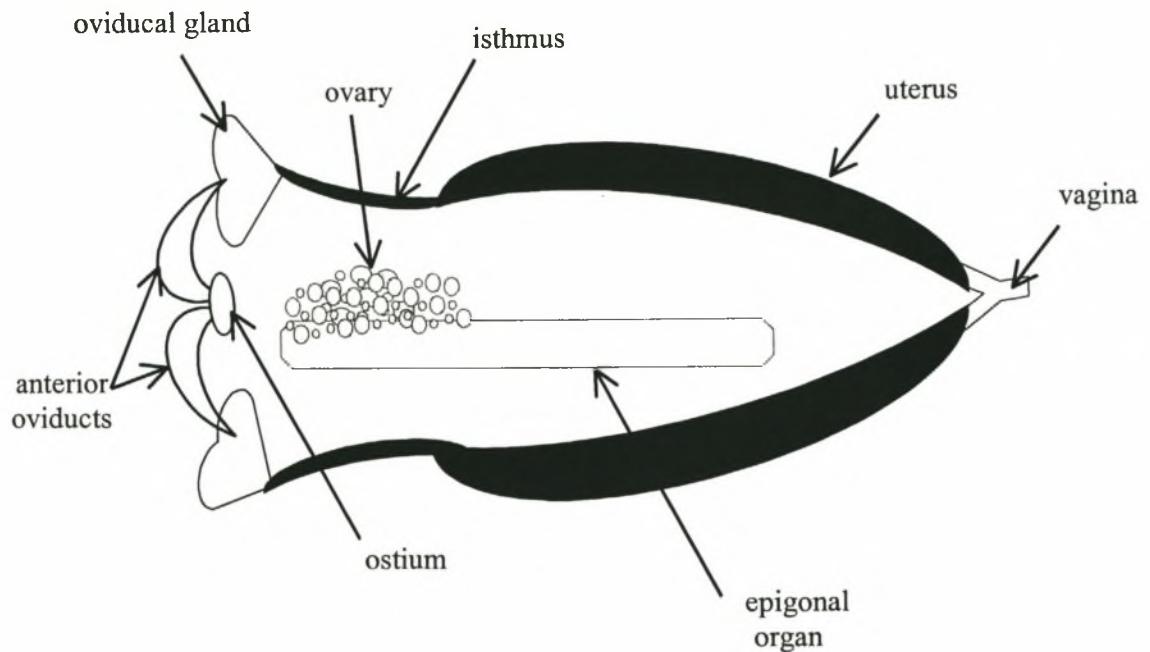


Figure 4. Schematic representation of the reproductive system of a female shark, in this case with an external ovary. In many species the ovaries are structurally or functionally asymmetrical with the right ovary being either atretic, absent or less active in producing ova.

The second type of ovary is internal and is characteristic of lamnids and alopiids, where it is contained within the epigonal organ that completely envelops the ovary. Numerous oocytes of all sizes develop around a lumen through which they are discharged during ovulation. Lamnids and alopiids have an efferent pore leading from the ovary towards the ostium. This pore enlarges as the ovary matures and reaches its maximum size when ovulation is at a peak (Gilmore 1993). When reproductively active, the ovary is greatly enlarged containing numerous ova of various sizes, as seen in *Odontaspis (=Carcharias) taurus* (Gilmore *et al.* 1983). In the basking shark, *C. maximus*, the ovary contains innumerable minute ova among a couple of larger ones at different stages of growth and corpora lutea, which are opaque, lenticular in shape, not spherical, and lighter coloured in comparison with the ova (Matthews 1950).

#### **2.4.3 Oviduct**

The female elasmobranch oviduct is compartmentalised into five divisions that will briefly be discussed individually. They are the ostium, anterior oviducts, oviducal glands, isthmus and uterus.

#### **2.4.4 Ostium**

The first part of the oviduct that the ovum enters after ovulation, is the ostium. It is formed by the fusion of the proximal ends of the oviduct and receives the ova from the ovary. In most species of shark it is lined with epithelium that are ciliated to facilitate ova transport. In *C. maximus*, the fusion of the proximal ends of the oviduct is more substantial than in most sharks, forming an efferent ovarian pore (Gilmore 1993). This special fusion brings the ostium, which becomes wider during the breeding season, in close proximity with the ovary and so eliminates the necessity of ciliary action to transport ova (Matthews 1950).

#### **2.4.5 Anterior oviducts**

The anterior oviducts bifurcate away from the ostium and transport the ova to the oviducal glands (Hamlett & Koob 1999). This section of the oviduct is ciliated in *S. canicula* (Metten 1939) and *C. taurus* (Gilmore *et al.* 1983). The anterior oviducts are normally of equal length but exceptions do occur as found in *I. omanensis*. In this species some females possess an oviduct (usually left) that is half the length of the other one and usually contain no eggs or embryos (Fishelson & Baranes 1998). Similar abnormalities have also been documented for *S. canicula* (Ellis & Shackley 1997). In *C. taurus* these ducts are also assumed to be the site

where fertilization occurs (Gilmore 1993), although Gilmore *et al.* (1983) previously reported it to most likely happen in the oviducal gland.

#### **2.4.6 Oviducal gland**

The oviducal gland is responsible for secreting albumin, mucus and an eggshell in all shark species, except in a few species where there is no shell. Much variation exists within Elasmobranchii concerning many aspects of the shell including its shape, size and composition. For example, six types of egg capsules are produced in *C. taurus* (Gilmore *et al.* 1983) and some of the other lamnid sharks (Fujita 1981; Gruber & Compagno 1981; Gilmore 1983; Gilmore *et al.* 1983). The term, oviducal gland, as opposed to nidamental or shell gland, is used here based on Pratt's (1979) reasoning concerning the true meaning of the words. The size of the glands are correlated with the stage of the reproductive cycle, as seen in *C. taurus* (Gilmore *et al.* 1983), where they reach their maximum size just prior to ovulation (Hamlett & Koob 1999). This is due to the storage of egg capsule precursors that are synthesised over a sometimes-extended period of time, before they are rapidly released and used when an ovum passes through the gland. This applies to both oviparous and viviparous forms except where no egg capsule is formed, like in *Urolophus jamaicensis* (Hamlett & Koob 1999).

Previously only two zones were recognised in the oviducal gland, namely the albumin-secreting zone and shell-secreting zone (Prasad 1945a,b; Pratt 1979). Hamlett *et al.* (1998) recently introduced a more detailed zone classification. The albumin-secreting zone is now subdivided into a club zone and papillary zone. The baffle zone replaces the shell-secreting zone and the fourth zone is the terminal zone. These divisions are not always clearly visible in cross section, as is seen in *C. maximus* with its more homogenous appearance (Matthews 1950).

The oviducal gland is believed to be the site where fertilization of ova takes place (Metten 1939; Prasad 1945a,b; Pratt 1979; Parsons 1983). Another function of the oviducal glands, that of a receptaculum seminis, has already been discussed above. Metten (1939) and Pratt (1979) both hold that sperm are retained in the shell-secreting part (posterior) of the gland.

#### **2.4.7 Isthmus**

The encapsulated ova leave the oviducal gland and enter the isthmus, an especially elastic part of the oviduct that connects to the uterus (Gilmore *et al.* 1983). In most cases the isthmus is lined with longitudinal folds and furrows (Pratt 1979). One of its functions is to act as a sphincter to isolate the contents of the uterus (Hamlett & Koob 1999), which is especially

handy in species like *Torpedo spp.*, *C. taurus* and *R. terraenovae* where the uterus contains some amount of histotroph.

#### **2.4.8 Uterus**

The final and largest part of the oviduct is the uterus, which has been very well studied in terms of function related to the degree of complexity and the various degrees of viviparity exhibited among the elasmobranchs. In oviparous sharks the uterus serves as no more than a passage and its wall is smooth and covered with flattened epithelium (Matthews 1950; Hoar 1969). On the other hand, in viviparous sharks like *C. maximus*, the wall of the uterus contains villuslike appendages (Matthews 1950). Our current knowledge of uterine involvement and specialisations for viviparity is largely based on microscopic work and so the physiological and biochemical mechanisms involved are yet to be clarified (Hamlett & Koob 1999). The details concerning the uterus and viviparity will not be covered here, but dedicated reviews on the topic include Amoroso (1960), Breeder & Rosen (1966), Dodd (1983), Hamlett & Koob (1999), Hoar (1969), Otake (1990), Wourms (1977), Wourms (1981) and Wourms *et al.* (1988). The size of the uterus is strongly influenced by the presence or absence of eggs and embryonic growth and, hence, varies throughout the reproductive season, as noted in *C. taurus* (Gilmore *et al.* 1983).

Functional asymmetry is reported for *S. squatina* where there are more embryos in the right arm of the uterus than in the left, whereas *S. oculata* is fully symmetrical in this respect (Capapè *et al.* 1990). *R. terraenovae* mostly also have equal numbers of embryos in each uterus, but sometimes there is a marked difference in this respect between the two uteri (Parsons 1983).

#### **2.4.9 Classifying stages of reproductive maturity**

Most authors make use of more than one parameter to determine the stage of reproductive maturity in an animal, but the most widely used indicator of maturity is the presence of vitellogenic follicles in the ovary (Pratt 1979). Other parameters used by authors include the extent of development of the ovary, size of the oviducal gland, width and extent of development of a flattened uterus, the presence of uterine eggs or embryos and fresh mating scars (Olsen 1954; Springer 1960; Wass 1973; Bass *et al.* 1975; Pratt 1979; Parsons 1983; Castro *et al.* 1988; Capapè *et al.* 1990; Freer 1992; Yano 1993).

In some species of shark, for example *P. glauca*, *S. squatina* and *S. oculata*, there is a distinct period of sub-adulthood. During this time, which lasts for more than a year in *P.*

*glaуca*, the vagina and other organs required for copulation are developed but the ovaries are not yet developed, making the accurate assessment of maturity rather difficult (Pratt 1979).

Growth of the ovary in *P. glauca* is too regular to be used as an index of maturity since it shows no sudden increase when plotted over body size (Pratt 1979). In *R. terraenovae*, on the other hand, ovarian growth is too variable even for individuals of the same size examined in the same month (Parsons 1983). Correlation between the morphological condition of the ovary and its reproductive status is still sparse.

#### 2.4.10 Oogenesis and cyclic activity in the ovary

When not actively breeding, some carcharhinids have ovaries that may consist of a 'pool' of small ova between 2 - 8 mm diameter, until rapid vitellogenesis occurs in some of them as the time of ovulation approaches, as seen in *R. terraenovae* (Parsons 1983). In *P. glauca* the ovary of a gravid female with full-term in-utero embryos contained more than 1000 follicles, mostly smaller than 1 mm, as well as the next generation's 123 vitellogenic ova, varying in diameter from 6 to 20 mm (Pratt 1979). Ovarian follicles of most sharks with annual cycles undergo vitellogenesis concurrently with gestation. The next generation of ova also develops pre-partum in some batoids, as in *S. squatina* and *S. oculata*, but vitellogenesis is semi-delayed until halfway through gestation (Capapè *et al.* 1990).

The ova ovulated by female elasmobranchs, whether oviparous, ooviviparous or viviparous, are typically large and abundantly yolked (Hamlett & Koob 1999). The only exceptions are some viviparous forms that rely primarily on uterine derivatives for embryonic development (Hamlett & Koob 1999).

There are three major patterns of seasonal reproduction in sharks (Wourms 1977). Some are reproductively active throughout the year with no sign of seasonal effects, for example *C. anguinus*, *Heterodontus portusjacksoni* (cited in Wourms 1977), *S. canicula* (Sumpter & Dodd 1979), *I. omanensis* (Fishelson & Baranes 1998), *P. glauca* (Gubanov & Grigor'yev 1975), *Alopias superciliosus* (Gruber & Compagno 1981), *Carcharhinus falciformes* (Compagno 1984) and *Carcharhinus dussumieri* (Teshima & Mizue 1972). Other species may have a poorly defined annual cycle, being reproductively active throughout the year with one or two peaks of activity. Examples include *R. erinacea* and *Hydrolagus colliei* (cited in Wourms 1977). A third category described by Wourms (1977) has a well-defined annual or biennial cycle. Examples are shown in Table 2.

The reproductive state of an animal can be described in terms of its reproductive morphology and endocrinology. The ovaries and compartments of the oviduct display varying sizes and properties throughout a reproductive cycle and allows for classification into

Table 2. Summary of the duration of reproductive cycles in some elasmobranchs.

Species	Cycle period	Reference
<i>Torpedo torpedo</i>	Annual	Cited in Capapè <i>et al.</i> (1990)
<i>Rhizoprionodon acutus</i>	Annual	Bass <i>et al.</i> (1975)
<i>Carcharias taurus</i>	Annual	Gilmore (1993)
<i>Mustelus canis</i>	Annual	Hisaw & Abramowitz (1939)
<i>Galeorhinus galeus</i>	Annual, bi- or triennial	Walker (1999)
<i>Torpedo marmorata</i>	Bi-or triennial	Capapè <i>et al.</i> (1990)
<i>Squatina</i> spp.	Biennial	Capapè <i>et al.</i> (1990)
<i>Squalus acanthias</i>	Biennial	Jones & Geen (1977)
<i>Prionace glauca</i>	Biennial	Pratt (1979)
<i>Carcharhinus plumbeus</i>	Biennial	Springer (1960)
<i>Carcharhinus limbatus</i>	Biennial	Compagno (1984)
<i>Carcharhinus obscurus</i>	Biennial	Compagno (1984)

different stages to define such a cycle. For example, the width of the anterior oviduct of *I. omanensis* varies from 3.5 - 6.0 mm in resting adult females and up to 18 - 22 mm during gestation (Fishelson & Baranes 1998). Also, ovarian activity (vitellogenesis) in *C. taurus* begins between January and April and lasts until September when the active right ovary is greatly enlarged containing numerous follicles of various sizes (Gilmore *et al.* 1983). When *R. terraenovae* females are found between vitellogenic activity cycles, the ovary consists of  $\pm$  30 oocytes just larger than 2 mm. As ovulation approaches, rapid yolk deposition ensues in four to eight of these oocytes which consequently enlarge (Parsons 1983). The changes that the ovary goes through in a reproductive cycle are most often quantified by a GSI as was explained for the males. Manire *et al.* (1995) gives a good example of how combinations of measurements of the different compartments of the oviduct are used on the placentially viviparous *S. tiburo*. Nine stages of reproductive activity were identified, namely mating, preovulation, ovulation, postovulation, early pregnancy, implantation, late pregnancy, parturition and postpartum. Each stage has a corresponding follicle size, an overall uterine condition and absence or presence of sperm in the oviducal gland, uterine ova, uterine embryos and placentas (Manire *et al.* 1995). The frequency of occurrence of each stage differs from month to month, indicating and describing the cycle for that species.

#### **2.4.11 Female reproductive endocrine cycles**

For a recent review covering cycles, source, regulation and function of female elasmobranch reproductive endocrinology, see Hamlett & Koob (1999). There are only five species of shark for which the seasonal fluctuations of circulating reproductive steroid hormones have been reasonably thoroughly described, namely *R. erinacea*, *S. acanthias*, *S. tiburo* (cited in Hamlett & Koob 1999), *S. canicula* (Sumpter & Dodd 1979) and *D. sabina* (Tricas *et al.* 2000). Table 3 summarises some available maximum and minimum values of the three main sex steroids, namely testosterone, estradiol and progesterone, present in mature female sharks of oviparous, aplacental viviparous and placental viviparous species. Placental viviparous species, except for *S. tiburo*, seem to have the lowest testosterone and estradiol levels.

Even though the actions of these three hormones are closely related to specific events in the reproductive cycle, their temporal patterns and associated functions differ among species (Tricas *et al.* 2000). Appendix A summarises the stage of the reproductive cycle of different species where testosterone, estradiol and progesterone have elevated and reduced levels. In *S. canicula*, testosterone and estradiol fluctuates together very closely with each other as well as with the GSI (Sumpter & Dodd 1979). These two steroids are both controlled by pituitary

Table 3. Maximum and minimum testosterone, estradiol and progesterone levels (ng/ml) in mature female sharks representing oviparous, aplacental viviparous and placental viviparous species. Data from this study are included in **bold**.

Species	Reprod. mode	Testosterone Min	Max	Estradiol Min	Max	Progesterone Min	Max
<i>Raja radiata</i> <sup>a</sup>	O	0.2	6.0				
<i>Raja ocellata</i> <sup>a</sup>	O		5.9				
<i>Scyliorhinus canicula</i> <sup>c</sup>	O	1	6.5	5	40		
<i>Torpedo marmorata</i> <sup>d</sup>	AV		24				73
<i>Squalus acanthias</i> <sup>e</sup>	AV	0.1	10	0.1	10	<1	6
<i>Dasyatis sabina</i> <sup>f</sup>	AV	0.005	7.8	0.006	9.7	0.03	0.63
<b><i>Galeorhinus galeus</i></b>	<b>AV</b>	<b>0.24</b>	<b>18.9</b>	<b>0.35</b>	<b>18.5</b>	<b>0.16</b>	<b>13.41</b>
<i>N.brevirostris</i> <sup>g</sup>	PV	0.15	0.8	0.89	2.9	0.12	0.22
<i>Carcharhinus acronotus</i> <sup>g</sup>	PV	0.05	0.78	0.38	1.4	0.06	0.12
<i>Rhizoprionodon porosus</i> <sup>g</sup>	PV	0.02	0.07	1.25	4.5	0.01	0.18
<i>Carcharhinus limbatus</i> <sup>g</sup>	PV	0	0.03	0	0.07	0.08	4.9
<i>Sphyrna tiburo</i> <sup>h</sup>	PV	0.01	93	0.06	37	0.18	44

**Data from this study in bold**

<sup>a</sup> Idler & Truscott (1966)

O = oviparous

<sup>b</sup> Koob *et al.* (1986)

AV = aplacental viviparous

<sup>c</sup> Sumpter & Dodd (1979)

PV = placental viviparous

<sup>d</sup> Lupo di Prisco *et al.* (1967)

<sup>e</sup> Tsang & Callard (1987b)

<sup>f</sup> Tricas *et al.* (2000)

<sup>g</sup> Rasmussen & Gruber (1990)

<sup>h</sup> Manire *et al.* (1995)

gonadotropin (Sumpter & Dodd 1979).  $17\beta$ -Estradiol is the main estrogen in the plasma (Sumpter & Dodd 1979) and directly affects the secretory activity of the oviduct (Dodd *et al.* 1960). An initial elevation in estradiol levels in *D. sabina* occurs pre-ovulatory as the oocytes approach final maturation. This probably served to increase the synthesis and uptake of vitellogenin like in *S. tiburo* and *R. erinacea* (cited in Tricas *et al.* 2000). A second estradiol surge occurs during the final stages of embryonic development and parturition, but its function is not clear yet (Tricas *et al.* 2000).

There is a peak in testosterone levels of *Torpedo marmorata* that occurs after and between gestations (Lupo di Prisco *et al.* 1967). This peak coincides with the time when growth of the oocytes resumes, since it is blocked until after gestation (cited in Capapè *et al.* 1990). Something noteworthy about *T. marmorata* is that the highest testosterone levels are found in immature females (Lupo di Prisco *et al.* 1967).

In their study of 11 carcharhinoid species, Rasmussen & Gruber (1990) identified two groups of sharks based on their levels of estradiol. One group, including *Carcharhinus perezi* and *Carcharhinus limbatus* had a low range of estradiol levels (0.002-0.065 ng/ml) and the other, which include *Negaprion brevirostris*, *Carcharhinus acronotus*, *Rhizoprionodon porosus* and *Sphyrna* spp., had a relatively high range of estradiol levels (0.38-4.5 ng/ml). Unlike *S. acanthias* where progesterone remains high throughout pregnancy, it remains low and stable in most of the placental carcharhinoids (Rasmussen & Gruber 1990). Two exceptions to this rule were recently mated *C. limbatus* and *N. brevirostris* who had very high progesterone levels in circulation that was correlated with high testosterone and estradiol levels (Rasmussen & Gruber 1990, 1993). These authors suggest that the estradiol might regulate the reproductive cycle in these species (Rasmussen & Gruber 1993).

In general, it seems that in oviparous skates and ratfishes, the estradiol levels remain high and testosterone levels remain low until egg laying takes place, after which estradiol drops and testosterone rises for most of the egg-laying period (Rasmussen & Muru 1992). This inverse relationship between estradiol and testosterone is also suggested by data for *C. taurus* (aplacental viviparous) and *C. plumbeus* (placental viviparous) (Rasmussen & Muru 1992). In contrast to this inverse relationship between testosterone and estradiol, there are oviparous (*R. erinacea* and *S. canicula*), aplacental viviparous (*D. sabina*, *S. acanthias*) and placental viviparous (*C. acronotus*, *C. limbatus* and *S. tiburo*) species where testosterone and estradiol levels fluctuate together very closely. Therefore, at this stage there seems to be no set functional roles for these steroids within the reproductive modes of elasmobranchs. Much

variation exists at species level and our lack of understanding of the diversity of strategies employed by sharks and the hormonal control thereof is emphasised.

## 2.5 MALE-FEMALE REPRODUCTIVE ASYNCHRONY

Conspecific males and females are often not in reproductive synchrony and care should be taken when considering the reproductive cycle of a species, to clarify whether the cycle pertains to a single sex or both. When spermatogenesis in males does not peak at the same time as ovulation occurs in the females, some sort of strategy is needed to ensure successful insemination of the ova for the next generation. One solution is for sperm to be stored by the males until mating can commence as in *R. terraenovae* (Parsons 1983). Alternatively, another helpful strategy to increase insemination success, especially in sparsely occurring-, pelagic- or low-density species (Pratt 1993) is sperm storage in the oviducal glands of females. In this way the females can acquire sperm from a male and store it until she ovulates and fertilisation can take place. Reproductive success is now elevated without the need for synchronous reproductive activity between the sexes.

Sperm storage in the oviducal gland was first reported in *S. canicula* (Metten 1939) and thereafter also in at least 19 other species (Prasad 1945a,b; Prasad 1948; TeWinkel 1956; Pratt 1979; Gilmore *et al.* 1983; Castro *et al.* 1988; Peres & Vooren 1991; Pratt 1993; Fishelson & Baranes 1998). The longest reported time for sperm storage was recorded when a *Scyliorhinus retifer* female produced fertile ova having been separated from male sharks for more than two years and four months (Castro *et al.* 1988).

Sexual segregation for the biggest part of the year is documented for a number of species. Examples include *P. glauca* (Gubanov & Grigor'yev 1975), *G. galeus* (Freer 1992), *C. plumbeus* (Springer 1960), *Sphyrna lewini* and *S. tiburo* (Compagno 1984) and *Alopias vulpinus* (Gubanov 1972). Successful reproduction now requires well-timed or fine tuned reproductive strategies, especially regarding mating. In all of these species, the females are known to store sperm, which plays a role of varying importance in different species. In female *D. sabina*, where both sexes are equally abundant throughout the year, there is no sperm storage (Tricas *et al.* 2000). In *A. vulpinus* the storage of sperm is brief and insemination reportedly takes place almost immediately (Pratt 1993). Both sexes of *P. glauca* are reproductively active all year round (Gubanov & Grigor'yev 1975). Without the need for reproductive synchrony, the only restriction these pelagic fish must overcome in order to procreate is probably locating a mate in the open sea.

## 2.6 GLOBAL INTRASPECIFIC VARIATION

As our knowledge of shark reproduction increases, so also must our awareness of the differences that occur between geographically separate populations of the same species around the globe. More and more aspects of life history are being added to the list of intraspecific variables and it is important for local fisheries managers to bear this in mind and not to generalise too freely. For instance, it would be inaccurate to compare seasonality in different populations of a species on a global level without bearing in mind that at any one time, any perceived differences may well be intraspecific and not necessarily seasonal. A wide range of factors can be responsible for such differences, for example local abundance / scarcity of one sex (e.g. *Carcharhinus plumbeus*, (Clark & Von Schmidt 1965), temperature (e.g. *S. canicula*, (Dobson & Dodd 1977b) and local climate (*S. acanthias*, (Parsons & Grier 1992).

*G. galeus* is a global species whose biology is reasonably well understood. It displays variation in many aspects of its biology as summarised in Table 4 (data from Walker (1999) except where stated otherwise).

Wass (1973) reported on a number of varying aspects of the reproductive biology of *C. plumbeus* (= *C. milberti*). The Hawaiian population of *C. plumbeus* reaches a considerably smaller maximum size and matures at a much smaller PCL (pre-caudal length) than their western North Atlantic counterparts. The population in Mauritius and Seychelles grow even larger than the North Atlantic population. These size differences are ascribed to the different temperature regimes of these geographically distinct areas (Wass 1973). The sex ratio at birth is 1:1 for the populations from Hawaii (Wass 1973) and East China Sea (Taniuchi 1971), while it was reported to be female biased 5:1 (Springer 1960) and 6:1 (Clark & Von Schmidt 1965) in the western North Atlantic and the central Gulf coast of Florida, respectively. Gestation takes nine months off Florida and 11-12 months off South Africa and the South China Sea (Compagno 1984). *C. plumbeus* young are born in April off Senegal and in summer (June to August) off the western North Atlantic coast (Compagno 1984). Other aspects that were reported that also varied among *C. plumbeus* populations were size at birth, mating season and litter size.

Some other examples of varying aspects within species include *S. squatina*, where parturition occurs in July in England and between December and February in the Mediterranean (Breeder & Rosen 1966). Also in *P. glauca* the time of parturition varies from between December and April in the Pacific (Suda 1953) to between April and July in parts of the Atlantic (Pratt 1979). *Carcharhinus altimus* gives birth in the Mediterranean from August

Table 4. Summary of *Galeorhinus galeus* data from different populations around the globe (taken from Walker 1999) except where stated otherwise). Sizes are in cm except where stated otherwise. Results from this study are added in **bold**.

	South Africa	Australia	California	Brazil	Tunisia
Mating season	<b>May-Jul</b>	May-Jun	Apr-May	May-Jun	May-Jun
Litter size	8-20	17-41	6-52	4-41	10-41
Ave. litter size	<b>26</b>	28	35	23	30
Embryo size range (mm)	8	±7	23-26	2-4	-
Parturition	Dec-Jan	Dec	May-Jul	Nov-Dec	Apr
Size at birth	30	30	35-37	30-31	37
Female cycle	<b>triennial</b>	biennial	annual	triennial	Annual
Gestation (months)	<b>12-15</b>	12	12	12	-
Mature size M	-	132	155	117	-
Mature size F	-	-	-	128	-
Smallest mature M	<b>118</b>	120	135	107	125
Smallest mature F	<b>131</b>	135	150	118	140
Max size M	170	171	185	148	158
Max size F	173	174	195	155	200

to September and in Madagascar from September to October (Compagno 1984). *Carcharhinus brevipinna* is born during the period from autumn to winter (March to June) in South Africa, around summer (June) off Senegal and from spring to early summer (March to June) in the Gulf of Mexico (Compagno 1984). Off South Africa and in the Gulf of Mexico, *C. leucas* is born between late spring and early summer, but off Nicaragua there is no specific birthing season (Compagno 1984). In *C. limbatus*, parturition occurs from October to November in South Africa, Madagascar and India and from April to June in the northern Atlantic Ocean (Compagno 1984). *Negaprion acutidens* young appear between October and November off Madagascar and between December and January in French Polynesia (Johnson 1978; Compagno 1984). For *Rhizoprionodon acutus* liberation of young occurs in summer (January) off South Africa and the eastern Atlantic, and in winter (January) off India (Compagno 1984).

Gilmore (1993) states, without giving details, that a very predictable environmental factor governs the mating activity and gonadal condition of *C. taurus* observed in both Florida (late February-April) and North Carolina (late April-early May). There appears to be a two to four week delay in development between the populations from these two locations. In both these areas, the males and females aggregate each year at exactly the same time, at exactly the same place (Gilmore 1993). Mating of *C. leucas* is a year-round affair while in the eastern Gulf of Mexico it is restricted to between June and July (Parsons & Grier 1992).

The *R. terraenovae* population at the central gulf of Florida has a late winter/early spring (February-March) mating- and pupping season (Clark & Von Schmidt 1965). In comparison, the populations of the north central Gulf of Mexico have a late spring to summer activity (Late-May to July) (Parsons 1983). In *C. plumbeus* mating takes place during July and August in Hawaii (Wass 1973), June off the Florida coast (Springer 1960) and between June and July in the East China Sea (Taniuchi 1971).

The fecundity of *S. acanthias* varies globally between three and five embryos per year in the Northeast Atlantic and 12 embryos per year in the Sea of Japan (Ketchen 1972). The litter size of *P. glauca* varies more drastically even in the same area (6-58 off Southern Africa) (Bass *et al.* 1975). Therefore, to compare global populations in this respect would be futile. Female size is thought to be responsible for this variation (Bass *et al.* 1975), but Gubanov & Grigor'yev (1975) reckons that the number of offspring is not correlated to female size. The largest number of offspring observed from a single *P. glauca* female was 135 (Gubanov & Grigor'yev 1975). Off South Africa, *Carcharhinus obscurus* has a higher number of offspring than the Florida population (Compagno 1984).

*P. glauca* from the equatorial zone were considerably larger than conspecifics sampled further south and the size composition of this globally abundant shark varies widely between populations (Gubanov & Grigor'yev 1975). A similar variation exists for *Rhizoprionodon acutus* (Springer 1964).

Gestation also varies globally between conspecific shark populations. *Carcharhinus melanopterus* has a gestation period of 16 months in the Red Sea, while at Aldabra Atoll gestation takes 10 - 11 months (Stevens 1984).

In some cases, the variations are not so much seasonal as it is due to the hemisphere where they occur (e.g. parturition of *C. limbatus*). What happens in summer in the Northern Hemisphere, happens in summer in the Southern Hemisphere. It should be noted, however, that in many of these cases mentioned, the variations are beyond this effect of hemisphere and these are the cases that should be borne in mind. Some of these aspects serve as parameters that are crucial for fisheries management and necessitates investigation for specific fisheries operating in specific areas.

### 2.6.1 South African shark populations

It is especially crucial in countries where shark fishing is an established industry, like South Africa, Japan, Australia and the USA, to investigate and know the reproduction and other biological aspects of their own shark populations. *G. galeus* is a principle species for South African and other fisheries around the world (Ripley 1946; Olsen 1954; Peres & Vooren 1991; Freer 1992), and much of what we know about this species is owed to its commercial importance. Up to 1975, nothing was known of the breeding biology of the southern African *G. galeus*, except that it is live bearing (Bass *et al.* 1975). These authors also commented on its viability as a commercially harvestable source due to its abundance around the South African coast. McClusky (1988) and Freer (1992) are the only contributors of biological data on the South African *G. galeus*. Bass *et al.* (1975) provides much data on other South African shark species, but still there remain some gaps in our knowledge of our local sharks.

### 2.6.2 Justification: Why understand reproductive cycles?

Reproduction is generally geared to produce offspring into a seasonally unstable environment that would offer them the greatest opportunity for their survival and development (Hoar 1969). The importance of understanding reproductive cycles, especially of commercially harvested animals, lies in the attempt to manage the harvesting of the resource in such a way as to minimise interference with their breeding activities and so obtain sustainability. An unfortunate reality is that it seems as if the breeding aggregations have

become the most attractive fishing grounds due to easy accessibility and high concentration of individuals there.

To ensure healthy, sustainable global shark resource management, many gaps that exist in our descriptive knowledge of shark biology will have to be filled. Primarily, the aspects that are utilised by fisheries management bodies need to be addressed first. This study was therefore firstly aimed at identifying and describing the reproductive cycles of both sexes of *G. galeus* off the southern coast of South Africa. A further aim was to obtain hormonal data that would contribute to the currently poor understanding of the endocrinology of shark reproduction.

### 3. Seasonal reproduction in the male soupfin shark, *Galeorhinus galeus.*

#### 3.1 INTRODUCTION

The living elasmobranchs are comprised of more than 800 species of sharks and rays (Compagno 1977). They exhibit unique reproductive strategies for example internal fertilisation, viviparity, placental mechanisms for foetal maintenance and the patterns of development of the reproductive tract that are more closely associated with amniotes than with other living fish species (Wourms 1977). Unique as they are on one level, elasmobranchs display diverse strategies within the group regarding some aspects of their reproduction. For instance, some species are oviparous where the embryos are deposited in the ocean after only a brief incubation in the mother and are nourished solely by the yolk contained within their eggshells. Other species are ovoviviparous where the embryos are nourished by their egg yolk or uterine secretions (sometimes both) and remain in the mother until full term. Still others are placentally viviparous where embryonic development is solely dependent on maternal nourishment via a placenta until parturition. The majority of elasmobranchs with more than two thirds of all sharks fall into one of the last two categories (Otake 1990).

Diversity in elasmobranch reproduction is also seen in the gonadal morphology where three types of testes can be found within this group, namely radial, diametric and compound as illustrated in Figure 3 (Pratt 1988). Diametric testes are found among carcharhinids and sphyraenids and refer to the lateral development of follicles across the diameter of the cylindrical testes. The germinal zone in this case is situated disto-laterally on the testes from where development is directed medially. Radial testes are also cylindrical and are found among lamnids and alopiids. Here the testis is divided into a highly variable number of lobes, each with a centrally situated germinal zone. Follicular development then ensues radially from the centre outward. The third type of testis, namely compound, is found in rajids and constitutes a combination of the radial and diametric modes of follicular development. This type of testis is less cylindrical, being laterally compressed, and has a lobular appearance on the surface. It consists of islands of germinal centra, all situated on the disto-lateral surface. Follicular development then spreads along the testicular surface from the centre of each island to where their borders meet, from where it submerges medially away from the surface (Pratt 1988).

In contrast to the case in amniotes where spermatogenesis takes place in seminiferous tubules, it happens in elasmobranchs in units called spermatocysts (Callard 1991), previously also called ampullae. The spermatocysts contain the sex cells that develop synchronously within an individual testis from spermatogonia to mature spermatozoa, which are then expelled through the efferent ducts. In cross section, the diametric type of testis can easily be divided microscopically into zones according to the different stages of spermatogenesis present in the spermatocysts. The spermatocysts containing spermatogonia form a band adjacent to the germinal zone. This is bordered by a zone of spermatocysts containing primary and subsequently secondary spermatocytes. Thereafter, moving in the direction of the epigonal organ, a zone of spermiogenic cysts are found from where mature sperm are eventually discharged out of the testes via the efferent ducts.

Elasmobranchs are generally considered to be seasonal breeders (Hamlett 1999) but the spermatogenic cycles of male sharks have been described in only a handful of species. These include *Squalus acanthias* (Simpson & Wardle 1967), *Scyliorhinus canicula* (cited in Hamlett 1999), *Mustelus manazo* and *Mustelus griseus* (Teshima 1981), *Sphyraena tiburo* (Parsons & Grier 1992), *Carcharhinus limbatus* (cited in Parsons & Grier 1992) and *Dasyatis sabina* (Maruska *et al.* 1996). The cycle is annual in all the above cases. There are also species, for example *Cetorhinus maximus* (Matthews 1950), *Squatina californica* (Natanson & Cailliet 1986) and *Prionace glauca* (Pratt 1979), in which the males of a population maintain reproductive potential throughout the year and hence no obvious seasonal fluctuation in sperm production is evident. Seasonal reproduction in males is mostly described in terms of a gonado-somatic index (GSI) or, to a lesser extent, on a histological basis where the temporal change in spermatogenic composition of the testis is described.

Most elasmobranchs inhabit fluctuating environments in terms of temperature, photoperiods, salinity and food availability. These environmental cues influence spermatogenic activity in the testes of male elasmobranchs to almost always follow a cyclic pattern (Dodd 1972). The gonads and other reproductive organs are closely linked to the environment by means of the endocrine system with steroid hormones being the main effectors of the impulses caused by the environmental changes. Increased levels of testosterone are normally associated with the onset of spermatocyst development and spermatogenesis after which the testosterone levels normally decrease again (Garnier *et al.* 1989; Manire & Rasmussen 1997; Tricas *et al.* 2000). Information on circulating steroid hormone levels has been limited to only a few species and much work needs to be done before we can fully understand the hormonal control of elasmobranch reproduction.

South Africa has had a commercial shark fishing industry since the Second World War and has seen a sharp decline in the total annual shark catches over the past few decades (Freer 1992). A very rich variety of species frequent the South African coastline and creates a unique platform for shark research. One of the main contributors towards our biological knowledge of the local species is Bass *et al.* (1975). Profitable as it is, this record is still rather incomplete, especially concerning reproduction among sharks. A good understanding of reproduction is essential for the fisheries authorities to successfully implement management programs, an issue that justifies serious attention that is already overdue.

The soupfin shark, *Galeorhinus galeus* (Triakidae), is one of the principal species of the local shark fisheries and its status has been described as fragile and in need of proper management (Freer 1992). It is a long-lived, circum-global species that occurs anywhere from the surf zone up to depths of about 470 m (Compagno 1984). The intraspecific differences surrounding many aspects of reproduction among the different *G. galeus* populations around the world (see Table 4) (Freer 1992; Walker 1999) necessitates that investigations be focussed on population level and calls for cautious generalisation between global populations. Previous contributions toward the reproduction of South African *G. galeus* have not conclusively shown seasonality in the testes of these males (McClusky 1988; Freer 1992). McClusky (1988), in a study of the macroscopic and microscopic anatomy of the male reproductive system, included preliminary seasonal observations that were based on data collected only over a 5-month period. This incomplete sample allowed only preliminary suggestions (McClusky 1988). Freer (1992) suggested that the males are ready for mating in May and gave no further description of the male cycle. In essence, it is not known for sure whether the South African *G. galeus* males indeed have a cyclic pattern of spermatogenesis in their testes and what that cycle looks like if it exists. According to Pratt (1979) spermatogenesis in male *G. australis* (= *galeus*) is not seasonal, but Peres & Vooren (1991) and Olsen (1954) report an annual cycle in the testes of males of this species from Brazil and Australia respectively. It could therefore be hypothesised that the behaviour of spermatogenesis is species-specific and that the South African male *G. galeus* also has an annual cycle of spermatogenesis.

The aim of this study is to identify and describe the male reproductive cycle for the South African population of *G. galeus*. This would be done on both histological and endocrinological level. In doing so this study also provides the first quantitative data of testosterone concentrations for a member of the Triakidae family.

## 3.2 MATERIALS AND METHODS

### 3.2.1 Collection of samples

The male *G. galeus* specimens for this study were collected at Stilbay ( $34^{\circ} 24'S$ ;  $21^{\circ} 25'E$ ), Kalkbay ( $34^{\circ} 8'S$ ;  $18^{\circ} 27'E$ ) and Gansbay ( $34^{\circ} 35'S$ ;  $19^{\circ} 21'E$ ) along the south coast of South Africa as illustrated in Figure 5. These collection sites constitute the main commercial harvesting areas for this species in South Africa where they occur. Environmental data were obtained for Gansbay from the weather bureau with the help of Dr. Michael Scholl and is illustrated in Figure 6.

A total of 70 male *G. galeus* were obtained for this study from the local fisheries or fishermen between April 1998 and July 2000. The sharks are caught on hand lines by fishermen and brought ashore to be gutted.

As soon as the sharks were landed, blood samples were collected by direct cardiac puncture using a heparinised needle and syringe and transferred to vac-u-test tubes and transported on ice to the laboratory. Here the whole blood was briefly centrifuged and the plasma collected and stored at  $-20^{\circ}\text{C}$  for subsequent testosterone assays. The complete reproductive system, consisting of the testes and their associated epigonal organs, the vasa efferentia, an epididymis, vas deferens and both vesiculae seminales, was removed by dissection for each specimen and stored in 4% phosphate buffered formaldehyde solution for subsequent morphometric and histological examination.

### 3.2.2 Morphometrics & maturity

The total length (TL) for each specimen was measured to the nearest 1 cm along a straight line from the tip of the snout to the tip of the upper caudal lobe. The pre-caudal length (PCL) for each specimen was measured from the tip of the snout to the pre-caudal notch. The inner clasper length (ICL) was measured along the medial edge from the tip to the anterior end of the cloacal opening as described in McClusky (1988) to the nearest 5 mm using a ruler. The length, width and height of a single testis per individual were measured to the nearest 1 mm using vernier callipers and its volume calculated.

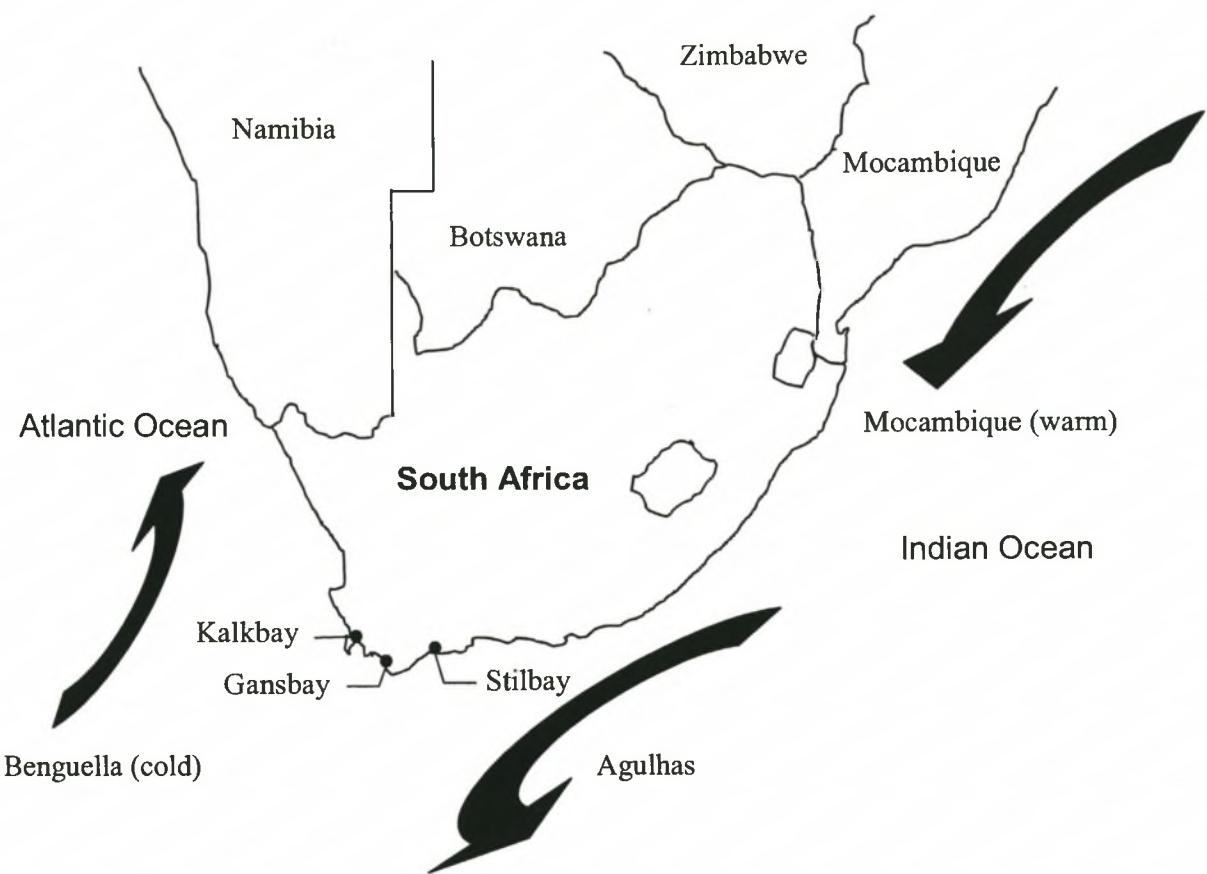


Figure 5. Geographical distribution of the coastal towns in South Africa where *Galeorhinus galeus* samples were collected for this study.

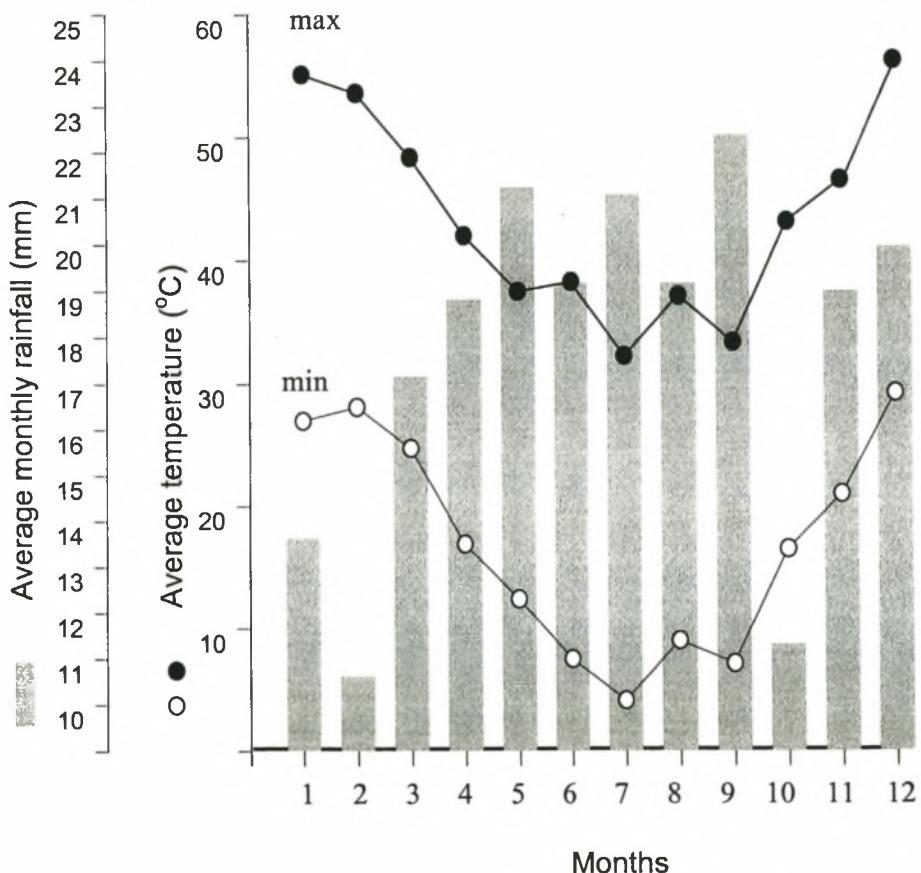


Figure 6. Seasonal variation in mean monthly maximum (●) and minimum (○) temperatures and rainfall (■) recorded at Gansbay ( $34^{\circ} 35'S$ ,  $19^{\circ} 21'E$ ).

The testes in this species have an almost cylindrical shape, so their respective volumes (in cm<sup>3</sup>) were calculated according to the formula:

$$\text{Volume} = L \times \pi \times r^2 \text{ where } L \text{ is the length of the testis and } r \text{ is the average between the width and height of the testis}$$

A gonado-somatic index (GSI) was calculated for each individual using the following formula:

$$\text{GSI} = \frac{\text{testis volume}}{\text{TL}}$$

Although hormonal and morphological data were collected for all the specimens, only the data from reproductively mature animals were used for analysis of the reproductive cycle. A wide range of determinants has previously been used to indicate maturity. These include the degree of calcification of the claspers (Springer 1960; Castro *et al.* 1988), relative growth of the claspers (Templeman 1944; Wass 1973; Parsons 1983; Castro *et al.* 1988; Capapè *et al.* 1990; Moreno & Moron 1992), the degree of folding of the deferent ducts (Moreno & Moron 1992), testis size (Wass 1973), siphon-sac development (Clark & Von Schmidt 1965; Parsons 1983), presence of sperm in seminal vesicles and sperm sacs (Templeman 1944; Kauffman 1950; Olsen 1954; Yano 1993), presence of spermatophores (Pratt 1979) and the presence of all the stages of spermatogenesis in histological cross section of the testis (Olsen 1954). In the present study male sharks were considered mature based on the relative growth of their claspers, testis volume and when histological cross sections of the testes showed all the stages of spermatogenic development.

### 3.2.3 Histology

A directly transverse block (5-7 mm thick) was removed from the middle of one of the testes from each male (Teshima 1981; McClusky 1988) and washed in running tap water for at least eight hours. These tissue blocks were dehydrated and impregnated using an automated Shandon tissue processor according to the following routine: 70% ethanol for three hours, 90% ethanol for one and a half hours (x2), 100% ethanol for one and a half hours (x2), 50% toluene in ethanol for one and a half hours, 100% toluene for one and a half hours, 100% toluene for two hours, 50% toluene in wax for two hours, 100% wax for five hours and another 100% wax for three hours. Each block was then embedded in paraffin wax (56C).

Histological sections of 10 µm were taken with a rotary microtome and stained with Harris's hematoxylin and eosin (Stevens 1977).

For each testis slide a continuos dorso-ventral series of digital images from the germinal zone all the way across to the epigonal organ, was captured through a microscope under 40x magnification. This series of images were then combined into a single, composite image using Microsoft PowerPoint and the cross-sectional areas of the different gametogenic zones subsequently determined using Leica Qwin image analysis software. The contribution of the area of each spermatogenic stage of each testis was quantified as its percentage of the total testis area in the composite images. In cases where the divisions between spermatocysts containing the different stages was not very clear, three or four sets of measurements were taken and their combined averages of each stage was then used as representative of that specimen.

An adaptation of the spermatogenic classification system of McClusky (1988) was used to define the three major groupings of spermatogenic stages used in this study. Firstly, 'spermatogonial cysts' are those that contain primary spermatogonial clusters and unilayered and multilayered spermatogonia (Figure 7a,b). The primary spermatogonial clusters are either embedded in the connective tissue matrix or become enveloped by epithelial cells to form a cyst. When these enveloped spermatogonia move away from the germinal zone of the testis and undergo mitotic divisions, a lumen starts forming in the cyst around which the spermatogonia and Sertoli cells are arranged in concentric layers. The next category is the 'spermatocytic cysts', which include cysts that contain primary and secondary spermatocytes (Figure 7c). The cysts with primary spermatocytes gradually loose their lumens because of swelling and they are distinguished from secondary spermatocytes by the flaky chromatin of their larger nuclei. After the first meiotic division, where the primary spermatocytes become secondary spermatocytes, the cysts increase further in size. The last category is the 'spermiogenic cysts', which includes cysts containing the various stages of spermatid development, i.e. spermatids, immature spermatozoa and mature spermatozoa (Figure 7d-f) (McClusky 1988).

After having quantified the proportions in which the different spermatogenic stages are present, it becomes possible to detect any seasonal changes in the proportionate composition of the different spermatogenic stages in the testes over the reproductive cycle. This method of describing the seasonal cycle of spermatogenesis in male sharks was first reported in *S. acanthias* (Simpson & Wardle 1967). Since then it has been used

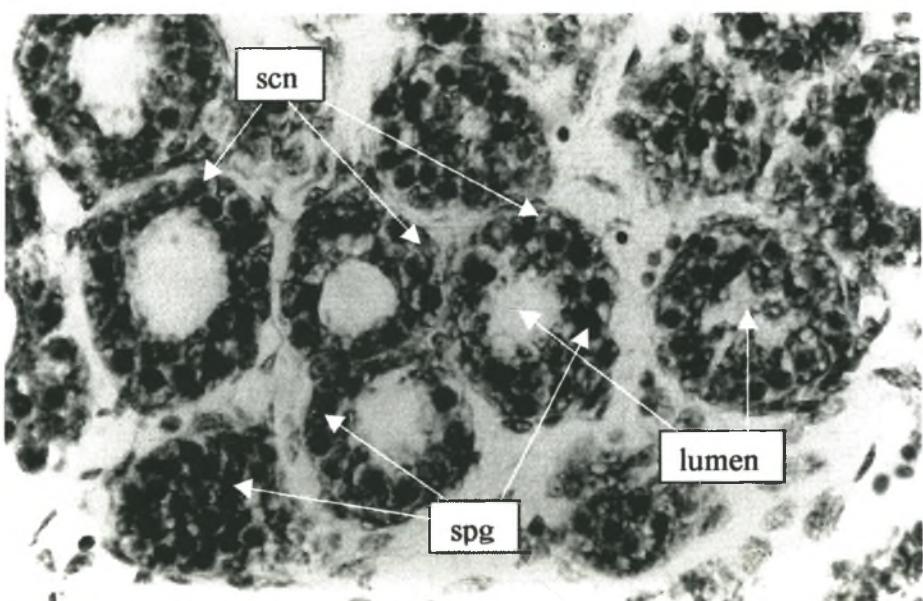


Figure 7a. Light micrograph of unilayered spermatogonial cysts in the testes of *Galeorhinus galeus* as described by McClusky (1988) under 40x magnification.  
(spg = spermatogonia; scn = Sertoli cell nuclei)

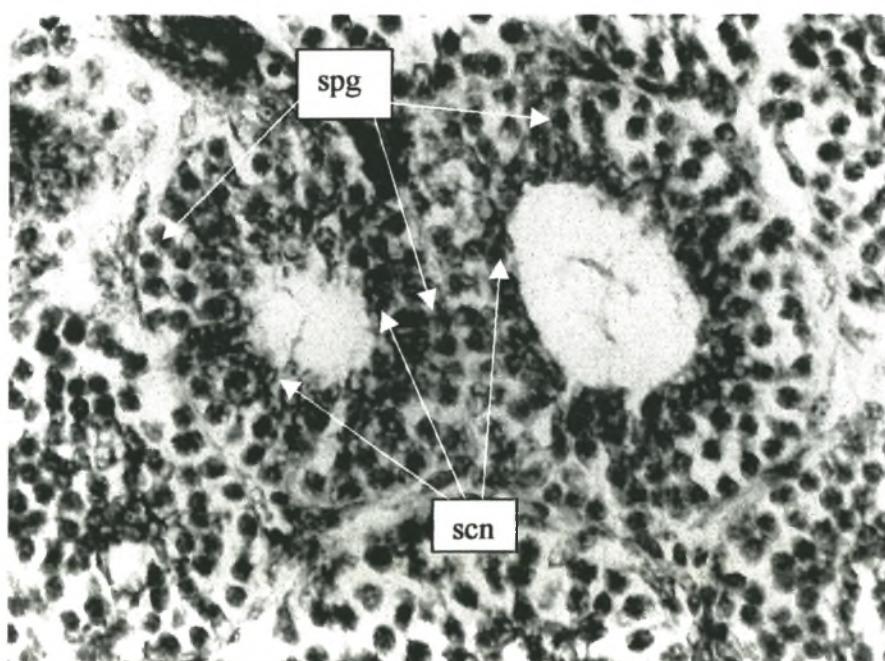


Figure 7b. Light micrograph of multilayered spermatogonial cysts in the testes of *Galeorhinus galeus* as described by McClusky (1988) under 40x magnification.  
(spg = spermatogonia; scn = Sertoli cell nuclei)

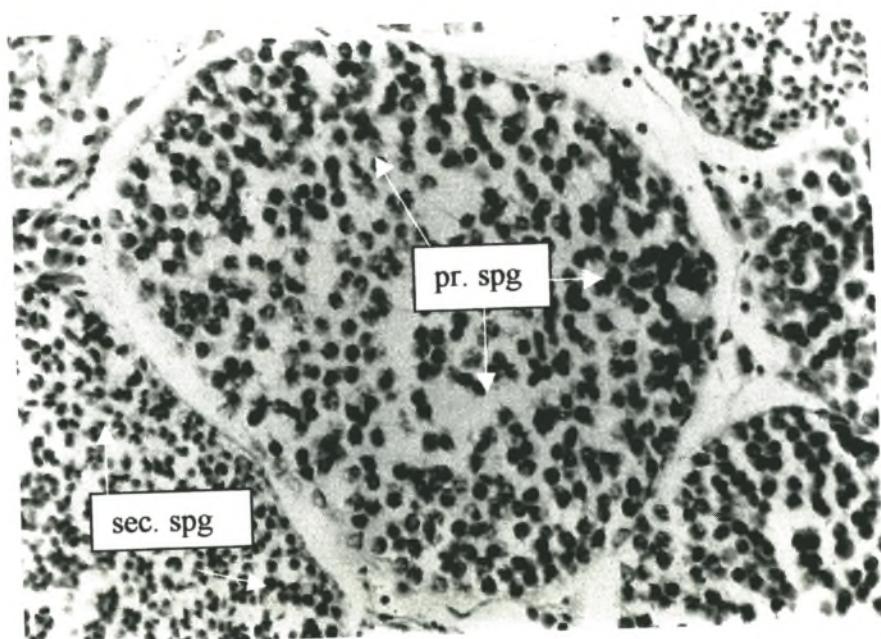


Figure 7c. Light micrograph of primary and secondary spermatocytic cysts in the testes of *Galeorhinus galeus* as described by McClusky (1988) under 40x magnification.  
(pr. spc = primary spermatocytes; sec. spc = secondary spermatocytes)

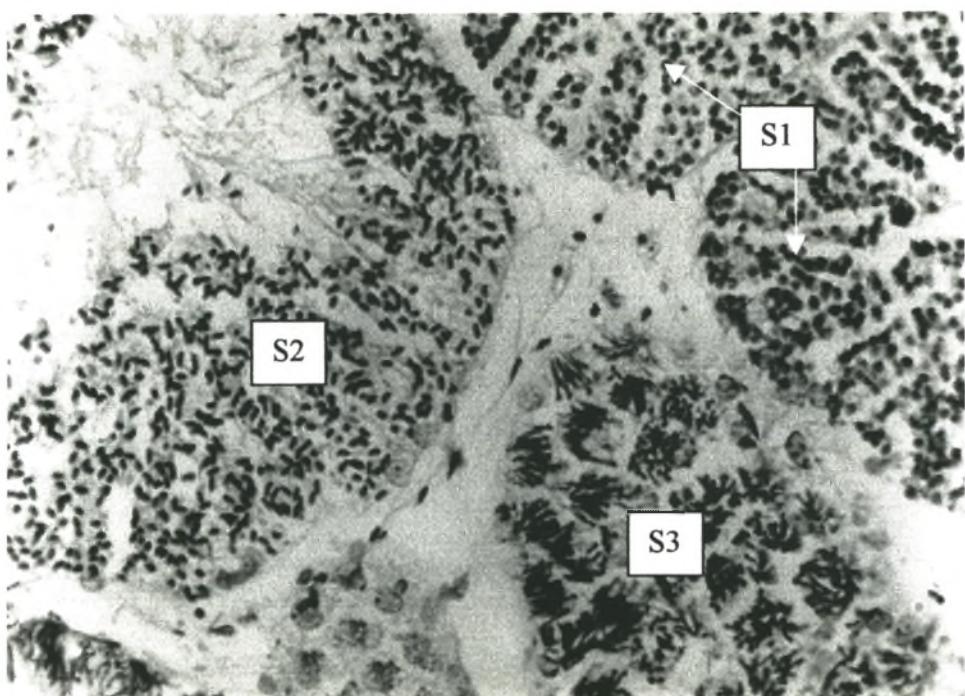


Figure 7d. Light micrograph of stage 1, 2 and 3 spermiogenic cysts in the testes of *Galeorhinus galeus* as described by McClusky (1988) under 40x magnification.  
(S1 - S3 = spermiogenic stages 1 to 3 (McClusky 1988)).

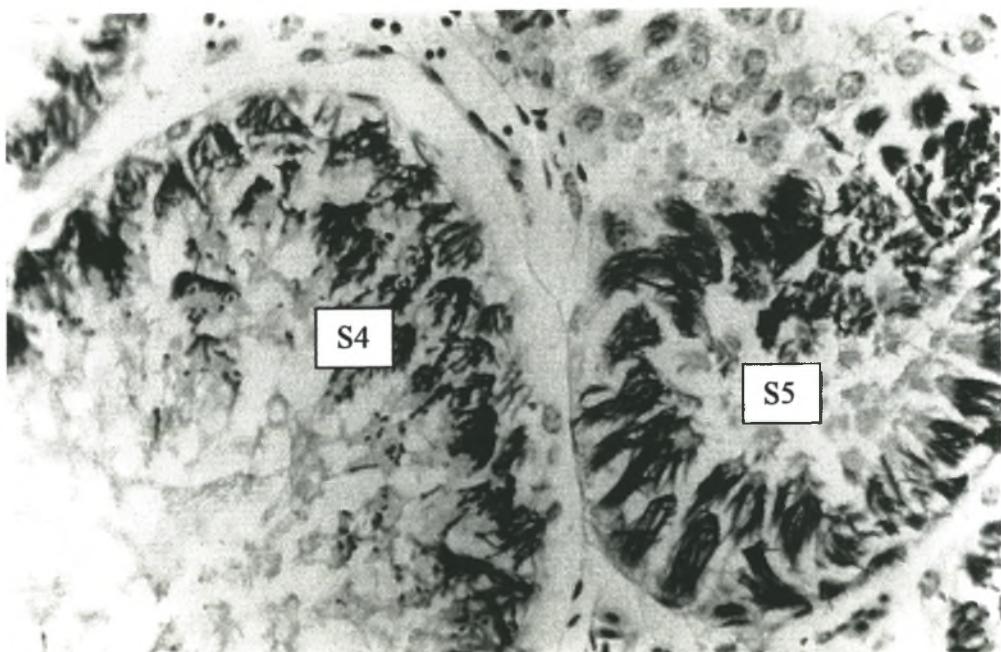


Figure 7e. Light micrograph of stage 4, 5 and 6 spermatogenic cysts in the testes of *Galeorhinus galeus* as described by McClusky (1988) under 40x magnification.  
(S4 - S6 = spermatogenic stages 4 to 6 (McClusky 1988)).

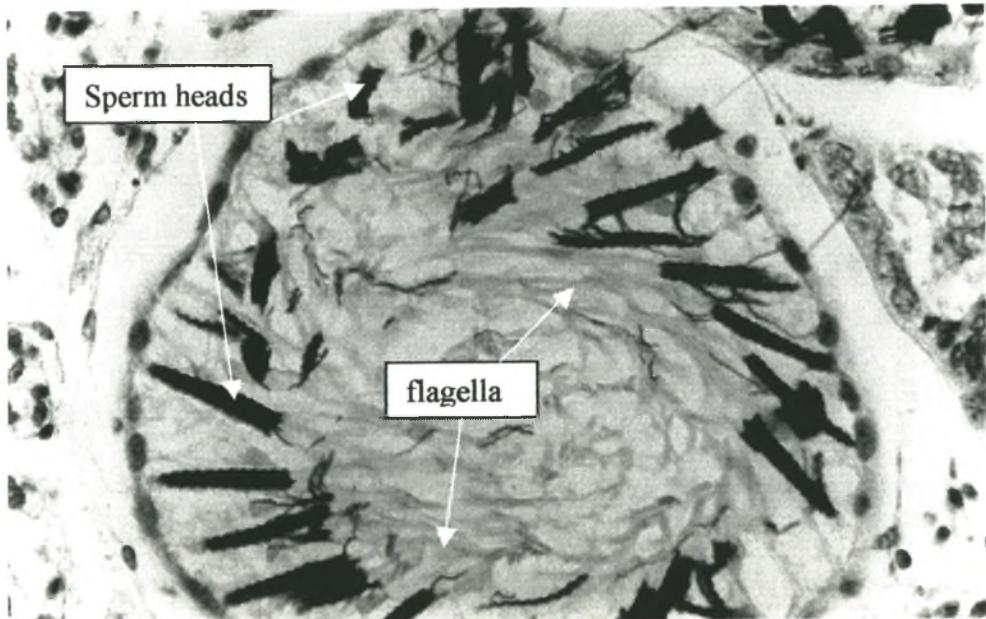


Figure 7f. Light micrograph of ripe sperm bundles in the testes of *Galeorhinus galeus* with their darkly stained 'heads' surrounding the pool of 'tails' under 40x magnification.

in only a few other species, namely *S. canicula*, *M. griseus*, *M. manazo*, *S. tiburo*, *C. limbatus* (cited in Parsons & Grier 1992) and *D. sabina* (Maruska *et al.* 1996).

### 3.2.4 Hormone extractions and analysis

The steroids were first extracted from the plasma samples using diethyl ether. 230 $\mu$ l serum and 1 ml diethyl ether (1:4.3 v/v) was vortexed for 20 seconds and then shaken for 15 minutes. It was then centrifuged for five minutes at 2000g and the aqueous phase (plasma) subsequently frozen in a -80°C freezer. The upper organic phase was decanted and evaporated until dry under a stream of air. A second 1 ml of diethyl ether was added to the plasma once it was thawed and the process was repeated. The dried extracts were reconstituted by sonication into 230  $\mu$ l steroid-free, 0.1% human serum albumin solution in a Sonicor sonicator (Sonicor Instrument Corporation, New York) for 12 minutes. Extraction efficiency was determined by adding known amounts of tritiated ( $H^3$ ) testosterone to four random aliquots of serum, extracting them together and determining the respective recoveries by scintillation counts against a 100% tritiated control. Extraction efficiency of testosterone was 84%.

The testosterone concentration of each serum sample was determined in duplicate using a testosterone-ELISA kit (IBL RE52151). In brief, the procedure involves the addition of 25  $\mu$ l of the standards and samples onto a 96-well plate that has been coated with anti-testosterone antibodies. They are allowed to incubate at room temperature for five minutes after which 200  $\mu$ l of enzyme conjugate is added to each well. After a 60-minute incubation at room temperature the plate is washed three times in the wash buffer and 200  $\mu$ l of TMB substrate solution is added to each well. After a final 15-minute incubation at room temperature, the TMB-stop solution is added (100  $\mu$ l) and the optical density is measured at 450 nm.

A dilution test in 0.1% human serum albumin/PBS was done for three samples at five or six dilutions each to determine the concentrations where detection fell within the appropriate 5-95% limits of the standard curve for this assay. The mean recovery over a concentration range from 0.3 ng/ml to 9.8 ng/ml was  $103.60\% \pm 11.02\%$  (mean  $\pm$  SD; n=17). Intra-assay variability was validated using four samples that were assayed in triplicate and resulted in a coefficient of variance (CV) of 3.4%. Inter-assay variability was determined using three samples that were assayed twice on different assays and resulted in a CV of  $12.27 \pm 3.7\%$ . Testosterone demonstrated complete parallelism in the ELISA when compared to the standards, supporting the assumption that the extraction procedure eliminated any major interfering substances. Validation procedures of this kind have been reported previously

(Manire *et al.* 1995; Manire & Rasmussen 1997; Snelson *et al.* 1997; Tricas *et al.* 2000) and proved the validity of this assay for shark steroid hormones.

### 3.2.5 Statistical analyses

A very typical problem when studying seasonal cycles in sharks is to obtain a large enough sample size from both sexes that represents at least one entire reproductive cycle (Wourms 1977). Another issue regarding the statistical analyses of periodically sampled data, especially that of hormone production, is that individuals are not always in synchrony with the rest of their population and this often results in monthly data sets not being normally distributed (Tricas *et al.* 2000). The monthly data sets for % spermatocytic cysts and % spermiogenic cysts were normally distributed and were tested for significant variation ( $P < 0.05$ ) using the One Way Analysis of Variance (ANOVA), followed by the Tukey Test method for the pairwise comparisons. The monthly data sets for % spermatogonial cysts and testosterone concentrations were not normally distributed and could not be normalised by transformation. These data sets were therefore tested for significant variation ( $P < 0.05$ ) using Kruskal-Wallis One Way Analysis of Variance on Ranks (KW-ANOVA), followed by Dunn's test for the pairwise comparisons. Associations between morphological-, histological- and endocrinological data were determined using the Pearson Product Moment Correlation method ( $P < 0.05$ ).

## 3.3 RESULTS

### 3.3.1 Morphometrics & maturity

The mean clasper length for mature males is  $16.3 \pm 1.0$  cm ( $n = 4$ ) with lengths ranging from 14cm to 18cm. A sharp increase in clasper length occurs when males grow larger than 118cm (see Figure 8). A marked increase in testis volume also occurs when TL exceeds 118cm (see Figure 9). There is no strong temporal variation in the volume of the testes, although it seems to be on the decline from autumn toward mid-winter (see Figure 10). The GSI ranged from 0.001 to 0.017 in immature males and from 0.21 to 2.33 in mature males and also increased abruptly at TL greater than 118cm (see Figure 11). There was no strong seasonal variation in the GSI throughout the year, but there is a decline in its values from autumn towards winter (see Figure 12), which is very similar to the trend showed by testis volume.

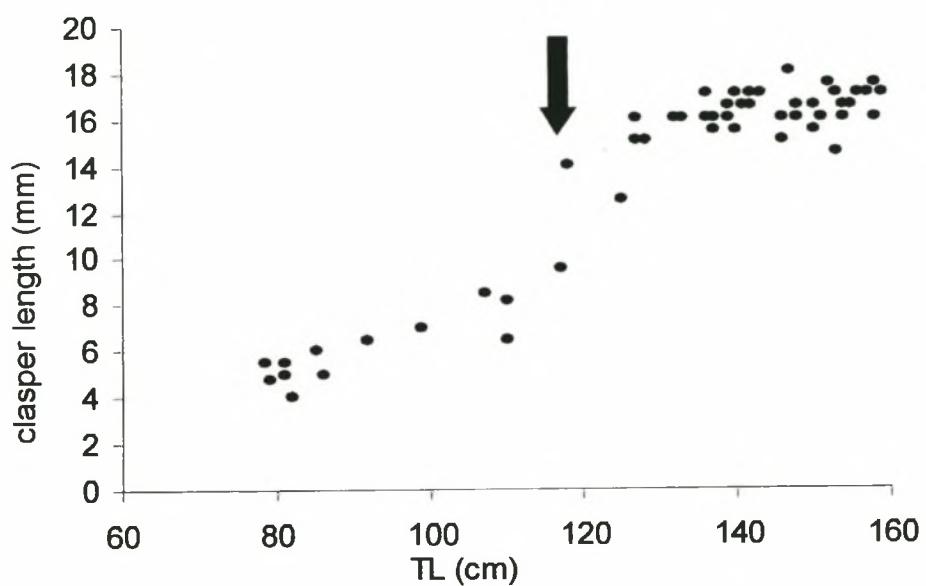


Figure 8. Clasper length vs. TL of male *Galeorhinus galeus*. The arrow indicates a TL of 118cm beyond which the clasper length increases dramatically.

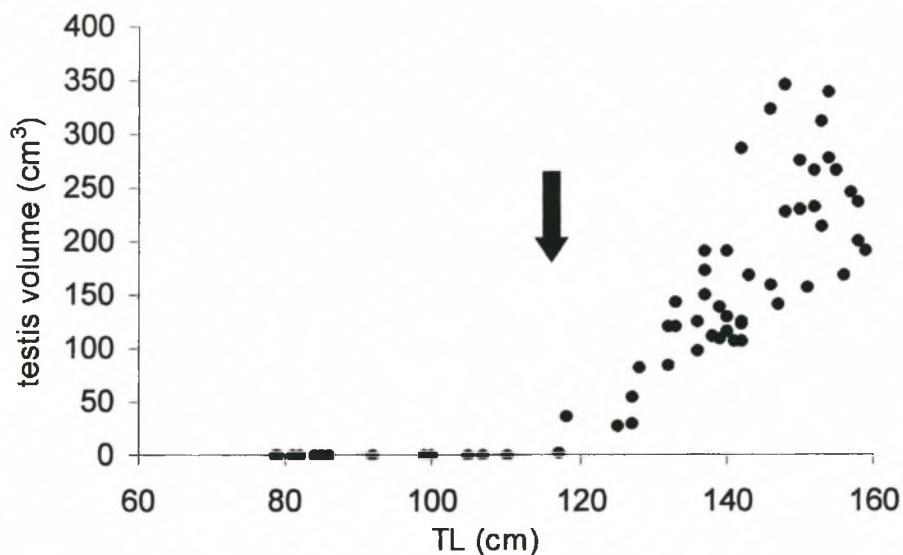


Figure 9. Testis volume (in  $\text{cm}^3$ ) vs. TL of male *Galeorhinus galeus*. The arrow indicates a TL of 118cm beyond which the clasper length increases dramatically.

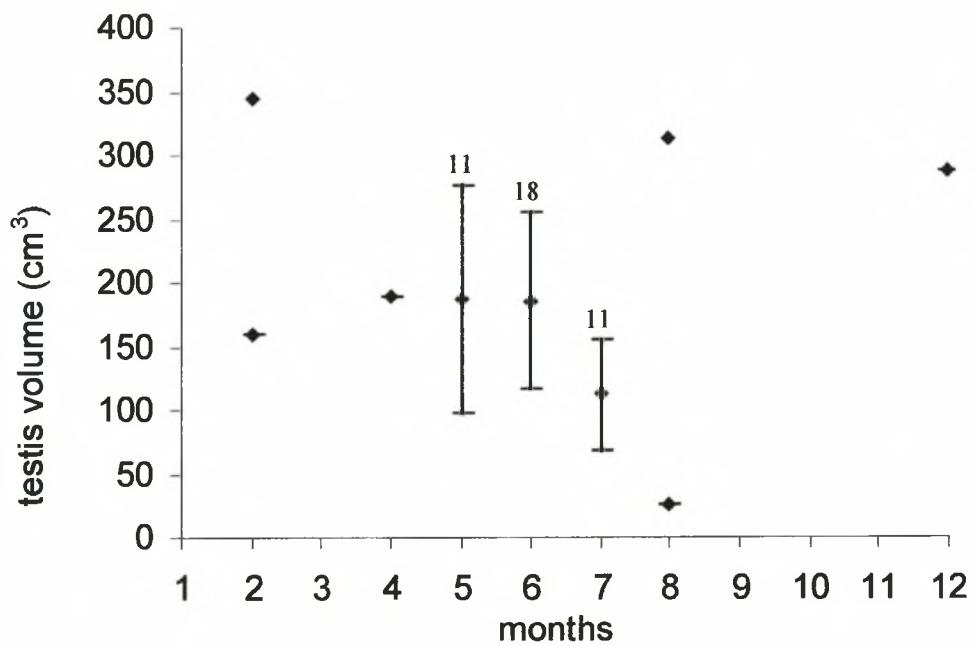


Figure 10. Seasonal variation of the mean testis volume ( $\text{cm}^3$ ) of male *Galeorhinus galeus* (mean  $\pm$  SD where  $n > 8$ ). The numbers on the graph represent n.

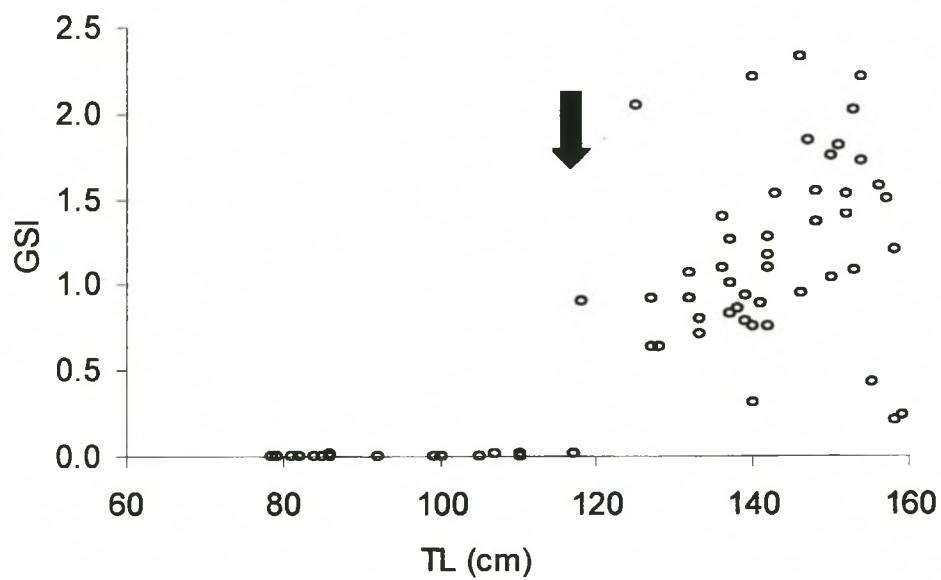


Figure 11. Relationship between the GSI and TL of male *Galeorhinus galeus*. The arrow indicates a TL of 118 cm beyond which the GSI increases dramatically.

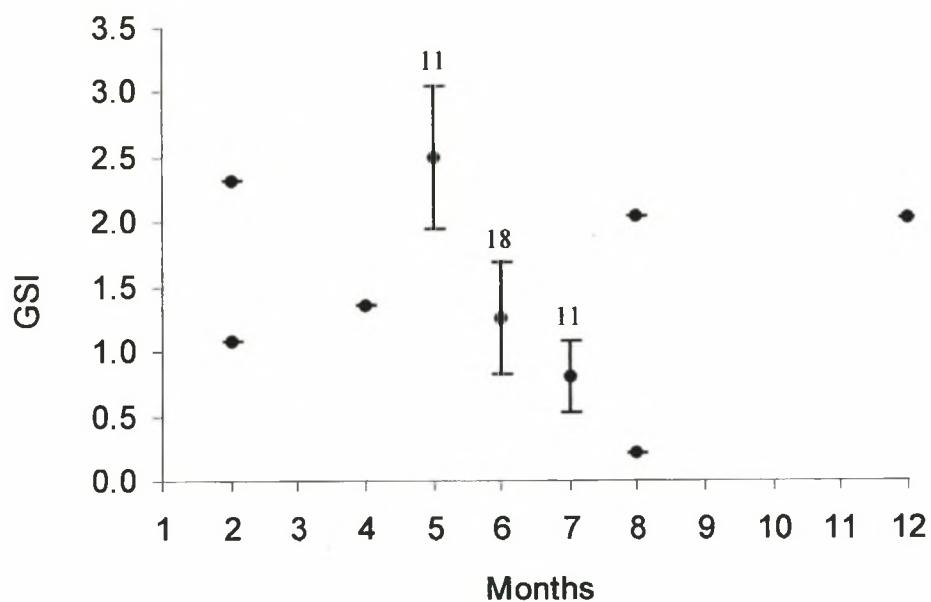


Figure 12. Seasonal variation of the mean GSI values of mature males (mean  $\pm$  SD where  $n > 8$ ). The numbers on the graph represent n.

### 3.3.2 Histology

The immature testis is markedly underdeveloped (see Figure 13a) and is little more than a mixture of some germinal and connective tissue. In the mature testis the different stages are clearly distinguishable and there is an ordered dorso-ventral progression from spermatogonial cysts to spermatocytic cysts and from spermatocytic cysts to spermigenic cysts (Figure 13b).

### 3.3.3 Seasonal spermatogenic activity

Data for similar times of the year display some variation, even when sampled on the same day and illustrates a measure of asynchrony in the reproductive conditions of individuals from the same populations. The data for the contributions of the respective spermatogenic stages to the testes' composition (see Figure 14a) all showed highly significant statistical differences between the means of the monthly data sets ( $P < 0.001$ ). There was no significant month-to-month variation observed for the % spermatogonial cysts (Dunn's method of pairwise comparison), but there was a trend for the values to decrease from higher values in autumn toward winter to reach a minimum in July ( $10.5\% \pm 2.3$ ). The % spermatocytic cysts was low in autumn after which there is a gradual monthly increase (Tukey Test,  $df = 4$ , ANOVA  $P < 0.05$ ) until a peak is reached in July ( $73.6\% \pm 9.8$ ). The % spermigenic cysts in the testes is high from February to April and decreases significantly (Tukey Test,  $df = 4$ , ANOVA  $P < 0.05$ ) until a minimum value is reached in August ( $3\% \pm 3.3$ ).

In terms of temporal variation in the testis as a whole, the annual changes that the testes undergo can be described as follows: during the autumn months the testis consists mainly of spermatogonial and spermigenic cysts. As winter approaches we see the % spermatogonial cysts decreasing as the spermatogonia start developing into spermatocytes, resulting in a gradual increase in % spermatocytic cysts. The middle of winter (July) sees % spermatogonial cysts at their minimum and % spermatocytic cysts at their maximum prevalence. Development continues so that the majority of spermatocytes become spermigenic between February and April, after which the % spermigenic cysts decline to reach its minimum in August.

### 3.3.4 Circulating hormone levels

The highest concentration of testosterone recorded in an immature shark was 12.1 ng/ml. However, for most of these juveniles the testosterone levels were under 2 ng/ml and did not seem to follow any seasonal patterns. The values of testosterone concentrations in mature animals were substantially higher than those recorded in the juveniles and ranged from 54.3 ng/ml to 1497.7 ng/ml. There is a highly significant statistical difference between the

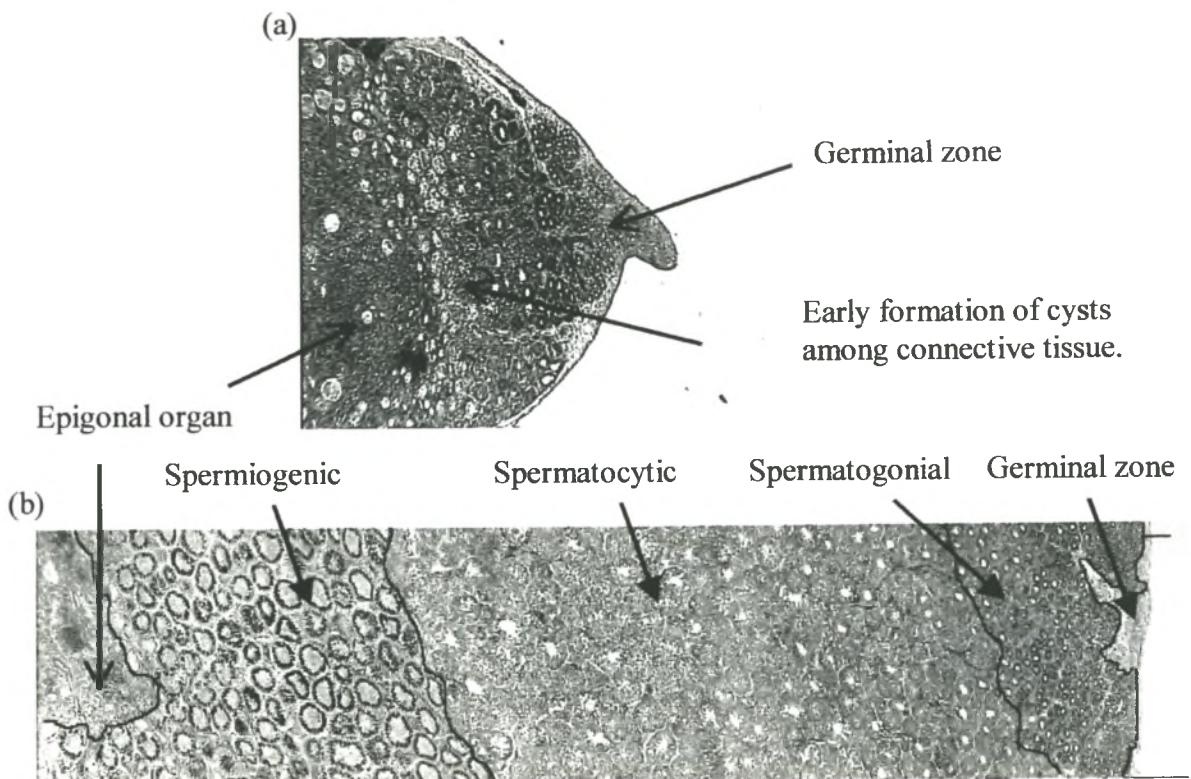


Figure 13. The organization of the testes of immature (a) and mature (b) *Galeorhinus galeus*.  
The lines were drawn to indicate the different spermatogenic zones more clearly.

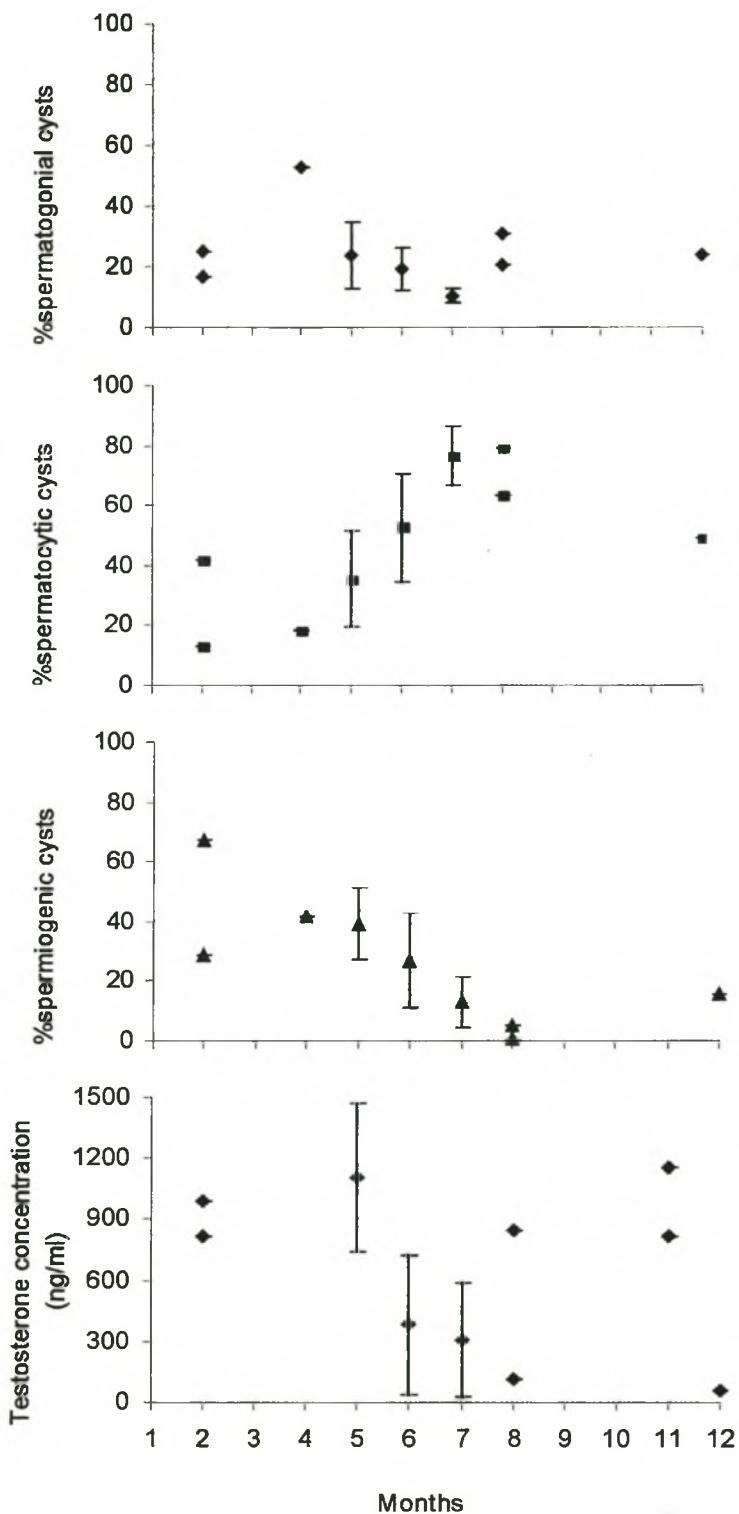


Figure 14. Combined illustration of the seasonal variation in the % spermigenic cysts (a), spermatocytic cysts (b) and % spermatogonial cysts (c) occupying the testes and testosterone concentration (ng/ml) (d) (mean  $\pm$  SD where  $n>8$ ).

means of the monthly data sets for the mature sharks (KW-ANOVA  $P < 0.001$ ). Relatively high testosterone levels occurred in early-autumn (February), which coincided with high prevalence of spermatogonial and spermiogenic cysts. The highest monthly median occurred in May (1254.7 ng/ml) as the % spermatogonial cysts and % spermiogenic cysts were decreasing and % spermatocytic cysts gradually increasing. Both low and high testosterone levels were found during winter months (June - August), but with the foregoing declining trend it can be suggested that the lowest median testosterone levels could probably be expected during July and August. This would then coincide with the minimum occurrence of spermatogonial- and spermiogenic cysts and the peak in the % spermatocytic cysts (see Figure 14).

### **3.3.5 Morphological and endocrine correlations**

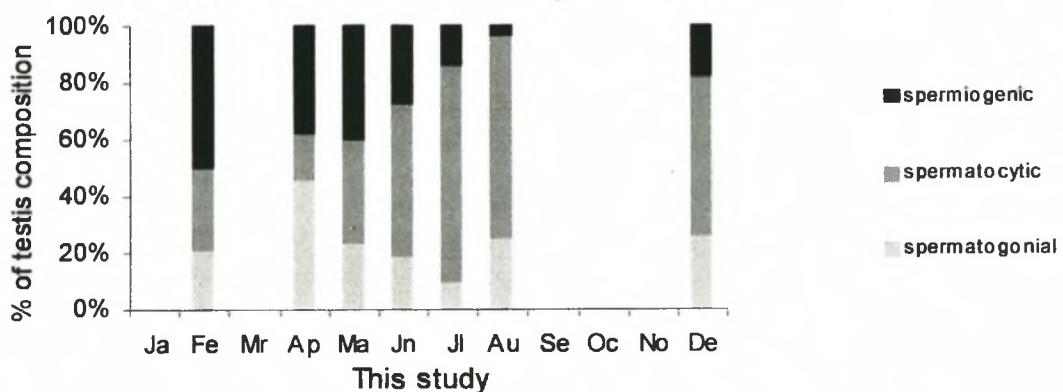
There are highly significant negative correlations between the % spermatocytic cysts and the spermatogonial ( $r = -0.64$ ,  $P < 0.001$ ) and spermiogenic percentages ( $r = -0.90$ ,  $P < 0.001$ ) respectively. The % spermiogenic cysts also showed significant but weak correlation with testosterone levels ( $r = 0.40$ ;  $P < 0.05$ ). Testosterone concentrations correlated weakly but significantly with total length ( $r = 0.38$ ;  $P < 0.05$ ) and the volume of the testis ( $r = 0.46$ ;  $P < 0.05$ ).

### **3.3.6 Intra- & interspecific comparisons**

As is seen in Figure 15, there are striking similarities between the results obtained in this study compared to that of McClusky (1988) for seasonal variation of the different spermatogenic stages. McClusky (1988) did not distinguish between the % spermatogonial cysts and % spermatocytic cysts in his analysis. However, the % spermiogenic cysts can be compared between these two studies and they show a very similar trend to decrease from the summer and autumn months to reach a minimum in July. When looking at the results of McClusky (1988), the testis composition for the month of May for two consecutive years look quite different (Figure 15b).

The extent of variation in the composition of the testes throughout the male reproductive cycle is not as severe in *G. galeus* when compared with another triakid, *M. griseus* (Figure 16). In *M. griseus* it is especially the spermatocytic and spermiogenic components of the testes that vary extensively throughout the cycle.

(a)



(b)

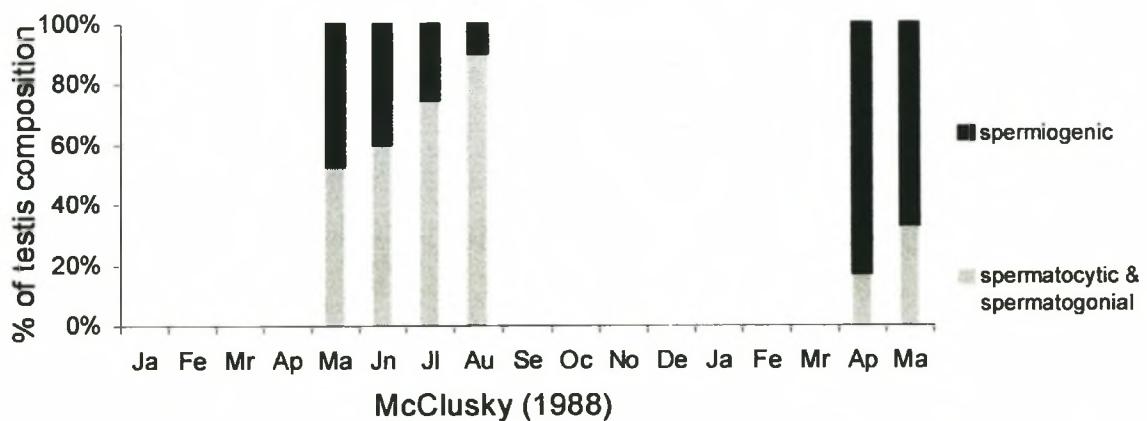


Figure 15. Comparison between the results of the seasonal variation in composition of the testis of *Galeorhinus galeus* obtained from this study and that obtained by McClusky (1988). These different studies show remarkably similar trends in the seasonal variation of especially spermiogenic activity.

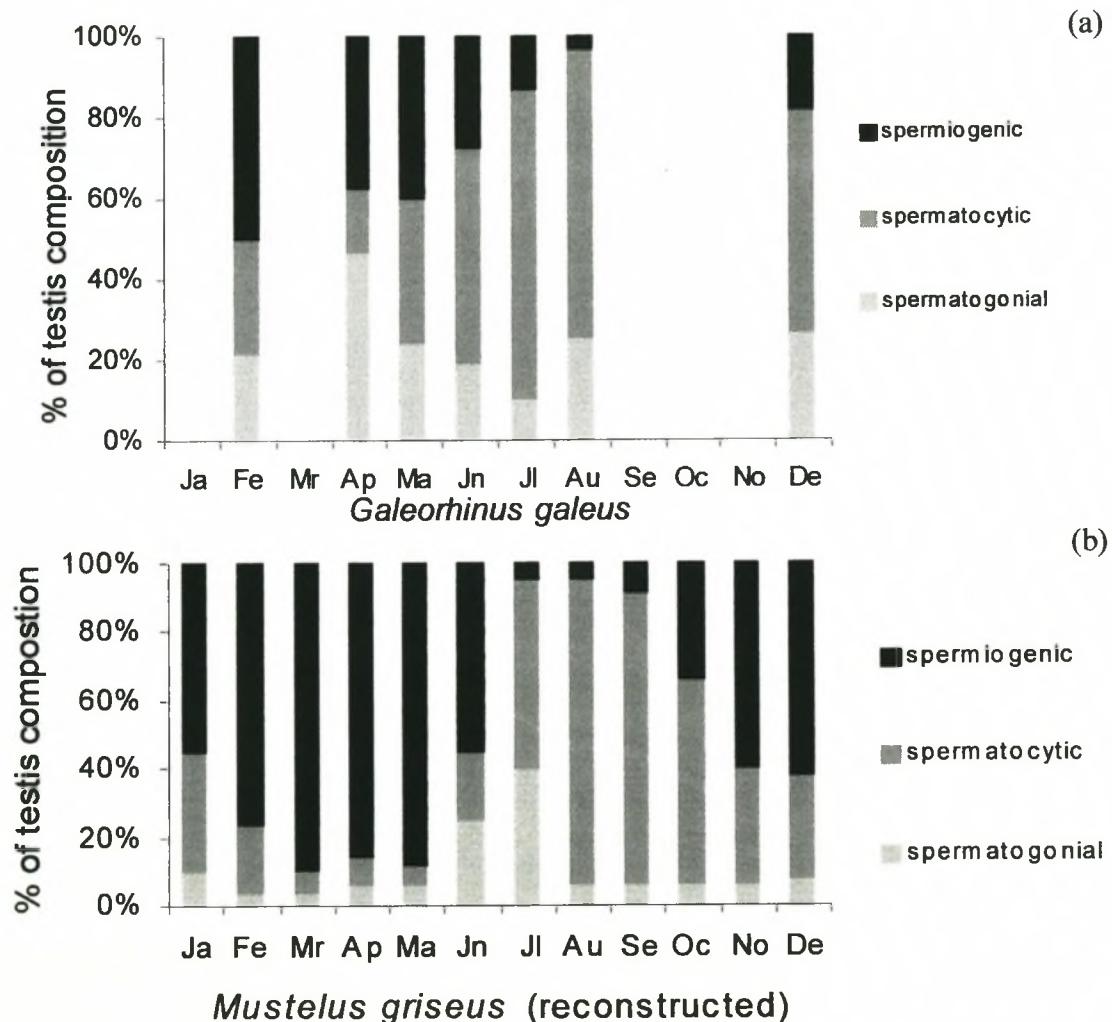


Figure 16. Comparison of the variation in the different spermatogenic stages between *Mustelus griseus* and *Galeorhinus galeus*. The variation of the spermatogenic cyst composition in *M. griseus* is much more extensive than in *G. galeus*.

### 3.3.7 The spermatogenic cycle of *G. galeus*.

Figure 17 shows the suggested reproductive cycle of the South African male *G. galeus* based on a combination of the findings of this study and that of McClusky (1988) and Freer (1992). The previous studies on the South African *G. galeus* have suggested, based on the GSI, that the mating season is from May to July (McClusky 1988; Freer 1992) and was confirmed by the histological and hormonal results of this study. Therefore, the mating season of *G. galeus* seems to start when GSI is low and testosterone concentrations and spermiogenic activity are decreasing. Spermatogenesis starts around April. The vesiculae seminales of *G. galeus* are also full at this time (McClusky 1988; Freer 1992) and therefore indicates a full 12-month cycle in the testis of *G. galeus*. The start of spermiogenesis is some time after August but it remains to be determined exactly how long after.

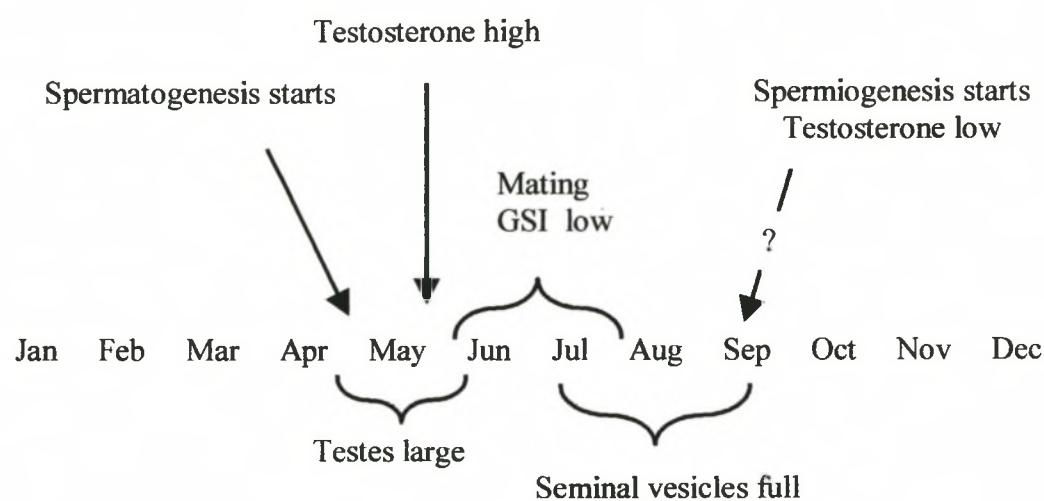


Figure 17. A schematic representation of the suggested reproductive cycle of the South African male *Galeorhinus galeus* based on a combination of the findings of this study, McClusky (1988) and Freer (1992).

### 3.4 DISCUSSION

The soupfin shark, *G. galeus*, is a globally distributed, coastal-pelagic species that is of great economic value to many shark fisheries around the world. Due to its importance to fisheries, this species has been the subject of many previous studies and its biology is considered to be reasonably well understood. Reproductive activity in male sharks takes on a cyclical nature in most species, as is expressed in the change of spermatogenic activity of the testes throughout a reproductive season. Even so, detailed descriptions of these spermatogenic cycles only exist for very few species. One of these is the soupfin shark, but due to evident global intraspecific variation regarding many aspects of their reproduction, dedicated studies on specific populations are required to assess the situation as, in this case, the South African population. The results obtained in this study identifies and describes the annual cycle of spermatogenic activity of the South African soupfin shark, *G. galeus*. Accounts of seasonal variation in serum testosterone concentrations of sharks are very rare and this study, as a first report for triakid hormone levels, should aid in our understanding of the endocrine control of shark reproduction.

The cyclical patterns that were observed in this study were often not very synchronised and a few possible reasons for this will be discussed first. Water temperature is known to be the main environmental influence on spermatogenic development in *S. canicula* (Dobson & Dodd 1977b) and the migratory behaviour of *G. galeus* (Olsen 1954). The average temperatures of the same months in different years are not the same (pers. observation) and could lead to a phase shift from year to year in the cycle of the testis. This would cause the monthly data to be more scattered, especially since sampling for this study was done over a stretch of 27 months. Evidence for this can be seen in the difference in the results of testis composition in *G. galeus* for the month of May for two consecutive years reported by McClusky (1988) (see Figure 15b). The different geographical areas sampled during this study and their respective temperature regimes might also have some influence on the variation in the data. Samples from Gansbay, being closer to the cold Benguela current and on average most likely colder than Stilbay, could be out of phase with the specimens from Stilbay. A similar account is the two- to three-month phase difference in the reproductive cycle of *G. galeus* between populations from Southeast Australia and South Australia, the water being slightly warmer at the latter location (Olsen 1954). These locations in Australia are, however, almost 2000 km away from each other so it is likely that Stilbay and Gansbay are too close (<200 km) to cause a significant difference. It would need specific testing to determine the level of influence of temperature on the variation displayed in the results.

Another possible contributor towards the scattered data could be asynchrony between the readiness of the sex cells and the secondary sex organs in *G. galeus*, as was also reported for *P. glauca* (Pratt 1979). This could lead to diminished confidence in the parameters used in determining maturity. In this present study, a 125 cm male had mature testes with all the stages of spermatogenesis present, but its claspers were not calcified and it would probably not have been able to mate yet. It is therefore possible that this particular 25 cm male might not actually be fully sexually mature yet and that the parameters to determine maturity in this present study are not strict enough. This would result in the possible inclusion of data from immature specimens, which would be reflected as low reproductive activity.

A third possible explanation arises from the positive correlation that exists between TL and testosterone concentration and testis volume, respectively. This suggests that if the smaller specimens are indeed fully mature, according to the parameters used in this study, they might not be sexually active yet. Nothing has been reported about the type of social structures that apply when the mature female and male schools aggregate to mate. It is therefore conceivable that the smaller males simply cannot access females until they are of a competitive body size. This mature but inactive reproductive state might then show up in the results as lower values in the hormone levels and, naturally, also in the tissues and structures that are affected by testosterone concentrations. Future studies would do well to investigate the influence of male body size on, for example, mating success of *G. galeus*.

The histological evidence and testis size both indicated that 118 cm TL is where sexual maturity is reached. This is smaller than the 128 cm and 123 cm TL at first maturity that was previously reported for South African *G. galeus* (Freer 1992). The former author based the onset of maturity on when the clasper length reached its mature size. Clasper length is the most widely used indicator of reproductive maturity in sharks (Pratt 1979). The sharp increase in clasper length after a TL of 118 cm in this study is therefore consistent with the other previously used indicators to determine the size at maturity. *G. galeus* males from Brazil reach maturity at a TL of 107 cm, which is considerably smaller than its South African counterparts. This difference in size at maturity is not unexpected if one considers that the maximum body size reported for the Brazilian population is also the smallest found among all the *G. galeus* populations around the world. The body size range of the South African *G. galeus* is very similar to that of the Australian population (see Table 4). In the present study, the range of the inner clasper length in mature sharks is very similar to the range that was reported by McClusky (1988) in an earlier study of the South African *G. galeus*. This range does not, however, agree with another study on the South African population where maturity

was reported to set in at a clasper length of 6.4cm with no subsequent development of the claspers in larger animals (Freer 1992). It is not clear why the data from the latter study differs so markedly from the current study and that of McClusky (1988). The range for the Brazilian *G. galeus*, based on the outer clasper length, is from 10.5 cm to 14 cm with a mean of 12 cm. This is also comparable with the data from this study (range 14 cm to 18 cm; mean 16 cm) because the outer clasper length is measured along the lateral edge of the clasper from its tip to the base of the pelvic fin and is therefore shorter.

McClusky (1988) reported some month-to-month variation in the GSI of *G. galeus*. No indication was given as to whether there were significant differences between the months, but the trend was that GSI was relatively high in autumn (April) and decreased toward a minimum level in June (early winter). Freer (1992) calculated the GSI for *G. galeus* as testis weight as a percentage of body weight and found that the highest monthly mean occurred in May and decreased to a minimum in July and August after which it increased again. It seems as if weight is a stronger indicator than volume of seasonal fluctuations in the testis, since both Freer (1992) and McClusky (1988) reveal the same trend based on testis weight. Regardless of how it was calculated, the GSI of the South African *G. galeus* seems to be at a minimum during winter.

It is clear that the seasonal variation in the volume of the testis of *G. galeus* is not very clearly expressed, especially when compared to other triakids. *M. manazo* and *M. griseus* both show very distinct peaks in the GSI at about December (early summer) and very distinct lows around July (mid-winter) (Teshima 1981). The differences between the high and low GSI values for these two species are also very prominent. In *Mustelus antarcticus* a distinct peak occurs in August which rapidly falls to reach its lowest levels in October to January (Lenanton *et al.* 1990). The GSI values, which is calculated by the same formula used by McClusky (1988) and Teshima (1981), forms a distinct peak in August although the variation is not as extensive as in the other two mustelid species (Lenanton *et al.* 1990). Similar seasonal variation in relative testis sizes with large ranges between minimum and maximum have also been reported in other species, for example *D. sabina* (Maruska *et al.* 1996) and *Rhizoprionodon terraenovae* (Parsons 1983) with more examples of shark species, whose GSI ranges are known, listed in Parsons & Grier (1992).

The diametric type of testis that is characteristic of carcharhinoid sharks (Pratt 1988), which includes *G. galeus*, makes it possible to describe and quantify the condition of the testis histologically. A detailed macroscopic and microscopic description of the reproductive

system and spermatogenesis of *G. galeus* was done by McClusky (1988) and should be consulted for details.

The pattern observed for the spermatogonial cysts suggests that the spermatogenic cycle starts around April. The decrease in % spermatogonial cysts thereafter is due to the subsequent development of the spermatogonia to become spermatocytes. The peak in % spermatocytic cysts about three months after the seemingly high percentage of spermatogonial cysts in April tells of this progression of development. By the time the % spermatocytic cysts peak, the % spermatogonial cysts is very low again, probably because most spermatogonia have by now developed into spermatocytes. This situation would probably remain until the next cycle starts the following autumn with the next peak in % spermatogonial cysts. The same shift in the peaks of the different stages is seen in *D. sabina* (Maruska *et al.* 1996), *M. manazo* and *M. griseus* (Teshima 1981) where the males have a clear annual cycle in spermatogenesis. In *M. manazo* and *M. griseus* the peaks in % spermatogonia and % spermatocysts are about a month apart (Teshima 1981), suggesting a more rapid development from spermatogonia to spermatocytes in these species than in *G. galeus* where it seems to take up to three months (see Figure 16).

The % spermigenic cysts peaks at the same time of the year as % spermatogonial cysts which suggests that it takes a full year for spermatogonia to become spermigenic. It seems that growth from spermatogonia to spermatocytes is more rapid than for spermatocytes to become spermigenic since the peaks of the former two stages are closer to each other. Spermatocytes dominate the testes for six to eight months in *S. acanthias* (Simpson & Wardle 1967) whereas in *M. manazo* and *M. griseus* the peak in % spermatocytes lasts only for about three months (see Figure 16b) after which it declines due to the cysts' becoming spermigenic (Teshima 1981). It remains to be determined which pattern of development applies in male *G. galeus* when larger data sets are available for the months from August to April.

The proportions of the testis taken up by spermatogonia and spermatocytes differ markedly between species. The large proportion of spermatogonial cysts recorded in *D. sabina* (Maruska *et al.* 1996) is not seen in this study for *G. galeus*. The spermatogonial cysts constitute the smallest proportion of the testis of all the stages in both *G. galeus* (this study) and *M. griseus* (Teshima 1981), whereas in *D. sabina* it seems to take up most of the testis for the biggest part of the year (Maruska *et al.* 1996). Also, in *G. galeus* the spermatocytic cysts seem to dominate the testis for most of the year (this study), whereas in *D. sabina* there seems to be a very low proportion of spermatocytes present (Maruska *et al.* 1996). It could be that the lack of data for some of the months in this study, especially summer, simply would not

show an abundant presence of spermatogonia if it existed. Nevertheless, the high proportion of spermatocytic cysts in *G. galeus* indicates that the variation that exists on the level of testicular composition is most probably accounted for by species differences. This is confirmed by the close resemblance between the trends in the current data (see Figure 15) and previous data for the South African *G. galeus* from McClusky (1988). The remarkable similarity in the monthly changes in especially spermiogenic activity between these two different studies confirms that this is indeed the pattern for the South African *G. galeus* and that the spermiogenic activity decreases from summer towards late winter.

Based on these monthly changes in the proportionate representation of the different spermatogenic stages in the testis, it is evident that an annual spermatogenic cycle is present in the testes of *G. galeus*.

This study is the first account of circulating hormone levels in *G. galeus* or any other shark from the family Triakidae. In general there is very little information available on plasma hormone levels in male elasmobranchs (Hamlett 1999) and consequently a poor understanding of the endocrine control of their reproduction.

The range between minimum and maximum testosterone values is very extensive in *G. galeus* compared to the findings in other studies that are summarised in Table 1. An almost 10-fold range was previously reported in *Raja ocellata* (Idler & Truscott 1966), but it is considerably less than the almost 30-fold range observed in *G. galeus* (this study). The minimum and maximum levels recorded in this study are also markedly higher than any previously recorded for elasmobranchs. The 358 ng/ml recorded in *Carcharhinus leucas* was, until now, one of the highest levels ever recorded for any vertebrate (Rasmussen & Gruber 1990). The fact that the maximum level in *G. galeus* is four-fold that of the previously top-scoring carcharhinoid, *C. leucas*, tempts to raise suspicion on the accuracy of the results. The fact of the matter is that the highest testosterone result is not an outlying data point. Just under half of the testosterone values recorded in this study were higher than that of *C. leucas*. With the appropriate validations for the assay being satisfactory, the only sensible conclusion to make is that the testosterone levels in *G. galeus* are indeed very high. Future contributions on elasmobranch hormone levels will eventually elucidate the true extent of variation in hormone levels among sharks.

The observed peak in testosterone concentration seems to be reasonably brief when one considers the sudden decrease in the two months thereafter. This is consistent with the trends in testosterone fluctuations from other studies where testosterone peaks never seem to last longer than two or three months (Garnier *et al.* 1989; Manire & Rasmussen 1997; Tricas *et al.*

2000). Unfortunately it is not possible to tell from the results of this study whether this peak in testosterone concentration during May is the only one in the spermatogenic cycle of *G. galeus*. A single peak in testosterone is found in the reproductive cycle of *S. tiburo* (Manire *et al.* 1997), while additional milder secondary peaks are reported for *S. canicula* (Garnier *et al.* 1989) and *D. sabina* (Tricas *et al.* 2000). It is therefore not possible to determine, based on the current data, whether *G. galeus* has only this single testosterone peak in its reproductive cycle or whether there are more, secondary testosterone peaks at other times of the year.

The peak in testosterone coinciding with the peak in % spermatogonial cysts suggests that testosterone plays a role in initiating the development of spermatogonia at the onset of the reproductive cycle. This is also the time when spermatocyte development is increasing in *G. galeus* and agrees with what has been reported in *D. sabina* (Tricas *et al.* 2000) and *S. tiburo* (Manire & Rasmussen 1997). Therefore, there seems to be a strong link between testosterone levels in male elasmobranchs and the initiation of the spermatogenic cycle as well as the onset of spermatocyte development. The *in vitro* evidence of testosterone production by the Sertoli cells in spermatocysts (cited in Tricas *et al.* 2000) suggests that this could probably be expected in all elasmobranchs.

The coinciding peaks of % spermigenic cysts and testosterone in *G. galeus* (Figure 14a,d) are similar to what happens in *D. sabina* (Tricas *et al.* 2000). In *D. sabina* the peak in spermogenesis also coincides with the peak in % spermatogonial cysts just like in *G. galeus*. There is a possibility though that the testosterone peak at this stage of the cycle is functionally related to mating and that it is not only related to the spermatogonial or spermigenic peaks at the onset or end of each cycle. The mating season for the Australian population of *G. galeus* is around May and June (Olsen 1954) which is about the same time of the year that the testosterone levels in the South African population start declining. In both *S. tiburo* and *S. acanthias* the mating season is accompanied by declining testosterone levels (Manire & Rasmussen 1997). This suggests that the mating season of the South African *G. galeus* is probably also around May and June when testosterone levels are declining and just after the peak in spermigenic activity. High testosterone levels drive the initiation and prevalence of aggressive sexual behaviour in *D. sabina* (Tricas *et al.* 2000) and it might also, at least partially, be the case for *G. galeus*. Testosterone therefore probably initiates mating behaviour, as in *D. sabina*, but does not seem to play a major role in sustaining it.

### **3.5 CONCLUSION: THE SEASONAL CYCLE OF THE MALE G. GALEUS.**

The seasonal cycle in the testes of *G. galeus* from South Australia has been well documented (Olsen 1954) and its similarities with this study and previous studies, aid in clarifying the cycle on the South African *G. galeus*.

The previous studies on the South African *G. galeus* have suggested the mating season to be from May to July, based on the GSI which was low during those months (McClusky 1988; Freer 1992). Although the relevance of GSI as an indicator of mating season has since been questioned (Maruska *et al.* 1996), the histological and hormonal results of this study seem to agree with the findings of McClusky (1988) and Freer (1992) that were based on GSI. Therefore, the mating season of *G. galeus* seems to start when GSI is low and testosterone concentrations and spermiogenic activity are decreasing. This differs slightly from the case in *M. manazo* and *M. griseus* where spermiogenesis is already at a minimum by the time mating starts (Teshima 1981).

The start of spermatogenesis is suggested to be around April. In *D. sabina* the decline in spermatogonial cysts and coincident increase in spermatocytic cysts marked the onset of spermatogenesis (Maruska *et al.* 1996). For *G. galeus* this would be just prior to the mating season, as is also found in *M. manazo* and *M. griseus* (Teshima 1981). The vesiculae seminales of *G. galeus* are also full at this time (McClusky 1988; Freer 1992) and therefore indicates a full 12-month cycle in the testis of *G. galeus* as was also reported for the Brazilian population of this species (Peres & Vooren 1991).

Based on the low proportion of spermiogenic cysts present in August, spermiogenesis is suggested to resume at that time (McClusky 1988, this study). However, in *M. griseus* the low proportion of spermiogenic cysts lasts for three months. More complete sampling of *G. galeus* is therefore needed, especially for the months following August, in order to determine more accurately when spermiogenic activity starts.

## 4. Seasonal reproduction in the female soupfin shark, *Galeorhinus galeus.*

### 4.1 INTRODUCTION

The living elasmobranchs are a diverse group that includes over 800 species of sharks and rays (Compagno 1977). Some unique aspects of their reproduction, for example internal fertilisation, viviparity, placental mechanisms for foetal maintenance and the patterns of development of the reproductive tract have closer association with what is found in amphibians and amniotes than with teleosts (Wourms 1977). Yet, within the elasmobranchs diverse strategies are displayed regarding some aspects of their reproduction, for example reproductive mode (Otake 1990). Some species are oviparous and, after only a brief incubation in the mother, the embryos are released in the ocean and left to be nourished solely by the yolk contained within their eggshells. Other species are ooviviparous and carry the embryo's until full term while they are nourished either by their egg yolk or uterine secretions (sometimes both). Still others are placentally viviparous where a placenta facilitates maternal nourishment of the embryos until parturition. Only 1/3 of all sharks is oviparous, leaving an array of species somewhere in a continuum between minimal and substantial maternal investment in embryonic development (Otake 1990). These and other reproductively related issues have fascinated researchers for a long time, not only because of their phylogenetic significance but also the strategies employed by them that have usually been associated only with higher vertebrates, for example internal fertilisation, highly specialised forms of viviparity and vertebrate endocrinology (Wourms 1977). However, when compared to teleosts, our knowledge of shark reproduction is at best fragmentary, selective and mostly only descriptive (Dodd 1983).

Morphologically, female elasmobranchs belong to one of two groups on the basis of their ovaries. Carcharhinids and sphyrids have external ovaries that are found on the distal surface of the epigonal organ. Typically only the right ovary is present, consisting of follicles that are confined under a single layer of generative tissue through which the ova are discharged during ovulation. The second type of ovary is internal and is characteristic of lamnids and alopids. In this case it is contained within the epigonal organ that completely envelops the ovary. Numerous oocytes of all sizes develop around a lumen through which they are discharged during ovulation. In the basking shark, *Cetorhinus maximus*, the ovary contains innumerable minute ova among a couple of larger ones at different stages of growth (Matthews 1950). Elasmobranch ova, regardless of what the reproductive mode is, are

typically large and abundantly yolked except for some viviparous forms that rely primarily on uterine derivatives for embryonic development (Hamlett & Koob 1999).

A wide range of determinants has previously been used to indicate maturity. These include the extent of development of the ovary, size of the oviducal gland, width and extent of development of a flattened uterus, the presence of uterine eggs or embryos and fresh mating scars (Olsen 1954; Springer 1960; Wass 1973; Bass *et al.* 1975; Pratt 1979; Parsons 1983; Castro *et al.* 1988; Capapè *et al.* 1990; Freer 1992; Yano 1993). However, the most widely used parameter is the presence of vitellogenic ovarian follicles (Pratt 1979). When plotted against body size, the diameter of the largest ovarian follicles serves as a good indicator of maturity (Pratt 1979; Parsons 1983; Yano 1993). However, ovarian follicle diameter might not be a good indicator on its own and should be used in conjunction with other parameters to prevent females with mature but inactive ovaries from being classified as immature. The condition of the ovary can also be taken into account. The mature ovary has yellow, yolk-filled ova while an immature ovary has white follicles and tends to be homogenous in texture (Capapè *et al.* 1990; Yano 1993). The ovary of *Galeorhinus galeus* is considered mature when it contains ovarian follicles of various sizes (McClusky 1988). The oviducal gland undergoes accelerated growth after maturity is reached and is also often used as an indicator of sexual maturity (Castro *et al.* 1988). The condition and size of the isthmus and uterus are also useful in distinguishing immaturity from inactivity.

The state of the reproductive activity of a mature animal can be described in terms of its reproductive morphology and endocrinology. The ovaries and compartments of the oviduct display varying sizes and properties throughout a reproductive cycle and allows for classification into different stages to define such a cycle. For example, in *Iago omanensis* the width of the anterior oviduct ranges between 3.5 - 6.0 mm in resting adult females and between 18 - 22 mm during gestation (Fishelson & Baranes 1998). Also, in *Carcharias taurus* the active right ovary is greatly enlarged and contains numerous follicles of various sizes during vitellogenesis (Gilmore *et al.* 1983). The ovaries of *Rhizoprionodon terraenovae* change markedly from consisting of about 30 small oocytes between vittelogenic cycles to having four to eight large, vitellogenic follicles near ovulation (Parsons 1983). In *Prionace glauca* the ovary of a gravid female with full-term in-utero embryos contained more than 1000 follicles, mostly smaller than 1 mm, as well as the next generation's 123 vitellogenic ova, varying in diameter from 6 to 20 mm (Pratt 1979). The same situation of concurrent vitellogenesis with embryonic development is true for a number of species that have annual reproductive cycles. Generally, the changes of the ovary during a reproductive cycle are most

often quantified by a gonado-somatic index (GSI), which is a parameter of the ovary divided into the same parameter of the whole animal, for example weight.

Our knowledge of the seasonal fluctuations of reproductive steroid hormones is sparse and has only been adequately described in *Raja erinacea* (Koob *et al.* 1986), *Squalus acanthias* (Tsang & Callard 1987b), *Sphyrna tiburo* (Manire *et al.* 1995) and *Dasyatis sabina* (Tricas *et al.* 2000). As a result, the endocrinological aspects of reproduction of sharks are still rather ill understood.

Three major patterns of seasonal reproduction are found in sharks (Wourms 1977). Some maintain reproductively activity throughout the year with no sign of seasonal occurrences, for example *Chlamydoselachus anguinus*, *Heterodontus portusjacksoni* (cited in Wourms 1977), *Scyliorhinus canicula* (Sumpter & Dodd 1979), *I. omanensis* (Fishelson & Baranes 1998), *P. glauca* (Gubanov & Grigor'yev 1975), *Alopias superciliosus* (Gruber & Compagno 1981), *Carcharhinus falciformes* (Compagno 1984) and *Carcharhinus dussumieri* (Teshima & Mizue 1972). Other species are reproductively active throughout the year with one or two mild peaks in activity. Examples include *R. erinacea* and *Hydrolagus colliei* (cited in Wourms 1977). The last category of female sharks has a well-defined annual or biennial cycle (Wourms 1977). Examples are listed in Table 2. Such studies are mostly based on temporal variation in the gonado-somatic index (GSI), ovarian follicle size or the uterine content (i.e. stage of embryonic development).

The soupfin shark, *G. galeus*, is a circumglobal species that has enjoyed a fair amount of attention mainly due to its economic importance in many countries (Compagno 1984). Studies on their reproduction have been done for populations from Brazil, Tunisia, Australia, California and South Africa (Walker 1999). This has brought to light the many differences that occur among different *G. galeus* populations, as summarised in Table 4, expressing the need for focussed studies on each of the populations separately. For example, an annual reproductive cycle has been reported for Tunisia (cited in Walker 1999) and California (Ripley 1946), a biennial cycle in the Australian population (Olsen 1984) while a three-year cycle is proposed for the Brazilian stocks (Peres & Vooren 1991).

Our knowledge of the reproduction of the South African *G. galeus* females is sparse (Walker 1999). The main sources of information have been McClusky (1988) and Freer (1992) but in these studies there were not enough samples available to describe the female reproductive cycle. The main problem is the seasonal migratory behaviour of this species that causes them to be absent from the sampling areas during certain times of the year (Walker 1999).

This study was therefore aimed mainly at describing the reproductive cycle of the South African female *G. galeus* on both a morphological and endocrinological basis. In doing so, this is the first report of female plasma concentrations for testosterone, 17- $\beta$ -estradiol and progesterone in a triakid shark. It is also the first account of employing an enzyme-linked immunosorbent assay (ELISA) for determining steroid hormone concentrations for sharks. This technique has very seldom been used before to quantify steroid hormones in non-mammalian animals and has many advantages over the more traditional radio-immuno assay. Lastly, this study is also an attempt to elucidate the specific reproductive functions of the circulating steroid hormones during the reproductive cycle of the female *G. galeus* and in doing so, contribute to the currently incomplete understanding of the reproductive endocrinology of female sharks.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Collection of material

The female *G. galeus* specimens used for this study were collected at Stilbay ( $34^{\circ} 24'S$ ;  $21^{\circ} 25'E$ ), Kalkbay ( $34^{\circ} 8'S$ ;  $18^{\circ} 27'E$ ) and Gansbay ( $34^{\circ} 35'S$ ;  $19^{\circ} 21'E$ ) along the south coast of South Africa. These collection sites, as illustrated in Figure 5, constitute the main commercial harvesting areas for this species in South Africa where they occur. Environmental data were obtained for Gansbay from the weather bureau with the help of Dr. Michael Scholl and is illustrated in Figure 6.

A total of 74 female *G. galeus* were obtained for this study from the local fisheries or fishermen between April 1998 and July 2000. The sharks are caught on hand lines by fishermen and brought ashore to be gutted. As soon as the sharks were landed, blood samples were collected by direct cardiac puncture using a heparinised needle and syringe and transferred to vac-u-test tubes and transported on ice to the laboratory. Here the whole blood was briefly centrifuged and the plasma collected and stored at  $-20^{\circ}\text{C}$  for subsequent testosterone, estradiol and progesterone assays.

The complete reproductive system for each individual, consisting of the ovary, ostium and two anterior oviducts, oviducal glands, isthmi and uteri, was removed by dissection for each specimen and stored in 4% phosphate-buffered formaldehyde solution for subsequent morphometric and histological examination.

#### 4.2.2 Morphometrics and maturity

The total length (TL) for each specimen was measured along a straight line from the tip of the snout to the tip of the upper caudal lobe. The precaudal length for each specimen was measured from the tip of the snout to the precaudal notch. The applicable measurements of each component of the reproductive system of female *G. galeus* were measured with a digital vernier calipers or a ruler (for measurements > 15 cm). The mean diameter of the largest follicles present in each ovary was recorded to the nearest 0.5 mm and the presence/absence of vitellogenic follicles in the ovary were noted. Vitellogenic follicles can be recognised by their distinct yellow colour compared to the white, translucent appearance of non-vitellogenic follicles (Peres & Vooren 1991). The length, width and height of the oviducal gland and width of the anterior oviduct were measured to the nearest 0.1 mm. The length and width of both the isthmus and uterus were recorded as well as the number and size, to the nearest 1 mm, of embryo's where present.

Although hormonal and morphological data were collected for all the specimens, only the data from reproductively mature animals were used for analysis of the reproductive cycle. Female sharks were considered mature when they were gravid or, in the absence of uterine ova or embryos, had well developed ovaries with various sized follicles, large, well developed oviducal glands and developed, distinguishable anterior oviducts, isthmi and uteri (Olsen 1954; Pratt 1979; Castro *et al.* 1988; McClusky 1988). All these requirements had to be met for a female to be classified as mature and borderline cases were considered immature.

#### 4.2.3 Histology

A directly transverse block (5-7 mm thick) that included both zones of the oviducal gland was removed from the middle of one of the oviducal glands of a subset of females. These tissue blocks were washed in running tap water for at least eight hours and then dehydrated and impregnated using an automated Shandon tissue processor according to the following routine: 70% ethanol for three hours, 90% ethanol for one and a half hours (x2), 100% ethanol for one and a half hours (x2), 50% toluene in ethanol for one and a half hours, 100% toluene for one and a half hours, 100% toluene for two hours, 50% toluene in wax for two hours, 100% wax for five hours and another 100% wax for three hours. Each block was then embedded in paraffin wax (56C). Histological sections of 10 µm were taken with a rotary microtome and stained with Harris's hematoxylin and eosin (Stevens 1977). After mounting the sections on microscope slides, they were examined to look for any signs of sperm presence.

#### 4.2.4 Hormone extractions and analyses

The steroids were first extracted from the plasma samples using diethyl ether. 230 µl serum and 1 ml diethyl ether (1:4.3 v/v) was vortexed for 20 seconds and then shaken for 15 minutes. It was then centrifuged for five minutes at 2000g and the aqueous phase (plasma) subsequently frozen in a -80°C freezer. The upper organic phase was decanted and evaporated until dry under a stream of air. A second 1 ml of diethyl ether was added to the plasma once it was thawed and the process was repeated. The dried extracts were reconstituted by sonication into 230 µl steroid-free, 0.1% human serum albumin solution in a Sonicor sonicator (Sonicor Instrument Corporation, New York) for 12 minutes. Extraction efficiency was determined by adding known amounts of tritiated ( $H^3$ ) testosterone to four random aliquots of serum, extracting them together and determining the respective recoveries by scintillation counts against a 100% tritiated control. Extraction efficiency of testosterone was 84%.

The testosterone, estradiol and progesterone concentrations of each serum sample were determined in duplicate using ELISA kits from IBL, Hamburg, Germany (RE52151, RE52041 and RE52231). In brief, the procedure involves the addition of 25 µl of the standards and samples onto a 96-well plate that has been coated with anti-testosterone antibodies. They are allowed to incubate at room temperature for five minutes after which 200 µl of enzyme conjugate is added to each well. After a 60-minute incubation at room temperature the plate is washed three times in the wash buffer and 200 µl of TMB substrate solution is added to each well. After a final 15-minute incubation at room temperature, the TMB-stop solution is added (100 µl) and the optical density is measured at 450 nm.

The % recovery, %intra-assay variance and %interassay variance are summarised in Table 5. These validations proved the validity of this assay for shark steroid hormones. The dilutions for testing parallelism were made in 0.1% human serum albumin/PBS on three samples at five or six dilutions each. Intra-assay variability was validated using four samples that were assayed in triplicate. Interassay variability was determined using three samples that were assayed twice on different assays. Testosterone, estradiol and progesterone demonstrated complete parallelism in the ELISA when compared to the standards, supporting the assumption that the extraction procedure eliminated any major interfering substances. Validation procedures of this kind have been reported previously (Manire *et al.* 1995; Manire & Rasmussen 1997; Snelson *et al.* 1997; Tricas *et al.* 2000) and proved the validity of this assay for shark steroid hormones.

Table 5. Validation results of the enzyme-linked immunosorbent assay used in this study for quantifying testosterone, estradiol and progesterone in *Galeorhinus galeus*.

	Recovery range	% recovery (n)	Intra-assay	Interassay
	(ng/ml)		%CV	%CV
Testosterone	0.3 - 9.8	103.6±11.0 (17)	3.4	12.3
Estradiol	0.02-1.20	110.7±12.8 (16)	11.7	7.1
Progesterone	0.3-8.6	93.3±14.9 (15)	10.8	12.7

#### 4.2.5 Statistics

A very common problem when studying seasonal cycles in sharks is to obtain a large enough sample size from both sexes that represents at least one entire reproductive cycle (Wourms 1977). Another issue regarding the statistical analyses of periodically sampled data, especially that of hormone production, is that individuals are not always in synchrony with the rest of their population and this often results in monthly data sets not being normally distributed (Tricas *et al.* 2000). The monthly data sets were analysed for normal distribution with the Kolmogorov-Smirnov normality test. Normally distributed data sets were tested for significant variation of the monthly means using One Way Analysis of Variance (ANOVA). The monthly data sets that were not normally distributed were tested for significant variation of their medians using Kruskal-Wallis One Way Analysis of Variance on Ranks (KW-ANOVA). Where variation was significant ( $P<0.05$ ), pairwise comparisons were done with Tukey's test (parametric) and Dunn's test (non-parametric) to isolate the groups that differed significantly. Associations between morphological-, histological- and endocrinological data were determined using the Pearson Product Moment Correlation method ( $P<0.05$ ). Females were lastly categorised into one of four groups: 1) NGR - non-gravid; 2) EGR - early-gravid where no developing embryos can be seen with the naked eye; 3) LGR - late-gravid where developing embryos can be seen and measured; 4) IMM - immature females. Steroid hormone concentrations and some morphological aspects were tested for significant variation amongst these four categories using Kruskal-Wallis One Way Analysis of Variance on Ranks (KW-ANOVA) after which Dunn's test was done to isolate the groups that differed significantly.

### 4.3 RESULTS

#### 4.3.1 Maturity

Of the 74 females sampled in this study, 36 were mature. The smallest mature female recorded in this study was 131 cm TL. There were, however, nine individuals that were larger than 131 cm TL that were not mature yet, the largest one being 142 cm TL. They could therefore not be considered mature plainly for being larger than 131 cm and necessitated that each female had to be analysed individually according to the various predetermined considerations.

### 4.3.2 Morphometrics

#### OVARIAN FOLLICLES

The mean diameters of the largest ovarian follicles formed two size groups, namely those that were smaller than 20 mm and those that were larger than 37 mm (see Figure 18). All non-pregnant females and 26% (6/23) of the pregnant females had follicle diameters smaller than 20 mm. The follicle diameters of the remaining 74% pregnant females were all larger than 37 mm.

With the exception of three specimens of which one was gravid, all the mature ovaries sampled in this study had vitellogenic follicles.

The monthly data sets for mean ovarian follicle diameter displayed significant variation between their monthly medians (KW-ANOVA  $P<0.05$ ). The biggest follicles in the ovary occur from mid- to late-winter (July-August), but there seems to also be a milder peak in ovarian follicle diameter in the middle of summer (January) (see Figure 19). The large ovarian follicles in August coincide with uterine eggs that have no visible embryos yet. All females, with the exception of only four in the summer, can be grouped into one of three categories: A) non-gravid females that have small ( $<20$  mm) ovarian follicles; B) gravid females with large ovarian follicles and no visible embryos in their uteri; and C) gravid females with small ovarian follicles and visible embryos in their uteri (see Figure 20).

#### OVIDUCAL GLANDS

The oviducal gland size was determined by the product of the length, width and height of the oviducal glands. This measure correlated highly significantly with the TL ( $r = 0.695$ ,  $P<0.001$ ) and had to be divided by TL for each individual to correct for the influence of body size on oviducal gland size. The natural logarithm was then calculated for this size index and was analysed for significant variance. The medians of the monthly data sets varied significantly (KW-ANOVA  $P<0.05$ ) and is graphically presented in Figure 21. There were no significantly different pairs of monthly data sets but there seem to be peaks in the oviducal gland size both in summer (December-January) and late-winter (August), although the winter peak seems to be higher (Figure 21).

Sperm was found in the oviducal glands of each of a sub-sample of females ( $n = 4$ ) as is seen in the example shown in Figure 22. The presence of sperm in the oviducal glands was not investigated in terms of temporal variation but only reported to confirm the storage of sperm by female *G. galeus*.

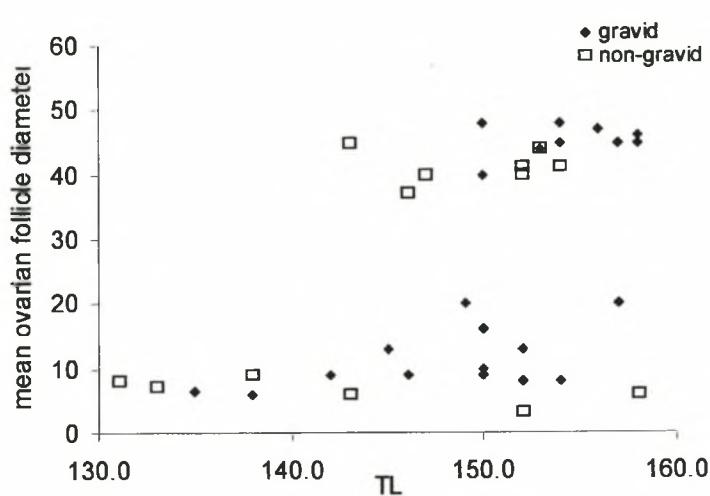


Figure 18. Means of the largest ovarian follicles of mature *Galeorhinus galeus* (mm) plotted against total length. Two distinct follicle size groups are noticeable.

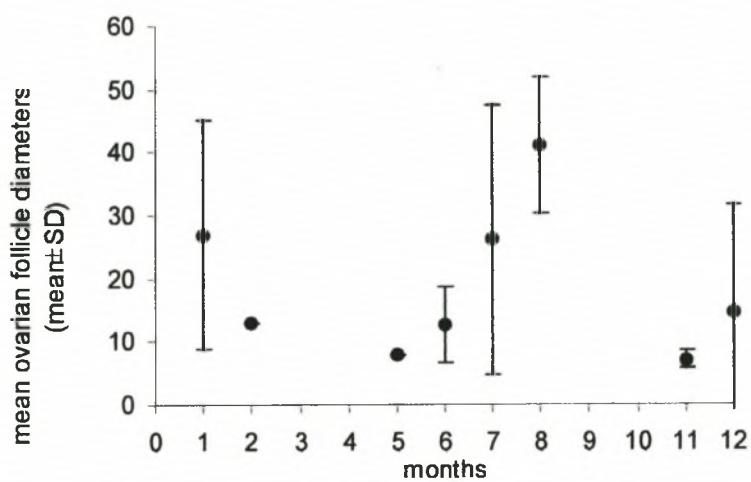


Figure 19. Seasonal variation of the mean ovarian follicle size (mm) of mature *Galeorhinus galeus* (mean  $\pm$  SD).

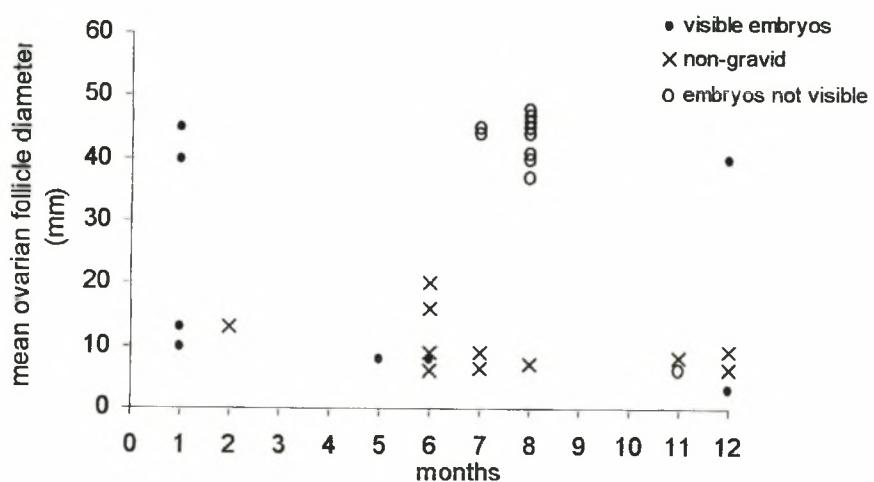


Figure 20. Seasonal distribution of the three categories of ovarian follicles found among mature *Galeorhinus galeus* females.  $\times$  = non-gravid females;  $\circ$  = early gravid females whose embryos are not yet visible with the naked eye;  $\bullet$  = late-gravid females whose embryos are visible with the naked eye.

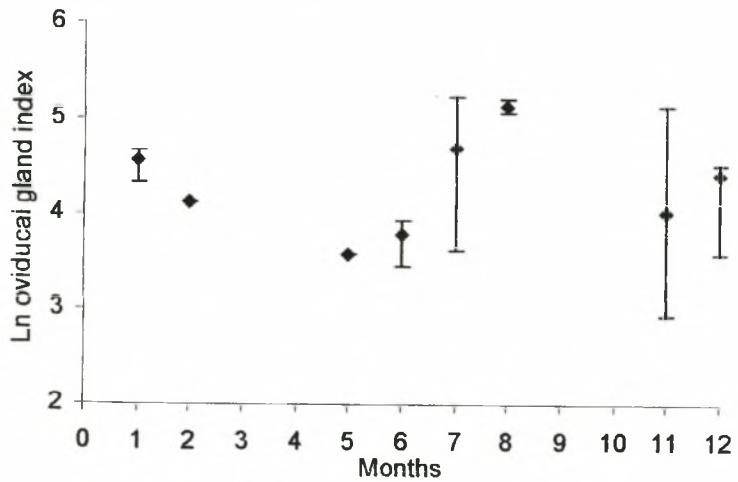


Figure 21. Seasonal variation in the size of the oviducal glands of *Galeorhinus galeus*. The graph represents the natural logarithm of the size index of the oviducal gland against months, indicating the median and 25<sup>th</sup> to 75<sup>th</sup> percentiles.

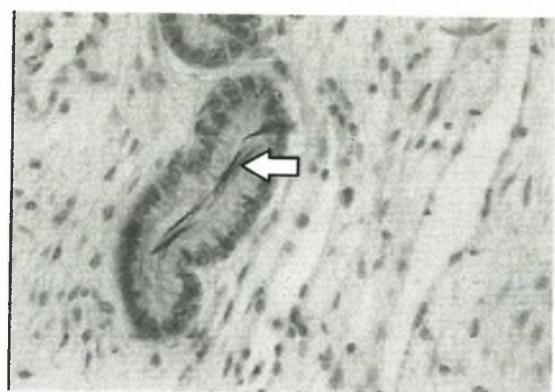


Figure 22. Digital micrograph of a 10  $\mu\text{m}$  section through the oviducal gland of *Galeorhinus galeus* showing the presence of sperm in one of the glandular tubes.

## PREGNANCY

A total of 23 females sampled during this study were gravid of which the smallest one was 143 cm. The average number of embryo's per pregnant female in this study is 26.4 (range 15 to 34; n=12). Embryos were either very small (~29 mm) or close to full-term size (~285mm) around December and January. Around the middle of the year they were typically mid-term size (~170mm).

### **4.3.3 Seasonal variation in plasma hormone concentrations**

#### TESTOSTERONE

There was significant seasonal variation between the monthly medians for testosterone concentration (KW-ANOVA P<0.05) with individual concentrations ranging from 0.24 ng/ml to 18.9 ng/ml (Figure 23a). Values predominantly remain below 0.3 ng/ml throughout the year until early winter (June) where a significant increase is seen (Dunn's test, P < 0.05) that peaks in August (median 16.3 ng/ml). The slightly elevated testosterone concentrations in November suggest that testosterone slowly decreases after its peak in August towards the summer months.

#### ESTRADIOL

The seasonal variation between the monthly means of estradiol concentrations was significant (ANOVA P<0.05). Individual concentrations ranged from 0.35 ng/ml to 18.5 ng/ml (Figure 23b). Estradiol concentrations were predominantly low (<7 ng/ml) over most of the year except for the distinct peak over the winter months (June to August). There were, however, low concentrations measured for these winter months as well.

#### PROGESTERONE

The individual concentrations that were measured for progesterone ranged between 0.16 ng/ml and 13.4 ng/ml although the majority of individuals measured progesterone concentrations of below 6 ng/ml. There was no seasonal pattern in the variation of the monthly data sets, except to note that a high individual value was recorded both in the beginning of summer (December) and mid-winter (July) (Figure 23c). This caused the data for progesterone concentration to display an almost bimodal distribution.

#### IMMATURE FEMALES

The ranges of the concentrations recorded in this study of immature *G. galeus* females are summarised in Table 6. There seems to be a reversal of rank in seasonal trends of the steroid

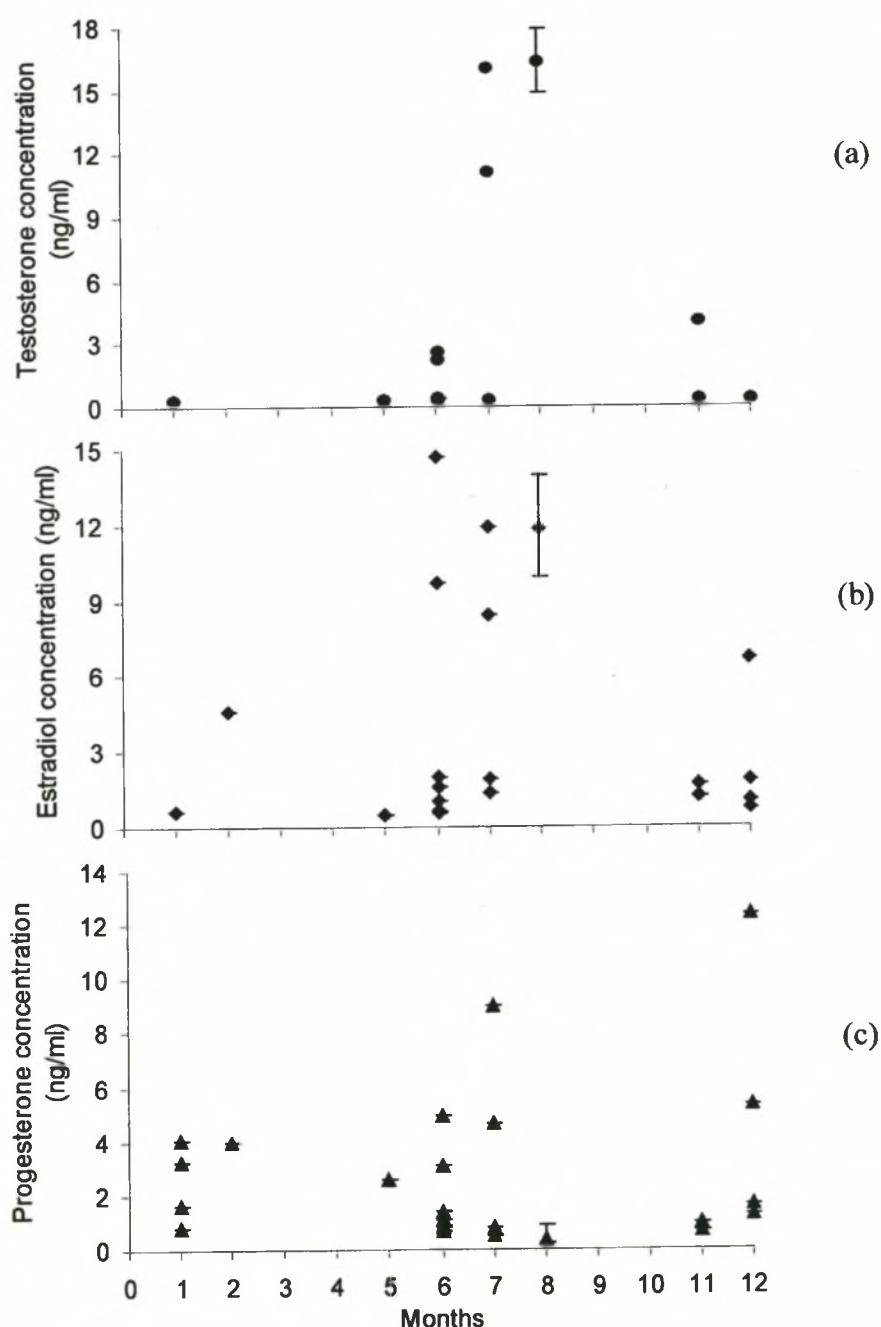


Figure 23. Seasonal variation in the concentrations of testosterone (a), estradiol (b) and progesterone (c) of **mature** female *Galeorhinus galeus* (a & c: median, 25<sup>th</sup> to 75<sup>th</sup> percentiles where n>8, b: mean  $\pm$ SD where n>8).

Table 6. Summary of the respective ranges recorded for each hormone in **immature female** *Galeorhinus galeus*. The concentrations are all given in ng/ml.

Steroid (ng/ml)	Range	
	min	max
Testosterone	0.17	0.66
Estradiol	0.04	1.65
Progesterone	0.33	14.94

levels of immature specimens (see Figure 24). Unlike the case with mature females, testosterone levels show the least seasonal variation of the three hormones (Figure 24a). Variation in testosterone levels (Table 6) is too small to suggest even a slight seasonal trend in immature females. Estradiol levels in immature females seem to show a similar seasonal trend to what was found in mature females with some values indicating a peak at the same time of the year as the estradiol values of mature females (Figure 24b). The fluctuation is very small, though, and the estradiol cycle in immature females is therefore considered to be much weaker. Progesterone levels, on the other hand, show an almost identical bimodal trend to that found in the mature females (Figure 24c). Progesterone concentrations therefore seem to fluctuate seasonally even in immature females. This could suggest that the role of progesterone starts even before reproductive maturity is reached and continues during the reproductive cycle of mature females.

#### 4.3.4 Morphological and endocrine correlations

##### MORPHOLOGICAL CORRELATIONS

On a morphological level, a highly significant correlation was found between the mean ovarian follicle diameter and the oviducal gland index ( $r = 0.79; P < 0.001$ ). Ovarian follicles were found to be the largest in early-gravid females (EGR) and was highly significantly larger than in non-gravid females (NGR) and immature females (IMM) ( $P < 0.001$ ). The late-gravid females (LGR) were the group with the second largest ovarian follicles and was also highly significantly larger than in NGR and IMM females ( $P < 0.001$ ).

The oviducal gland size index was highly significantly larger in EGR females than in NGR and IMM females ( $P < 0.001$ ). The LGR females had the second-largest oviducal glands and IMM females the smallest (highly significantly) of all four groups ( $P < 0.001$ ).

##### TESTOSTERONE

EGR females have highly significantly increased testosterone concentrations above that of NGR, LGR and IMM females (KW-ANOVA  $P < 0.001$ ) (see Figure 25). There is no statistical difference in testosterone concentrations between these latter three groups. Testosterone also positively correlates highly significantly with ovarian follicle diameter ( $r = 0.82; P < 0.001$ ), oviducal gland size ( $r = 0.77; P < 0.001$ ) and estradiol concentration ( $r = 0.84; P < 0.001$ ).

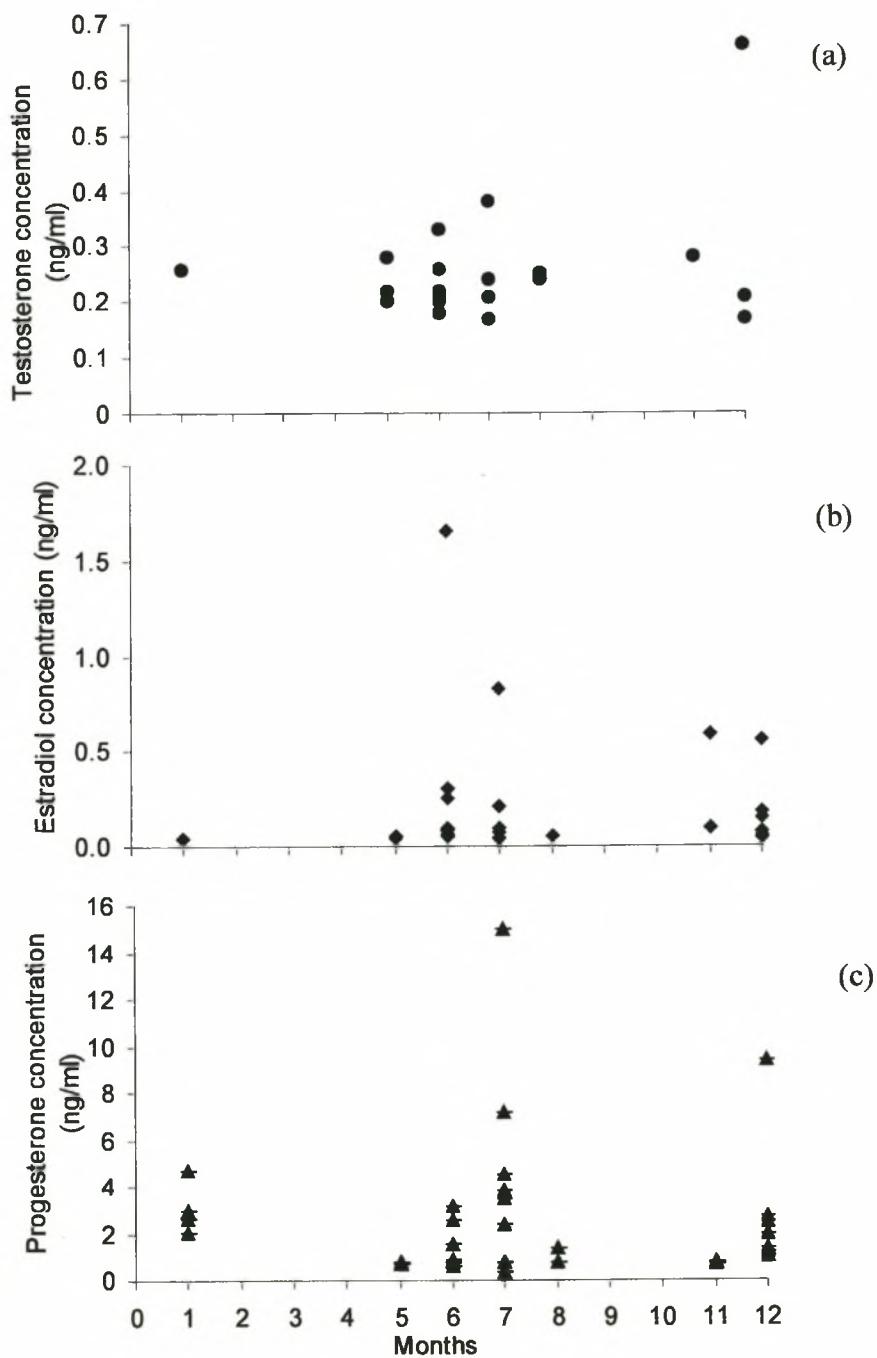


Figure 24. Seasonal variation in the concentrations of testosterone, estradiol and progesterone of immature *Galeorhinus galeus* females.

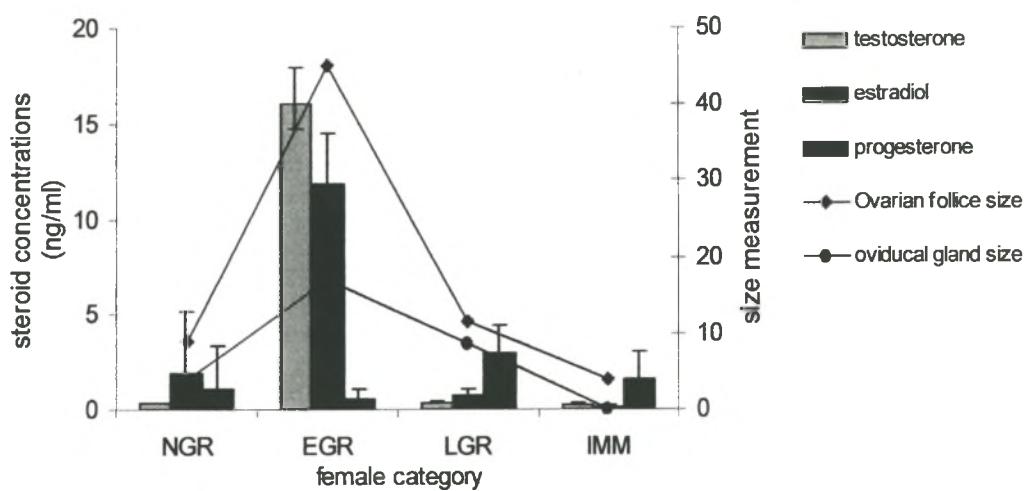


Figure 25. Variation in the size of the ovarian follicles (mm) and oviducal glands (index units) and concentrations (ng/ml median, 25%, 75%) of testosterone (□), estradiol (▨) and progesterone (■) between functionally grouped *Galeorhinus galeus* females. The females are categorized into non-gravid (NGR), early-gravid (EGR), late-gravid (LGR) and immature (IMM).

## ESTRADIOL

LGR and IMM females have estradiol concentrations that are highly significantly lower than that of EGR (highest) and NGR (second-highest) females (ANOVA  $P<0.001$ ) (see Figure 25). Estradiol also positively correlates highly significantly with ovarian follicle diameter ( $r = 0.69$ ;  $P<0.001$ ) and oviducal gland size ( $r = 0.64$ ;  $P<0.001$ ) although these correlation coefficients are lower than those of testosterone.

## PROGESTERONE

In order of rank, LGR females, whose embryos are visible and busy developing, had the highest progesterone levels followed by NGR, IMM and lastly EGR females (KW-ANOVA  $P<0.05$ ) (see Figure 25). The only significant pairwise differences were between EGR females and LGR and IMM females respectively ( $P<0.05$ ). There was no correlation between progesterone concentration and any morphological- or hormonal measurement.

## 4.4 DISCUSSION

This study is firstly an attempt to describe seasonality in the reproductive cycle and associated circulating reproductive hormones in the South African female soupfin shark, *G. galeus*. Secondly, this study was aimed at elucidating and inferring respective functions of testosterone, 17- $\beta$ -estradiol and progesterone during the reproductive events in this species. Reports on some aspects of the reproduction of the South African *G. galeus* exist (McClusky 1988; Freer 1992), but little is known about the seasonal cycles involved (Walker 1999). Both McClusky (1988) and Freer (1992) make only preliminary findings. Studies that aim to describe reproductive cycles in sharks are often difficult and as a result seasonal cycles in spermatogenesis and oogenesis have been thoroughly described in only a handful of the over 380 shark species (Hisaw & Abramowitz 1939; Simpson & Wardle 1967; Pratt 1979; Teshima 1981; Parsons & Grier 1992; Maruska *et al.* 1996). In reality, seasonal reproductive cycles may be a much more common phenomenon than is suggested by these few reports (Parsons & Grier 1992).

The smallest mature female recorded in this study of 131 cm TL is slightly smaller than, but still comparable to, the 134 cm TL that was recorded previously for South African female *G. galeus* (Freer 1992) (see Table 4 for summary). Maturity in these females was determined in terms of oviducal gland development and granular texture of the ovaries (Freer 1992). The measures used in this study to determine maturity are probably more strict than those used by Freer (1992) and it is surprising that the minimum mature size is not larger than that reported by Freer (1992). In the Australian *G. galeus* females, maturity is first reached at 135 cm TL

(Olsen 1954) which is also comparable to what is found in the South African stocks. The method used by Olsen (1954) is much the same as the combination of aspects considered in this study. The Brazilian *G. galeus* females first mature at 118 cm based on colour and size of the ovarian follicles and the width of the uterus and oviducal glands (Peres & Vooren 1991). This is much smaller compared to the South African and Australian populations, but the Brazilian stock is comparatively smaller in all the measured parameters listed in Table 4.

There seems to be quite a large window of time within which maturity is reached in female *G. galeus*. The largest immature female from this study was 142 cm TL, which, according to growth parameters in Olsen (1954), would be more than two years older than the smallest mature specimens. This means that all females are mature at a body size 8% larger than the smallest mature body size. For the Brazilian stock the largest immature female was 128 cm TL (Peres & Vooren 1991), which is also 8% larger than those at the minimum mature size. In *Galeorhinus japonicus* this figure is markedly higher at 21% (Chen & Mizue 1973). This window of reaching maturity is smaller than what is found in other triakids. The last females of *Mustelus griseus*, *Mustelus manazo* and *Mustelus lenticulatus* to mature, do so at body sizes 12%, 11% and 15%, respectively, larger than that of the smallest mature females (Teshima 1981; Massey & Francis 1989). For *Triakis semifasciata*, this figure is 29%, which translates to a difference of five years between the smallest mature and largest immature females (Kusher *et al.* 1992).

The two classes of ovarian follicle size observed in this study is similar to what was found in the Australian population and it can be assumed that the smaller size group (<20 mm) would not ovulate during that breeding season (Olsen 1954). Ovarian follicles of *G. galeus* are ovulated when they are between 45 mm (Olsen 1954) and 55 mm (Peres & Vooren 1991) in diameter. In the Australian *G. galeus* population, ovulation occurs from October to January (Walker 1999). In the South African population, the peak in the size of the ovarian follicles at the beginning of August (Figure 19) and the fact that they are the expected size for ovulation, suggests that ovulation starts in August. The uterine eggs in those specimens had no visible sign yet of embryonic growth and it can be concluded that they are indeed very recently ovulated. This also fits the description for the condition of females of *S. tiburo* when they are busy ovulating (Manire *et al.* 1995). Due to the lack of samples for the months of September and October, it cannot be determined exactly how long ovulation carries on for, but it seems likely that it could stretch over four or perhaps five months, as is the above-mentioned case in the Australian *G. galeus* (Walker 1999). The large ovarian follicles that occur in summer could be a further indication of this. In a close relative, *M. lenticulatus*,

ovulation was estimated to take place over a period of six months, but this seems to be an extreme case in terms of having an extended ovulation period (Francis & Mace 1980).

Beyond the population level, this prolonged ovulation period seems to apply also to the level of the individual. The newly fertilised eggs mostly coincided with large ovarian follicles, indicating that ovulation was still in progress at that time in those specimens. If this extended period during which a female ovulates is long enough, it could explain the differences in the lengths between embryos from the anterior and posterior ends of the same uterus that are summarised in Table 7. The larger, and obviously older, embryos were always found in the posterior portion of the uterus. This issue was not investigated in the current study. It seems quite likely, therefore, considering the extent of the size differences depicted in Table 7, that the ovulation period of the South African *G. galeus* is indeed extended for the individual and occurs from August to as late as December for the population.

Two ovarian follicle size classes were described for the non-gravid Brazilian females that consisted of the first-year non-gravid group with small ovarian follicles and the second-year non-gravid group with large ovarian follicles (Peres & Vooren 1991). Their simultaneous occurrence was interpreted as a three-year reproductive cycle for those females (Peres & Vooren 1991). During the female's first year after parturition, vitellogenesis is slow and the oviduct is in a resting phase (first-year non-gravid group). The following year sees the production of large ovarian follicles, due to accelerated vitellogenesis, and preparation of the uteri and oviducal glands for ovulation (second-year non-gravid group). Ovulation occurs at the end of that year (November-December) and heralds the third and last year of the cycle for a 12-month gestation period (gravid group) which terminates with parturition in November-December. Peres & Vooren (1991) argues that the 6-month gestation period suggested by Olsen (1954) is too short in the light of their more recent findings and therefore reinterprets the findings on the Australian population to also have a three year female reproductive cycle.

At first glance there seems to be a critical difference between the findings of the South African *G. galeus* population and that of the Brazilian population. In the Brazilian population it was found that the non-gravid females had both large and small ovarian follicles and gravid females having exclusively small ovarian follicles that were opaque white and did not grow (Peres & Vooren 1991). This seems to be exactly the opposite of what was found during this study. In the South African population the gravid females have both large ( $>37$  mm diameter) and small ( $<20$  mm diameter) ovarian follicles while the non-gravid females have exclusively small ovarian follicles ( $<20$  mm diameter) (see Figure 20). The rather large group of females sampled in August can explain this contrast in ovarian follicle size-groupings. These females

Table 7. Differences in length between embryos from the anterior and posterior ends of the same uterus from different global populations of *Galeorhinus galeus*.

Population	Size difference (mm)	Reference
California	26	Ripley (1946)
Australia	30	Olsen (1954)
Brazil	20-40	Peres & Vooren (1991)
South Africa	8	Freer (1992)

were caught in the middle of their ovulation period and would therefore be classified as gravid (having uterine ova) but with large ovarian follicles (still to be ovulated). A short time earlier, before ovulation had commenced, they would have represented the (second-year) non-gravid group with large ovarian follicles of Peres & Vooren (1991). The only difference is that in the Brazilian study, the females were yet to ovulate whereas in the present study ovulation had started already. This means that the South African *G. galeus* females, although it looks slightly different, actually have the same three categories as in the Brazilian population. These findings seem to fit the model for a three-year reproductive cycle in the females consisting of slow vitellogenesis in the first year, accelerated vitellogenesis and ovulation during the second year and embryonic development during the third year ending with parturition. All three categories of females seem to be present throughout the year even in the limited sample size of this study but more data are required to confirm these findings.

There are three specimens from late-December and early-January that seems to be exceptions to the rule, being gravid with small embryos but having large ovarian follicles. There are two possible explanations for this phenomenon. Firstly, it is possible that those females are still ovulating, hence the large ovarian follicles. The embryos would then have developed from ova that were ovulated first in that breeding season. This would mean that the furthest developed embryos are between 30 mm and 45 mm larger than the youngest ones. This is possible considering the in-utero range of embryo sizes that was discussed earlier. However, the fact that the biggest such difference yet recorded for South African *G. galeus* is only 8 mm might indicate that those embryos from late-December and early-January are too big already to be considered part of the same clutch as the large ovarian follicles still to be ovulated. A second possible explanation is that those embryos and ovarian follicles are indeed from separate clutches and that the ovary is not going to enter its rest period as was also reported by Olsen (1954, 1984). Freer (1992) also found females from South Africa that had both embryos and viable ova and concluded that not all the females enter that resting period. Those large ovarian follicles will then be ovulated at the beginning of the next breeding season to begin a new gestation period straight away.

It has been suggested that mating and ovulation coincides between May and July in the South African *G. galeus* (McClusky 1988). However, the timing of ovulation was based on a very limited sample size and was reported as inconclusive (McClusky 1988). It seems now, based on the above-mentioned commencement of the ovulation period in August, that mating precedes ovulation by between two and five months, which is similar to what was reported for the Brazilian *G. galeus* (Peres & Vooren 1991). Such circumstances would then

necessitate storage of sperm by the female from the time of mating until ovulation for subsequent fertilisation. Storage of sperm has been documented for females of various shark species (Metten 1939; Prasad 1945a,b; Prasad 1948; TeWinkel 1956; Pratt 1979; Gilmore *et al.* 1983; Castro *et al.* 1988; Pratt 1993; Fishelson & Baranes 1998), including the Brazilian *G. galeus* (Peres & Vooren 1991). This was confirmed in this study for the South African population by the sub-sample of females who all had sperm in their oviducal glands. It is therefore quite possible that mating may occur between May and July as has been previously reported (McClusky 1988; Freer 1992), but not coinciding with ovulation leading to the need for sperm to be stored in the female until ovulation and subsequent fertilisation.

Oviducal glands in sharks reach their peak size just before ovulation (Hamlett & Koob 1999), as has been reported for the Brazilian *G. galeus* population (Peres & Vooren 1991). The peak in the size of the oviducal glands in the present study suggests that ovulation starts not long after August. It is not possible to tell how long this peak lasts for, but it could be prolonged as is suggested by the still relatively large oviducal glands in the summer. However, these large oviducal glands in summer could also be due to enlarged oviducal glands as a result of the foregoing ovulation event. Again, the lack of samples for September and October makes it impossible to tell conclusively, but it would have helped to determine over how many months ovulation takes place and for how long after ovulation the oviducal gland remains enlarged.

Parturition for the South African population occurs around mid-summer (December-January) but some still heavily pregnant females are sometimes caught as late as February and March (Freer 1992). This is reconfirmed by the few specimens in this study whose embryo sizes were measured. In summer, embryos were either very small or close to full-term size while in winter the two specimens measured then were exactly the expected half-term size.

Freer (1992) suggested that, based on circumstantial evidence, gestation could not be less than six months. The seasonal size distribution of embryos in this study, with both small and near-term embryos occurring in the summer, suggests that the gestation period for the South African *G. galeus* is not less than 12 months. Ovulation and parturition occur simultaneously in the Californian and Brazilian populations and a gestation period of 12 months apply in those populations (Ripley 1946; Peres & Vooren 1991). There is no conclusion yet as to how long the gestation period of the Australian *G. galeus* population actually is, but the latest opinion is that it is at least 12 months (Walker 1999). With parturition around the middle of summer and ovulation suggested to be anytime from August to December, it can be

suggested that the gestation period of the South African *G. galeus* is longer than 12 months. Assuming immediate fertilisation after ovulation, a gestation period of close on 15 months seems the most likely. It is, in fact, not uncommon for conspecific shark populations to differ in the length of their gestation periods. In *Carcharhinus melanopterus* and *Carcharhinus plumbeus* the length of gestation differs by five months and three months, respectively, between different populations (Compagno 1984; Stevens 1984). Also in the more closely related *Mustelus antarcticus* a difference of up to four months is found in the length of the gestation period between populations from south-eastern Australia and south-western Australia (cited in Lenanton *et al.* 1990). The southeastern waters of Australia are colder than off the more western states (Olsen 1954). This temperature difference causes a phase difference between *G. galeus* populations from these different waters (Olsen 1954) and could possibly also be responsible for the different lengths of gestation with the colder environment slowing down intrauterine development. Generally, the events of the reproductive cycle of female *G. galeus* do not seem to be as synchronised as is the case in some other sharks. In *S. tiburo*, for instance, mating, ovulation, pregnancy and parturition were so synchronised throughout the reproductive cycle that their timing among different populations could be distinguished by as little as seven to ten days (Manire *et al.* 1995). The seemingly unsynchronised results from this study could, at least in part, be accounted for by geographically separated sampling areas (Olsen 1954) and possible different temperature cycles that were encountered over the long 27-month sampling period. It is possible, however, that it is simply due to species differences and that the cycle may indeed be less synchronised in *G. galeus*.

As far as circulating steroid hormones are concerned, information on their levels in elasmobranchs is sparse and the seasonal fluctuations thereof have only been adequately described in very few species. There is clearly a need for more data of this kind in order to make comparisons between elasmobranch groups and working towards a better understanding of the functions of the sex steroids during the elasmobranch reproductive cycle and in the different reproductive modes. This study provides the first data on female steroid hormone concentrations and its temporal variations in a member of the family Triakidae. It is also an attempt to elucidate some of the functions of steroid hormones in elasmobranch reproduction.

The steroid hormone levels found in mature female *G. galeus* are comparable to some but seems higher than most that have been previously recorded for other elasmobranchs, as is summarised in Table 3. The species that have the closest corresponding hormone levels are

also aplacentally viviparous, but it would be premature to relate steroid hormone ranges with reproductive mode at this stage.

An inverse relationship is found between estradiol and testosterone fluctuations in some species (*C. taurus*, *C. plumbeus*) (Rasmussen & Muru 1992) while there are other species whose testosterone and estradiol levels fluctuate together very closely (Sumpter & Dodd 1979; Koob *et al.* 1986; Tsang & Callard 1987b; Rasmussen & Gruber 1990; Manire *et al.* 1995; Snelson *et al.* 1997). The coincident fluctuations of testosterone and estradiol in this study are similar to what is found among this latter group of sharks. However, in some *D. sabina* populations there is a second peak in estradiol and testosterone levels (Snelson *et al.* 1997) whereas in *G. galeus* there is only one. Admittedly, the data in this study lack samples for some months, but there is no indication of any other peaks in steroid levels other than the one in August.

The fluctuations in progesterone are not as strongly expressed as in testosterone and estradiol, but there seems to be mild elevations of progesterone in both summer and winter. It is interesting to note that progesterone has two peaks whereas testosterone and estradiol only have the one peak in winter. Such a bimodal seasonal distribution for progesterone has not been reported before for any other shark species. The range of progesterone variation in *S. acanthias* (<1 ng/ml to 6 ng/ml) is very similar to that of *G. galeus* in this study if the three high values are ignored. This further confirms that a definite seasonal cycle is indeed operating in the concentrations of progesterone, but that it fluctuates within a smaller range than testosterone and estradiol.

The absence of any detectable seasonal variation in immature females' testosterone levels (Table 6) suggests that testosterone only starts appearing in circulation once maturity is reached. This is not unexpected since the ovary is considered to be the main source of circulating steroid hormones and one will not expect to find much circulating testosterone until there are larger follicles in the ovary (Hamlett & Koob 1999).

The weak fluctuations of estradiol suggest that this steroid starts being synthesised before testosterone. It has been shown from both oviparous and viviparous species that smaller ovarian (pre-ovulatory) follicles predominantly produce estradiol and that the larger follicles predominantly produce testosterone (Hamlett & Koob 1999). These ovaries are therefore clearly busy developing and the presence of a few small follicles could be responsible for the slight fluctuations in the immature females. It is interesting to note, though, that the seasonal influence of the reproductive cycle seems to already be at work in these immature females.

The striking similarity between the range of progesterone fluctuations in immature females and mature females suggests that the immature ovary synthesises progesterone as readily as the mature one. Corpora lutea can therefore not be the main sites of progesterone production as was found to be the case in *S. acanthias* (Tsang & Callard 1987a). The main origin of progesterone in *G. galeus* is therefore still speculative. Unlike the weakly expressed seasonal cycle of estradiol, the fluctuations of progesterone in immature females are as strongly expressed as in the mature females. This suggests that progesterone is the first one of the three steroids investigated in this study to get actively synthesised and testosterone the last.

The function of testosterone in female shark reproduction is still very ill understood (Hamlett & Koob 1999). Our current knowledge of testosterone function does not extend beyond some developmental processes of the uterus and histotroph nourishment of embryos as in *D. sabina* (Snelson *et al.* 1997). There is, however, a definite indication that testosterone plays a significant role in EGR females. Physiologically this means that testosterone is involved in the final stages of vitellogenesis and ovulation in *G. galeus* females. Since the ovary is the main site of steroid production (Hamlett & Koob 1999) the tight correlation between testosterone and ovarian follicle size is probably more effect than cause. On the other hand, oviducal gland size is likely to be an effect of high testosterone concentrations, preparing and storing eggshell precursors for when ovulation occurs (Gilmore *et al.* 1983). The strong positive correlation between testosterone and estradiol (this study) suggests that these two steroids function very similarly. If the level of correlation between hormone levels and morphological conditions can be an indication of its function, then testosterone seems to play a more important role than estradiol. The combined behaviour of these two steroids also differs a lot among elasmobranch species. There are oviparous (*R. erinacea* and *S. canicula*), aplacental viviparous (*D. sabina*, *S. acanthias*) and placental viviparous (*Carcharhinus acronotus*, *Carcharhinus limbatus* and *S. tiburo*) species where testosterone and estradiol levels fluctuate together very closely. In contrast to this co-fluctuation of testosterone and estradiol levels, inverse fluctuations occur in oviparous skates and raffles where estradiol levels remain high and testosterone levels remain low until egg laying takes place, after which estradiol drops and testosterone rises for most of the egg-laying period (Rasmussen & Muru 1992). This inverse relationship between estradiol and testosterone has also been reported for *C. taurus* (aplacental viviparous) and *C. plumbeus* (placental viviparous) (Rasmussen & Muru 1992). The fact that testosterone and estradiol covary so tightly, causes difficulty in distinguishing their respective functions from the current data. More intense experimentation would be needed to solve this issue.

The main organs targeted by steroid hormones are the liver, ovary and the oviduct (Callard *et al.* 1991). The liver is the site of vitellogenin synthesis (Tata & Smith 1979) and the ovary is the site of vitellogenin uptake by the follicles (Wallace & Bergink 1974). This latter function is known to be regulated by estradiol (Craik 1978). The correlation between ovarian follicle diameter and the peak in estradiol levels just prior to the proposed time of ovulation in *G. galeus* (this study) suggests a similar function to what has been reported in other elasmobranchs. Elevated levels of estradiol just prior to ovulation, due to its synthesis by the mature follicle (Tsang & Callard 1983), has also been recorded in *D. sabina* (Snelson *et al.* 1997), *S. tiburo* (Manire *et al.* 1995), *S. acanthias* (Tsang & Callard 1987b), *S. canicula* (Sumpter & Dodd 1979) and *R. erinacea* (Koob *et al.* 1986). This pre-ovulatory peak in estradiol seems to be the case regardless of reproductive mode since it is seen in oviparous and aplacental and placental viviparous species and seemingly functions to prepare the oviduct for ovulation. Estradiol has previously been shown to be involved in regulating the development of oviducal glands (Koob *et al.* 1986) and other parts of the oviduct as in *R. erinacea* (Reese & Callard 1987) and some sharks (Dodd & Goddard 1961). This seems to also be the case with *G. galeus* as shown by the correlation of estradiol concentrations with the size of the oviducal gland (this study). It is therefore not surprising that LGR females, who do not need any preparation of oviducal glands and other parts of the oviduct anymore, have the lowest estradiol concentrations, only slightly higher than in IMM females.

Estradiol has also been linked to parturition (Tricas *et al.* 2000) and the embryonic transition from yolk-dependence to histotroph-dependence (Snelson *et al.* 1997), respectively, in different populations of *D. sabina*. The lack of data for some months of the year makes it impossible to tell whether such function exists for estradiol in *G. galeus* females.

The function of progesterone is clearly different from that of testosterone and estradiol. It shows the same temporal fluctuations in immature and mature individuals and there are no correlations with testosterone and estradiol or any of their correlates. Elevated progesterone levels that coincides with peaks in testosterone and estradiol has been recorded in recently mated *C. limbatus* and *Negaprion brevirostris* (Rasmussen & Gruber 1990, 1993) and in *D. sabina* (Tricas *et al.* 2000). However, unlike *S. acanthias* where progesterone remains high throughout pregnancy, it remains low and stable in most of the placental carcharhinoids (Rasmussen & Gruber 1990). In these sharks the fluctuations of progesterone are inverse to that of testosterone and estradiol. In *G. galeus* there is neither an inverse, nor direct relationship of progesterone with testosterone and estradiol and points toward a function in this species that is independent of the placental carcharhinoids. This difference could be

based on reproductive mode but it is not certain. The only indication of the function of progesterone is linked to embryonic development, since the highest concentrations were found among LGR females (who have visible embryos). Progesterone is considered to regulate compartmentalisation of the uterus by inhibiting myometrial contractions in placental sharks (Callard *et al.* 1992) and encapsulation and oviposition of fertilised ova in oviparous species (Callard *et al.* 1992; Koob *et al.* 1986). Possible functions of progesterone that could be related to (not necessarily exclusively) the aplacental reproductive mode includes inhibition of estradiol-induced vitellogenin synthesis (cited in Tsang & Callard 1987b; Callard *et al.* 1992), the maintenance of pregnancy (Tsang & Callard 1987b; Callard *et al.* 1992), influences on the condition of the reproductive tract (Callard *et al.* 1993), ovulation (Tsang & Callard 1987b; Manire *et al.* 1995; Snelson *et al.* 1997; Tricas *et al.* 2000) and with parturition (Snelson *et al.* 1997; Tricas *et al.* 2000). None of these possible functions have been demonstrated experimentally yet (Hamlett & Koob 1999) and, in the absence of any correlation in this study, none seem to apply to *G. galeus*. It could, however, be possible that some parameter not quantified in this study does correlate with progesterone, but this is yet to be shown.

#### **4.5 CONCLUSION: SEASONAL CYCLE OF THE FEMALE *G. GALEUS***

In conclusion, even though the limited sample size in this study prevented firm conclusions on a statistical basis, there seems to be only one plausible model that fits the data. All the evidence suggests that the South African female *G. galeus* has a triennial reproductive cycle. The only other known report of a female elasmobranch with a cycle that is longer than two years is that for *C. anguineus*, which is thought to be at least three and a half years (Tanaka *et al.* 1990). This length of reproductive cycle for *G. galeus* is the same as what has been suggested for the Brazilian population (Peres & Vooren 1991) but different from the annual cycle reported for the Californian (Ripley 1946) and Mediterranean (cited in Walker 1999) populations and the biennial cycle of the Australian population. This could be due to some factor related to the different hemispheres that these populations occur in, but that is not known for certain and is highly speculative. This does, however, imply that the populations with the triennial female cycle have a lower fecundity and could be more susceptible to over-exploitation.

Based on a limited sample size in a previous study, mating and ovulation has been suggested to coincide between May and July (McClusky 1988). In this study, however, there is much evidence to suggest that ovulation starts two to three months after mating and may even be prolonged on the population level. Such an asynchrony between males and females

is very plausible since sperm is present in the oviducal glands of the females and will fertilise the ova as they pass through. A gestation period of between 12 and 15 months follows fertilisation and consummates in parturition the following summer. The year following the pregnancy is normally an inactive resting period as far as reproduction is concerned. This is mostly the case, but there are exceptions where accelerated vitellogenesis starts in the next generation's ovarian follicles straight away (Freer 1992, this study).

Testosterone and estradiol concentrations go through one peak in the year and fluctuate together in *G. galeus*. Their function is clearly related to reproductive activity, since the levels of these two hormones in immature females are very low and lack any temporal fluctuation. Elevated levels of testosterone and estradiol in mature female *G. galeus* are related to the conditions just prior to ovulation and with oviducal gland development. Progesterone concentrations show two peaks in the year and hold the same temporal distribution in immature females as in mature ones. Its only known function is related to embryonic development, but there seems to also be a different function at work even before reproductive maturity is reached.

## 5. Discussion and conclusions

The variety of reproductive cycles that are found among elasmobranchs is of such extent that it is clearly impossible to predict its presence and length on the basis of phylogenetic relatedness. Just within the family Triakidae we find sharks that have well-defined annual cycles like *Triakis semifasciata* (Kusher *et al.* 1992), *Mustelus antarcticus* (Lenanton *et al.* 1990) *Mustelus canis* (Hisaw & Abramowitz 1939) and *Mustelus lenticulatus* (Francis & Mace 1980), and others with no seasonal fluctuation in their reproductive activity like *Iago omanensis* (Fishelson & Baranes 1998). In this study the reproductive cycle of male and female *G. galeus* was determined and the first quantified hormone levels for a triakid shark are reported.

A summary illustrating how the male and female reproductive cycles run together is shown in figure 26. The histological results of the testes from this study were remarkably similar to that of McClusky (1988). The initial aim was to have samples that represent the entire reproductive season, but samples were not available all year round. Although the data are not as complete as desired, the trends suggested by McClusky (1988) for the South African male population are expanded and complemented by the findings of this study. The fluctuations in the proportions of each spermatogenic stage occupied in the testis indicated that spermatogenesis follows an annual cycle that starts around April. Spermiogenesis is also at a peak at this time as would be expected in an annual cycle. Mating occurs shortly thereafter from May to July and the next spermiogenic cycle would start again probably some months after August. The spermatogenic cycle of *G. galeus* is more weakly expressed when compared to that of *M. manazo* and *M. griseus* where the fluctuations of the spermatogenic stages throughout the reproductive cycle in the testis are far more extensive (Teshima 1981).

The South African *G. galeus* females follow a triennial reproductive cycle as was also reported for the Brazilian stocks (Peres & Vooren 1991). Again the lack of a more complete sample hindered conclusions on a statistical level, but comparisons with other studies revealed that this is indeed the only plausible model for the findings of this study. The timing of mating was not specifically investigated in the females of this study, but other data from this study suggests that mating occurs between May and July. After mating had taken place the sperm is stored in the oviducal glands of the female until a protracted time of ovulation ensues after August possibly until about December.

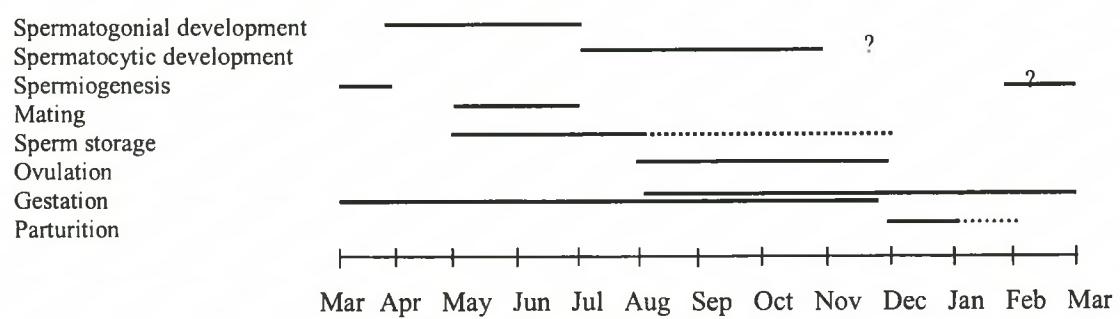


Figure 26. Combined summary of the male and female reproductive cycles of *Galeorhinus galeus*. Exact beginnings and endings of the stages of the cycles are indicated with '?'.

A gestation period of up to 15 months takes the newly fertilised ovum to parturition that following summer. This is different to what is found in the Californian and Brazilian populations where ovulation and parturition coincides to give a 12-month gestation period (Ripley 1946; Peres & Vooren 1991). The year following that summer of parturition is one of reproductive inactivity marked by slow or no vitellogenesis. It is then only in the second year after parturition that vitellogenesis starts again in the ovarian follicles of the next generation.

This study is the first account of reproductive steroid hormone levels in a triakid. The concentrations of testosterone measured here for *G. galeus* males were much higher than in any previously reported non-mammalian. Hormone data exists only for a few species and it is quite possible that other species could have similar high circulating steroid levels, but that it has just not been investigated yet. There is only a single detected peak in testosterone levels of *G. galeus* that coincides with the onset of the spermatogenic cycle, spermatocyte development and initiation of aggressive mating behaviour. In female *G. galeus*, testosterone and estradiol fluctuate together and show a single peak around August. These elevated levels are linked to the development of ovarian follicles and oviducal glands. Progesterone functions differently from testosterone and estradiol and shows a bimodal temporal pattern that looks the same in mature and immature females. Its only function in the reproduction of *G. galeus* seems to be related to embryonic development.

The effectiveness of the enzyme-linked immunosorbent assay for determining steroid hormone levels in sharks was shown here for the first time. This assay is more cost-effective compared to the more frequently used radio-immuno assay and has some important advantages. It makes it possible to quantify steroids in samples even when they are limited to small volumes and it alleviates the use of radioactive and biologically hazardous materials. This would hopefully pave the way for more non-mammalian hormonal determinations in the future, especially on small animals where blood volume has since now been a limiting factor and destructive sampling was required to obtain enough blood.

**APPENDIX A**

Summary of where in the reproductive cycle of different species elevated and reduced levels of testosterone, estradiol and progesterone occurs.

**Mating**

- Estradiol - *S. tiburo, N. brevirostris, C. limbatus*
- Testosterone - *S. tiburo, N. brevirostris, C. limbatus*
- Progesterone - *N. brevirostris, C. limbatus*

**Preovulation**

- Estradiol - *R. erinacea, S. canicula, S. tiburo, D. sabina, S. acanthias, C. limbatus, C. acronotus*
- Testosterone - *R. erinacea, S. canicula, S. tiburo, S. acanthias, C. limbatus, C. acronotus*
- Progesterone - *R. erinacea, S. canicula, S. tiburo, C. limbatus*
- Progesterone - *S. acanthias*

**Ovulation**

- Estradiol - *D. sabina*
- Testosterone - *D. sabina*
- Progesterone - *S. tiburo, D. sabina*

**Early pregnancy**

- Estradiol - *R. porosus*
- Estradiol - *S. tiburo, S. acanthias*
- Testosterone - *S. tiburo, S. acanthias, T. marmorata, R. porosus*
- Progesterone - *S. tiburo, S. acanthias, T. marmorata*

**Late pregnancy**

- Estradiol - *R. porosus*
- Estradiol - *S. acanthias*
- Testosterone - *S. acanthias, T. marmorata, R. porosus*
- Progesterone - *S. acanthias*
- Progesterone - *S. tiburo*

**Parturition / egg laying**

- Estradiol - *D. sabina, S. acanthias*
- Estradiol - *S. canicula, S. tiburo*
- Testosterone - *S. acanthias*
- Testosterone - *S. canicula, S. tiburo*
- Progesterone - *D. sabina*
- Progesterone - *S. acanthias*

**Post partum / intergestation**

- Estradiol - *N. brevirostris*
- Testosterone - *T. marmorata*
- Progesterone - *N. brevirostris*

## References

- AMOROSO, E.C. 1960. Viviparity in fishes. *Symposium of the Zoological Society of London* 1: 153-181.
- BACKUS, R.H. 1957. Notes on western north Atlantic sharks. *Copeia* 1957: 246-248.
- BASS, A.J., D'AUBREY, J.D. & KISTNASAMY, N. 1975. Sharks of the East Coast of Southern Africa. III. The families Carcharhinidae (excluding *Mustelus* and *Carcharhinus*) and Sphyrnidae. *Oceanographic Research Institute Investigational Report* 38: 1-100.
- BREEDER, C.M. & ROSEN D.E. 1966. *Modes of reproduction in fishes*. Natural History Press, Garden City, New York.
- CALLARD, G.V. 1991. Reproduction in male elasmobranchs. In: *Comparative Physiology*, (ed.) R.K.H. Kinne, Vol. 10, pp. 104-154. Karger, Basel.
- CALLARD, I.P., ETHERIDGE, K., GIANNOUKOS, G., LAMB, T. & PEREZ, L. 1991. The role of steroids in reproduction in female elasmobranchs and reptiles. *Journal of Steroid Biochemistry and Molecular Biology* 40: 571-575
- CALLARD, I.P., FILETI, L.A., PEREZ, L.E., SORBERA, L.A., GIANMOUKOS, G., KLOSTERMAN, L.L., TSANG, P. & McCACKEN, J.A. 1992. Role of the corpus luteum and progesterone in the evolution of vertebrate viviparity. *American Zoologist* 32: 264-275.
- CALLARD, I.P., FILETI, L.A. & KOOB, T.J. 1993. Ovarian steroid synthesis and the hormonal control of the elasmobranch reproductive tract. *Environmental Biology of Fishes* 38: 175-185.
- CAPAPÈ, C., QUIGNARD, J.P. & MELLINGER, J. 1990. Reproduction and development of two angel sharks, *Squatina squatina* and *S. oculata* (Pisces: Squatinidae), off Tunisian coasts: semi-delayed vitellogenesis, lack of egg capsules and lecithotrophy. *Journal of Fish Biology* 37: 347-356.
- CASEY, J.G. & PRATT H.L., JR. 1985. Distribution of the white shark, *Carcharodon carcharias*, in the western North Atlantic. *Memoirs of the Southern California Academy of Science* 9: 2-14.
- CASTRO, J.I., BUBUCIS, P.M. & OVERSTROM, N.A. 1988. The reproductive biology of the chain dogfish, *Scyliorhinus retifer*. *Copeia* 1988: 740-746.

- CHEN, C-T. & MIZUE, K. 1973. Reproduction of *Galeorhinus japonicus*. *Bulletin of the Faculty of Fisheries, Nagasaki University* **36**: 37-51.
- CHEN, C-T., LEU, T.C. & JOUNG, S.J. 1988. Notes on reproduction in the scalloped hammerhead, *Sphyrna lewini*, in northeastern Taiwan waters. *Fishery Bulletin* **86**: 389-393.
- CHIEFFI, G. 1967. The reproductive system of elasmobranchs: developmental and endocrinological aspects. In: *Sharks, skates and rays*, (eds) P.W. Gilbert, R.F. Mathewson & D.P. Rall, pp. 553-580. Johns Hopkins Press, Baltimore.
- CHIEFFI, G. & LUPO DI PRISCO, C. 1961. Identification of estradiol-17B, testosterone, and its precursors from *Scyliorhinus stellaris* testes. *Nature* **190**: 169-170.
- CLARK, E. & VON SCHMIDT, K. 1965. Sharks of the Central Gulf coast of Florida. *Bulletin of Marine Science* **15**: 13-83.
- COMPAGNO, L.J.V. 1973. Interrelationships of living elasmobranchs. In: *Interrelationships of fishes*, (eds) P.H. Greenwood, R.S. Miles & C. Patterson, pp. 15-61. Academic Press, London.
- COMPAGNO, L.J.V. 1977. Phyletic relationships of living sharks and rays. *American Zoologist* **17**: 303-322.
- COMPAGNO, L.J.V. 1984. FAO species catalogue. Vol. 4. Sharks of the world. An annotated and illustrated catalogue of shark species known to date. Part 2. Carcharhiniformes. In: *United Nations Food and Agriculture Organization Fisheries Synopsis No. 125*, Vol. 4, pp. 251-655.
- CRAIK, J.C.A. 1978. The effects of oestrogen treatment on certain plasma constituents associated with vitellogenesis in the elasmobranch, *Scyliorhinus canicula* L. *General and Comparative Endocrinology* **35**: 455-464.
- DOBSON, S. & DODD, J.M. 1977a. Endocrine control of the testis in the dogfish *Scyliorhinus canicula* L. II. Histological and ultrastructural changes in the testes after partial hypophysectomy (ventral lobectomy). *General and Comparative Endocrinology* **32**: 53-71.
- DOBSON, S. & DODD, J.M. 1977b. The roles of temperature and photoperiod in the response of the testis of the dogfish, *Scyliorhinus canicula* L. to partial hypophysectomy (ventral lobectomy). *General and Comparative Endocrinology* **32**: 114-115.
- DODD, J.M. 1972. Ovarian control in cyclostomes and elasmobranchs. *American Zoologist* **12**: 325-339.

- DODD, J.M. 1975. The hormones of sex and reproduction and their effects in fish and lower chordates: Twenty years on. *American Zoologist* **15**: 137-171.
- DODD, J.M. 1983. Reproduction in cartilaginous fishes. In: *Fish Physiology*, (eds) W.S. Hoar, D.J. Randall & E.M. Donaldson, Vol. IX A, pp. 31-95. Academic Press, New York.
- DODD, J.M. & GODDARD, C.K. 1961. Some effects of oestradiol benzoate on the reproductive ducts of the female dogfish, *Scyliorhinus caniculus*. *Proceedings of the Zoological Society of London* **137**: 325-331.
- DODD, J.M., EVENNETT, P.J. & GODDARD, C.K. 1960. Reproductive endocrinology in cyclostomes and elasmobranchs. *Symposium of the Zoological Society of London* **1**: 77-103.
- DONALDSON, E.M. 1973. Reproductive endocrinology of fishes. *American Zoologist* **13**: 909-927.
- ELLIS, J.R. & SHACKLEY, S.E. 1997. The reproductive biology of *Scyliorhinus canicula* in the British Channel. *United Kingdom Journal of Fish Biology* **51**: 361-372.
- FISHELSON, L. & BARANES, A. 1998. Observations on the Oman shark, *Iago omanensis* (Triakidae), with emphasis on the morphological and cytological changes of the oviduct and yolk sac during gestation. *Journal of Morphology* **236**: 151-165.
- FRANCIS, M.P. & MACE, J.T. 1980. Reproductive biology of *Mustelus lenticulatus* from Kaikoura and Nelson. *New Zealand Journal of Marine and Freshwater Research* **14**: 303-311.
- FREER, D.W.L. 1992. The commercial fishery for sharks in the South-western Cape, with an analysis of the biology of the two principle target species, *Callorhynchus capensis* Dumeril and *Galeorhinus galeus* Linn. M.Sc. Thesis. University of Cape Town, Cape Town, 103 pp.
- FUJITA, K. 1981. Oviparous embryos of the pseudocarcharhinid shark, *Pseudocarcharias kamoharai*, from the central Pacific. *Japanese Journal of Ichthyology* **28**: 37-44.
- GARNIER, D.H., COQUIL, C. & CHAUVIN, J. 1989. Seasonal variation of plasma and testicular sex steroid levels in a selachian (*Scyliorhinus canicula*). *General and Comparative Endocrinology* **74**: 298.
- GILMORE, R.G. 1983. Observations on the embryos of the longfin mako, *Isurus paucus*, and the bigeye thresher, *Alopias superciliosus*. *Copeia* **1983**: 375-382.
- GILMORE, R.G. 1993. Reproductive biology of lamnid sharks. *Environmental Biology of Fishes* **38**: 95-114.

- GILMORE, R.G., DODRILL, J.W. & LINLEY, P.A. 1983. Reproduction and embryonic development of the sand tiger shark, *Odontaspis taurus* (Rafinesque). *Fishery Bulletin* **81**: 201-225.
- GRUBER, S.H. & COMPAGNO, L.J.V. 1981. Taxonomic status and biology of the bigeye thresher, *Alopias superciliosus*. *Fishery Bulletin* **79**: 617-640.
- GUBANOV, Y.P. 1972. On the biology of the thresher shark *Alopias vulpinus* (Bonnaterre) in the northwest Indian Ocean. *Journal of Ichthyology* **12**: 591-600.
- GUBANOV, Y.P. 1978. The reproduction of some species of pelagic sharks from the equatorial zone of the Indian Ocean. *Journal of Ichthyology* **18**: 781-792.
- GUBANOV, Y.P. & GRIGOR'YEV, V.N. 1975. Observations on the distribution and biology of the blue shark *Prionace glauca* (Carcharhinidae) of the Indian Ocean. *Journal of Ichthyology* **15**: 37-43.
- HAMLETT, W.C. 1999. Male reproductive system. In: *Sharks, skates and rays. The biology of elasmobranch fishes*, (ed.) W.C. Hamlett, pp. 444-470. The John Hopkins University Press, Baltimore.
- HAMLETT, W.C. & KOOB, T.J. 1999. Female reproductive system. In: *Sharks, skates and rays. The biology of elasmobranch fishes*, (ed.) W.C. Hamlett, pp. 398-443. The John Hopkins University Press, Baltimore.
- HAMLETT, W.C., KNIGHT, D.P., KOOB, T., JEZIOR, M., LUONG, T., ROZYCKI, T., BRUNETTE, N. & HYSELL, M. 1998. Survey of oviducal gland structures and function in elasmobranchs. *Journal of Experimental Zoology* **282**: 399-420.
- HISAW, F.L. & ABRAMOWITZ, A.A. 1939. "Physiology of reproduction in the dogfishes, *Mustelus canis* and *Squalus acanthias*." Report of the Woods Hole Oceanographic Institute.
- HOAR, W.S. 1969. Reproduction. In: *Fish physiology*, (eds) W.S. Hoar & D.J. RANDALL, Vol. 3, pp. 1-72. Academic Press, New York.
- IDLER, D.R. & TRUSCOTT, B. 1966. Identification and quantification of testosterone in peripheral plasma of skate. *General and Comparative Endocrinology* **7**: 375-383.
- JOHNSON, R.H. 1978. *Sharks of tropical and temperate seas*. Les Editions du Pacifique, Papeete, Tahiti. 170pp.
- JONES, B.C. & GEEN, G.H. 1977. Reproduction and embryonic development of spiny dogfish (*Squalus acanthias*) in the Strait of Georgia, British Columbia. *Journal of the Fisheries Research Board of Canada* **34**: 1286-1292.

- KAUFFMAN, D.E. 1950. "Notes of the biology of the tiger shark (*Galeocerdo arcticus*) from Philippine waters." United States Fish and Wildlife Service Research Report, Vol. 16, pp. 10.
- KETCHEN, K.S. 1972. Size at maturity, fecundity and embryonic growth of the spiny dogfish (*Squalus acanthias*) in British Columbia waters. *Journal of the Fisheries Research Board of Canada* **29**: 1717-1723.
- KOOB, T.J., TSANG, P. & CALLARD, I.P. 1986. Plasma estradiol, testosterone and progesterone levels during ovulatory cycle of the little skate, *Raja erinacea*. *Biology of Reproduction* **35**: 267-275.
- KUSHER, D.I., SMITH, S.E. & CAILLIET, G.M. 1992. Validated age and growth of the leopard shark, *Triakis semifasciata*, with comments on reproduction. *Environmental Biology of Fishes* **35**: 187-203.
- LENANTON, R.C.J., HEALD, D.I., PLATELL, M., CLIFF, M. & SHAW, J. 1990. Aspects of the reproductive biology of the gummy shark, *Mustelus antarcticus* Gunther, from waters off the south coast of Western Australia. *Australian Journal of Marine and Freshwater Research* **41**: 807-822.
- LUPO DI PRISCO, C., VELLANO, C. & CHIEFFI, G. 1967. Steroid hormones in the plasma of the elasmobranch *Torpedo marmorata* at various stages of the sexual cycle. *General and Comparative Endocrinology* **8**: 325-331.
- MANIRE, C.A. & RASMUSSEN, L.E.L. 1997. Serum concentrations of steroid hormones in the mature male bonnethead shark, *Sphyrna tiburo*. *General and Comparative Endocrinology* **107**: 414-420.
- MANIRE, C.A., RASMUSSEN, L.E.L., HESS, D.L. & HUETER, R.E. 1995. Serum steroid hormones and the reproductive cycle of the female bonnethead shark, *Sphyrna tiburo*. *General and Comparative Endocrinology* **97**: 366-376.
- MANN, T. 1960. Serotonin (5-hydroxytryptamine) in the male reproductive tract of the spiny dogfish. *Nature* **188**: 941-942.
- MARUSKA, K.P., COWIE, E.G. & TRICAS, T.C. 1996. Periodic gonadal activity and protracted mating in elasmobranch fishes. *Journal of Experimental Zoology* **276**: 219-232.
- MASSEY, B.R. & FRANCIS, M.P. 1989. Commercial catch composition and reproductive biology of rig (*Mustelus lenticulatus*) from Pegasus Bay, Canterbury, New Zealand. *New Zealand Journal of Marine and Freshwater Research* **23**: 113-120.

- MATTHEWS, L.H. 1950. Reproduction in the basking shark, *Cetorhinus maximus* (Gunner). *Philosophical Transactions of the Royal Society of London, Series B* **234**: 247-316.
- McCLUSKY, L.M. 1988. Aspects of the reproductive biology of the male soupfin shark, *Galeorhinus galeus*, with notes on female reproduction. M.Sc. Thesis. University of the Western Cape, Cape Town, South Africa., 114pp.
- MERRIMAN, D. & OLSEN, Y.H. 1949. The angel shark, *Squatina dumeril*, in southern New England waters. *Copeia* **1949**: 221.
- METTEN, H. 1939. Studies on the reproduction of the dogfish. *Philosophical Transactions of the Royal Society of London, Series B* **230**: 217-238.
- MORENO, J.A. & MORON, J. 1992. Reproductive biology of the bigeye thresher shark, *Alopias superciliosus* (Lowe, 1839). *Australian Journal of Marine and Freshwater Research* **43**: 77-86.
- MOULTON, P.L., SADDLER, S.R. & KNUCKEY, I.A. 1989. New time-at-liberty record set by tagged school shark *Galeorhinus galeus* caught off Southern Australia. *North American Journal of Fisheries Management* **9**: 254-255.
- MOULTON, P.L., WALKER, T.I. & SADDLER, S.R. 1992. Age and growth studies of gummy shark, *Mustelus antarcticus* Gunther, and school shark, *Galeorhinus galeus* (Linnaeus), from Southern Australian waters. *Australian Journal of Marine and Freshwater Research* **43**: 1241-1267.
- NATANSON, L.J. & CAILLIET, G.M. 1986. Reproduction and development of the pacific angel shark, *Squatina californica*, off Santa Barbara, California. *Copeia* **1986**: 987-994.
- OFFICER, R.A., GASON, A.S., WALKER, T.I. & CLEMENT, J.G. 1996. Sources of variation in counts of growth increments in vertebrae from gummy shark, *Mustelus antarcticus*, and school shark, *Galeorhinus galeus*: implications for age determination. *Canadian Journal of Fisheries and Aquatic Sciences* **53**: 1765-1777.
- OLSEN, A.M. 1954. The biology, migration and growth rate of the school shark, *Galeorhinus australis* (Macleay) (Carcharhinidae) in south-eastern Australian waters. *Australian Journal of Marine and Freshwater Research* **5**: 353-410.
- OLSEN, A.M. 1984. Species synopsis of school shark, *Galeorhinus australis* (Macleay, 1881). In: *United Nations Food and Agriculture Organization Fisheries Synopsis*, Vol. 139, pp. 42 pp., Rome.
- OTAKE, T. 1990. Classification of reproductive modes in sharks with comments on female reproductive tissues and structures. In: *Elasmobranchs as living resources: Advances*

- in the biology, ecology, systematics and the status of the fisheries.* (eds) H.L. Pratt, S.H. Gruber & T. Taniuchi, Vol. 90, pp. 111-129. National Oceanic and Atmospheric Administration Technical Report NMFS.
- PARSONS, G.R. 1983. The reproductive biology of the Atlantic sharpnose shark, *Rhizoprionodon terraenovae*. *Fishery Bulletin* **81**: 61-73.
- PARSONS, G.R. & GRIER, H.J. 1992. Seasonal changes in shark testicular structure and spermatogenesis. *Journal of Experimental Zoology* **261**: 173-184.
- PERES, M.B. & VOOREN, C.M. 1991. Sexual development, reproductive cycle and fecundity of the school shark, *Galeorhinus galeus* off southern Brazil. *Fishery Bulletin* **89**: 655-667.
- PRASAD, R.R. 1945a. Further observations on the structure and function of the nidamental glands of a few elasmobranchs of the Madras coast. *Proceedings of the Indian Academy of Science, Section B* **22**: 368-373.
- PRASAD, R.R. 1945b. The structure, phylogenetic significance and function of the nidamental glands of some elasmobranchs of the Madras coast. *Proceedings of the National Institute of Science, India. Part B, Biological Sciences* **11**: 282-302.
- PRASAD, R.R. 1948. Observations on the nidamental glands of *Hydrolagus colliei*, *Raja rhina* and *Platyrhinoidis triseriatus*. *Copeia* **1948**: 54-57.
- PRATT, H.L., Jr. 1979. Reproduction in the blue shark, *Prionace glauca*. *Fishery Bulletin* **77**: 445-470.
- PRATT, H.L., Jr. 1988. Elasmobranch gonad structure: a description and survey. *Copeia* **1988**: 719-729.
- PRATT, H.L., Jr. 1993. The storage of spermatozoa in the oviducal glands of western North Atlantic sharks. *Environmental Biology of Fishes* **38**: 139-149.
- RASMUSSEN, L.E.L. & GRUBER, S.H. 1990. Serum levels of circulating steroid hormones in free-ranging carcharhinoid sharks. In: *Elasmobranchs as living resources: Advances in the biology, ecology, systematics and the status of the fisheries*. (eds) H.L. Pratt, S.H. Gruber, & T. Taniuchi, Vol. 90, pp. 143-155. National Oceanic and Atmospheric Administration Technical Report NMFS.
- RASMUSSEN, L.E.L. & GRUBER, S.H. 1993. Serum concentrations of reproductively-related circulating steroid hormones in the free-ranging lemon shark, *Negaprion brevirostris*. *Environmental Biology of Fishes* **38**: 167-174.

- RASMUSSEN, L.E.L. & MURU, F.L. 1992. Long-term studies of serum concentrations of reproductively related steroid hormones in individual captive carcharhinids. *Australian Journal of Marine and Freshwater Research* **43**: 273-281.
- REESE, J.C. & CALLARD, I.P. 1987. Receptors for estradiol-17B in the oviduct of skate *Raja erinacea*. *Biological Bulletin* **27**: 28-29.
- RIPLEY, W.E. 1946. The soupfin shark and the fishery. *Fish Bulletin* **64**: 7-37.
- SIMPSON, T.H. & WARDLE, C.S. 1967. A seasonal cycle in the testis of the spurdog, *Squalus acanthias*, and the sites of 3-B-hydroxysteroid dehydrogenase activity. *Journal of the Marine Biological Association of the United Kingdom* **47**: 699-708.
- SNELSON, F.F., RASMUSSEN, L.E.L., JOHNSON, M.R. & HESS, D.L. 1997. Serum concentrations of steroid hormones during reproduction in the Atlantic stingray, *Dasyatis sabina*. *General and Comparative Endocrinology* **108**: 67-79.
- SPRINGER, S. 1960. Natural history of the sandbar shark, *Eulamia milberti*. *Fishery Bulletin* **61**: 1-38.
- SPRINGER, V.G. 1964. A revision of the carcharhinid shark genera *Scoliodon*, *Loxodon* and *Rhizoprionodon*. *Proceedings of the United States Natural Museum* **115**: 559-632.
- STEVENS, A. 1977. The haematoxylins. In: *Theory and practice of histological techniques*. (eds.) A. Bancroft & J.D. Stevens, pp. 85-94. Churchill Livingstone., Edinburgh, London & New York.
- STEVENS, J.D. 1984. Life-history and ecology of sharks at Aldabra Atoll, Indian Ocean. *Proceedings of the Royal Society of London, Series B* **222**: 79-106.
- STEVENS, J.D. 1990. Further results from a tagging study of pelagic sharks in the north-east Atlantic. *Journal of the Marine Biological Association of the United Kingdom* **70**: 707-720.
- STEVENS, J.D. & LYLE, J.M. 1989. Biology of three hammerhead sharks (*Eusphyra blochii*, *Sphyrna mokarran* and *S. lewini*) from northern Australia. *Australian Journal of Marine and Freshwater Research* **40**: 129-146.
- SUDA, A. 1953. Ecological study on the blue shark (*Prionace glauca* Linnè). *South Sea Area Fisheries Research Laboratory Report* **26**: 1-11.
- SUMPTER, J.P. & DODD, J.M. 1979. The annual reproductive cycle of the female lesser spotted dogfish, *Scyliorhinus canicula* L., and its endocrine control. *Journal of Fish Biology* **15**: 687-695.

- TANAKA, S., SHIOBARA, Y., HIOKI, S., ABE, H., NISHI, G., YANO, K. & SUZUKI, K. 1990. The reproductive biology of the frilled shark, *Chlamydoselachus anguineus*, from Suruga Bay, Japan. *Japanese Journal of Ichthyology* **37**: 273-291.
- TANIUCHI, T. 1971. Reproduction of the sandbar shark, *Carcharhinus milberti*, in the East China Sea. *Japanese Journal of Ichthyology* **18**: 94-98.
- TATA, J.R. & SMITH, D.F. 1979. Vitellogenesis: a versatile model for hormonal regulation of gene expression. *Cell* **9**: 1-14.
- TEMPLEMAN, W. 1944. The life-history of the spiny dogfish (*Squalus acanthias*) and the vitamin A values of dogfish liver oil. *Department of Natural Resources, Newfoundland Fisheries Research Bulletin* **15**: 1-102.
- TEMPLEMAN, W. 1963. Distribution of sharks of the Canadian Atlantic (with special reference to Newfoundland waters). *Bulletin of the Fisheries Research Board Canada* **140**: 1-77.
- TESHIMA, K. 1981. Studies on the reproduction of Japanese dogfishes, *Mustelus manazo* and *M. griseus*. *Journal of the Shimonoseki University of Fisheries* **29**: 113-199.
- TESHIMA, K. & MIZUE, K. 1972. Studies on sharks 1. Reproduction in the female sumitsuki shark, *Carcharhinus dussumieri*. *Marine Biology* **14**: 222-231.
- TEWINKEL, L.E. 1956. Spermatozoa in the oviducal gland of the smooth dogfish, *Mustelus canis*. *Biological Bulletin* **102**: 314.
- TEWINKEL, L.E. 1963. Notes on the smooth dogfish, *Mustelus canis*, during the first three months of gestation. II. Structural modification of yolk-sacs and yolk-absorptive function. *Journal of Experimental Zoology* **152**: 123-137.
- TRICAS, T.C., MARUSKA, K.P. & RASMUSSEN, L.E.L. 2000. Annual cycles of steroid hormone production, gonad development, and reproductive behavior in the Atlantic stingray. *General and Comparative Endocrinology* **118**: 209-225.
- TSANG, P.C.W. & CALLARD, I.P. 1983. In vitro steroid production by ovarian granulosa cells of *Squalus acanthias*. *Biological Bulletin* **23**: 78-79.
- TSANG, P.W.C. & CALLARD, I.P. 1987a. Luteal progesterone production and regulation in the viviparous dogfish *Squalus acanthias*. *Journal of Experimental Biology* **241**: 377-382.
- TSANG, P.C.W. & CALLARD, I.P. 1987b. Morphological and endocrine correlates of the reproductive cycle of the aplacental viviparous dogfish, *Squalus acanthias*. *General and Comparative Endocrinology* **66**: 182-189.

- VAN DER ELST, R. 1981. *A guide to the common sea fishes of Southern Africa*. Struik, Cape Town. 367.
- WALKER, T. 1999. *Galeorhinus galeus* fisheries of the world. In: *Case studies on the management of Elasmobranch fisheries*. (ed.) R. Shotton, Vol. 378 Part 2, pp. 745-773. FAO Technical Paper, Rome.
- WALLACE, R.A. & BERGINK, E.W. 1974. Amphibian vitellogenin: properties, hormonal regulation of hepatic synthesis and ovarian uptake and conversion to yolk proteins. *American Zoologist* **15**: 1159-1175.
- WASS, R.C. 1973. Size, growth and reproduction of the sandbar shark, *Carcharhinus milberti*, in Hawaii. *Pacific Science* **27**: 305-318.
- WOURMS, J.P. 1977. Reproduction and development in chondrichthyan fishes. *American Zoologist* **17**: 379-410.
- WOURMS, J.P. 1981. Viviparity: The maternal-fetal relationship in fishes. *American Zoologist* **21**: 473-515.
- WOURMS, J.P., GROVE, B.D. & LOMBARDI, J. 1988. The maternal-embryonic relationship in viviparous fishes. In: *Fish Physiology*, (eds) W.S. Hoar & D.J. Randall, Vol. 11, pp. 1-134. Academic Press, San Diego.
- YANO, K. 1993. Reproductive biology of the slender smoothhound, *Gollum attenuatus*, collected from New Zealand waters. *Environmental Biology of Fishes* **38**: 59-71.